ijlpr 2020; doi 10.22376/ijpbs/lpr.2020.10.4.L121-133

International Journal of Life science and Pharma Research

Research Article

Biotechnology for Prospective Medical Science

Characterization of Purified Collagen from Marine Squid *Uroteuthis Duvauceli*

Jency George* and Dr. Manjusha W. A.

**Research Scholar, Register no: 18113082022004, Interdisciplinary Research Centre, Department of Biotechnology, Malankara Catholic College, Manonmaniam Sundaranar University, Abishekapatti, Tirunelveli - 627 012, Tamil Nadu, India.*

Head and Assistant Professor of Biotechnology Department, Malankara Catholic College, Manonmaniam Sundaranar University, Abishekapatti, Tirunelveli - 627 012, Tamil Nadu, India.

Abstract: Collagen is the essential and most abundant structural protein found in the animal kingdom, involving almost 25-30 % of the entire body protein content. It is normally found in fibrous tissues, for example, skin, tendons, ligaments, skin, corneas, ligament, bones, veins, teeth, and so on. The skin of the squid is rich in collagen and is an excellent source of raw materials that can be used for the development of collagen products. As such, the use of *Uroteuthis duvauceli* skin is an alternative source of collagen may be an effective way to obtain high-value-added products. In this study, - the isolated Type I collagen was found in *Uroteuthis duvauceli* (skin and muscle). Additionally, the characterization of the Type I collagen from *Uroteuthis duvauceli* (skin and muscle) was examined through various techniques such as SEM, FTIR, XRD, TGA. 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 fibrillar structures of collagen samples were observed by SEM. FTIR investigation showed the existence of helical arrangements of the type I collagen from skin and muscle. UV spectrum of Type1 collagen from *Uroteuthis duvauceli* skin and muscle has a maximum absorption peak near 230 nm and a minor absorption peak near 280 nm. In XRD, type1 collagen from *Uroteuthis duvauceli* skin and muscle one wide peak was obtained following with characteristic diffraction peaks of collagen. The results showed that type I collagen from skin and muscle had slight differences in molecular weight, amino acid composition, morphological structures, and thermal stability. 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 biomedical field.

Keywords: Collagen, *Uroteuthis duvauceli*, Scanning Electron Microscope, Fourier-transform infrared spectroscopy, X-ray powder diffraction

***Corresponding Author**

Jency George , Research Scholar, Interdisciplinary Research Centre, Department of Biotechnology, Malankara Catholic College, MS University, Tirunelveli, India.Pincode: 629153

Recieved On 05 July 2020
Revised On 21 August 2020
Accepted On 27 August 2020
Published On 06 October 2020

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Jency George and Dr. Manjusha W. A. , Characterization of Purified Collagen From Marine Squid Uroteuthis Duvauceli.(2020).Int. J. Life Sci. Pharma Res.10(4), L121-133 http://dx.doi.org/10.22376/ijpbs/lpr.2020.10.4.L121-133

1. INTRODUCTION

Collagen is the essential and most abundant structural protein found in the animal kingdom, involving almost 25-30% of the entire body protein content[1]. It is normally found in fibrous tissues, for example, skin, tendons, ligaments, skin, corneas, ligament, bones, veins, teeth, and so on. It exists overwhelmingly in the form of a network like structure or as extracellular fibrils. Twenty-eight kinds of collagen have been recognized, each having a unique kind amino acid sequence, structure, and function. Among the collagen types, type I is the most encouraging as far as its attractive possibilities. The structural characteristics of collagen have permitted researchers to seclude it from crude materials with acetic acid, resulting in acid-soluble collagen (ASC). Pepsin is used to digest peptide chains in the telopeptide region of collagen molecules, and the obtained collagen is referred to as pepsin-soluble collagen (PSC)[2]. These two types of collagen have particular physicochemical properties. Due to collagen tensile strength and fibrous structure, collagen gives skin strength and elasticity, besides strengthening blood vessels and playing a major role in tissue development. Such capability of collagen has tremendous bearing on anti-aging treatment, cosmetic surgery, burn surgery, and even in weight management, leaving aside its industrial usages [3,4]. In general, the application of collagen can be classified into biomedical and non-biomedical sections. There is no uncertainty in the way that using collagen as biomaterial is effective. Moreover, collagen has provided relief from long courses of treatment in the clinical circle, where it has significantly reduced the time of healing as well as the severity of pain that is associated with traditional treatment procedures. For example, percutaneous collagen induction, which is an alternative to laser resurfacing, has been gaining popularity due to the advantages like preservation of the epidermis, thicker skin, a short healing phase and usage of only local anesthesia[5]. Altogether it is a safe, non-surgical procedure that softens lines and furrows on the face and acts as an agent of beautification even in older age, besides repairing the scars from the accident and untimely wrinkles. Despite the fact that the collagen has been proved to play an important role as biomaterial, there are disadvantages of its use too. Though it is currently used for cosmetic and burn surgery, people with a high rate of allergic reactions can suffer from a prolonged period of side effects. Also, there is three percent of the population who are sensitive to collagen. As well as, collagen cannot provide a permanent solution to aging, since it breaks down in the human body like any other protein. For instance, a bovine collagen injection to eliminate wrinkles can last from six weeks to a year, after which the patient will need another injection to regenerate its effect. There are several methods for deciding collagen and collagen types. The most widely recognized being founded on the quantitation of hydroxyproline, which represents around 10% of the collagen molecule. The amino acid composition can be recognized by High Performance Liquid Chromatography (HPLC). Determination of the molar proportion of specific collagen types includes separation of the peptide mixture created by enzymatic digestion, utilizing different partition techniques, for example, Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) or HPLC and their recognition by Mass Spectrometry (MS), Liquid chromatography/electrospray ionization-mass spectrometers (LC/ESI-MS) and High execution fluid chromatography coupled mass spectrometry (HPLC-MS/MS)[6] which empower the examination of marker peptides in peptide mixture delivered by cyanogen bromide/trypsin processing. Another scientific technique includes the radioactive naming of proline and protein immunoassay by collagen- specific. The Indian Ocean Squid (*Uroteuthis duvauceli*), is an Indo-West Pacific species of squid with a wide range through the Indian Ocean to Malaysia and the South China Sea, and is also present in the Red Sea and the Arabian Sea[7]. The skin of the squid is rich in collagen and is an excellent source of raw materials that can be used for the development of collagen products. As such, the use of *Uroteuthis duvauceli* skin as an alternative source of collagen may be an effective way to obtain high-value-added products. In this study, - the isolated Type I collagen found in *Uroteuthis duvauceli*. Additionally, the characterization of the Type I collagen was examined through the techniques such as SEM, FTIR, XRD, TGA.

2. MATERIALS AND METHODS

2.1 *Characterization Of Isolated Collagens*

2.1.1 *Determination of Hydroxyproline:*

Hydroxyproline content was determined by the technique for Neuman and Logan method[8]. Collagen samples were taken into a round bottom flask and 100 ml of 6 N HCl solutions was added and boiled at 100 °C for 16 h. The cooled hydrolysate was transferred to a volumetric flask and diluted with distilled water. Hydroxyproline standard solution was prepared by dissolving 100 mg standard of hydroxyproline in distilled water. 4ml of the final dilution was taken in the test tube. Then 2 ml of oxidant solution (Chloramines-T) was added and left to stand for 25 min to the mixture. After that 2 ml of color reagent (4-dimethylaminobenzaldehyde solution) was added and mixed. This mixture was placed in a water bath at 60 °C for 15 min. Hydroxyproline oxidized with chloramine-T to pyrrole and red-purple colour developed after addition of 4-dimethylaminobenzaldehyde, was measured spectrophotometrically at 560 nm (U1800, Shimadzu, Japan, UV–VIS Spectrophotometer), according to Kolar (1990)[9]. Hydroxyproline content in the sample was calculated from the standard curve.

2.1.2 *SDS-PAGE:*

Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970)[10] using 10 % separation gel and 1 % stacking gel. Collagen (10 mg) was dissolved in 1.0 ml of the sample buffer (Tris–HCl, pH 6.8 containing 2-mercaptoethanol, sucrose, bromophenol blue, 5 % SDS) and heated at 50 °C for 10 min. Then, 20 µl was loaded in each well along with high molecular weight protein markers. Electrophoresis was carried out at 50mA initially and then at 100 mA. 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.

2.1.3 *Effect of pH on solubility:*

Four (4) ml of collagen solutions from skin and muscle (3 mg/ml) respectively were transferred to 15 ml centrifuge

tubes and either 6 N NaOH or 6 N HCl added to obtain the final pH, ranging from 1 to 10. The volume of the solution was made up to 10 ml with deionized water previously adjusted to the same pH as the collagen solution. The solutions were centrifuged at 20,000 g for 30 min and the protein content of the supernatant determined. Protein solubility was calculated using the following equation[11].

Solubility= (protein content of the supernatant)/ (total protein content of the sample)
Relative solubility= (solubility at given pH)/ (highest solubility in the range of pH)

2.1.4 *Effect of NaCl on solubility:*

Four (4 ml) collagen solutions from skin and muscle (3 mg/ml) respectively were mixed with 1 ml of NaCl in 0.5 M acetic acid to give final concentrations of 0 to 12%. The mixture was stirred for 30 min, followed by centrifugation at 20,000 g for 30 min. Protein content in the supernatant was measured and the relative solubility calculated using the following equation[11].

Solubility= (protein content of the supernatant)/ (total protein content of the sample)
Relative solubility= (solubility at given pH)/ (highest solubility in the range of pH)

2.1.5 *Thermogravimetric Analysis:*

The behavior of the samples with temperature was studied using TA Instrument (STA 6000, PerkinElmer) in a nitrogen atmosphere. TGA curves were taken in the range 25–900 °C at a heating rate of 5 °C /min [12].

2.1.6 *Scanning Electron Microscope:*

The microstructure of samples was analyzed with a JEOL electron microscope JSM7600F, using the technique of secondary electrons with a voltage of 5 kV. The size of the samples used in SEM was 1 cm × 1 cm × 0.5 cm, and no sample was covered with a conductive material; they were placed directly in the sample holder. EDS technique was also employed for determining the main compounds of different superficial zones [12].

2.1.7 *Fourier Transform Infrared (FTIR) Spectra:*

Infrared spectra from 400 to 4000 cm^{-1} were obtained with the KBr disc method using an infrared spectrophotometer (IRAffinity-1S, SHIMADZU). The number and location of the amide band were provided by Fourier self-deconvolution[13], which was conducted using a resolution enhancement factor of 2.1 and half-height bandwidth of 13.5 cm^{-1}[14].

2.1.8 *X-Ray Diffraction:*

The powders of collagens were tested by an X-ray diffractometer D8 Advance of Bruker AXS, with Cu K radiation (λ = 0.154 nm) and 2θ from 18^0 to 80^0, to obtain structural information on an atomic scale from both materials (Muscle and skin)[13].

2.1.9 UV Absorption Measurement:

The ultraviolet absorption spectra of extracted collagens were recorded individually by a UV spectrophotometer (UV 1800, Shimadzu, Japan). The collagen samples were prepared by dissolution in 0.5 M acetic acid solution with a sample/solution ratio of 1:1000 (w/v)[12].

3. STATISTICAL EVALUATION

Data are given as the mean ± standard deviation of at least three independent experiments. Statistical significance was evaluated through one-way analysis of variance (ANOVA). $P<0.05$ is represented a statistically significant difference. These calculations were performed with MINTAB Release 14 Minitab Inc. software.

4. RESULTS

4.1 *Characterization Of Isolated Collagens*

4.1.1 *Hydroxyproline content:*

The assessment of hydroxyproline and the collagen content in skin and muscle are shown in Table 1**.** All results were subjected to analysis of variance (ANOVA) and are given as mean ± S.D. from triplicate determinations. Hydroxyproline and collagen content were significantly higher in skin tissue ($P < 0.05$) than muscle (Table 1).

Table 1: Hydroxyproline content (g/100 g) and collagen content(% of total protein) from squid skin and muscle

Tissue	Hydroxyproline	Collagen
Skin	0.20 ± 0.060	17.8 ± 0.36
Muscle	0.14 ± 0.048	12.1 ± 0.10

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

4.1.2 *Effect of pH on collagen solubility:*

The effect of pH on the solubility of type1 collagen from muscle and type1 collagen from the skin was studied. The highest solubility of type1 collagen from *Uroteuthis duvauceli* skin and muscle was found at pH 3 and pH 4 respectively (Figure 1). In type1 collagen from muscle, at above pH 3, there was a sharp decrease in pH. Low solubility was observed at pH 7-10. In type1 collagen from skin, at above pH 4, there was a sharp decrease in pH. Low solubility was observed at pH 8-10. There was no major difference in solubility between type1 collagen from *Uroteuthis duvauceli* skin and muscle at various pH. The result suggested that both type1 collagen from *Uroteuthis duvauceli* skin and muscle showed higher solubility in acidic conditions than the alkaline condition (Figure 1).

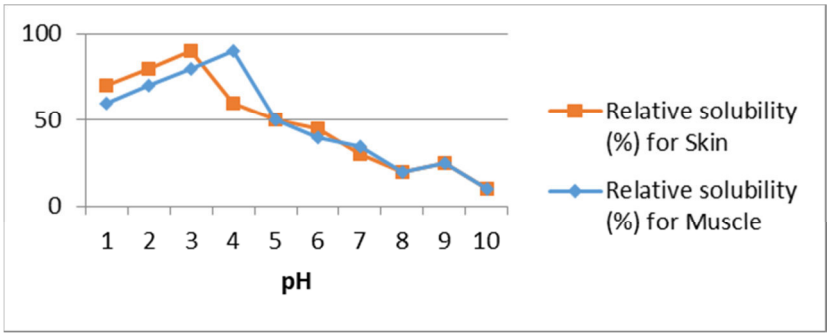


Fig 1: Effect of pH: Relative solubility of collagen type I from skin and muscle of *Uroteuthis duvauceli*

4.1.3 *Effect of NaCl on collagen solubility:*

Solubility of Type I collagen from *Uroteuthis duvauceli* skin and muscle at different NaCl concentrations are shown in Figures 2a and 2b, respectively. The highest solubility of type1 collagen from *Uroteuthis duvauceli* skin and muscle was found at 2 % NaCl concentration. There was decrease in solubility observed for collagen type I (Muscle) and collagen type I(Skin) in 0.5 M acetic acid with an 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. The decrease of collagen solubility at high NaCl concentration was thought to be mainly due to the phenomenon of salting out (Figure 2a and 2b).

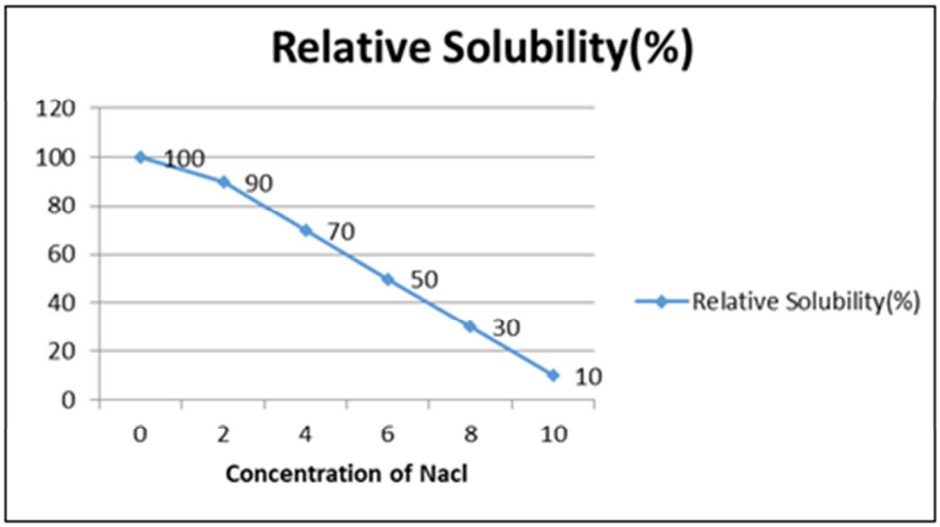


Fig 2a: Relative Solubility of Type I collagen from skin of *Uroteuthis duvauceli*

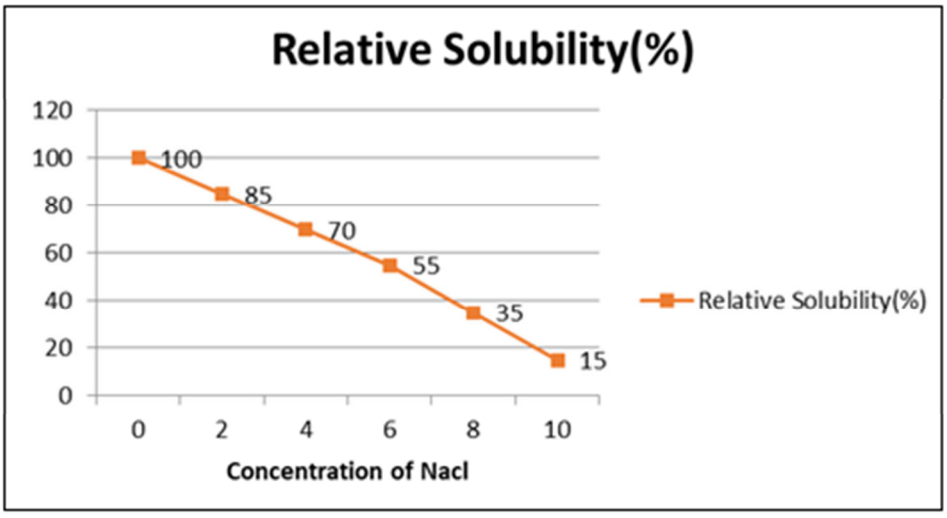


Fig 2b: Relative Solubility of Type I collagen from muscle of *Uroteuthis duvauceli*

4.1.4 *Molecular weight analysis by SDS-PAGE:*

SDS-PAGE band is usually applied to determine the type and constituents of collagen on the subunit composition, electrophoretic mobility and potency of the band. The presence of α1 and α2 chains in muscle and skin collagens were a typical pattern of Type I collagen (Figure 11). 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. There were no significant differences among the molecular weights of these two subunits of skin and muscle collagens. The molecular weights of skin and muscle subunits were about 120 KDa for α1 and 115 KDa for α2. Nevertheless, the band intensity of β and γ-chains from type I collagen in skin was higher than that of type I collagen in muscle. Therefore, it could be concluded that the intra and inter- molecular cross links of collagens were higher in type I collagen in skin than in type I collagen in muscle (Figure 3).

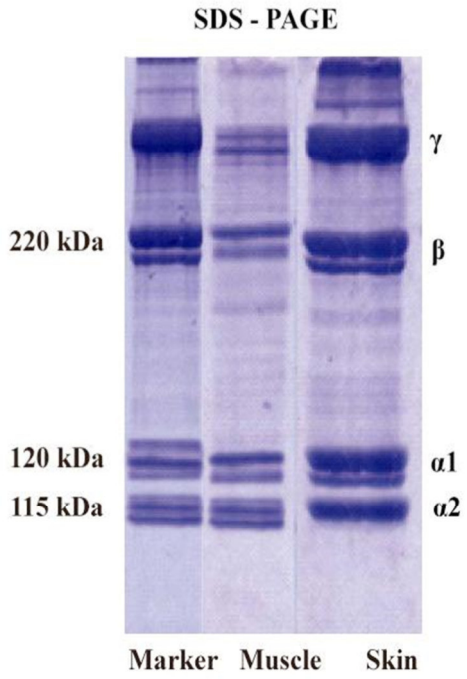
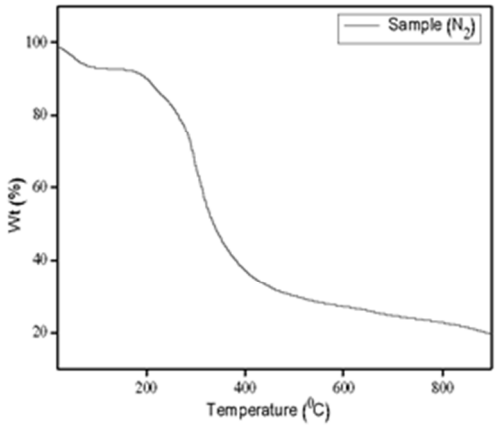


Fig 3: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of muscle and skin collagen from *Uroteuthis duvauceli* on 7.5% gel under reducing condition.

4.1.5 *Thermogravimetric Analysis:*

Thermal properties of the Type I collagen from skin and muscle were studied using thermogravimetry analysis (TGA). TGA curves indicate the weight loss evolution of collagen with increasing temperature were presented in Figure 4a and 4b. The collagen type I from *Uroteuthis duvauceli* skin and muscle degradation occurs in the range of 370-400 °C and 360-400 °C, respectively (Figure 4a and 4b).



TGA : N_2 atmosphere.
Temperature: r.t. to 900 ^{0}C
Scan Rate : 5 ^{0}C/min

Fig 4a: The Collagen type I from *Uroteuthis duvauceli* (skin) degradation occurs in the range o370-400 °C

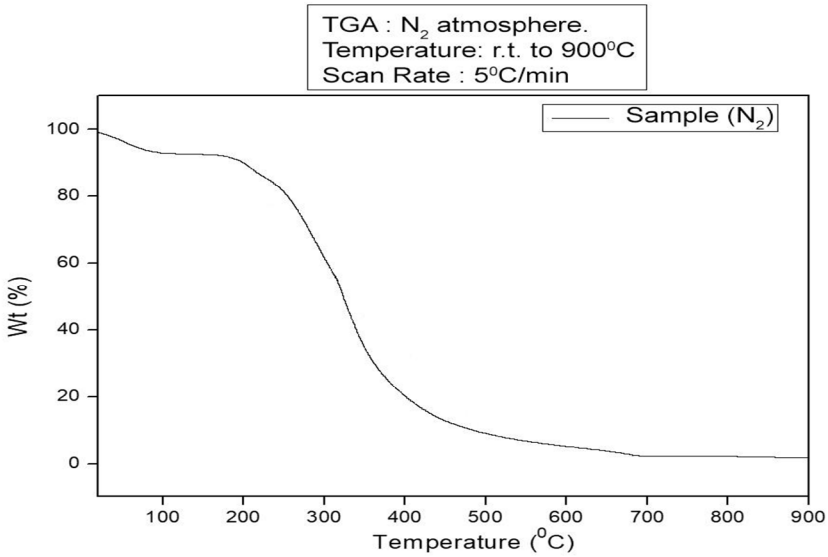


Fig 4b: The collagen type I from *Uroteuthis duvauceli* (muscle) degradation occurs in the range of 360-400 °C

4.1.6 FTIR-Type I collagen from the Muscle of Uroteuthis duvauceli

FTIR spectra of Type I collagen from the skin and muscle of squid are depicted in Figure 5a and 5b: Table 2. In FTIR spectra the amide A bands were observed at a wavenumber of 3402 cm^{-1} and 3309 cm^{-1} in type I collagen from skin and muscle respectively. A free N-H stretching coupled with hydrogen bond occurs in this range of 3400–3440 cm^{-1}. This result indicated that the N-H groups of this collagen were involved in hydrogen bonding. The amide B band positions of Type I collagen from the skin and muscle were found at wavenumbers of 2926 and 2927 cm^{-1}, respectively, representing the CH_2- asymmetric stretching. The amide I band was observed at wave numbers of 1648 to 1653 cm^{-1}, mainly coupled with the stretching vibrations of the carbonyl group. The amide II band of Type I collagen from the skin and muscle was situated at a wavenumber of 1538 and 1541 cm^{-1}, respectively, while the amide III band of Type I collagen from the muscle and skin was located at wavenumbers of 1236 and 1238 cm^{-1}, respectively. The amide II and amide III bands represent N-H bending vibrations coupled with C-N stretching vibration and C-H stretching, respectively. The absorption 1236 and 1238 cm^{-1} bands (amide III) of Type I collagen from the skin and muscle demonstrated the presence of a helical structure. In addition, the absorption peaks around 1434–1440 cm^{-1} were also observed in Type I collagen from the skin and muscle. This corresponded well to the CH_2 bending vibration (Figure 5a ad 5b) (Table 2).

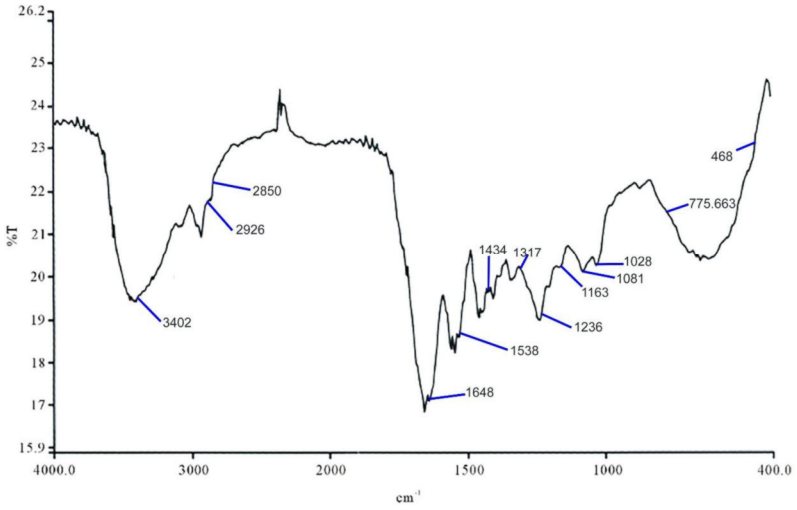


Fig 5a: Fourier transform infrared spectroscopy (FTIR) spectra of Type I collagen from the skin of squid Uroteuthis duvauceli

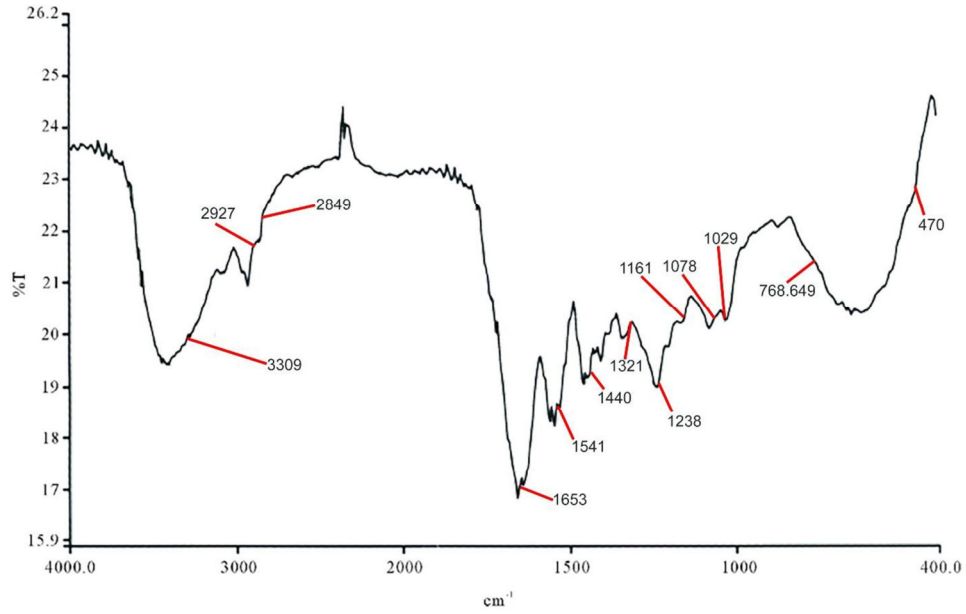


Fig 5b: Fourier transform infrared spectroscopy (FTIR) spectra of Type I collagen from the muscle of squid *Uroteuthis duvauceli*

Table 2: General peak assignment of the FTIR spectra consist of type I collagen from ski and muscle of *Uroteuthis duvauceli*.

Peak Wavenumber (cm^{-1})			Peak Assignments
Type I Collagen	Skin	Muscle	
3420	3402	3309	Amide A: mainly N-H stretching coupled with hydrogen bond
2928	2926	2927	Amide B: CH_2-asymmetric stretching
2853	2850	2849	CH_3-asymmetric stretching mainly protein
1646	1648	1653	Amide I: C=O stretching/ hydrogen bond coupled with COO^-
1536	1538	1541	Amide II: N-H bond coupled with C-N stretching
1436	1434	1440	CH_2 bending vibration
1319	1317	1321	CH_2 wagging of proline
1236	1236	1238	Amide III: N-H Bend coupled with C-N stretching
1160	1163	1161	COO-C asymmetric stretching
1079	1081	1078	PO2-symmetric stretching
1032	1028	1029	C-O stretching/C-O band
779,667	775, 663	768, 649	Skeletal stretching
473	468	470	Out of plane band

4.1.7 *Scanning Electron microscopy:*

Scanning electron microscopy (SEM) images of Type I collagen from the squid *Uroteuthis duvauceli* (skin and muscle) were shown in Figures 6a and 6b, respectively. Type I collagen in skin, the fibrillar structures of collagen were observed. The irregular, wavy collagen fibers were found to be arranged in single. The porous structure of collagen was clearly visible and the collagen surface was viewed as rough and uneven. Whilst, Type I collagen from muscle was found to have a composite mesh work form and contact with some fibrils. Such collagen fibrils formed bundles which diversified in width, thickness and twisted with each other. The figure showed a porous matrix with good interconnectivity in Type I collagen from skin and muscle (Figure 6a and 6b).

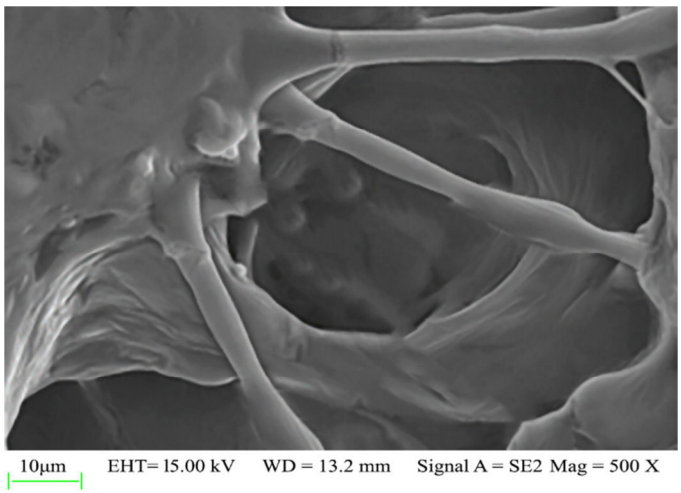


Fig 6a: Scanning electron microscopy images of Type I Collagen from the skin of *Uroteuthis duvauceli*

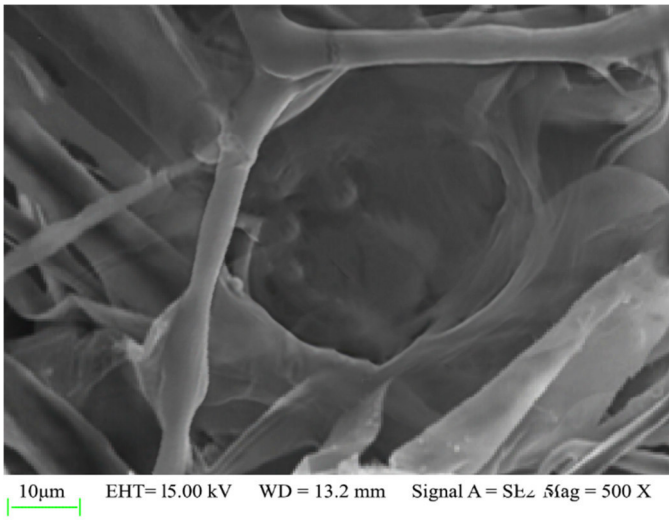


Fig 6b: Scanning electron microscopy images of Type I collagen from muscle of *Uroteuthis duvauceli*

4.1.8 *X-Ray Diffraction Analysis*:

The X-ray diffraction spectra of type1 collagen from *Uroteuthis duvauceli* skin and muscle are shown in Figures 7a and 7b, respectively and their peak values are presented in Table 3. In type1 collagen from *Uroteuthis duvauceli* skin and muscle one sharp peak was obtained which was found to be in accordance with characteristic diffraction peak of collagen. From Bragg's equation, d (A°) = λ/2sinθ, (λ= 1.54 A°), the minimum values (d) of the repeat spacing was calculated. 'd' is the interplanar distance. The d of the wide peak type1 collagen from *Uroteuthis duvauceli* skin was 4.8 Å and that of the type1 collagen from *Uroteuthis duvauceli* muscle was 4.16 Å (Table 3), (Figure 7a and 7b).

Table 3: Peak assignments type1 collagen from *Uroteuthis duvauceli* skin and muscle

Samples	Diffraction angle (2θ)	d (A°)
Type I collagen from *Uroteuthis duvauceli* skin	18.7	4.8
Type I collagen from *Uroteuthis duvauceli* muscle	21.6	4.16

d(Å) = λ/2sinθ (λ = 1.54 Å)

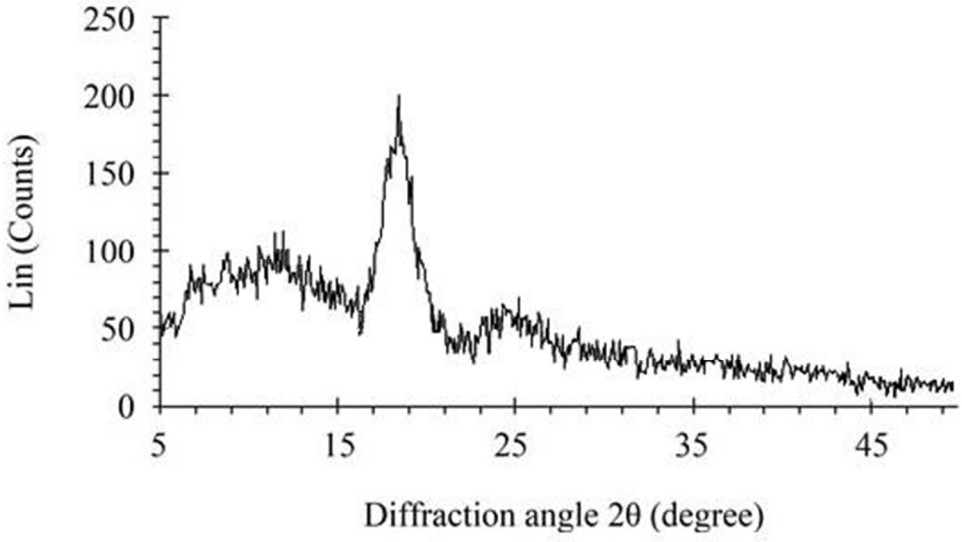


Fig 7a: X-Ray Diffraction Analysis of Type I collagen from the skin of *Uroteuthis duvauceli*

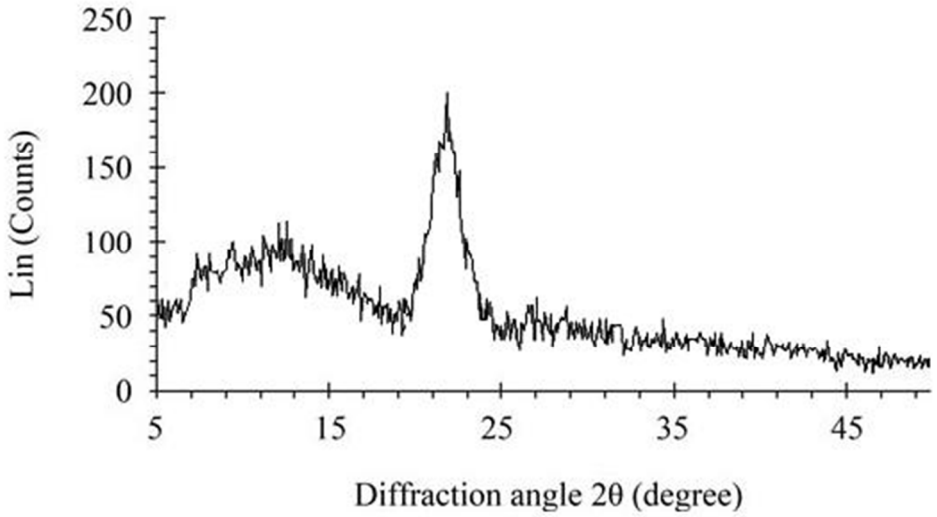


Fig 7b: X-Ray Diffraction Analysis of Type I collagen from the muscle of *Uroteuthis duvauceli*

4.1.9 *UV absorption spectrum:*

The UV absorption spectrums of type1 collagen from *Uroteuthis duvauceli* skin and muscle at the wavelengths between 200–400 nm were presented in Figure 8a and 8b: Table 4 and 5. The maximum absorptions of type 1 collagen from *Uroteuthis duvauceli* skin and muscle, were determined at the wavelengths of 220 - 240 nm region. Type1 collagen from *Uroteuthis duvauceli* skin and muscle has a maximum absorption peak near 230 nm and a minor absorption peak near 280 nm. The triple helical collagen has a maximum absorbance peak near 230 nm (Table 4,5), (Figure 8a ad 8b).

Table 4: UV absorption spectrum of Type1 collagen from *Uroteuthis duvauceli* skin

No.	Wavelength nm.	Absorbance	Description
1	227	0.8	Associated with the groups C=O, –COOH, $CONH_2$ in polypeptide chains of Type I collagen
2	274	0.3	Presence of aromatic (tyrosine and phenylalanine) and tryptophan amino acids

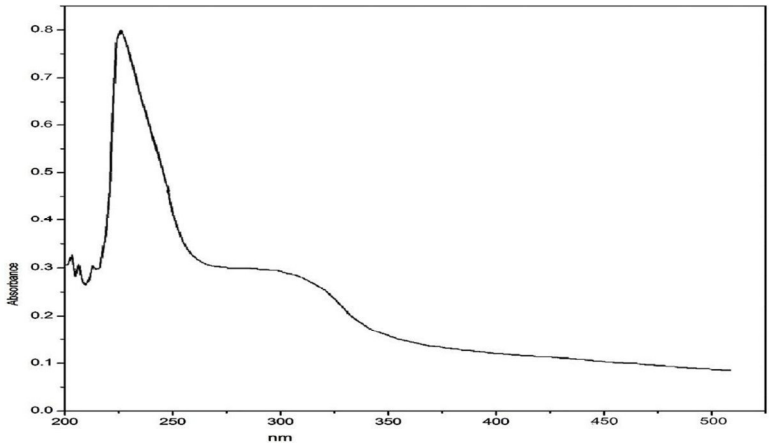


Fig 8a: UV absorption spectrum of Type1 collagen from the skin of *Uroteuthis duvauceli*

Table 5: UV absorption spectrum of Type1 collagen from the skin of *Uroteuthis duvauceli*

No.	Wavelength nm.	Absorbance	Description
1	220	0.8	Associated with the groups C=O, –COOH, $CONH_2$ in polypeptide chains of Type I collagen
2	270	0.3	Presence of aromatic (tyrosine and phenylalanine) and tryptophan amino acids

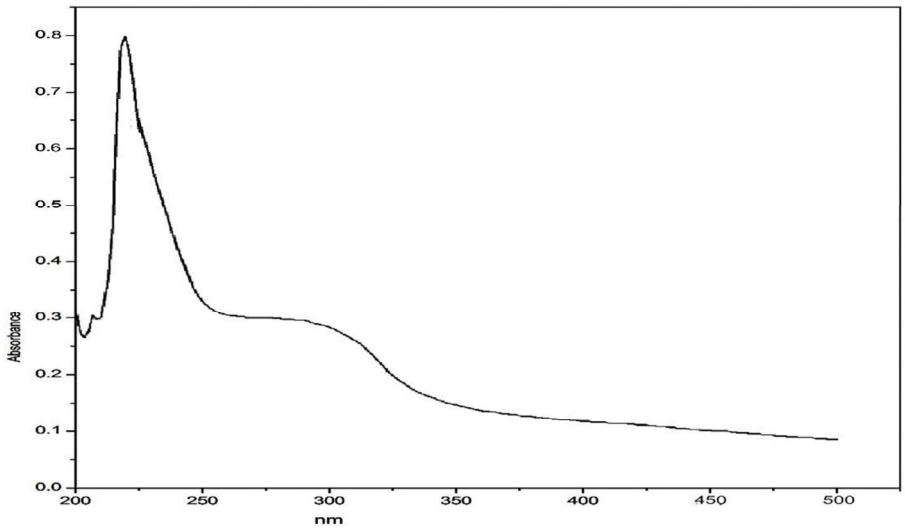


Fig 8b: UV absorption spectrum of Type1 collagen from the muscle of *Uroteuthis duvauceli*

5. DISCUSSION

Squid contains an abundant source of protein in its skin and muscle. The chemical composition and evaluation of collagen from skin and muscle (*Uroteuthis duvauceli*) is investigated in this current study. The extracted type I collagen from skin was found to contain 6.4±0.08percent moisture, 90.2±0.1percent protein, 0.8±0.08 percent fat and 0.85±0.04 percent ash contents and the results extracted type I collagen from muscle was found to contain 5.7±0.1percent moisture, 92.5±0.1percent protein, 0.84±0.03 percent fat and 0.7±0.08 percent ash contents. Suyama Kobayashi (1980)[13] analyzed eight species of squid and observed that moisture varied from 75–80 %, crude protein 16–21 % and ash from 1–2 % with 1 % crude fat. The chemical constituents of cephalopods are dependent upon the factors such as species, growth stage, habitat, season and the anatomical location of the cephalopod [14,15,16]. In this study, hydroxyproline content in collagen was significantly higher in skin tissue ($P < 0.05$) than muscle of *Uroteuthis duvauceli*. The strength of collagen is dependent upon the number of amino acid residues [17] and it plays an important role in stabilizing the triple helical structure of collagen[18]. Nagai *et al*. (2008)[19] reported that lower the imino acid content lower denaturation temperature than their hydroxylation. The molecular structure of collagen is conserved by the restrictions on the modifications of the secondary structure of the polypeptide chain, crosslinked by pyridine rings of proline and hydroxyproline. 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. These results showed that 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[20]. There was a decrease in solubility observed for collagen type I (Muscle) and collagen type I(Skin) in 0.5 M acetic acid with an 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. 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 [21]. An ionic strength increase has the ability can intensify the hydrophobic-hydrophobic interactions of protein chains and increase the competition for water with the ionic salts and to form protein precipitation[22]. 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 found that the collagen appeared as trimers having two α1 and one α2 chains. Collagen from the skin of ocellate puffer fish [23], striped catfish [24] , Nile perch [25 and] outer skin of *Sepia pharaonis* (Jayalekhshmi *et al*, 2017)[26] have also been classified as type I collagen. They all consisted of two α chains (α1 and α2), β and γ components. The γ chain had a high molecular weight of approximately 220 kDa, whereas the β and α chain had molecular weights below 200 kDa. In addition, the presence of β components affirmed that the collagen contains more intermolecular cross-links. The γ chain had the ability to renature the collagen and their presence represented the three chains of collagen are intramolecularly crosslinked[27]. Other than the cross-linked chains, the collagen contained two α bands- α1 and α2 segments, representing the typical of Type I collagen. Collagen isolated from the skin of Baltic cod had α chains with molecular weight below 116 kDa[28]. Muyonga *et al.* (2004) [29 -] isolated the Type I skin collagen from Nile perch and characterized the collagen as containing two α1 and one α2 chains. In addition, observed the molecular weights of α subunits of α1 and α2 were 115 KDa and 120 KDa respectively. Ogawa *et al.* 2004[30], isolated the skin and bone collagen from the marine fish, dark drum and sheepshead . Whereas found out both had resemblance in electrophoretic band with molecular weights of α1 fractions as 130 kDa and α2 fractions as 110 kD. This could be concluded that the molecular weights for α1 and α2 subunits that the skin and muscle collagens of *Uroteuthis duvauceli* were typical of Type I collagen. Determination of the thermal stability of collagen by using TGA, is an important characteristic to deal with as it will influence the temperature that can be used in collagen processing into structures and its biomedical applications. In this work, the collagen type I from *Uroteuthis duvauceli* skin and muscle degradation occurs in the range of 370-400 °C and 360-390 °C, respectively. The curve lies in the 370 °C to 390 °C range, corresponding to collagen degradation, as identified by Horn *et al* (2009) [31] Su Jin Yang *et al* (2012) [32]. extracted the collagens from squid (Todarodes pacificus) skin and Alaska pollack (Theragra chalcogramma) skin and found out the decomposition rate of temperature for the collagen was in the range of 300 °C and showed adequate for their thermal stabilities.

FTIR is used to identify the different types of collagen and is also used to compare the collagen composition [33]. In this case of *Uroteuthis duvauceli*, FTIR spectra the amide A bands were observed at wavenumber of 3402 cm-1 and 3309 cm^{-1} in type I collagen from muscle and skin respectively. The amide A band is generally associated with N-H stretching vibration and it occurs in the wavenumber range 3,400-3,440 cm-1. The amide B band positions of Type I collagen from the muscle and skin of *Uroteuthis duvauceli* were found at wavenumbers of 2926 and 2927 cm^{-1}, respectively, amide B band represents the asymmetrical stretch of CH2 [34]. The amide I band of U*roteuthis duvauceli* (skin and muscle) were f at wavenumbers of 1648 cm^{-1} to 1653 cm-1. Payne and Veis, 1988 [35] reported that the amide I band showed the frequencies in the range from 1600-1700 cm^{-1}, which is mainly coupled with stretching vibrations of the carbonyl groups (C=O bond) coupled with polypeptide backbone and is an important factor in determining the peptide secondary structure[36]. The amide II band of Type I collagen from the muscle and skin was situated at a wavenumber of 1538 and 1541 cm-1, respectively, while the amide III band of Type I collagen from the muscle and skin was located at wavenumbers of 1236 and 1238 cm-1, respectively, associated with N-H bending vibration coupled with CN stretching vibration[37]. The absorption 1236 and 1238 cm−1 bands (amide III) of Type I collagen from the muscle and skin of *Uroteuthis duvauceli* demonstrated the presence of a helical structure. The fibrillar structures of collagen samples were observed by SEM[38]. *Uroteuthis duvauceli* muscle, the fibrillar structure of type I collagen was observed. The irregular, wavy collagen fibers were found to be arranged in single. The normal porous structure of collagen was distinctly visible and the collagen surface was found to be irregular and unequal. Whilst, Type I collagen from skin was determined to have a complicated meshwork form and interacted with some fibrils. Such collagen fibrils are formed as bundles as well as they vary in width, thickness and also cross-linked with each other. In addition, both showed a porous matrix with good interconnectivity. Vairamani Shanmugam[39] *et al*, 2012, that the SEM ultrastructure of the resultant fibrils was similar with those of collagens from the outer skin of *Sepiella inermis*. In XRD, type1 collagen from *Uroteuthis duvauceli* skin and muscle one wide peak was obtained which was found to be in accordance with characteristic diffraction peak of collagen. The d of the wide peak type1 collagen from *Uroteuthis duvauceli* skin was 4.8 Å and that of the type1 collagen from *Uroteuthis duvauceli* muscle was 4.16 Å. which were related to the single left-hand helix chain. The collagen extracted had a tri-helix structure [40]. UV spectrum of Type1 collagen from *Uroteuthis duvauceli* skin and muscle has a maximum absorption peak near 230 nm and a minor absorption peak near 280 nm. Kittiphattanabawon[41] *et al*, 2015 reported that the collagen from the skin of Splendid squid (*Loligo formosana*) has a UV absorption peak at 232 nm. The maximum absorbance at 220–240 nm is related with the groups C=O, –COOH, $CO\text{-}NH_2$ along with polypeptide chains of collagen[42]. The maximum absorption wavelength of protein in the near ultraviolet region is 280nm because of the absorbance (280 nm) of aromatic amino acids such as Phe, Trp and Tyr[43]. The numbers of aromatic (tyrosine and phenylalanine) and tryptophan amino acids contribute to the ultraviolet absorption at 280 nm[43].

5. CONCLUSION

In summary, Type I collagens were successfully isolated from the skin and muscle of *Uroteuthis duvauceli*. 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. 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 fibrillar structures of collagen samples were observed by SEM. FTIR investigations showed the existence of helical arrangements of the type I collagen from skin and muscle. UV spectrum of Type1 collagen from *Uroteuthis duvauceli* skin and muscle has a maximum absorption peak near 230 nm and a minor absorption peak near 280 nm. The results showed that type I collagen from skin and muscle had slight differences in molecular weights, amino acid composition, morphological structures, and thermal stability. From this study, these results could provide a valuable scientific basis for the study of the texture and development of squid. The results suggested that the marine squid collagen is an alternative source of collagen for further application in food, pharmaceutical industries, cosmetic, suitable biomaterial, and other applications.

9. REFERENCES

1. Di Lullo GA, Sweeney SM, Körkkö J, Ala-Kokko L, San Antonio JD. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem. 2002;277(6):4223-31. doi: 10.1074/jbc.M110709200, PMID 11704682.
2. Nalinanon S, Benjakul S, Kishimura H. Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (Thunnus alalunga). Food Chem. 2010;121(1):49-55. doi: 10.1016/j.foodchem.2009.11.089.
3. Moreno HM, Montero MP, Gómez-Guillén MC, Fernández-Martín F, Mørkøre T, Borderías J. Collagen characteristics of farmed *Atlantic salmon* with firm and soft fillet texture. Food Chem. 2012;134(2):678-85. doi: 10.1016/j.foodchem.2012.02.160, PMID 23107678.
4. Buehler MJ. Nature designs tough collagen: explaining the nanostructure of collagen fibrils. Proc Natl Acad Sci U S A. 2006: ? 103 (33) ,12285-12290:12285-90. doi: 10.1073/pnas.0603216103, PMID 16895989.
5. Fratzl. Collagen: structure and mechanics. In: Fratzl P, editor. Available from: https://www.springer.com/gp/book/9780387739052, S pringer, new-York, NY (2008); 2008.
6. Fernandes D. Percutaneous collagen induction: an alternative to laser resurfacing. Aesthet Surg J. 2002 May;22(3):307-9. doi: 10.1067/maj.2002.126195, PMID 19331986.
7. Pataridis S, Eckhardt A, Mikulíková K, Sedláková P, Mikšík I. Identification of collagen types in tissues using HPLC-MS/MS. J Sep Sci. 2008 October;31(20):3483-8. doi: 10.1002/jssc.200800351, PMID 18837476.
8. Neuman RE, Logan MA. The determination of hydroxyproline. J Biol Chem. 1950 May;184(1):299-306. PMID 15421999.
9. Kolar K. Colorimetric determination of hydroxyproline as measure of collagen content in meat and meat products: NMKL collaborative study. J Assoc Off Anal Chem. 1990;73(1):54-7. doi: 10.1093/jaoac/73.1.54, PMID 2312514.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 May 7;227(5259):680-5. doi: 10.1038/227680a0, PMID 5432063.
11. Wichuda J, Sunthorn C, Busarakum P. Comparison of the properties of collagen extracted from dried jellyfish and dried squid. Afr J Biotechnol. 2016 April;15(16):642-8. doi: 10.5897/AJB2016.15210.
12. Wang T, Li Q. G-f, Z., Zhou, G., Yu, X., Zhang, J. Comparative evaluation of a biomimetic collagen/hydroxyapatite/b-tricalcium phosphate scaffold in alveolar ridge preservation with Bio-Oss Collagen. Front. Mater. Sci. 2016, 10: 122–133. doi:10.1007/s11706-016-0333-0.
13. Suyama M, Kobayashi H. Free amino acids and quaternary ammonium bases in the mantle muscle of squids [Decapoda]. Bull Jpn Soc Sci Fish. 1980;46:1261-1264(10).
14. Kreuzer R. Cephalopods: handling, processing and products. FAO Fish Tech Pap. 1984;254:108.
15. Sinanoglou VJ, Miniadis-Meimaroglou S. Fatty acid of neutral and polar lipids of (edible) Mediterranean cephalopods. Food Res Int. 1998;31(6-7):467-73. doi: 10.1016/S0963-9969(99)00014-9.
16. Ozogul Y, Duysak O, Ozogul F, Özkütük AS, Türeli C. Seasonal effects in the nutritional quality of the body structural tissue of cephalopods. Food Chem. 2008;108(3):847-52. doi: 10.1016/j.foodchem.2007.11.048, PMID 26065744.
17. Ikoma T, Kobayashi H, Tanaka J, Walsh D, Mann S. Physical properties of type I collagen extracted from fish scales of Pagrus major and Oreochromis niloticas. Int J Biol Macromol. 2003;32(3-5):199-204. doi: 10.1016/S0141-8130(03)00054-0.
18. Ramachandran GN. Stereochemistry of collagen. Int J Pept Protein Res. 1988;31(1):1-16. doi: 10.1111/j.1399-3011.1988.tb00001.x, PMID 3284833.
19. Nagai T, Suzuki N, Nagashima T. Collagen from common minke whale (Balaenoptera acutorostrata) unesu. Food Chem. 2008;111(2):296-301.

6. ACKNOWLEDGEMENT

We would like to extend our thanks and gratitude to the Malankara Catholic College, Department of Biotechnology, Manonmaniam Sundaranar University for providing all the research requirements and harnessing all laboratory capabilities to support scientific research.

7. 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.

8. CONFLICT OF INTEREST

Conflicts of interest declared none.

doi: 10.1016/j.foodchem.2008.03.087, PMID 26047426.
20. Hebert EJ, Grimsley GR, Hartley RW, Horn G, Schell D, Garcia S, Both V, Sevcik J, Pace CN. Purification of ribonucleases Sa, Sa2, and Sa3 after expression in Escherichia coli. Protein Expr Purif. 1997;11(2):162-8. doi: 10.1006/prep.1997.0776, PMID 9367812.
21. Li ZR, Wang B, Chi CF, Zhang QH, Gong YD, Tang JJ, Luo HY, Ding GF. Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (Scomberomorous niphonius). Food Hydrocoll. 2013;31(1):103-13. doi: 10.1016/j.foodhyd.2012.10.001.
22. Wu QQ, Li T, Wang B, Ding GF. Preparation and characterization of acid and pepsin-soluble collagens from scales of croceine and redlip croakers. Food Sci Biotechnol. 2015;24(6):2003-10. doi: 10.1007/s10068-015-0264-z.
23. Nagai T, Araki Y, Suzuki N. Collagen of the skin of ocellate puffer fish (Takifugu rubripes). Food Chem. 2002 Aug 1;78(2):173-7. doi: 10.1016/S0308-8146(01)00396-X.
24. Singh P, Benjakul S, Maqsood S, Kishimura H. Isolation and characterisation of collagen extracted from the skin of striped catfish (Pangasianodon hypophthalmus). Food Chem. 2011;124(1):97-105. doi: 10.1016/j.foodchem.2010.05.111.
25. Muyonga JH, Cole CGB, Duodu KG. Characterisation of acid soluble collagen from skins of young and adult Nile perch (Lates niloticus). Food Chem. 2004;85(1):81-9. doi: 10.1016/j.foodchem.2003.06.006.
26. Krishnamoorthi J, Ramasamy P, Shanmugam V, Shanmugam A. Isolation and partial characterization of collagen from outer skin of Sepia pharaonis (Ehrenberg, 1831) from Puducherry coast. Biochem Biophys Rep. 2017 February 27;10:39-45. doi: 10.1016/j.bbrep.2017.02.006, PMID 28955735.
27. Lewis MS, Piez KA. The characterization of collagen from the skin of the dogfish shark, Squalus acanthias. J Biol Chem. 1964;239(10):3336-40. PMID 14245383.
28. Skierka E, Sadowska M. The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (Gadus morhua). Food Chem. 2007;105(3):1302-6. doi: 10.1016/j.foodchem.2007.04.030.
29. Muyonga JH, Cole CGB, Duodu KG. Characterisation of acid soluble collagen from skins of young and adult Nile perch (Lates niloticus). Food Chem. 2004 March;85(1):81-9. doi: 10.1016/j.foodchem.2003.06.006.
30. Ogawa M, Moody MW, Portier RJ, Bell J, Schexnayder MA, Losso JN. Biochemical properties of black drum and Sheepshead sea bream skin collagen. J Agric Food Chem. 2003 December 31;51(27):8088-92. doi: 10.1021/jf034350r, PMID 14690401.
31. Horn MM, Martins VCA, de Guzzi Plepis AMG. Interaction of anionic collagen with chitosan: effect on thermal and morphological characteristics. Carbohydr Polym. 2009;77(2):239-43. doi: 10.1016/j.carbpol.2008.12.039.
32. Yang SJ, Hong J-H. Extraction and Physicochemical Properties of Collagen from Squid (Todarodes pacificus) skin and Alaska pollack (Theragra chalcogramma) skin. Korean journal of food and cookery science. December 2012;28(6):711-9. doi: 10.9724/kfcs.2012.28.6.711.
33. Belbachir K, Noreen R, Gouspillou G, Petibois C. Collagen types analysis and differentiation by FTIR spectroscopy. Anal Bioanal Chem. 2009;395(3):829-37. doi: 10.1007/s00216-009-3019-y, PMID 19685340.
34. Montero P, Jiménez-Colmenero F, Borderìas J. Effect of pH and the presence of NaCl on some hydration properties of collagenous material from trout (Salmo irideus Gibb) muscle and skin. J Sci Food Agric. 1991;54(1):137-46. doi: 10.1002/jsfa.2740540115.
35. Payne KJ, Veis A. Fourier transform ir spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for conformational studies. Biopolymers. 1988 November;27(11):1749-60. doi: 10.1002/bip.360271105, PMID 3233328.
36. Surewicz WK, Mantsch HH. New insight into protein secondary structure from resolution-enhanced infrared spectra. Biochim Biophys Acta. 1988 January;952(2):115-30. doi: 10.1016/0167-4838(88)90107-0, PMID 3276352.
37. Barth A, Zscherp C. What vibrations tell us about proteins. Q Rev Biophys. 2002 November;35(4):369-430. doi: 10.1017/S0033583502003815, PMID 12621861.
38. Raspanti M, Strocchi SG R, Ruggeri A. Different fibrillar architectures coexisting in Haversian bone. Ital J Anat Embryol = Archivio italiano di anatomia ed embriologia. 1995 February;100;Suppl 1:103-12.
39. Shanmugam, V, Ramasamy P, Subhapradha N. Extraction, structural and physical characterization of type I collagen from the outer skin of Sepiella inermis (Orbigny, 1848). Afr J Biotechnol. 2012 October;11(78):14326-37. doi: 10.5897/AJB12.444.
40. Zhang F, Wang A, Li Z, He S, Shao L. Preparation and characterisation of collagen from freshwater fish scales. Food Nutr Sci. 2011;02(8):818-23. doi: 10.4236/fns.2011.28112.
41. Kittiphattanabawon P, Nalinanon S, Benjakul S, Kishimura H. Characteristics of pepsin-solubilised collagen from the skin of splendid squid (loligo formosana). J Chem. December 2015;2015, 8 pages. doi: 10.1155/2015/482354.
42. Pal GK, Suresh PV. Comparative assessment of physico-chemical characteristics and fibril formation capacity of thermostable carp scales collagen. Mater Sci Eng C. 2017;70(1):32-40. doi: 10.1016/j.msec.2016.08.047, PMID 27770898.
43. Li ZR, Wang B, Chi CF, Zhang QH, Gong YD, Tang JJ, Luo HY, Ding GF. Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (Scomberomorous niphonius). Food Hydrocoll. 2013;31(1):103-13. doi: 10.1016/j.foodhyd.2012.10.001.