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Extraction and characterization of acid soluble collagen (ASC) from airbladder of striped cat fish (*Pangasius hypophthalmus*)

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Abstract

The aim of the present study was to extract high pure acid soluble collagen (ASC) from the airbladder of striped cat fish (*Pangasius hypophthalmus*). ASC was extracted and its physico-chemical characterization was carried out to confirm the purity and structural integrity of extracted collagen. The yield of ASC extract was determined to be 73.4±0.8% of the dry weight of raw material. Amino acid analysis revealed the glycine content as 243.0±1.3 residues/1000 residues, imino acids as 186.1±1.4 residues/1000 residues respectively and was shown to be devoid of non-collagenous amino acids such as tryptophan. Electrophoretic analysis confirmed the subunit pattern of type I collagen and showed that it also contained β and γ subunits. UV/Vis absorption and FT-IR analysis demonstrated the primary and secondary structural integrity of collagen and also indicated that no denaturation had occurred during extraction process. $^1\text{H-NMR}$ analysis validated the structural integrity of collagen triple helix. The denaturation temperature (T_d) of ASC was assessed as 33.3 °C. The air bladder collagen extracted from striped cat fish is of high purity and belongs to type I with intact triple helical structure.

Keywords: Collagen, air bladder, striped cat fish, *Pangasius hypophthalmus*

1. Introduction

Collagen is the most abundantly available structural protein, naturally occurring in the connective tissues of vertebrates. It is one of the most widely used biopolymers in the formulation of nutraceuticals and pharmaceuticals, since it possesses unique amino acid composition and comprise of several bio-active peptides upon enzymatic digestion. Interestingly, collagen and its peptides possess excellent biocompatibility, biodegradability, weak antigenicity, potential antioxidant [Liu *et al.*, 2012] ^[25] and anti-inflammatory properties. Earlier studies in our laboratory have indicated that hydrolysates and peptides prepared from fish collagen are effective in ameliorating oxidative stress in experimentally induced inflammatory conditions [Hema *et al.*, 2016] ^[13].

Growing demand for collagen based healthcare products such as food and beverages, tissue regenerative biomaterials, wound dressings and cosmetics leads to the exponential growth of global collagen market, it is anticipated to reach 6.63 billion USD by 2025 [Grand view research, 2017]. Presently Bovine hide and skin are being considered as dominating sources in the collagen industry [Bussiness wire, 2017]. The outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza [Veeruraj *et al.*, 2015] ^[40] have diminished the demand for mammalian collagen. Meantime, the concern of halal origin collagen for Muslim community also played a role in the same. The growth of collagen industry is expected to be driven by marine resources in recent future, mainly due to its better biocompatibility and cost effectiveness as compared to collagen derived from mammalian sources. Several fish processing industries are located in the coastal areas of Kerala, where an enormous quantity of by-products (skin, scales, fins, and airbladder) are generated during processing and filleting. These by-products can be used as a potential source for collagen extraction, which would also be a possible step taken towards fishery waste management.

The fish species used in the present study, striped cat fish (*P hypophthalmus*) has large air bladder with thick outer wall, which may be associated with the collagen cross-links,

especially cross-linking caused by hydroxylysine. Recently, pepsin-soluble collagen was extracted from the swim bladders of catfish and bighead carp, the yields of 40% and 59% respectively [Bama *et al.*, 2010; Liu *et al.*, 2012] ^[2, 25]. Nowadays, striped cat fish (*Phyphthalmus*) has become most trendy among the consumers worldwide. In India this species is being widely cultured in many states such as Andhra Pradesh, West Bengal, Tamil Nadu, Odisha, Maharashtra, Uttar Pradesh, Karnataka, Kerala, Bihar, and Rajasthan. However, there is no information available regarding the chemical composition and molecular properties of collagen from the air bladder of striped cat fish (*P hypophthalmus*) to the best of our knowledge. In the present study, an attempt has been made to extract, purify and characterize acid soluble collagen from the airbladder of *P. hypophthalmus*.

2. Materials & Methods

2.1 Chemicals and sample used

Fish samples (*P hypophthalmus*) were collected from West Godavari District, Andhra Pradesh and the air bladders were carved out from each fish. The samples were stored at -20 °C for less than one month before starting with extraction process. Amino acid standards (AAS-18) were acquired from Sigma-Aldrich GmbH (Steinheim, Germany). Novex-Tris-Glycine precast gels (4-12%), Novex Tris-Glycine SDS Sample Buffer, Novex Tris-Glycine SDS Running Buffer, and See Blue Plus2 pre-stained protein standard (Invitrogen) were procured from Thermo fisher Scientific.

2.2 Methods

2.2.1 Proximate composition of raw materials

The raw materials were analysed for its proximate composition including moisture content, total protein content using micro kjeldahl method, total fat content using soxhlet method (SocsPlus Pelican Equipment) and ash content [AOAC, 2000] ^[1].

2.2.2 Extraction of collagen by acetic acid treatment

ASC extraction was done from the air bladder of *P hypophthalmus* [Bama *et al.*, 2010] ^[2]. Alkali pre-treated sample was treated with 0.5 M acetic acid with gentle stirring for another 72 h under refrigerated condition. The supernatant was collected after homogenization of the tissue. Collagen content was precipitated using salting out protein precipitation method. The extract was dialysed against 0.5 M acetic acid for 72 h and then against distilled water until the extract attains neutral pH. Each processing step was carried out at 4 °C. The extract was lyophilized to obtain collagen powder.

2.2.3 Yield of crude extract

The yield of crude extract was calculated according to the dry weight of raw materials using the following equation [Chen *et al.*, 2016; Kaewdang *et al.*, 2014] ^[3, 19].

$$\text{Yield (\%)} = \left[\frac{\text{Weight of lyophilized collagen}}{\text{Dry weight of airbladder}} \right] \times 100$$

2.2.4 Determination of total protein content in the extract

The determination of crude protein content in the ASC was carried out using micro-kjeldahl method [AOAC 2000] ^[1]. Percentage of purity of ASC was determined from the collagen content in the extract. The collagen content was calculated from the percentage of hydroxyproline content

using the following relationship [Edwards and O'Brien, 1980] ^[8]. Hydroxyproline content in the purified ASC was determined using amino acid analyzer as explained later in this paper. Previous reports suggest the hydroxyproline content of fish collagen approximately 8-9 g hydroxyproline/100g collagen. Thus 1 g hydroxyproline corresponds to 12.5% collagen [Sotelo *et al.*, 2016] ^[35].

$$\text{Collagen (g/100 g extract)} = \text{Hydroxyproline (g/100g extract)} \times 12.5 \text{ (conversion factor)}$$

2.2.5 Physicochemical characterization of collagen

2.2.5.1 Analysis of amino acid composition using HPLC method

Amino acid composition of ASC was determined using an amino acid analyzer HPLC (HITACHI L-2130) model equipped with anion exchange column (ShodexCXPak P4215), fluorescence detector (HITACHI L-2485) and column oven (HITACHI L-2350). The post-column derivatization was done using two Shimadzu LC-10AT VP pumps. The flow rate was constant at 0.4 ml/min, and the oven temperature was set at 60 °C. The fluorescence excitation and emission wavelengths were 340 nm and 450 nm, respectively [Ishida *et al.* 1981] ^[18].

Tryptophan content was estimated colorimetrically using a spectrophotometer (HITACHI U-2910) [Sastry & Tammaru, 1985] ^[31].

2.2.5.2 Analysis of UV/Vis absorption spectrum of collagen

UV/Vis absorption spectrum of ASC (1mg/ml) in 0.02M sodium acetate buffer (pH 4.8) containing 2M urea was obtained through a wavelength scan between a range of 200-700 nm with a scan speed of 2 nm/s at room temperature using a spectrophotometer (Shimadzu UV-1601). The base line of the scan was set with 0.02 M sodium acetate buffer (pH 4.8) containing 2M urea.

2.2.5.3 Analysis of electrophoretic pattern of collagen subunits

SDS-PAGE pattern of the ASC was analysed by following Laemmli's method. Precast gel with gradient gel strength (4-12%) was used for the separation of subunits using the discontinuous Tris-HCl/glycine buffer system. The gel after run was stained with Coomassie Brilliant Blue R-250 and destained to remove excess stain.

2.2.5.4 Analysis of Fourier Transformed Infrared Spectroscopy (FT-IR) of ASC

FTIR analysis of ASC was carried out using the spectrometer (PerkinElmer Spectrum 2) with attenuated total reflectance on diamond crystal. Sample analysis was carried out in the spectral range of 4000-400 cm⁻¹ and the signals were received in 32 scans at a resolution of 4 cm⁻¹.

2.2.5.5 Analysis of 1H Nuclear Magnetic Resonance (1H-NMR) of ASC

1H-NMR spectrum of ASC was obtained using a Bruker Avance III, 400MHz spectrometer equipped with BBO 400 MHz probe. All the chemical shifts were represented in parts per million (ppm) and D2O was used as solvent.

2.2.5.6 Determination of denaturation temperature

The temperature induced change in viscosity of ASC (0.1% collagen resuspended in 0.1M acetic acid) was measured using Brookfield Ultra Programmable Rheometer (Model: DV-III, Brookfield Engineering Laboratories Inc., MA USA) with spindle No. 41 and speed 100 rpm. Collagen solution was incubated at different temperatures from 4 to 50 °C in a water bath. The sample was held at each designated temperature for 30 min prior to the determination of viscosity. Each measurement was carried out in triplicates and the fractional viscosity was calculated using the following formula [Pati *et al.*, 2010]^[28].

$$\text{Fractional viscosity} = \frac{[(\text{Measured viscosity} - \text{Minimum viscosity})]}{(\text{Maximum viscosity} - \text{Minimum viscosity})}$$

2.2.6 Statistical analyses

Methods for collagen extraction and their characteristic analyses were carried out in triplicates. The data were presented as mean \pm Standard deviation.

3. Results and Discussion

3.1 Proximate composition of raw materials

The proximate composition of the raw materials was analyzed and the results are displayed in Table 1. According to the results airbladder was found to contain 73.9 \pm 0.5 percent moisture, 20.5 \pm 0.2 percent protein, 3.77 \pm 0.1 percent fat and 0.89 \pm 0.1 percent ash contents. Air bladder of bighead carp was reported to possess 75.2% moisture content [Liu *et al.*, 2012]^[25]. Moreover, swim bladder of yellow fin tuna was reported to contain 83.3% moisture, 12.09% protein, 1.44% fat and 0.29% ash [Kaewdang *et al.*, 2014]^[19].

Table 1: Proximate composition of airbladder of striped cat fish (*P hypophthalmus*)

Sample	Moisture	Total protein	Total fat	Ash
Air bladder (<i>P hypophthalmus</i>)	73.9 \pm 0.5	20.5 \pm 0.2	3.77 \pm 0.1	0.89 \pm 0.1

*Mean values \pm standard deviation, n=3. Values are expressed in terms of percentage.

3.2 Determination of Yield in the ASC

The lyophilized air bladder collagen from striped cat fish was white colored and fibrous in nature. Table 2 represents the yield of crude extract, total protein content and collagen content in the ASC. Yield of crude extract was determined from the dry weight of extract and raw material and the result was analyzed to be 73.4 \pm 1.2% (dry weight) which was higher than the yield of ASC from the swim bladders of yellowfin tuna (1.07%) [Kaewdang *et al.*, 2014]^[19] whereas, slightly less than that of the ASC from the airbladder of seabass (85.3%) [Sinthusamran *et al.*, 2013]^[33]. The results obtained in yield analysis, indicating the successful removal of non-collagenous impurities and tissue remnants during alkali pre-treatment of tissues. Difference in the yield of collagen extraction from different species is possibly related to the

inter chain cross-links at the telopeptide region of collagen. Acid treatment leads to the swelling of tissues and loosens the arrangement of fibrils making them more soluble without altering inter molecular cross links [Tamilmozhi *et al.*, 2013]^[37]. The total protein content of the extract was determined to be 97.9 \pm 0.9% (dry weight basis). The amount of hydroxy proline, the unique amino acid present in collagen, was 8.01%. Collagen content in ASC was determined as 100% from the level of from the hydroxyproline in the extract demonstrating the high purity of ASC extract. The results of the present study suggests that air bladder collagen can be efficiently isolated using acidic solvent. The purity of the ASC was confirmed by analysis of amino acid composition and UV/Vis absorption spectrum, proving the absence of non-collagenous amino acids.

Table 2: Yield and total protein content of acid soluble collagen extracted from the airbladder of striped cat fish (*P hypophthalmus*)

Samples	Yield	Total Protein	Collagen content
Acid soluble collagen	73.4 \pm 1.2	97.9 \pm 0.9	100 \pm 0.8

*Mean values \pm standard deviation, n=3. Values are expressed as Percentage.

3.3 Physico-chemical characterization of collagen

3.3.1 Analysis of amino acid composition using HPLC method

The chromatogram of amino acid analysis of ASC is given in Fig 2 and the compositional values of each amino acid including hydroxyproline is shown in Table 3. In the present study, the amount of glycine was found to be 1/3rd of total amino acids (243.0 \pm 1.3 glycine/1000 residues of amino acids). ASC from the swim bladders of yellow fin tuna was reported to have similar levels of glycine content (225 residues/1000 total residues) [Kaewdang *et al.*, 2014]^[19]. Comparable results were also reported in squid mantle was (256/1000 residues) [Cozza *et al.*, 2016]^[7] and skin of *E macrura* contains 278 glycine/1000 residues [Veeruraj *et al.*, 2013]^[41]. In general, collagen possess glycine in every third position in the repeating sequence of (-Gly-X-Y)_n amino acids except for the first 14 residues from N-terminus and first 10 residues from C-terminus in collagen [Foegeding *et al.*, 1996]^[9]. It facilitates the tight packaging of fibrous threads by twinning three fibers together so that the smallest side chain (H group) exhibit no steric hindrance in their

molecular structure.

Proline and hydroxyproline contents were recorded to be 106.2 \pm 0.9 & 80.1 \pm 0.5 residues/1000 total residues of amino acids. The swim bladder collagen from seabass was reported to possess 111 residues of proline and 83 residues of hydroxyproline [Sinthusamran *et al.*, 2013]^[33]. Scale collagen of rohu and catla were reported to contain 118 and 130 residues of proline, as well as 83-84 residues of hydroxyproline [Pati *et al.* 2010]^[28]. Similarly, skin collagen from yellow fin tuna exhibited 12.5% proline and 8% of hydroxy proline [Woo *et al.* 2008]^[43]. Skin collagen from big eye snapper and that of grass carp possessed 193 and 186 residues of imino acids [Kittiphattanabawon *et al.*, 2005; Zhang *et al.*, 2007]^[20, 44]. Increased degree of hydroxylation of proline residues contribute to the structural integrity and thermal stability of collagen molecule. This occurs through formation of hydrogen bonds with the hydroxyl groups of pyrrolidine ring which can impose restrictions on the conformational changes and thereby strengthening the secondary structure of the polypeptide chain [Holmgren *et al.* 1966]^[15]. The levels of hydroxylation also determines the gel

strength of both collagen and gelatin [Gilsenan & Ross-Murphy 2000; Gomez-Guillen *et al.* 2002] ^[11, 12]. The amounts of imino acids may be affected by the water temperature of living environment [Thuy *et al.* 2014] ^[38]. The ASC was also found to possess high levels of alanine, and glutamate, aspartate, lysine and arginine. Although, tryptophan, cysteine and methionine were found to be completely absent (Refer Fig 2). Sivakumar *et al.*, (2000) ^[34]

established the absence of cysteine in collagen from skin of Indian cat fish. The amounts of hydrophobic amino acids were found to be 616.3±1.8 residues/1000 residues in airbladder collagen. Peptide fragments composed of hydrophobic amino acids were reported to possess antioxidant activity and anti-hypertensive activity [Chen *et al.*, 2016; Li *et al.*, 2007] ^[3, 24].

Table 3: Determination of amino acid composition of acid soluble collagen (ASC) from the air bladder of striped cat fish (*P hypophthalmus*)

Amino acids	Composition
Aspartate	49.4±0.5
Threonine	31.1±0.4
Serine	33.9±0.7
Glutamate	103.7±0.4
Proline	106.2±0.9
Glycine	243.0±1.3
Alanine	105.8±0.8
Valine	20.2±0.3
Isoleucine	14.8±0.1
Leucine	23.0±0.2
Phenyl alanine	22.7±0.5
Histidine	29.7±0.5
Lysine	56.7±0.9
Arginine	78.8±0.8
Tyrosine	1.5±0.2
Hydroxyproline	80.1±0.5

*Mean values ± standard deviation, n=3. Values expressed as ‘residues/ 1000 residues of amino acids’.



Fig 1: Striped cat fish (*Pangasius hypophthalmus*) belongs to the order Siluriformes and is a member of Pangasidae family.

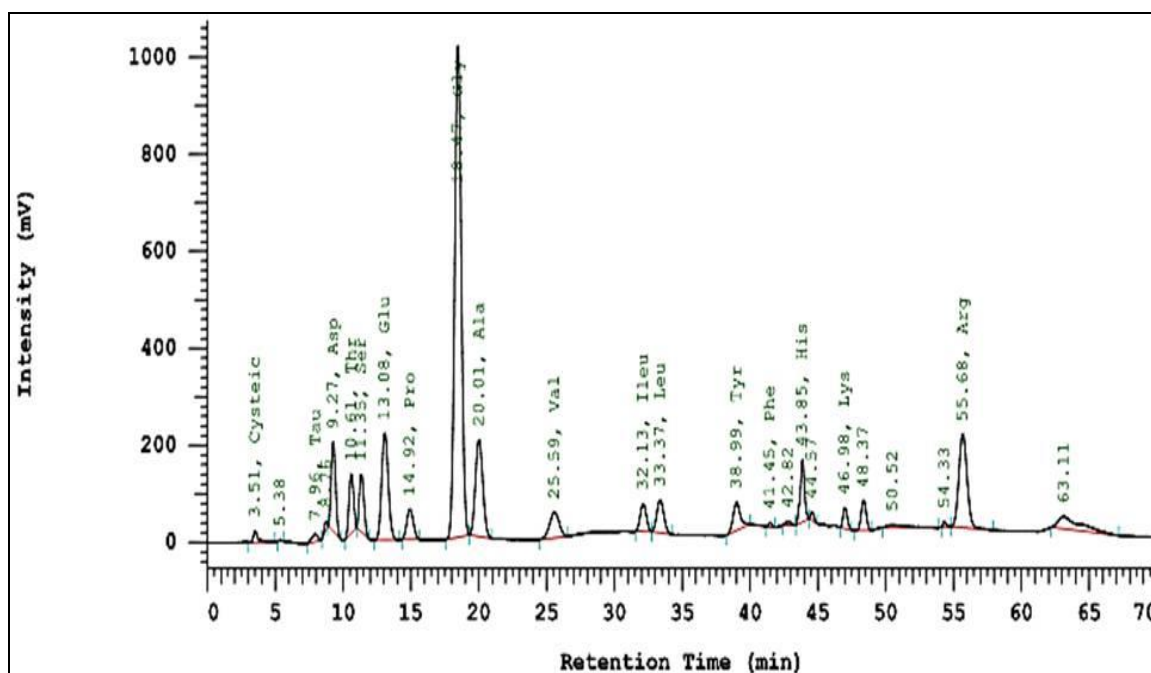


Fig 2: Chromatogram of amino acid analysis of collagen using High Performance Liquid Chromatography (HPLC) of acid soluble collagen (ASC) extracted from the airbladder of striped cat fish (*P hypophthalmus*).

3.3.2 Analysis of UV/Vis absorption spectrum of collagen

Spectra of wavelength scan of ASC is depicted in Fig 3. The extract was found to exhibit UV/Vis absorption bands in the range between 200-250 nm with maximum absorption at 224 nm that was found to be within the similar range as that of collagen from the swim bladders of yellow fin tuna [Kaewdang *et al.*, 2014] [19], skin of channel cat fish [Liu *et al.*, 2007] [26], and skin of balloon fish [Huang *et al.*, 2011]. The absorption band suggests the presence of peptide bond, in addition indicating that the keto, carboxylic acid and amide groups are accessible in the polypeptide chain [Veeruraj *et al.*,

2013] [41]. Generally, proteins show maximum absorption wavelength at 280 nm, which is an indication of the presence of aromatic amino acids such as tryptophan, tyrosine and phenyl alanine, possessing an absorption maximum at 275 and 258 nm respectively [Kozłowska *et al.*, 2015] [21]. The results obtained in the present study demonstrated the efficient removal of non-collagenous protein during the extraction procedure. The results also concurs with the amino acid analysis of collagen extract carried out in the study (Refer Fig 2).

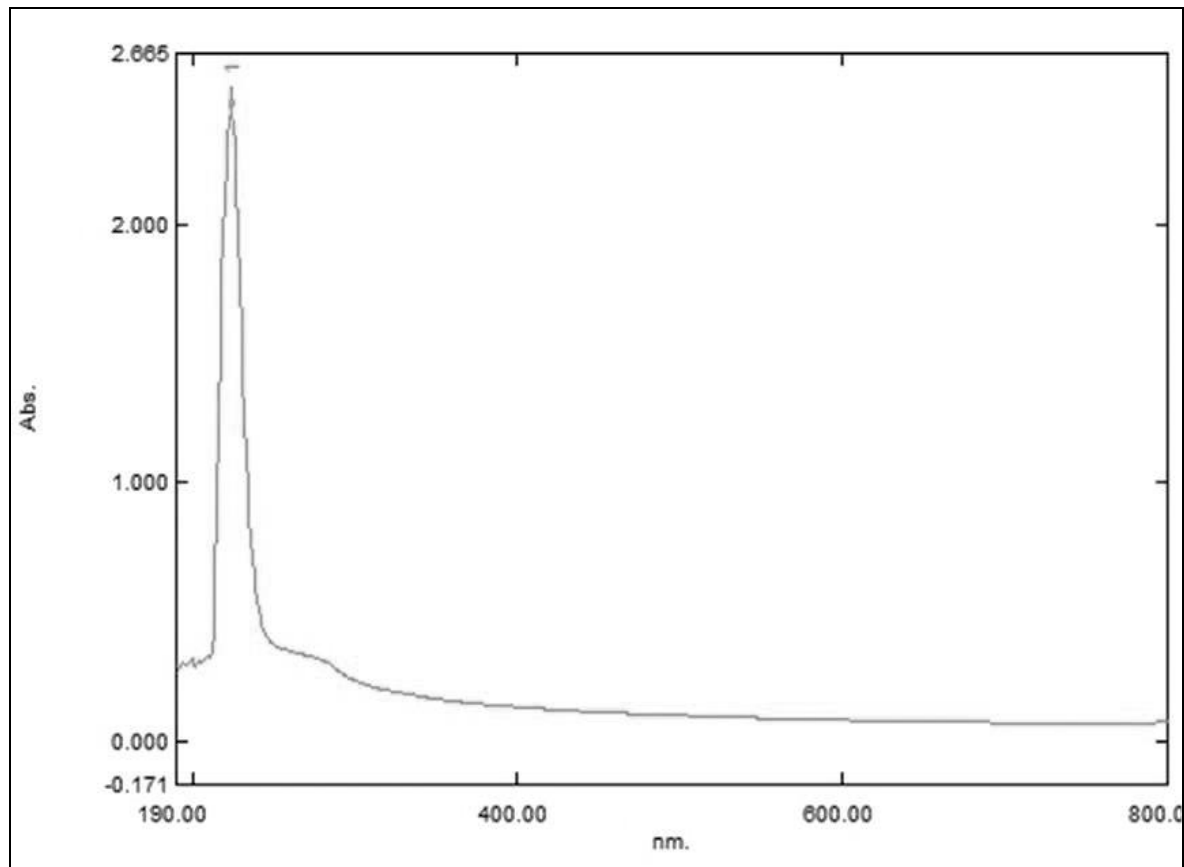


Fig 3: UV/Vis absorption spectrum of Acid soluble collagen (ASC) extracted from the air bladder of striped cat fish (*P hypophthalmus*).

3.3.3 Analysis of electrophoretic pattern of collagen subunits

The subunit pattern of ASC extracted from the air bladder of striped cat fish (*P hypophthalmus*) was analyzed using SDS-PAGE technique. Previous reports suggest the absence of intra molecular disulfide linkages in the airbladder collagen [Kaewdang *et al.*, 2014; Sinthusamran *et al.*, 2013] [19, 33], hence in the present study the sample digestion was carried out under a non-reducing condition. Fig 4 reveals the presence of two α subunits ($\alpha 1$ and $\alpha 2$) as major constituents with molecular weight of 98-100KDa, as well as their inter cross linked components, ' β ' dimers and ' γ ' trimers. The band width for γ band was comparatively small, mostly indicating the heat denaturation during digestion. The presence of two identical α chains demonstrate that the extracted collagen in the present study belongs to Type I [Pearson & Young, 1989; Wong, 1989]. Collagen extracted from air bladders of sea bass [Sinthusamran *et al.*, 2013] [33] and yellow fin tuna [Kaewdang *et al.*, 2014] [19] were reported to be identified as type I. Type I collagen from calf skin has given similar pattern as that obtained in the present study [Hema *et al.*, 2014] [14].

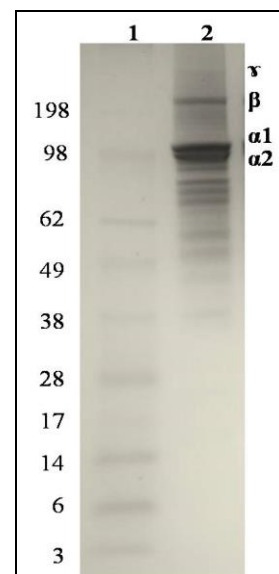


Fig 4: SDS PAGE pattern of acid soluble collagen (ASC) extracted from the air bladder of striped cat fish (*P. hypophthalmus*). Lane 1 represents Marker, Lane 2 represents ASC

3.3.4 Analysis of Fourier Transformed Infrared Spectroscopy (FT-IR) of collagen

Fig 5 shows the FT-IR spectrum of fish collagen extracted from the air bladders of striped cat fish. Usually the FTIR spectrum of type I collagen exhibits characteristic bands of amide A and B as well as amide I, II and III. The results obtained in the present study was found to possess all the aforementioned bands, which was similar with the previously reported data of collagen from swim bladders of yellow fin tuna [Kaewdang *et al.*, 2014]^[19], skin of tilapia [Chen *et al.*, 2016]^[3], skin of eel fish [Veeruraj *et al.*, 2013]^[41] and scale collagen from rohu and catla [Pati *et al.*, 2010]^[28]. In general, amide A band was characterized in the range of 3400-3440 cm⁻¹. The band for amide A was found to be at a lower frequencies at wave number of 3300 cm⁻¹ (Table 4), since the shift in position towards lower frequency indicates the existence of hydrogen bond with C=O group of peptide chain [Doyle *et al.*, 1975]^[7]. Amide B band was found in the position of wave number 2928 cm⁻¹ (Table 4). The wave numbers of amide I, II, and III bands directly indicating the helical configuration of collagen [Kumar *et al.*, 2017]^[23]. Amide I band usually fall in the range from 1600 to 1700

cm⁻¹ and can be used as a positive marker of the secondary structure of the peptide. In case of ASC peak for amide I band was found to occur at a wave number 1633 cm⁻¹ (Table 4). Difference in the band can be associated with the difference in molecular structure of collagen. An increased transmittance and broadening of band width of amide I occurs when collagen is denatured at higher temperature [Surewicz *et al.*, 1988]. The standard absorption range of amide II position occurs between 1550 and 1600 cm⁻¹, even though in the present study amide II band appeared at 1548 cm⁻¹ (Table 4). Amide III band (1235–1240 cm⁻¹) representing the deformation of N-H bond and stretching vibrations of C-H groups, was observed at a wavenumber 1237 cm⁻¹ (Table 4). The absorption peak around 1400-1446 cm⁻¹ were found representing pyrrolidine ring indicating the presence of proline and hydroxyproline [Kumar *et al.*, 2017]^[23]. The ratio of nearly 1, between the absorption of amide III band and that of the band between the wave numbers 1450-1454 cm⁻¹ indicating the intact structure of triple helical collagen [Plepis *et al.*, 1996]^[30]. In the present study, the ratio obtained was 1.01, demonstrates the triple helical structural integrity of extracted collagen.

Table 4: FT-IR Spectra peak locations and assignments for ASC from the air bladder of striped cat fish (*P hypophthalmus*)

Properties	Peak wave numbers (cm ⁻¹)	Peak region assignments
Amide A	3300	N—H stretching vibrations
Amide B	2928	Asymmetric stretch of --CH ₂
Amide I	1633	Stretching vibrations of carbonyl group (C=O)
Amide II	1548	Bending vibrations of N--H coupled with the stretching vibrations of C—N [Krimm & Bandekar, 1986] ^[22]
Amide III	1237	Stretching vibrations of C-H groups

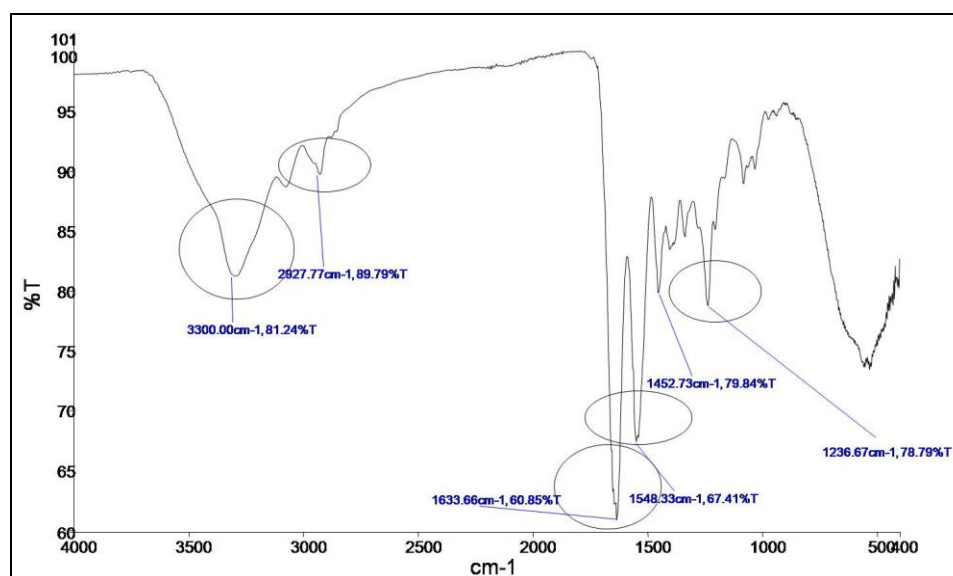


Fig 5: FT-IR spectrum of acid soluble collagen (ASC) from the air bladder of striped cat fish (*P hypophthalmus*).

3.3.5 Analysis of 1H-Nuclear Magnetic Resonance (1H-NMR) of collagen

1H-NMR is a useful technique to trace out the position of hydrogen atoms within the molecular construction of complex structures [Fullerton *et al.*, 2006]^[10]. Figure 6 shows the 1H NMR spectrum of ASC that reveals a major intense band between 4.5-5.0 ppm designating the presence of hydration water (D2O) in the molecule that can interact with the collagen surface along with structural water molecule, thereby stabilizing the helical structure [Fullerton *et al.*, 2006]^[10]. Similar band spectra werestated at 4.6-4.8 ppm in sailfish

collagen [Tamilmozhi *et al.*, 2013]^[37] also in squid mantle collagen [Montoya *et al.*, 2010]^[27]. The spectrum also indicated a major singlet band at 1.9 ppm indicating amide bond and several additional peaks at 1.3, 1.6 and 3.9 ppm representing the unfolding of protein and exploitation of amide groups and α -carbon protons within the polypeptide structure [Constantine *et al.*, 1992]^[5]. Chemical shift of imino group was observed to concur with the peak at 1.9 ppm. Also the peak corresponding to tryptophan (3.3 ppm) was absent in the structure, clearly indicating the complete removal of non-collagenous matter and structural moiety of collagen in ASC.

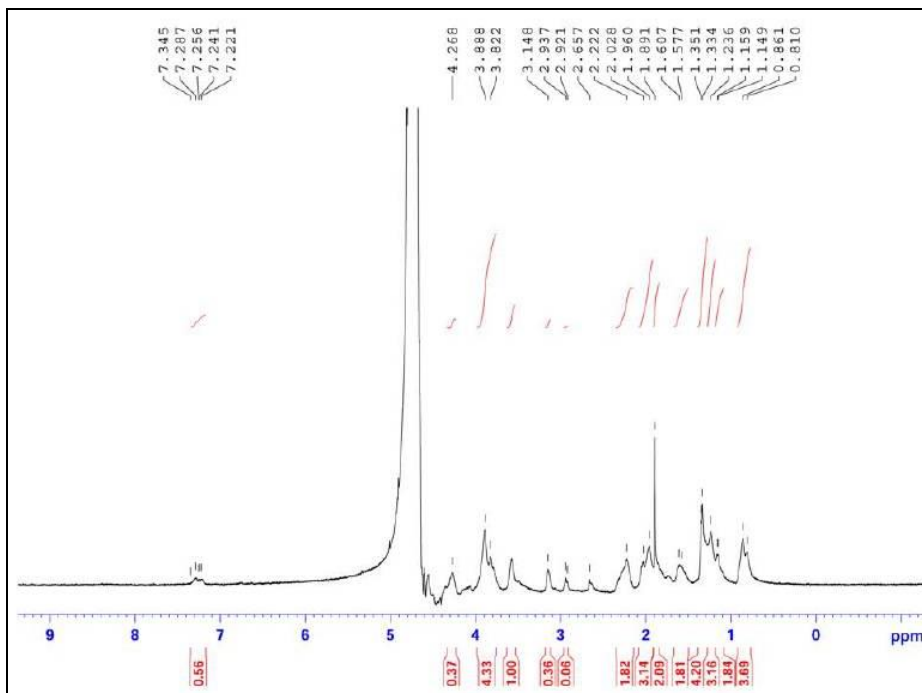


Fig 6: ¹H-Nuclear magnetic resonance (¹H-NMR) spectrum of acid soluble collagen from the air bladder of striped cat fish (*P hypophthalmus*).

3.3.6 Determination of denaturation temperature (Td) through change in viscosity

The intramolecular hydrogen bonds of collagen molecules breaks down gradually with increasing temperature which leads to the thermal denaturation of collagen structure to form a random coil structure. Which can be monitored by measuring the change in physical properties, such as viscosity, sedimentation, diffusion, light scattering and optical activity [Usha & Ramasami, 2004] [39]. In the present study the thermal denaturation temperature of ASC was determined by measuring the change in viscosity at different temperatures from 4-50 °C. Fig 7 demonstrates the graphical representation of fractional viscosity at different temperatures. The term denaturation temperature (Td) defines the temperature at

which the change in viscosity is half completed and can be determined by viscosity measurements at different temperatures. In the present study, the denaturation temperature of ASC was determined to be 33.3 °C, signifying the presence of higher levels of intramolecular hydrogen bonds. Quite similar results were reported in squid skin collagen (35.8 °C) [Veeruraj *et al.*, 2015] [40], scale collagen of rohu and catla (~35 °C), porcine skin collagen (37 °C) [Pati *et al.*, 2010] [28] and much higher than that of skin collagen from hammerhead shark (16.9 °C) [Chi *et al.*, 2014] [4]. The higher thermal stability of ASC was found to correlate with the higher levels of imino acids in the ASC as already discussed in this paper.

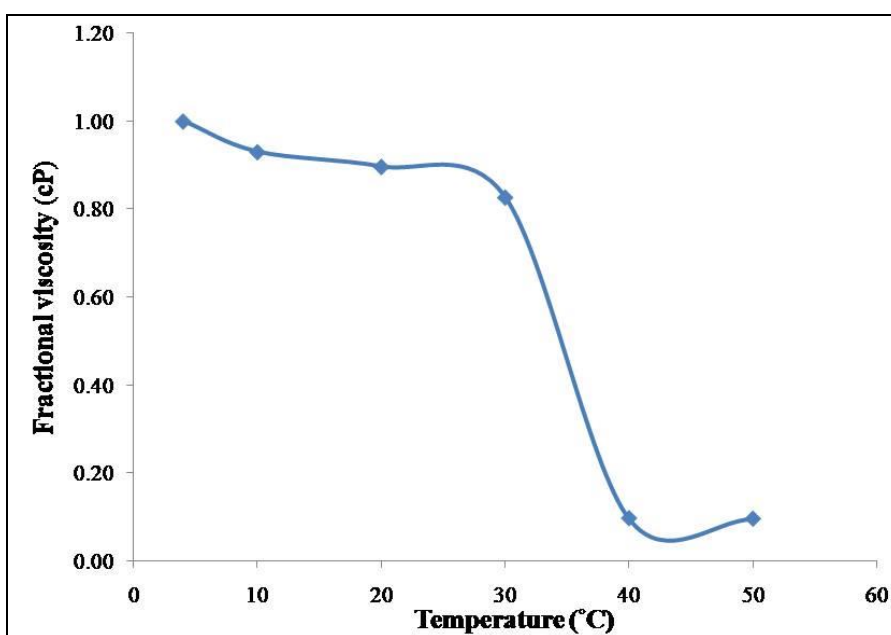


Fig 7: Graphical representation of fractional viscosity of acid soluble collagen (ASC) extracted from the air bladder of striped cat fish (*P hypophthalmus*). Denaturation temperature (Td) was determined to be 33.3 °C.

4. Conclusions

In the present study, an effective method has been developed for the preparation of high pure collagen with maximum yield from the air bladder of striped cat fish (*P hypophthalmus*). Biochemical and physico-chemical characterization of acid soluble collagen confirmed that it belongs to type I and is quite stable at room temperature. Moreover the purified air bladder collagen maintained its helical structure even after enduring the extraction and purification processes. Likewise the ASC extract possess higher levels of hydrophobic amino acids, demonstrating them as a possible source of bioactive peptides. As well, the higher levels of hydroxylation of imino acids make the collagen thermally stable above room temperature. Thus, the air bladder of striped cat fish can be considered as an efficient and reliable source of high quality type I collagen.

5. Conflict of interest

The authors declare that they have no conflict of interest.

6. Acknowledgement

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