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Research Article

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Extraction and Purification of Collagen from Marine Squid *Uroteuthis Duvauceli*

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Abstract: Collagen is a protein, which plays a significant role in the development of tissues and organs. These are used for cosmetic surgery and as wound healing aids in burn patients, remaking of bone and a wide assortment of dental, orthopedic and surgical purposes. These days' collagen is isolated from calf, porcine tissue and so forth. But it can cause the onset of diseases such as, for example, Bovine spongiform encephalopathy (BSE), Transmissible spongiform encephalopathy (TSE), Foot-and-mouth illness (FMD), and so forth and furthermore cultural and social concerns. As another option, collagen can be separated from marine squid. The aim of the present work is to isolate and partially characterize the collagen from the marine squid, *Uroteuthis duvauceli*. The confined collagen was evaluated by utilizing Bradford test and partially purified by using gel filtration on a Sepharose 4B column. These filtered collagens were affirmed by SDS PAGE. These days industrially accessible collagen in the market are costly and are not affordable to the common people. The present data on collagens from the marine squid, could help the future endeavors to unwind the therapeutically significant, more secure collagens from marine squid for their utilization in the biomedical field.

Keywords: Collagen, *Uroteuthis duvauceli*, Bradford assay, SDS PAGE, Sephadex G-100 gel filtration chromatography.

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1. INTRODUCTION

Osteoporosis is one of the dangerous conditions described by relaxing of the bones that makes them weak and gets broken effectively. It is generally predominant among old age individuals and is the most widely recognized reason for pathological bone fracture. At the point when the activity of osteoblast stops with increasing age the procedure of bone repairment likewise diminishes bringing about less bone mineral density. Mature bone is made out of proteins and minerals. Around 60% of the bone includes minerals, mainly calcium and phosphate. The remaining is water and network, which is shaped before the mineral is stored, and can be viewed as the framework for the bone. About 90% of the matrix proteins are collagen, which is the most copious protein in the body. Collagen is formed as chains (short bits of string) which twist into triple helices (strings). These line up and are fortified together into ropes (fibrils). The fibrils are arranged in layers, and mineral crystals will store between the layers. Collagen has a gel-like, smooth structure that spreads and holds bones together. It permits skimming and moving the bone without torment. Notwithstanding that, it encourages joints to move all the more effectively, diminishes torment frequently connected with maturing and even decreases the danger of joint crumbling. Collagen types III, VI, IX, X, XI, XII, and XIV are on the whole present in minute amounts in the mature ligament[1]. Type I collagen is the most bounteous collagen and the major structural protein of bone, skin, ligament, tendon, sclera, cornea and veins. Scientific studies uncovered that collagen has an assortment of medical advantages, from relieving joint agony to improving skin health, increment the bone mineral density and increment the action of the osteoblasts for bone repair in old age[2]. Squid are cephalopods in the superorder *Decapodiformes* with extended bodies, enormous eyes, eight arms and two appendages. Squid have a distinct head, bilateral symmetry, and a mantle. They are mostly delicate bodied and have a little inner skeleton as a rod like gladius or pen, made of chitin. Squid collagen was found to have a permeable structure which supports cell adhesion, morphogenesis, accumulation and development. The collagen separated from the Persian Gulf Squid was demonstrated to be appropriate to be utilized as a substrate in three-dimensional cell culture framework, which thus could be utilized adequately as an apparatus in biomedical research and the advancement of more up to date medications and treatment convention especially on account of disease. The collagen removed from the mantle of Jumbo squid (*Dosidicusgigas*) can possibly be utilized as a plasticizer agent and can be utilized for biofilm readiness when mixed with chitosan. Jumbo squid blade and skin collagen hydrolysates displayed antioxidant activity, anti-mutagenic and anti-proliferative activity. The enzymatic hydrolysates of the collagen obtained from the skin of squid (*Todarodespacificus*) demonstrated antioxidant, anti-elastase and tyrosinase inhibitory activities. The mantle of the squid *Loligo vulgaris* exhibited its potential as a substituted source of collagen-derived materials. Recently, the animal source had been utilized commercially for collagen extraction, particularly the porcine and cow-like skin and bones. But it can cause the onset of diseases such as, for example, Bovine spongiform encephalopathy (BSE), Transmissible spongiform encephalopathy (TSE), Foot-and-mouth illness (FMD), and so forth and furthermore cultural and social concerns. From that point forward genuine examinations have been completed on collagen extraction from marine vertebrates and invertebrates, for example, fishes, starfish, sponges, octopus, squid, sea urchin, cuttlefish, sea anemone, jellyfish, prawn and so on. Marine preparing squanders which are a risk to the environment can be used viably for the extraction of many value added products items which additionally incorporate collagen. The handling squanders of squid incorporates its skin, head, viscera, spines or pens, ink, appendages, blades, mantle, and so forth. Research has indicated that about 70% of the squid skin dry matter is collagen[3]. Because of the commercial significance and the presence of high protein content particularly collagen in the squid, it has been decided to investigate.

2. MATERIALS AND METHODS

2.1 *Collection and identification of Uroteuthis duvauceli (Indian Ocean Squid)*

Specimen of *Uroteuthis duvauceli* was collected from Vizhigam harbour, Trivandrum, Kerala, India in the month of February 2018. It was transported to the laboratory in dry ice and was frozen at -20 °C until use. The specimen identification was confirmed by the Research Centre of Central Marine Fisheries Research Institute (ICAR) Thiruvananthapuram, India. The Species was identified as *Uroteuthis duvauceli* Fig:1, Table 1.

2.2 *Skin and Muscle Preparation from Squid*

Skin and Muscle obtained from *Uroteuthis duvauceli* was dissected out, separated, washed in chilled distilled water, cut into small pieces using scissors and adhering particles were removed manually. Muscle, skin, gladius and fin were placed in polyethylene bags and then stored at -20°C until use[4].

2.3 *Determination of Proximate Composition in Skin and muscle samples*

Moisture, ash, fat and protein contents were determined from the *Uroteuthis duvauceli* by AOAC methods (2000)[5].

2.3.1 *Moisture*

Moisture was determined according to the AOAC (2000) method by drying[4]. A clean and dry petridish was cooled in desiccators and weighed (W1). Approximately 10-20g of the finely homogenized samples was evenly spread and weighed (W2). Petridish with the sample was dried in an oven at 105^{0}C, cooled in desiccators and weighed (W3). The process of heating and cooling were repeated to get a constant value. Results were expressed as percentage of wet weight.

$$\text{Moisture (\%)} = (W2 - W3)/ (W2 - W1) \times 100$$

2.3.2 *Protein*

Total protein content in the homogenized samples (0.2 g) was determined using Kjeldahl method (AOAC, 2000)[4]. Weighed and transferred 0.2 g of the sample into a Kjeldhal flask. A pinch of digestion mixture was added followed by 10mL of concentrated sulphuric acid. Kjeldhal flask was kept over a heater in order to heat the content until the solution became colorless. Cooled and made up to desired volume (100 mL) according to the protein content of the sample. Prepared blank with distilled water. A conversion factor (6.25) was used to convert total nitrogen to crude protein. Results were expressed as percentage on wet weight basis. Placed a conical flask having 10 mL of boric acid at the receiving end of the distillation apparatus and made sure that the tip of the condenser was slightly immersed in the boric acid. Pipetted out 5 mL of the digested sample and transferred in to the distillation apparatus and also added 10 mL of 40% NaOH followed by was distilled water for rinsing. Steam accelerated the liberation of nitrogen from the sample and it distilled and dissolved in the boric acid solution. Depending upon the concentration of nitrogen, colour of the solution turned green from pink. The solution in the receiving flask was green at this stage. Titrated the content against N/100 sulphuric acid until the original pink colour was restored. Noted the volume of acid used for titration. One mL of 0.01N sulphuric acid contains 0.14 mg Nitrogen. If the titre value of the sample after subtracting blank was X, then,

$$\text{Protein content (g 100g)} = X \times 0.14 \times 6.25 \times 100 / 1000 \times V1$$

Where,

V = Total volume of the digest
V1 = Volume of the digest for distillation
W = Weight of sample for digestion

2.3.3 *Fat*

The crude fat was determined by Soxhlet method using petroleum ether as solvent[6]. The estimation of crude fat content was carried out by continuous extraction of fat with petroleum ether according to AOAC (2000). 2 g (W1) of dried sample was weighed into a thimble and a cotton plug was kept on top of it. Thimble was placed in a Soxhelet apparatus and extracted with petroleum ether for 16 hrs. The apparatus was cooled and the solvent was filtered in to a pre-weighed conical flask (W2). The flask of the apparatus was washed with small quantities of ether and the washings were added to the above flask. The ether was removed by evaporation and the flask with fat was dried at 80-100 °C, cooled in a desiccator and weighed (W3).

$$\text{Fat content (g/100g)} = (W3 - W1) / (W1)$$

2.3.4 *Ash*

Ash content was determined by heating sample for 6 hrs in a furnace at 600°C (AOAC, 2000)[4]. Heated a platinum or porcelain crucible to 600 °C in a muffle furnace, cooled in desiccators and weighed (W1). Approximately 5-10 g of the dried sample was weighed and transferred into a platinum crucible (W2). The crucible along with sample was placed on a clay triangle and heated at a low flame until the material was charred. The charred material was kept inside the previously set muffle furnace and heated at 600°C for 3-4 hrs to get white or grayish white ash. The crucible was cooled in desiccators and weighed (W3). The crucible was again heated for further 30 min, cooled and weighed. Results were expressed as percentage of wet weight.

$$\text{Ash content (\%)} = (W3 - W1) / (W2 - W1)$$

2.4 *Bradford protein assay*

The protein contents of the samples were resolved utilizing the Coomassie Plus (Bradford) protein assay kit (Thermo Fisher, USA), with bovine serum albumin as a standard[3].

2.5 *Histological Studies*

Van Gieson's Stain used to differentiate collagen fibers with other tissue segments. The samples are deparaffinize and hydrated to distilled water. Soak samples in Wiegert's iron hematoxylin solution for 5 minutes. Then wash quickly in running tap water and flush in two changes of distilled water and add Van Gieson's stain for three minutes. After staining dehydrate in three changes of 95% alcohol and complete dehydration in three changes of absolute alcohol. Clear in three or four changes of xylene and mount with synthetic resin. The collagen strands and muscle filaments are obtained with red and yellow colors, respectively, and are used to observe by light microscope[7].

2.6 *Extraction of collagen*

Extractions steps were performed at 4°C with continuous mixing[8]. Skin and muscle pieces obtained from *Uroteuthis duvauceli* were cut into small pieces (1 to 2 cm) and afterward they were treated with 0.1N NaOH independently at a solid to solvent proportion of 1:10 (w/v) for 3 days. The alkali solution was changed each day. Following 3 days, alkali solution was disposed of; skin and muscle pieces were washed in sufficient measure of distilled water until a neutral pH was accomplished. After this, 10% butyl liquor was added at solid to solvent proportion of 1:10 (w/v) for 24hr to skin and muscle pieces for a defatting reason. After 24hr, skin and muscle pieces were washed in cold distilled water. Further, extraction was done in 0.5M acetic acid for 3 days. The viscous solution was centrifuged at 12,000 x g for 1hr, 4°C. The supernatant was gathered and pellets were stored in a beaker at 4°C for the extraction of pepsin soluble collagen. Salting-out was performed for the supernatant by adding Nacl to get a final concentration of 2.5 M in the presence of Tris-HCl (pH 7.5). The precipitates were gathered by

centrifugation at 12,000 x g for 1hr at 4°C. The resultant pellet was mixed in a minimum amount of 0.5 M acetic acid, dialyzed against 0.1M acetic acid and distilled water and lyophilized.

2.7 *Purification of crude collagen*

The collagen is purified with modification of the protocol followed by Saravanan *et al*.[9]through Sephadex G-100 gel filtration chromatography. The section (25cm × 2.5cm) was eluted with 0.1, 0.2, 0.4 and 0.6M phosphate buffer saline (pH 7.4) with a flow rate of 0.33ml/min and the portions were collected. The active fractions were pooled and stacked in a dialysis membrane and dialyzed against double distilled water at 4°C for 12h and freeze dried. All collected fractions were identified by an UV absorbance spectroscopy measured at 230nm.

2.8 *Determination of Proximate Composition in type I collagen*

Moisture, ash, fat and protein contents were determined in type I collagen of *Uroteuthis duvauceli* skin and muscle according to AOAC methods (2000) using automatic moisture analyzer (moisture content), Automatic Microkjedahl unit (crude protein), Soxhlet method (fat) and Muffle furnace (ash)[4,5].

2.9 *Determination of Hydroxyproline*

Hydroxyproline content was determined by technique for Neuman and Logan[10] method. Collagen tests were taken into a round bottom flask and 100ml of 6N HCl solutions was added boiled at 100°C for 16h. The cooled hydrolysate was moved to a volumetric flask and dissolved with distilled water. Hydroxyproline standard solution was prepared by dissolving 100mg standard Hydroxyproline in distilled water. Four ml of the final dilution was taken in the test tube; 2 ml of chloramines-T was added and left to stand for 25min to the mixture. 2ml of color reagent (4-dimethylaminobenzaldehyde arrangement) was added and mixed. This subsequent mixture was put in a water bath at 60°C for 15min. The blend was cooled and the absorbance of solution was noted at 558nm. Hydroxyproline content in the sample was calculated from the standard curve[5].

2.10 *Solubility of Collagen*

2.10.1 *Effect of pH on solubility*[12]

Four ml collagen solutions from skin and muscle samples (3 mg/ml) were transferred to 15ml centrifuge tubes and either 6N NaOH or 6N HCl added to get the final pH, ranging from 1 to 10. The volume of test solutions was made up to 10ml with deionized previously adjusted to the same pH as the collagen solution. The solutions were centrifuged at 20,000g for 30min and the protein content of the supernatant determined. Protein solubility were calculated by using the following equation:

Solubility= (protein content of the supernatant)/ (total protein content of the sample)

Relative solubility= (solubility at given pH)/ (highest solubility in the various range of pH)

2.10.2 *Effect of NaCl on solubility*[11]

Four ml collagen solutions from skin and muscle samples (3 mg/ml) were mixed with 1 ml of NaCl in 0.5 M acetic acid to give final concentrations of 0 to 12%. The mixture was mixed for 30min, followed by centrifugation at 20,000g for 30min. Protein content in the supernatant was estimated and the relative solubility calculated using the following equation.

Solubility= (protein content of the supernatant)/ (total protein content of the sample)

Relative solubility= (solubility at given pH)/ (highest solubility in the various range of pH)

2.11 *SDS-PAGE*[5]

Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970)[13] using 10% separation gel and 1% stacking gel. Collagen (10mg) samples were mixed in 1.0ml of the sample buffer (Tris–HCl, pH 6.8 containing 2-mercaptoethanol, sucrose, bromophenol blue, 5% SDS) and heated at 50oC for 10min. Then, 20µl was loaded in each well along with high molecular weight protein markers and the electrophoresis was carried out at 50mA initially and then at 100mA. Protein bands were stained with Coomassie Brilliant Blue R250 and destained using a solution containing water, methanol and acetic acid (5:4:1, v/v/v). The molecular weights of the collagen α-chains were determined by comparison with standard protein markers.

3. STATISTICAL ANALYSIS

Data are presented as the mean ± standard deviation of at least three independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant difference. These calculations have been performed with MINTABTM Release 14 Minitab Inc. software.

Fig 1. *Uroteuthis duvauceli* collected from Vizhinjam harbour, Kerala, India.

Table 1. Scientific Classification: *Uroteuthis duvauceli* (d'Orbigny, 1835)	
Kingdom	Animalia
Phylum	Mollusca
Class	Cephalopoda
Subclass	Coleoidea
Superorder	Decapodiformes
Order	Myopsida
Family	Loliginidae
Genus	*Uroteuthis*
Species	*Uroteuthis duvauceli*

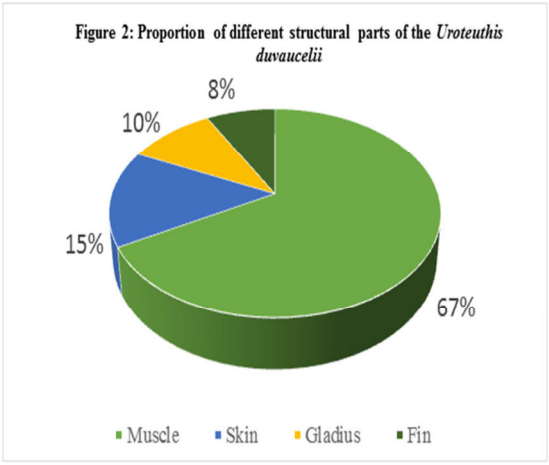


Fig 2. The yields of different structural parts of *Uroteuthis duvauceli*

Table :2 Chemical compositions in Skin of *Uroteuthis duvauceli*	
Proximate composition (%)	**Skin**
Moisture	71.83±0.4
Protein	25.5±0.2
Fat	1.13±0.05
Ash	1.2±0.1

Values are given as mean ± S.D; (n=3) P<0.05, statistically significant

Table 3. Chemical compositions in muscle of *Uroteuthis duvauceli*	
Proximate composition (%)	**Muscle**
Moisture	82.2±0.1
Protein	13.2±0.05
Fat	2.2±0.1
Ash	1.2±0.05

Values are given as mean ± S.D; (n=3) P<0.05, statistically significant

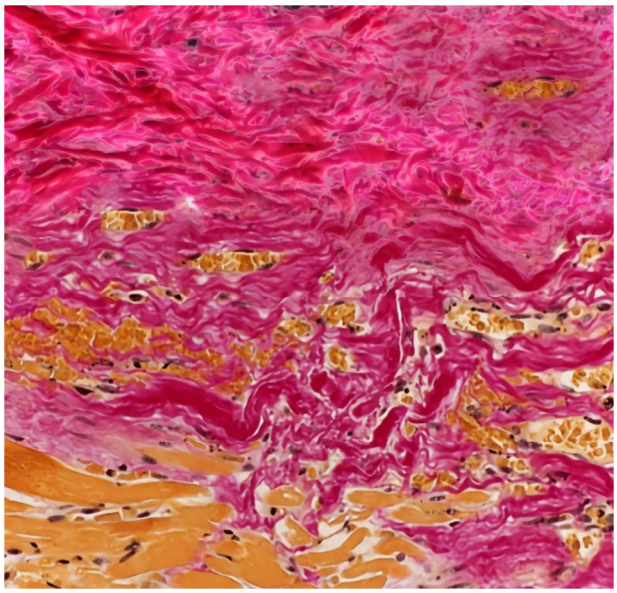


Fig 3. Photomicrograph of the squid skin colored with Van Gieson stain. Collagen and skin fibers were colored with pink and yellow, respectively.

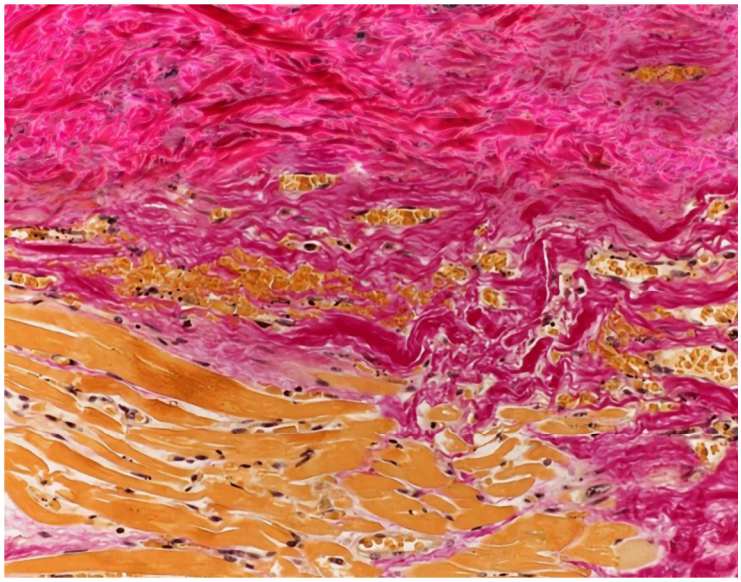


Fig 4. Photomicrograph of the squid muscle colored with Van Gieson stain. Collagen and muscle fibers were colored with pink and yellow, respectively.

Table 4. Chemical composition of extracted Type I collagen from Muscle

Proximate composition (%)	Skin
Moisture	6.4±0.08
Protein	90.2±0.1
Fat	0.8±0.08
Ash	0.85±0.04

Values are given as mean ± S.D; (n=3) P<0.05, statistically significant

Table 5. Chemical composition of extracted Type I collagen from skin

Proximate composition (%)	Muscle
Moisture	5.7±0.1
Protein	92.5±0.1
Fat	0.84±0.03
Ash	0.7±0.08

Values are given as mean ± S.D; (n=3) P<0.05, statistically significant

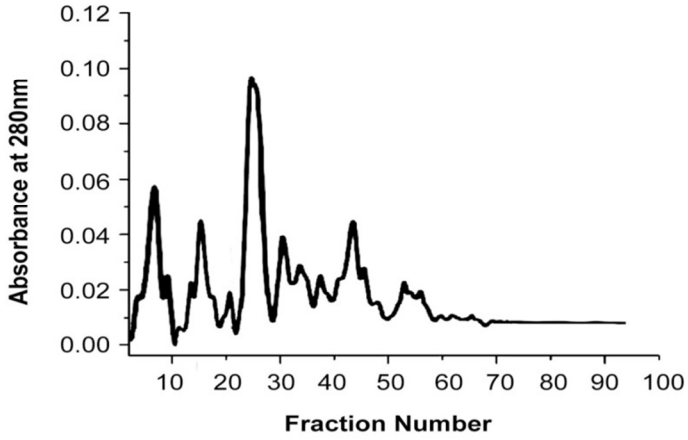


Fig 5. Purification of Type I collagen from Skin of *Uroteuthis duvauceli* by Gel filtration with Sephadex G-100

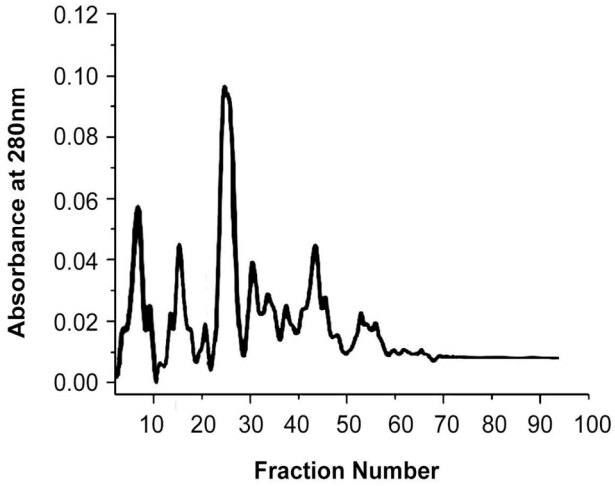


Fig 6. Purification of Type I collagen from muscle of *Uroteuthis duvauceli* by Gel filtration with Sephadex G-100

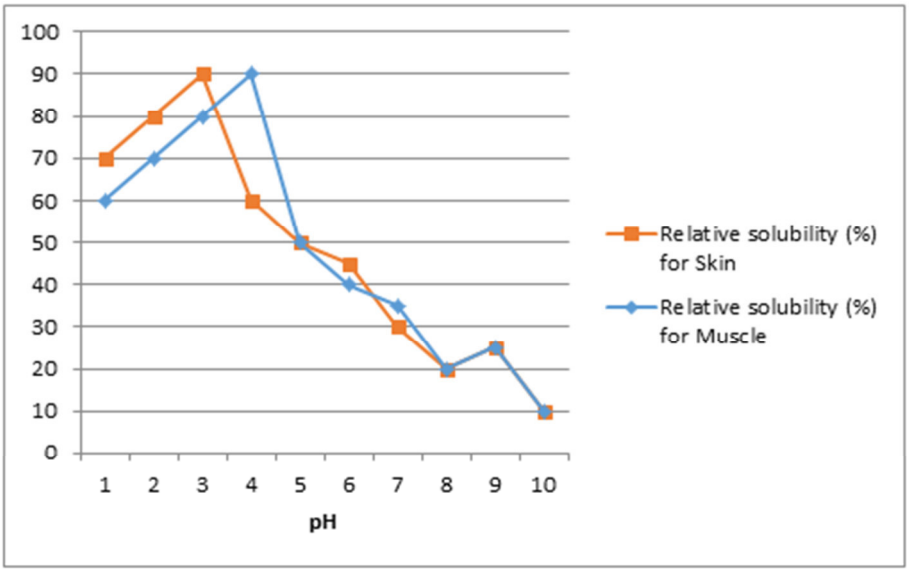


Fig 7. Effect of pH: Relative solubility of collagen type I from skin and muscle of *Uroteuthis duvauceli*

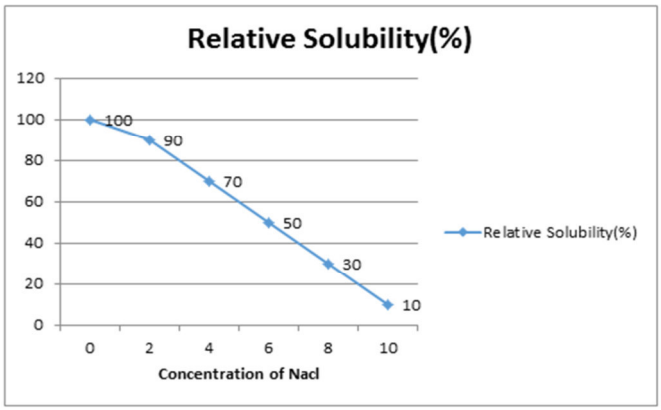


Fig 8. Relative Solubility of Type I collagen from *Uroteuthis duvauceli* muscle

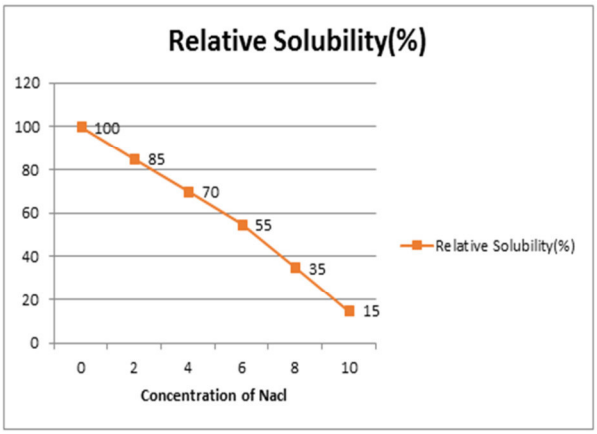
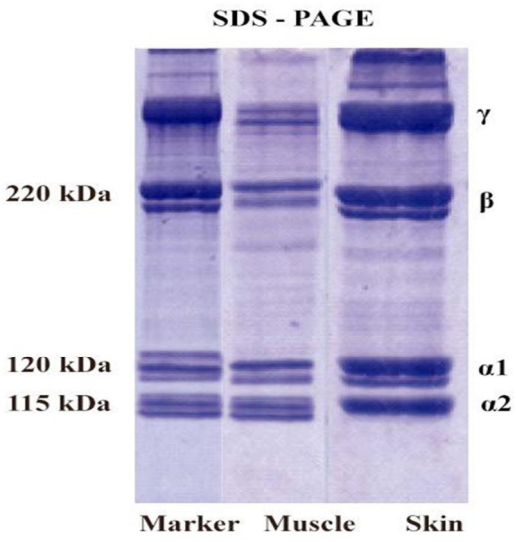


Fig 9. Relative Solubility of Type I collagen from *Uroteuthis duvauceli* muscle



(A) Molecular weight markers, (B) Skin collagen (C) Muscle collagen.

Fig 10. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of collagen from muscle and skin on 7.5% gel under reducing condition.

3. RESULTS AND DISCUSSION

In this study squid, *Uroteuthis duvauceli* skin and muscle tissues were used as raw material for type I collagen extraction. *Uroteuthis duvauceli* squids occur in temperate and tropical coastal waters[11] and are abundantly seen in Kerala coast. The main source of collagen is land animals previously is being superseded by other elective sources as a result of certain health related, religious and social concerns. Hence, this prompted us to extract the type I collagen from skin and muscle of the squid which could be promising to gain value added products and to clear the wastes caused by dumping of processing wastes of squid at coastal areas. *Uroteuthis duvauceli* were washed with potable water to clean the dust, dirt, sand and other extraneous matter. Samples were fed into a mechanical mincer and the muscle constituted 67% and

remaining were their frames (33%). The frames included skin (15%), gladius (10%) and fin (8%). The yields of different structural parts of *Uroteuthis duvauceli* are given in Fig:2. Shahidi (1994)[13]revealed that 25% of minced meat could be gotten by mechanical deboning of processing discards. Mechanical deboning process typically expels more wastes than manual separation and the wastes generated differ in amount and composition depending on the species and process employed. Another report expressed that the processing discards accounted upto75% of the total weight of fish in filleting operations[14]. Similar to it, the *Uroteuthis duvauceli* used in this study comprised high proportions of skin and mantle besides gladius and fin, as processing discards. These disposes of were discovered appropriate for collagen extraction. As the muscle of this squid was additionally utilized for collagen extraction. The proximate composition of *Uroteuthis duvauceli* skin and muscle were determined. The proximate composition studies revealed that skin was found to contain 71.83±0.4 percent moisture, 25.5±0.2 percent protein, 1.13±0.05 percent fat and 1.2±0.1 percent ash contents; and in muscle contained 82.2±0.1 percent moisture, 13.2±0.05 percent protein, 2.2±0.1 percent fat and 1.2±0.05 percent ash contents. The moisture content of *U.duvauceli* skin was lesser than muscle. The protein content of *U.duvauceli* skin was more than muscle whereas the fat content of *U.duvauceli* skin was found to be lesser than that of muscle, and ash content was found to be similar in both skin and muscle; and thus statistically significant (P<0.05).Similar finding was observed in the tentacles of European squid (*L. vulgaris*)[15]. Santoso *et al*., (2013)[16] investigated the proximate composition of Japanese common squid (*Todarodes pacificus*) and reported the content of 44.0 g 100g-1 of fat, 13.5 g 100g-1 of protein and 2.11 g 100g-1 of ash in dry matter. Remyakumari *et al*, (2018)[4] reported that the proximate composition of *Uroteuthis duvauceli* showed a content of 80.47% for moisture, 17.5% for protein, 0.52% for fat and 1.13% for ash, respectively. Relatively high moisture content was observed in *U. duvauceli* as compared to its total fat content and also revealed better content of protein and ash. Roper *et al*., (1984)[18] suggested that low fat content and high protein concentrations in cephalopods make them appropriate for human consumption especially for elderly population. Bano *et al*., (1992)[18] analyzed and compared the protein content of *Sepiella inermis* and *Sympleclotenllies oualaniensis* and found better protein content in *S. oualaniensis*. The present findings are in corroboration with the findings of Thanonkaew *et al*., (2006)[19]. Whereas the fat content of *U.duvauceli* skin was found to be lesser than that of muscle, and ash content was found to be similar in both skin and muscle. Nurjanah *et al* (2012)[20] reported that proximate analysis of cuttlefish contained 13.16 -13.51% proteins, 0.7-0.9% ash, and 0.8% fat and1-1.4% carbohydrate. Servet *et al* (2011)[15] studied the moisture, fat, protein and ash contents of tentacles were 80.72%, 1.44%, 16.16% and 1.63% while the same contents for mantle were 78.54%, 1.37%, 18.52% and 1.45% respectively in European squid. The chemical composition, size, and weight of cephalopods are dependent on growth stage, temperature, salinity, oxygen, light, food, competition, social interaction and sex [21,22]. The Bradford protein assay was developed by Marion M. Bradford[3]. It is a quick and precise spectroscopic analytical procedure used to quanify the concentration of protein in a solution. The reaction is based on the amino acid composition of the measured proteins. In this study the concentration of protein from *U.duvauceli* (Skin and Muscle) was calculated by using Bradford method. Mayra Lizett González-Félix *et al* (2014)[23], concentration of protein from jumbo squid (*Dosidicus gigas*) was calculated by using Bradford method. The protein contents from dried jellyfish and dried squid were determined using the Coomassie Plus (Bradford) protein assay kit (Thermo scientific, USA), with bovine serum albumin as a standard[24]. Sri Kumaran Nadarajah *et al*, (2017)[25], determined the total protein content of the whole squid ink by the method of Bradford. Van Gieson's stain is a mixture of picric acid and acid fuchsin. It is the simplest method to differentialte the collagen and other connective tissue by using staining. It was introduced to histology by American neuropsychiatrist and pathologist Ira Van Gieson[26]. In Histological studies of *Uroteuthis duvauceli*, the Van Gieson stain differentiated collagen fibers with other tissue components. The collagen fibers and muscle fibers were acquired with red and yellow colors, respectively and were observed using light microscopy. Fig 3 and 4. The collagen fibers and muscle fibers were acquired with red and yellow colors, respectively and were observed using light microscopy. Mizuta S *et al*, (2000)[27], studied the histochemical identification of a minor collagen in raw muscles of Decapod mollusks. In this study, Small pieces of the muscular tissue were dissected, fixed in Bouin's solution (Hoshino *et al*, 1998)[28] for 6 h, and embedded in paraffin (Parahisto, Nacalai Tesque, Japan). Four-micrometer sections were cut with a microtome. The prepared sections were stained with Van Gieson stain (Hoshino *et al*, 1998)[28] and observed with a light microscope. Muscle fibers and collagen fiber were stained yellow and red, respectively. Naveen Kumar *et al*, (2014)[29], used Verhoeff-Van Gieson staining to differentiate the collagen (pink colour) and elastic fibres (black colour) in horizontal (H) and vertical (V) sections of shoulder joint, wrist, ankle, forearm, and thigh areas. Their pattern of segmentation by tissue-quant software is shown in adjacent photographs. Ya'nan Yang *et al,* (2016)[30] studied the structure and type of collagen fiber in calipash were determined by van Gieson staining and Picrosirius red staining, which could contribute to the isolation of collagen from soft-shelled turtle Calipash (STCC). Muscle fibers and collagen fiber were stained yellow and red, respectively. Collagen bundles were observed from the skin of jellyfish *Rhopilema esculentum* under the light microscope by using histological analysis[33]. The yield of acid soluble collagens from the skin and muscle of squid *U.duvauceli* were extracted. The yield of extracted collagen was 0.33g/ml in muscle and 0.22g in skin. The total amount of collagen which was extracted from 100g of squid skin and 100g of muscle was 5.55g. Many studies have reported the yield of collagen from marine animals extracted by the aid of acetic acid as well as pepsin. Such increase in the solubility of collagen after pepsin treatment was also reported to increase by many authors[32,33,34]. Senaratne et al. (2006)[8] have reported that the yield of collagen was increased to 54.3% with the addition of 10% (w/v) pepsin in the brown backed toadfish skin. Pasiyappazham Ramasamy *et al*, (2017)[35]; reported that the collagen isolated from skin of squid *Sepioteuthis lessoniana* by acid-soluble and pepsin-soluble extraction. The results suggested that the yield of squid skin collagen can be effectively by extracting with 0.5 M acetic acid. The proximate composition of the extracted collagen from skin and muscle were analyzed and found to contain 5.7±0.1percent moisture, 92.5±0.1percent protein, 0.84±0.03percent fat and 0.7±0.08percent ash contents in skin and muscle contained 6.4±0.08 percent moisture, 90.2±0.1percent protein, 0.8±0.08percent fat and 0.85±0.04percent ash contents. Table 4 and 5. Gel filtration

chromatography is the easiest and mildest chromatography methods and isolates particles based on differences in size. Sephadex is used for rapid group separation such as desalting and buffer exchange and it is widely used in the marine organism purification field (Bougatef *et al*, 2010)[36]. Jai ganesh *et al*. (1975)[37]; and Vijaykrishnaraj *et al*. 2016[38]; used Sephadex G-25 to separate *Parastromateus Niger* viscera and mussel flavour, respectively. In this study also the lyophilized collagen from skin of *Uroteuthis duvauceli* were purified and fractionated by gel filtration chromatography with Sephadex G-100. The molecules separated fractions to range from 4kDa to 150kDa. Fig 5. Wu *et al.* (2014)[39]; was purified trypsin inhibitor from Yellowfin Tuna (*Thunnus Albacores*) , done by column chromatography with Sephacry S-200, Sephadex G-50, and DEAE-cellulose and found to have an apparent molecular weight of 7 × 104 Da. Mala Nurilmala *et al*, (2019)[40] reported that the collagen obtained from yellowfin tuna *Thunnus albacares* skin was further hydrolyzed with alcalase, and the peptides fractionated by gel filtration chromatography. The highest solubility for both skin and muscle type I collagen obtained from *Uroteuthis duvauceli* were found to be at pH 3 and pH 4 respectively. In type1 collagen from muscle, at above pH 3, there was a sharp decrease in pH and low solubility was observed at pH 7-10. In type1 collagen from skin, at above pH 4, there was a sharp decrease in pH and low solubility was observed at pH 8-10. Fig: 6. These results showed that the both skin and muscle type I collagen reached the pI in range (7-10), which results in protein precipitation and the net charge on protein becomes zero [41]. There was decrease in solubility observed for collagen type I (Muscle) and collagen type I(Skin) in 0.5M acetic acid with increase in NaCl concentration. There was a sharp decrease in solubility above 4% NaCl concentration in both Type I collagen from *Uroteuthis duvauceli* skin and muscle. Fig 7,8. This can be explained as; the decrease of collagen solubility at high NaCl concentration was thought to be mainly due to the phenomenon of salting out[41]. An ionic strength increase could enhance the hydrophobic-hydrophobic interactions of protein chains and increase the competition for water with the ionic salts, which led to protein precipitation[42]. Molecular structure of collagen consists of three polypeptide α-chains which are twisted together to form a triple helix. In the case of squid, the skin and muscle showed the typical SDS-PAGE pattern of type I collagen with two different α bands, α1 and α2 and also contains β and γ chains. The α2 unit was the minor component in muscle and skin and it seems that the collagen exists as trimers consisting of two α1 and one α2 chains Fig 9. Collagen from the skin of ocellate puffer fish[43], striped catfish[44], Nile perch[45], outer skin of *Sepia pharaonis*[46] have also been classified as type I collagen. They all consisted of two α chains (α1 and α2), β and γ components. Molecular structure of collagen consists of three polypeptide α-chains which are twisted together to form a triple helix. In this case of squid, the skin and muscle showed the typical SDS-PAGE pattern of type I collagen with two different α bands, α1 and α2 and also contains β and γ chains. The α2 unit was the minor component in muscle and skin and it seems that the collagen exists as trimers consisting of two α1 and one α2 chains. The γ chain had high molecular weight of approximately 220 kDa; while β and α chain had molecular weights below 200 kDa. The presence of βcomponent confirmed that the collagen contains more intermolecular cross-links. The γ component had the ability to renature the native collagen and their presence indicated that the three chains of collagen are intra-molecularly crosslinked[47]. Besides the cross-linked chains, skin and bone collagens consisted two distinct α bands corresponding to α1 and α2 components, which is typical of Type I collagen. Electrophoretic band pattern of bone collagen was similar to that of skin collagen. Muyonga *et al*. (2004)[45] was characterized the Type I skin collagen of Nile perch which consisted of two α1 and one α2 chains. The molecular weights of α subunits were 120 KDa for α1 and 115 KDa for α2. Skin and bone collagens separated from the marine fish, for example, dark drum and sheep shead also had similar electrophoretic patterns with molecular weights of α1 and α2 fractions as 130 and 110 kDa, respectively[48]. Collagen extracted from skin of Baltic cod also had α chains with molecular weight below 116 kDa (Skierka and Sadowska 2007)[34]. It was apparent from the molecular weights obtained for α1 and α2 subunits that the skin and bone collagens of leather jacket were typical of Type I collagen. *Presence* of another type of collagen was also documented by Kimura *et al*.[49] from the scale and bone of carp which consisted of (α1)2α2 as a main component and α1α2α3 as a minor component. The electrophoretic pattern of skin and bone collagen of leather jacket also showed a slight nearness of α3 subunit, as a minor component. As the α3 subunit relocates as similar as the way of migration of α1.

4. CONCLUSION

Uroteuthis duvauceli (Indian Ocean Squid) was used to extract type I collagen in this study. In histological studies the Van Gieson stain differentiated collagen fibers with other tissue components. The collagen fibers and muscle fibers were acquired with red and yellow colors, respectively and were observed using light microscopy. The collagen could be extracted from skin and muscle of squid by acetic acid digestion method and the lyophilized collagen was purified and fractionated by gel filtration chromatography with Sephadex G-100. The collagen consists of α1 and α2 were a typical pattern of Type I collagen, characterized by SDS PAGE. The hydroxyproline content of collagen from skin and muscle of squid was determined by used hydroxyproline assay. The collagenous appearance of collagen from skin and muscle of squid were observed by used light microscopy. Highest solubility of type1 collagen from *Uroteuthis duvauceli* skin and muscle was found at pH 3 and pH 4 respectively as well as type I collagen from *Uroteuthis duvauceli* skin and muscle showed highest solubility at 2% Nacl concentration.

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6. AUTHORS CONTRIBUTION STATEMENT

Ms. Jency George performed the experiments, conceptualized, gathered and analyzed the data. Dr. W.A Manjusha has given necessary inputs in designing the manuscript. Both the authors discussed the methodology and results and contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflicts of interest declared none.

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