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Determination of Electrophoretic Subunit Pattern and Peptide Mapping of Collagen and Collagen Peptides Extracted From Skin of Hammerhead Shark (*Sphyrnae mokkaran*)

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Abstract

SDS-PAGE is a simple moreover standard method for separation and identification of protein moiety based on their molecular size. In the present study, shark skin-acid soluble collagen (SSk-ASC) and shark skin-pepsin soluble collagen (SSk-PSC) were extracted from the skin of hammerhead shark. Subunit pattern of both extracted collagens were observed using SDS-PAGE. From the electrophoretic patterns both the extracts were identified as they belong to type I. Collagen peptides were prepared from SSk-ASC by enzymatic digestion, followed by subsequent fractionation using gel filtration chromatography and anion exchange chromatography. The relationship between molecular weight distributions along with the antioxidant activity of the fractions were measured using SDS-PAGE analysis and ABTS radical scavenging assay. The results of evaluation revealed that the fractions with smallest fragments exhibiting maximum antioxidant activity.

Introduction

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has become the simple moreover standard technique for the analysis of subunits pattern, also high resolution separation of proteins in a complex extract. Discontinuous system for peptide separation was first described by Laemmli [1]. Generally the technique is being used for the determination of molecular mass of protein of interest as well as it is the first step of western blotting method. The SDS-PAGE analysis for molecular weight determination was primarily done by Shapiro [2]. Reichel demonstrated the SDS-PAGE technique for detecting doping in athletes with recombinant erythropoietin by determining the difference in molecular mass between native and recombinant erythropoietin in urine [3]. Native SDS-PAGE is useful for electrophoretic separation of proteins with their active functional domains such as metal binding sites [4]. The method was found to be less precise for the determination of apolipo-proteins in triglyceride rich plasma lipoproteins [5].

Collagen is the triple helical fibrous protein present in the extracellular matrix of connective tissues of animals. There are several types of collagen identified from

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different sources which differ in their subunit pattern as well as molecular weight of subunits [6]. Electrophoretic pattern of type I collagen was previously reported with a typical (α1)2 α2 chains and Type V collagen with a typical α1α3α2 chains. The electrophoretic pattern of collagen reveals the presence of α subunits as well as their cross-linked high molecular weight dimers (β) below 200 kDa and trimers (ɤ) above 200 kDa [7]. The presence of cross-linked subunits in collagen represents its ability to renature the native structure similarly, indicates that the presence of intra-molecular cross-links [8]. The susceptibility of mammalian collagen to mediate the occurrence of transmissible diseases (TSE, BSE) has reduced its demand in society. Nowadays, fish origin collagen is being considered as a suitable alternative source by the scientific community [9].

Hammerhead shark belongs to the family Sphyrnidae are coastal pelagic semi-oceanic shark. It's fins are highly demanded worldwide for their high fin ray count [10]. There is an exponential growth in the international market demand for hammerhead shark in India. Besides their fins, shark meat and liver oil are widely used for human consumption.

Bioactive peptides derived from collagen were proven to have a positive impact on human health. Their bioactivities can be influenced by type of enzyme used for hydrolysis and all other physical hydrolytic conditions applied. Thus the proteolysis taken place at controllable condition can enhance the functional properties of pepties [11, 12]. Antioxidant peptides from plant and diary sources are widely used as dietary bioactive components. Gelatin hydrolysates from fish skin possess excellent bioavailability and antioxidant activity which is attributed to their unique amino acid composition as well as molecular weight distribution of small fragments [13]. In the present study, an attempt has been made to identify the type of the extracted collagen from the skin of hammerhead shark (*S mokkaran*), like wise the collagen peptides obtained through aforementioned methods were studied to determine the link between molecular weight and free radical scavenging activity.

Materials and Methods

Proteolytic enzymes (Pepsin, papain and protease) from Bacillus, 2, 2'-Azino-bis(3 ethylbenzothiazoline-6-sulfonic acid (ABTS), Butylated hydroxyanisol, Sephadex G-25, DEAE-Sephadex A-25, were purchased from Sigma Aldrich. Novex-Tris-Glycine precast gels (4-12%), Novex™Tris-Glycine SDS Sample Buffer, Novex™ Tris-Glycine SDS Running Buffer, and See Blue Plus2 prestained protein standard (Invitrogen) were procured from Thermofisher Scientific.

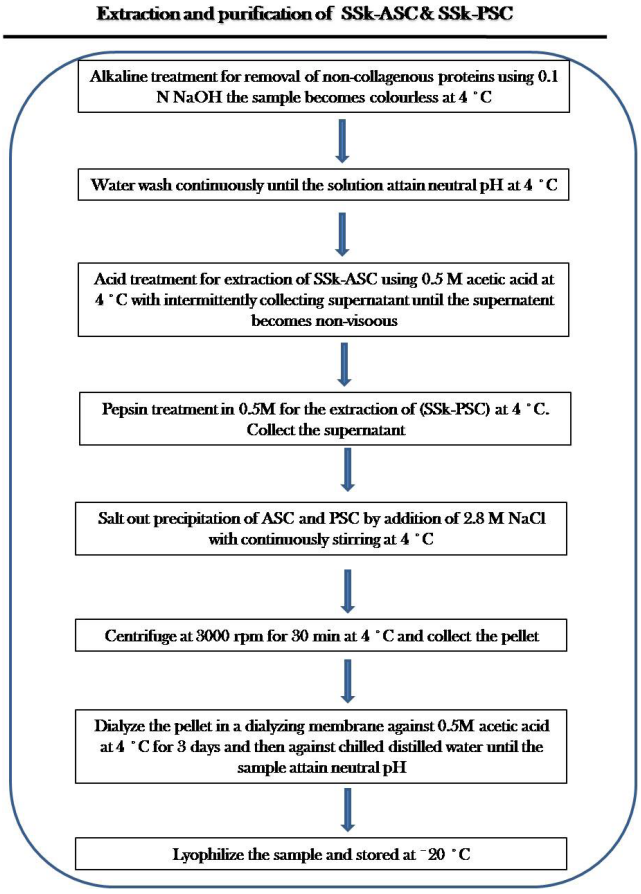
Skin of hammerhead shark was collected from the local market, it was cleaned under tap water, chopped and stored at -20°C. The image of the fish is shown in Figure 1.

Figure 1: Hammerhead Shark (*Sphyrnae mokkaran*)

Extraction of Collagen from the Skin

A method has been standardized for the extraction of acid soluble collagen (ASC) and pepsin soluble collagen (PSC). Briefly, the raw material (skin of hammerhead shark) was undergone alkaline treatment followed by water wash. The skin was then treated with diluted acetic acid for ASC extraction, the undissolved matter was then treated with pepsin in diluted acetic acid to extract the PSC. Extracted collagen samples were salted out, dialyzed and lyophilized. The detailed extraction and purification protocols of collagen from skin of hammer head shark (*S mokkaran*) are depicted in Plate 1.

Plate 1:



Preparation of Collagen Peptides

The extracted ASC was hydrolyzed by three consecutive proteolytic enzyme treatments, pepsin followed by papain and by protease. The hydrolyzed samples were fractionated by passing through sephadex G-25 gelfiltration column followed by DEAE-Sephadex A-25 anion exchange column and desalted by passing through sephadex G-25 column. The fractions were analyzed for antioxidant activity using ABTS radical scavenging activity and those with similar activity were pooled and lyophilized. The purification protocol of collagen peptides from Hammerhead shark skin collagen is depicted in Plate 2.

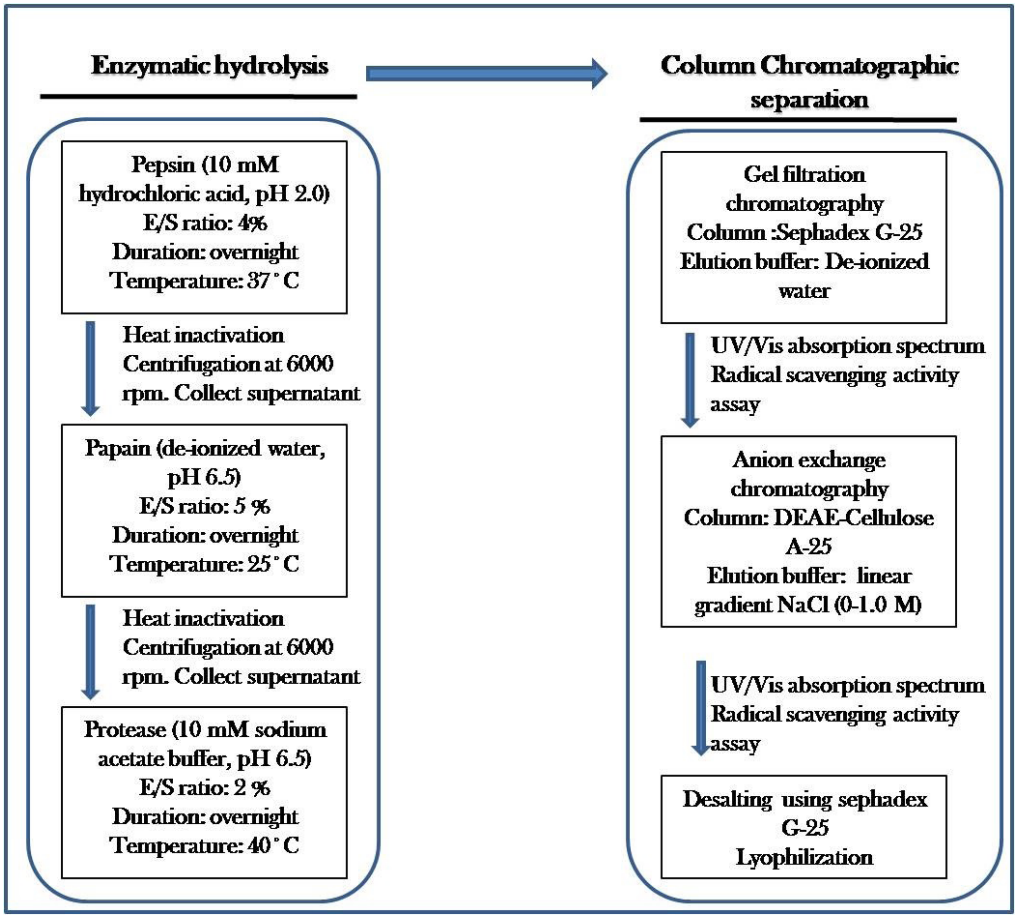
Determination of ABTS Radical Scavenging Activity of Collagen Peptide

The free radical scavenging activity of crude collagen hydrolysate and fractionated peptides were measured using 2, 2'-Azino-bis (3 ethylbenzothiazoline-6-sulfonic acid (ABTS) following Lee et al. [14] with slight modification using butylated hydroxylanisol (BHA) as standard. Activity was expressed as percentage of radical scavenging activity which is equivalent to that of µg/ml of BHA.

SDS-PAGE Subunit Pattern of Collagen and Collagen Peptides

SDS-PAGE apparatus used for the separation is Mini gel tank (Life Technologies) connected with a Power pack from Bio-rad. Electrophoretic separation of samples were carried out using 4-12% Novex-Tris-Glycine precast gels (Thermofisher Scientific), Novex-Tris-Glycine SDS Sample Buffer, Novex-Tris-Glycine SDS Running Buffer (Thermofisher Scientific), See Blue Plus2 prestained protein standard (Invitrogen). Both SSk-ASC and SSk-PSC (1 mg/ml) were dissolved in diluted acetic acid prior to digestion, similarly collagen peptides (CP I, CP II and CP III) were reconstituted in deionized water (1 mg/ml). Equal volume of sample buffer was added to each vials and digested in a boiling water bath for 10-15 min. 20 µl samples were then loaded in each well and were run at 80V for 2 hrs. The gel was stained using Coomassie

Plate 2: Preparation and purification of Antioxidant Collagen Peptide



brilliant blue dye for 1 min followed by destaining for the removal of excess stain on the gel by washing with destaining solution composed of 5% glacial acetic acid and 20% methanol.

Statistical Analysis

The antioxidant activity was measured using triplicate. The results are expressed as Mean±SD.

Results and Discussion

Extraction of Collagen from Skin

The lyophilized ASC and PSC were appeared white puffy and slightly hygroscopic in nature.

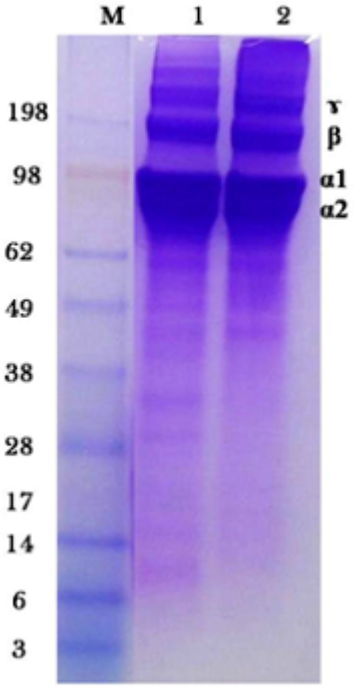
Subunit Pattern Analysis of Fish Collagen

The electrophoretic analysis of SSk revealed that both ASC and PSC were composed of two α subunits (α1 and α2) in an approximate ratio of 2:1, along with their inter cross-linked dimers (β) and trimers (ɤ) (Figure 2). Intensity difference between the bands suggests that the collagen composed of two identical subunits of α1 as well as one of that of α2. The electrophoretic position of subunits indicating that α1 has 98 kDa and α2 possess 89 kDa. In the present study the subunit pattern of both the collagen extract propose that they belong to type 1. The results matches with the previous reports of collagen from the skin of marine eel fish [15], skin of squid [9]. Similar work conducted by us with collagen from the swim bladder of striped cat fish exhibit slightly higher molecular weight (α1 -150 kDa and α2 -116 kDa) than that of skin collagen (unpublished data) which is matching with the report shown by [16]. According to Chen *et al*. molecular weight pattern of subunits α1 and α2 of ASC extracted from skin as well as scales of tilapia showed higher values than the data availed from current study [17].

Collagen Peptide Preparation

Lyophilized collagen hydrolysates appeared white granular, odorless, hygroscopic and completely soluble in water. Upon ultrafiltration the hydrolysate particles got

Figure 2: Electrophoretic Pattern of Fish Collagen Extracted from the Skin of Hammerhead shark (S. *Mokkarn*). Lane 1 Indicates Protein Marker, Lane 2 indicates ASC, Lane 3 indicates PSC



separated based on their molecular weight and ionic charge on the side chains of amino acids. The hygroscobicity of peptides were observed to be reduced for smaller fractions.

Radical Scavenging Activity of Collagen Peptide Fractions

The ABTS radical scavenging assay demonstrated the highest level activity compared to other activity assays. This actually measures the activity of both hydrophilic and hydrophobic amino acids [13, 18]. Since, collagen peptides are composed of both hydrophilic and hydrophobic amino acids ABTS assay might be most suitable for analyzing their antioxidant activity. And thus the activity was measured using ABTS radical scavenging assay.

The crude collagen hydrolysate was found to possess 88. 5% radical scavenging activity that is equivalent to 19. 7 µg/ml BHA (Figure 3). Primary isolation of antioxidant fragments from the crude hydrolysate was carried out by passing through Sephadex G-25. Four fractions were collected (F1-F4) and each fragments were analyzed for ABTS radical scavenging activity. Among the four, fractions with smaller fragments (F3 and F4) were displaying maximum activity (79. 1 and 75. 8 % respectively) which was equivalent to 18. 7 and 18. 3 µg/ml BHA (Refer Figure 3). Both the samples (F3 & F4) were pooled and it was further fractionated using DEAE-sephadex A-25 anion exchange column chromatography and 12 fractions were isolated (T1-T12) which were analyzed for their ABTS radical scavenging activity. T7 was expressed maximum activity. The results of the radical scavenging activity assay was given in Figure 4. The fractions with similar activity were pooled together into three fractions which were named as CP-I, CP-II, and CP-III. (T7 is named as CP-II). The ABTS radical scavenging activity of these 3 final fractions were also measured and CP-II exhibited maximum activity (Table). T7 was eluted with higher concentrations of NaCl, the fragments may be composed of neutral amino acids showed maximum activity. Intarasirisawata has reported similar results that is the peptides from skipjack roe hydrolysate were fractionated using cation exchange column and among the fractions neutral fragments exhibited maximum activity [19]. While treatment with different digestive enzymes, produces fragments with different biological properties which are determined by the site of digestion. The gelatin hydrolysates produced from the skin of unicorn leatherjacket produced anionic fragments with maximum radical scavenging activity the digestive enzyme used was glycyl endopeptidase [13].

Figure 3: Radical Scavenging Activity of Collagen Peptide Fractions after Gel Filtration Chromatographic Separation determined by ABTS Radical Scavenging assay. The Numbers on the Bar Indicates the Equivalent Concentration of BHA (µg) Corresponding to the Antioxidant Activity. Values Indicate Mean ± SD

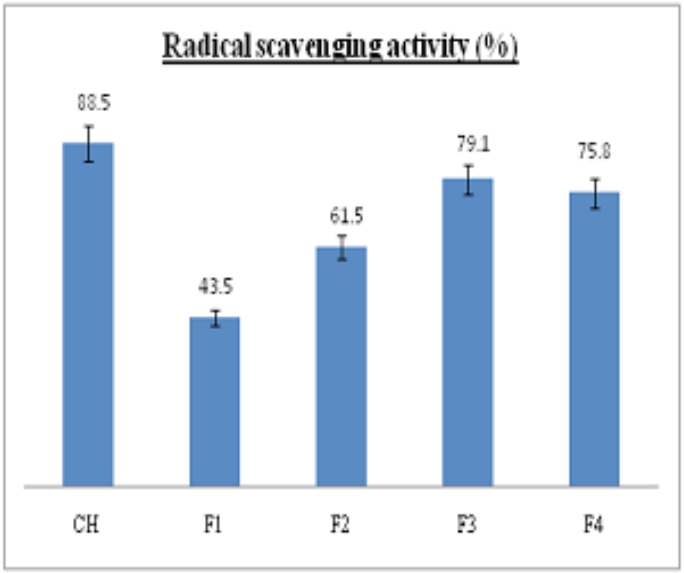


Figure 4: Radical Scavenging Activity of Collagen Peptide Fractions after Anion Exchange Chromatographic Separation Determined by ABTS Radical Scavenging Assay. The Numbers Indicates the Equivalent Concentration Of BHA (µg) Corresponding to the Antioxidant Activity. Values Indicate Mean ± SD.

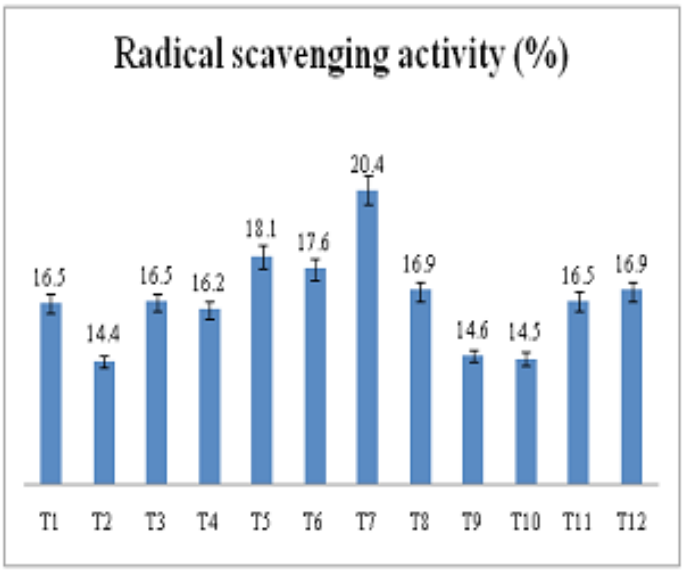


Table 1: Radical Scavenging Activity of Collagen Peptide Fractions Pooled from the Fractions Obtained from Anion Exchange Column Separation

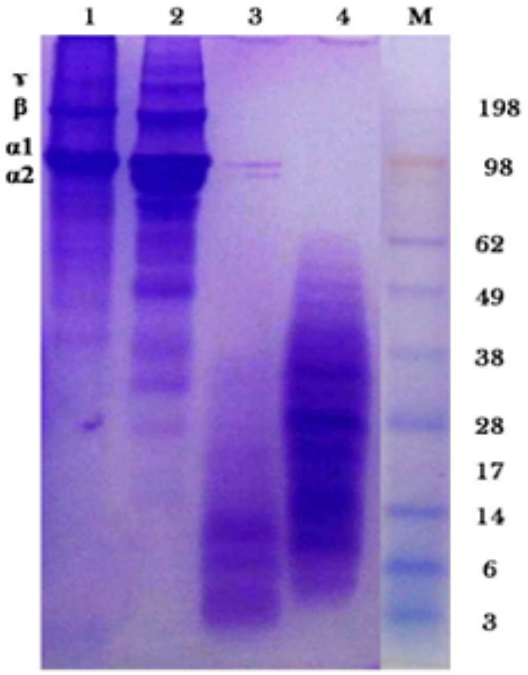
Collagen	Radical scavenging activity (%)
Collagen Hydrolysate (crude)	88.5±0.5 (equivalent to 19 µg BHA)
Peptide Fractions	
CPI	40.3 ±0.8(equivalent to 13 µg BHA)
CPII	94.6 ±1.1.(equivalent to 20 µg BHA)
CPIII	62.2 ±0.9(equivalent to 16 µg BHA)

*The Samples are Analyzed in Triplicates and Values are Expressed as Mean ± SD

Peptide Mapping of Enzyme Hydrolyzed and Fractionated ASC

Figure 5 demonstrated the electrophoretic pattern of ultrafiltered fractions of collagen peptides, revealed that enzymatic treatment partially digested the α1 and α2 also their crosslinked molecules. Meanwhile, the gel filtration chromatographic method separated the high molecular weight components completely. The first fraction CP-I was found to possess α1 and α2 along with cross linked subunits, along with fragments within a range of 14 kDa. The fractions CP II and CP III were found to contain smaller fragments (28 kDa - 3kDa) which was exhibiting maximum antioxidant properties. The results of the present study shown that the smaller fragments are responsible for their antioxidant property. According to Chi reports lower weight molecules exhibit the highest antioxidant activity [20]. The present observation on the molecular weight of fractions suggested that the presence of highly hydrophobic regions of collagen is possibly responsible for make it less susceptible to enzymatic or chemical digestion.

Figure 5: Electrophoretic Pattern of Fish Collagen Peptide (FCP) Fractions after Column Chromatographic Separation. Lane 1 Indicates Collagen Hydrolysate, Lane 2 Indicates CP I, Lane 3 Indicates CP II, Lane 4 Indicates CP III, Lane 5 Indicates Protein Marker



Conclusion

The electrophoretic pattern of collagen extract revealed that both SSk-ASC and SSk-PSC belong to type 1. The collagen peptides prepared from SSk-ASC by consecutive fragmentation using pepsin, papain and protease from Bacillus. Ultra filtration (gel filtration chromatography followed by anion exchange chromatography) isolated the moderately charged smaller peptide fragments with maximum radical scavenging activity. Peptide mapping using SDS-PAGE revealed that the fragment with maximum activity possess smallest fragments.

Conflict of Interest

The authors declare that they have no conflict of interest.

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