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Isolation and Characterization of Type V collagen from outer skin waste of *Loligo uyii*

Muthuvel ARUMUGAM* Thangappan AJITHKUMAR Thangavel BALASUBRAMANIAN
*Centre of Advanced Study in Marine Biology, Faculty of Marine Science, Annamalai University, Parangipettai, INDIA

*Corresponding Author
e-mail: aru_hep@yahoo.com

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Abstract

Type V like collagens is widely distributed in marine invertebrates, particularly crustaceans and molluscs. The present study the collagen was prepared from the outer skin of

L. uyii. The net yield of *L. uyii* acid soluble collagen estimated as 10.54% (ASC), subsequent digestion of residue with pepsin soluble collagen was yielded as 31.16% (PSC). SDS-PAGE analysis was revealed the chain composition of (α1)2α2 heterotrimer for ASC which similar to Japanese common squid as well as cuttle fish collagen. The ASC and PSC total protein was determined by Bradford method which contains 64.27µg/ml and 18.93µg/ml respectively. Fourier transforms infrared spectroscopy Analysis (FT-IR) of ASC and PSC is helpful in predication and confirmation of secondary structure of proteins. This report indicates that *L uyii* outer skin has potential to supplementing the skin of land vertebrates as a source of collagen.

Keywords: collagen; mollusk; FT-IR; SDS-PAGE; squid.

INTRODUCTION

Collagen, one of the major constituents of intramuscular connective tissue, has been shown to exist in different genetic forms. In fish species, collagen has been shown to play an important role in maintaining the structure of muscle in association with swimming movement and meat texture [1,2]

The collagen content of fish and seafood depends on the species, feeding regime, and seafood depends on the fish. In general, fish muscle contains 0.2-2.2% collagen in the case of Teleosts and upto10% in Elasmobranches [3]. Although higher collagen content contributes to the toughness of muscles, no such problems are encountered in fish connective tissue contributes little, if any, to the eating texture of cooked fish. However, some species of squid may develop tough, rubber texture upon heat processing [4].

Collagen is one of the long, fibrous structural proteins whose function is quite different from those of globular proteins such as enzymes. Tough bundles of collagen called collagen fibers are a major component of the extra-cellular matrix that support most tissues and gives cells structure from the outside, but collagen is also found inside certain cells. Collagen has great tensile strength, and is the main component of fascia, cartilage, ligament, tendons, bone and teeth. Along with soft keratin, it is responsible for skin strength and elasticity, and its degrading leads to wrinkles that accompany aging. It strengths blood vessels and play a role in tissue development. It is present in the cornea and lens of the eyes in crystalline forms. It is also used in cosmetics, surgery and burns surgery. When used cosmetically, there is a chance of allergic reactions causing prolonged redness; however, this can be virtually eliminated by simple and inconspicuous patch testing prior to cosmetic use.

Most medical collagen is derived from young beef cattle (Bovine) from certified BSE (Bovine spongiform encephalopathy) free animals due to their compatibility. Most manufacturers use donor animals from either "closed herds", or from countries which have never had a reported case of BSE such as Australia and New Zealand. Porcine (pig) tissue is also widely used for producing collagen sheet for a variety of surgical purposes. Due to the care in donor animal breeding and selection, as well as the technology used in the preparation of collagen from animal sources, the chance of immune reactions or disease transmission has been virtually eliminated. Alternatives using the patient's own fat, hyaluronic acid or polyacrylamide gel are readily available. Collagens are widely employed in the construction of artificial skin substitutes used in the management of severe burns. These collagens may be derived from bovine, equine or porcine, and even human, sources and are sometimes used in combination with silicones, glycosaminoglycans, fibroblasts, growth factors and other substances. Collagen is also sold commercially as a joint mobility supplement. This lacks supportive research as the proteins would

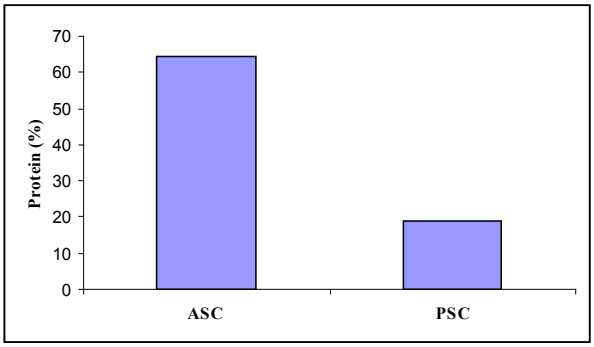

Fig 1. Shown the amount of protein in ASC and PSC of L. uyii.

just be broken down into its base amino acids during digestion, and could go to a variety of places besides the joints.

MATERIALS AND METHODS

Sample collection

L. uyii was collected from Cuddalore landing centre (Tamilnadu, East Coast of India) and the animals were kept in to the laboratory at -20ºC. The outer skins were removed, cut into small pieces and stored at -4ºC until used.

Preparation of collagen from the outer skin

The standard procedure was followed after suitable modification [5]. Briefly, outer skins of

L. uyii was extracted with 0.1M NaOH to remove non collagenous proteins for 3 days, then washed with distilled water and lyophilized. Lyophilized skins were treated with 0.5 M Acetic acid for 3 days and this extracts were centrifuged at 5000rpm for 1 hour. The residue was re extracted with the same solution for 2 day, and extract was centrifuged under the same conditions. Each solution was mixed and salted out by adding NaCl to a final concentration of 0.8M and followed by precipitation of collagen by the addition of NaCl (concentration of 2.3M) at a neutral pH 7.5. The resultant precipitates was obtained by centrifugation at 5000 rpm for 1 h and dissolved in 0.5M acetic acid dialyzed with 0.1M acetic acid and distilled water then lyophilized (ASC).

The residue from the acetic acid extractions was suspended in 0.5 M acetic acid and was digested with 10% (w/v) (Sigma) pepsin at 4ºC for 48 hours. The pepsin solubilized collagen was centrifuged at 5000rpm for 1 hour and the supernatants were dialyzed against 0.02M Na2HPo4 [pH 7.2] for 3 days with change of solution once per day. The precipitates obtained by centrifugation at 5000 rpm for 1 h. were dissolved in 0.5M acetic acid and salted out by adding NaCl to a final concentration of 0.8 M and followed by precipitation of collagen by further adding to final concentration of 2.3M NaCl in 0.05 M Tris-HCl [pH 7.5]. The resultant precipitate was obtained by centrifugation at 5000 rpm for 1 h and dissolved in 0.5M acetic acid; it was dialyzed against 0.1M acetic acid, distilled water and then lyophilized.

Estimation of collagen protein

The amount of Collagen protein was estimated by Bradford method with BSA used as standard [6].

SDS Polyacrylamide gel electrophoresis [SDS-PAGE]

SDS-PAGE was performed on gradient separating gel of 10% polyacrylamide using a 4% stacking gel according to previous method [7]. The collagen sample was mixed with 1.5M Tris HCl buffer (pH6.8) containing 10% SDS and 11.14% 2-mercaptoethanol, 40% glycerol and 0.02% bromo-phenol blue ,heated at100◦ c for 3min and electrophoresed at 50-100v in vertical slab gels. Samples of PSC, ASC, and standard were loaded on each gel. Gels were stained with 0.1%Coomassie Brilliant Blue R-250 in methanol / acetic acid / water 5:2:5 (v/v/v) and de-stained in 15% methanol / 7.5% acetic acid.

Fourier Transform- Infra Red Spectrum Analysis

FTIR absorption of ASC and PSC of L. uyii relied on at Bio red FT IR – 40 models, USA. Sample (10mg) was mixed with100mg of KBr, and compared to prepare as a salt disc (10mm) diameter for reading spectrum further by using KBr for pelleted forms of samples. FTIR spectrum of ASC and PSC was obtained and the effective peaked were assigned with that of standard collagen.

RESULTS

Isolation of collagen from L. uyii skin

The *L .uyii* outer skin was hardly solubilized with 0.5M acetic acid. The yield of ASC was very low than that of PSC and its value about 2% on the basis of lyophilized dry weight. On the other hand, by treating the residue with 10% (w/v) pepsin in 0.5M acetic acid, PSC was perfectly solubilized. PSC was precipitated with 0.8M Nacl in acid solution and followed by the addition of 2.3 M NaCl at neutral pH. PSC obtained was as a pinkish fiber, and it was supposed that a pigment, such as ommochrome, remained in this collagen sample. The yield of PSC was higher than that of ASC and it was obtained about 35% on the basis of lyophilized dry weight (Table 1).

Table 1. Shown the Yield of isolated collagen obtained from *Loligo uyii*

Source	Yield (%)		Net Yield (%)
	Acid soluble Collagen (ASC)%	Pepsin soluble Collagen (PSC)%	ASC+PSC
Loligo uyii	10.54%	31.16%	42%

SDS Polyacrylamide gel electrophoresis [SDS-PAGE]

When these collagens were examined by10% SDS-PAGE, PSC showed only a single α band and it seemed that this was α1. ASC was shown to comprise two α chains, α1 α2 (Fig. 2). Moreover, a great amount of β chains obtained in ASC .These collagens, were act as inter and intra-molecular cross linked components

Estimation of collagen protein

The amount of protein in ASC, PSC of L. uvii contains 64.27µg/ml,18.93µg/ml respectively and it represented in Fig.1.

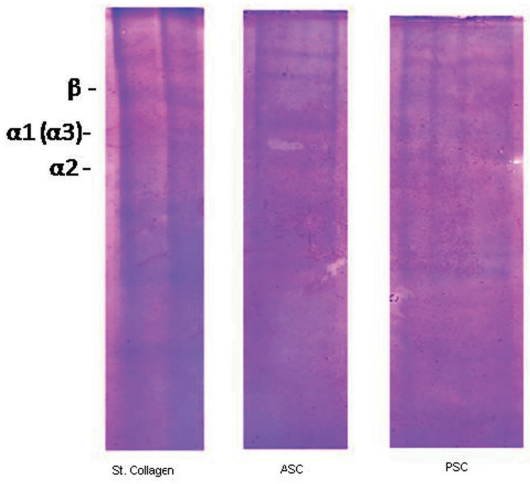

Fig.2. Protein pattern of ASC and PSC isolated from *Loligo uyii* (SDS-PAGE analysis) Lane 1: Standard Collagen; Lane 2: ASC; Lane 3: PSC

FT-IR (Fourier Transform – Infrared Spectrem)

The presence of secondary protein structure was assigned with standard collagen spectral range. From the FT-IR results, absorption of ASC and PSC corresponding to amide split at 1654 cm1and 1653 cm1 and in prominent absorption at 3441 cm1 and 3368 cm1 characteristic of collagens were identified. 1236 cm1 and 1238 cm1 range corresponds to the C-H stretching and N–H stretching (Fig.3). From the spectral data was helpful in predication and confirmed of secondary structure of proteins of isolated collagen from *L. uyii*.

DISCUSSION

The net yield of this collagen was estimated has 42% on the basis of the lyophilized dry weight value was higher than that of other sources like the jelly fish as (25.2 – 35.2 %) respectively [9-10]. Likewise net yield of the present collagen is also higher than that of Baltic cod *(Gadus morhua)* skin collagen (21.5%). Further, the net yield of *L. uyii* was higher than that of *Crassostrea gigas* which contains 11.1 percent [11].

The Collagen was extracted from *L. uyii* by limited pepsin and acetic acid digestion and it was found that comparatively large amount of collagen was obtained from *L. uyii* than that of *Sepia lycidas* (34.5%) [5]. This results indicates that the *L. uyii* outer skin was potential source of natural collagen and it also used as alternative source of collagen in at very lowest price. The higher amount of collagen present *L. uyii* may due to the presence of rich protein content of Cephalopod animals. This proposed type of V collagen was used as effective source of drug delivery [11].

Electrophoresis is a selective tool for the identification of different proteins. In the present study the results obtained from the PAGE of ASC and PSC are well defined and the bands migrated as like the standard collagen and respective protein bands.

α- chains were identified by SDS-PAGE analysis. From the electrophoretic pattern found that the *L.uyii* collagen had a chain composition of (α1)2α2 heteroprimer which was similar to other squid collagen [1,4,12]. ASC and PSC were shown to comprise two α chains such as α1 and α2. When compared with PSC a prominent β chain was obtained in ASC. The presence of collagen in *L.uyii* was poor in intra and inter- molecular cross linked components.

In vertebrates, type V collagen (particularly α1 chain) important for controlling fibril diameter in cartilage and cornea. Type V collagen are present in all animals having as an ancestor molecule largely unchanged during the course of phylogenitic evolution [13]. Type V collagen plays an important role in the maintenance of pregnancy and that decreased spontaneous abortion [14]. Type V

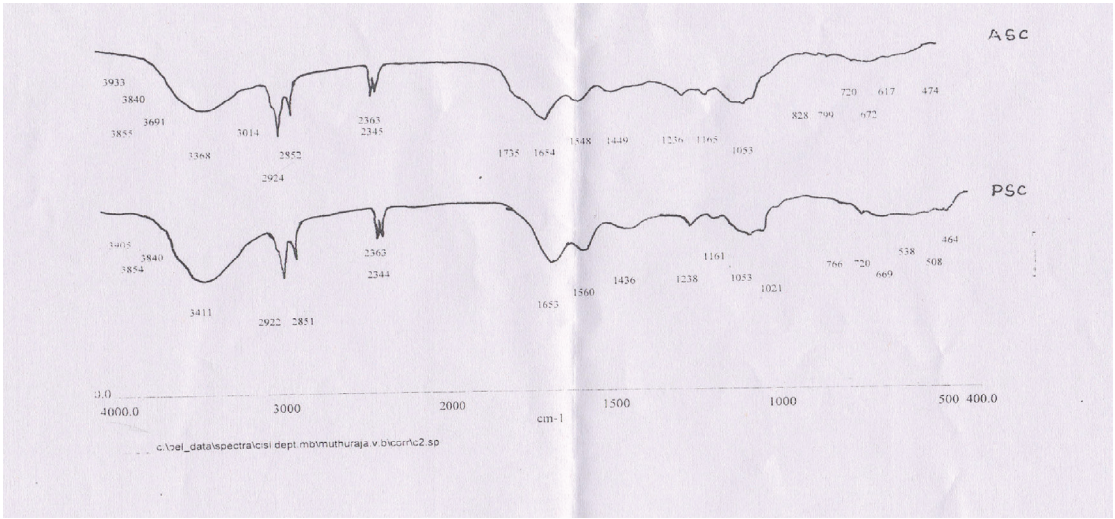

Fig.3. FT-IR spectra of ASC and PSC of *Loligo uyii*

collagen is able to promote apoptosis in cancer cells. The expression of other components required for the apoptotic activation, Type V collagen also induced apoptosis in 8701-BC breast cancer cells [15].

Further, the protein content of *L. uyii* was comparatively similar other mollscan species such as *Galloprovincialis* [10]. The isolated collagen of both ASC and PSC consist of type V collagen. Functionally, the type V collagen also controls the initiation of collagen fibril assembly [16]. SDS-PAGE pattern of PSC of *L. uyii* was similar to *Crassostrea gigas* α1 and α2 chains [15]. The protein profiles of *L. uyii* was much similar to other molluscs with consist of unique α1, α2 and β chains [1]. When compared to other mollusc *L. uyii* contains more amounts of β chains.

In the present study L. uyii contained very sharp peaks at 1653 cm1 and 1654 cm1 which responsible for amide split. This peak was descending down to 1236 cm1 and 1238 cm1 indicating the CH and NH stretching respectively. Further the sample shows the prominent absorption of characterized collagen was identified at 3441 cm1 and 3368 cm1. FT-IR showed that both ASC and PSC were type V with slight structural differences.

In the case of FT-IR of yellow fin tuna showed regions of amides A, I, II and III were 3427, 1651, 1544 and 1240 cm−1, respectively. Collagen solubility sharply decreased at over pH 4.0 and it was relatively low in the range of pH 5.0–9.0. Collagen solubility continuously decreased with increasing salt concentrations up to 4% and it was slightly changed at higher concentrations [17].

In the present study, the spectrum of standard collagen had definite band at 3370 cm1 and the spectrum of ASC and PSC *L. uyii* had characteristic band at 3341 cm1 and 3368 cm1 respectively. Another similarity between standard collagen and sample was the presence of two definite bands at 1653 cm1 and 1654 cm1. Whereas amide split occurred C-H stretching and N-H stretching appeared in the region of 1236 cm1 and 1238 cm1 respectively.

REFERENCE

[1] Yoshinaka. R., Mizuta. S., Itoh. Y., Sata. M., 1990, Two genetically distinct types of collagen in kumura prawn *Penaeus japanicus. Comparative Bio chemistry and Physiology,* 96B, 4 5 1 - 456.

[2] Kuo. J. D., Haultin. H.O Attalla. M.T., Pan. B.S., 1991 role of collagen and contractile elements in ultimate tensile strength of squid mantle. Food chem., 39, 1149- 1154.

[3] Sikorski. Z. E., Scott. D.N., and Buisson. B.S., 1984. The role of collagen in the quality and processing of fish. Critical reviews in food science and nutrition, 20, 301-343.

[4] Mizuta. S., Yoshinaka. R., Sato. M., Sakaguchi. M., 1994, Isolation and partial characterization of two distinct types of collagen in the Squid, Todarodes pacificus. Fisheries science, 60, 467-471.

[5] Nagai. T., 2000, Isolation and characterization of collagen from the outer skin waste materials of cuttle fish, *Sepia lycidas.* Food chemistry, 72, 425-429.

[6] Bradford,M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein is using the principles of protein –dye binding. Anal.biochem. 72, 248-252.

[7] Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

[8] Mimura. S., and Kimura. S., 1985. Jellyfish mesogloea collagen Chacterization of molecule as α1 α2 α3 heterotetramers. Journal of biological chemistry, 260, 15356.

[9] Nagai T. and N. Suzuki, 2000. Partial characterization of collagen from purple sea urchin (*Anthocidaris crassispina*) test. Inter. J. Food Sci. Tech., 35: 497-501.

[10] Mizuta. S., Mizagi. T., Yoshinika. R., 2005, Characterization of the qualitatively major collagen in the mantle of Oyster *Crassostrea gigas*. Fisheries science, 71, 229-235.

[11] Wolfgang, F. (1998). Collagen—biomaterial for drug delivery. European J. Pharm. Biopharm, 45, 113–136.

[12] Shadwick. R.E., 1985, Crosslinks and chemical characterization of cephalopod collagens. Biology of invertebrates collagen, (pp.337-343) new York, plenum press.

[13] Rigo, C, Hartmann, D.J, Bairati, A., 2002, Electrophorectic and immunochemical study of collagen from sepia officinalis cartilage. Biochimica biophysica acta, 1572, 77-84.

[14] Iwahashi. M. and Nakano. R., 1998. Decreased type V Collagen expression in human decidual tissues of spontaneous abortion during early pregnancy. Journal of clinical pathology, 51, 41-46.

[15] Ida-pucci-minafra, Cintia carella. Type V collagen induces apoptosis of 8701- BC breast cancer cells and enhances m-calpain expression, 2000, Breast cancer res 2000 , 2: E008.

[16] Wenstrup, R.J, J. B. Florer, E. W. Brunskill, S.M. Bell,I. Chervoneva and D. E. Birk (2004). Type V Collagen Controls the Initiation of Collagen Fibril Assembly. Journal of Biological Chemistry, 279: 53331-53337.

[17] Woo et al., 2008 J.W. Woo, S.J. Yu, S.M. Cho, Y.B. Lee and S.B. Kim, Extraction optimization and properties of collagen from yellowfin tuna (Thunnus albacares) dorsal skin, Food Hydrocolloids 22 (2008), pp. 879–887.