



# Extraction and characterization of collagen from the skin of *Pterygoplichthys pardalis* and its potential application in food industries

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**Abstract** The primary objective of this study was to extract collagen from underutilized fish species owing to its cost effective nature and also its ability to address the demand of type I collagen arising from food and pharmaceutical industries. Acid and pepsin soluble collagen (ppASC and ppPSC) were extracted from the skin of sucker catfish (*Pterygoplichthys pardalis*) with a yield of 19.6 and 23.8% on wet weight basis respectively. The same were characterized and confirmed as type I collagen by SDS-PAGE, FTIR and UV-Vis spectroscopy, amino acid analysis, and Zeta potential. Taking into consideration the application of collagen in food industry, a food product was developed by incorporating with fresh cheese. This fortification was found to be acceptable and had not altered the taste, odor and other sensory properties of the product.

**Keywords** Collagen · Type I · *Pterygoplichthys pardalis* · Fresh cheese

## Introduction

Collagen is a biopolymer which constitutes about 25–35% of the total protein in animals and the most abundant protein in skin, bones, connective tissues, including tendons and extracellular matrix (Gauza-Włodarczyk et al., 2017). So far, about 29 types of collagen (I-XXIX) have been recognized in miscellaneous tissues and among these; type I collagen is the ubiquitous type in the body. This fibrillar protein shows the highest degree of organized structure because of its strong and flexible nature followed by composition and arrangement of amino acids. At molecular level, type I collagen consists of 3 polypeptide chains intervened one another in right-handed direction, which contributes to the stability of the protein (Nazeer et al., 2014). Owing to its superior characteristics, the practical applications of type I collagen has increased in various commercial industries such as biomedical, cosmetic, pharmaceutical and food (Wu et al., 2017).

In general, collagen is extracted from earthbound mammals like bovine, porcine and horse (Ogawa et al., 2004) but now-a-days this practice has been facing serious hurdles due to their antipathetic reactions as well risk of spreading contagious diseases (Gauza-Włodarczyk et al., 2017). Moreover, concerns of various religious beliefs have also led to increased interest in obtaining collagen from alternative sources such as marine and fresh water fishes that serve as a suitable substitute for mammalian collagen as fish collagen also possess similar characteristics of porcine collagen. Prominent quantity of fish waste is being dumped at landfill sites, which may cause infections and effuse a nauseous odor (Kumar et al., 2012; Nazeer et al., 2014). To overcome this hazardous complication, fish processing wastes can be utilized as an alternative source to mammalian collagen and this has attracted the attention of

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the research society (Bhagwat and Dandge, 2016; Sampath Kumar and Nazeer, 2013).

*Pterygoplichthys pardalis* is an invasive sucker mouth armoured catfish exotic to Amazon river (Lujan et al., 2015) and prominently available in Kolleru lake, India. It is popularly called as “janitor fish/ leopard pleco” and was exported to various countries as ornamental aquarium fish because of its bottom-dwelling nature. During transportation it must have got released intentionally or accidentally into native water bodies and got established in various parts of the globe (Biju et al., 2015; Gibbs et al., 2013). Due to the survival ability in poor water conditions (German et al., 2010; Jumawan et al., 2010) and lack of native predators (Nico and Martin, 2001) lead to successfully invade non-native environments. Since the muscle content is very low and skin is covered with bony plates, the fish species are categorized as non-edible species and has no commercial importance. On the other hand, Ebenstein et al. (2015) reported observations on high percentage of collagen and hydroxyapatite availability in the dermal plates of *P. pardalis* based on preliminary analysis using FTIR spectroscopy and X-ray diffraction studies. Therefore, to address the problem at the local level and at large, the aim of this study was to extract and analyze acid and pepsin soluble collagen from the underutilized fish *Pterygoplichthys pardalis* in order to use it in food industries.

## Materials and methods

*Pterygoplichthys pardalis* was collected from Kolleru lake, Andhra Pradesh, India (16°65'N; 81°21'E). NaOH (Sodium hydroxide), NaCl (Sodium chloride), Acetic acid, butanol, Tetra acetyl ethylene diamine, Ammonium per sulphate, were of AR grade and purchased from Merck India Pvt. Ltd. Type I collagen was procured from Sigma-Aldrich.

### Pre-treatment

Freshly collected *P. pardalis* was cleaned, dissected and skin was separated from the body and subjected to 0.5 M NaOH to separate small size proteins from the tissue. This process was continued for 4 h with a change of solution for every 1 h for better results, which was followed by separating the tissue from NaOH using a cheese cloth. The collected tissue was washed with ddH<sub>2</sub>O for a few times until the tissue reached pH 7. The pretreatment was completed by dipping the tissue in 20% butanol for removing of fats for a period of 6 h. The defatted tissue was washed later and stored in – 20 °C until further used (Nazeer et al., 2014).

### Extraction procedure

Collagen was extracted from the defatted tissue using 0.5 M acetic acid (CH<sub>3</sub>COOH) under continuous stirring at 4 °C for a period of 3 days. Un-dissolved residue attained after acid extraction was suspended in 2 volumes of 0.5 M acetic acid containing 1.5% (w/w) pepsin for 30 h at 4 °C with continuous stirring. Later, the sample was filtered through a cheese cloth and salted out for precipitation of protein. The precipitate was collected after centrifuging it at 140×g for 30 min and suspended in 3 mL of CH<sub>3</sub>COOH. Extract was consecutively dialyzed with 0.1 M CH<sub>3</sub>COOH and double distilled water for 3 days and lyophilized (Mini Lyodel, India) before further experimentation.

### Characterization of collagen

Collagen was characterized using UV–Vis and FTIR spectroscopy as reported by Sampath Kumar and Nazeer (2013). Lyophilized collagen (1 mg) was suspended in 0.5 M CH<sub>3</sub>COOH and wave scan was noted by Shimadzu Corporation, Japan (UV-1800) from 200 to 420 nm. On the other hand, collagen spectrum was noted from 500 to 4000 cm<sup>-1</sup> at a data acquisition rate of 2 cm<sup>-1</sup> using ATIR spectrophotometer (Perkin Elmer).

### SDS PAGE analysis

Molecular weight of ppASC (*P. pardalis* acid soluble collagen) and ppPSC (*P. pardalis* pepsin soluble collagen) was confirmed upon comparing the samples with standard type I collagen and high molecular weight marker using the standard protocol of SDS PAGE (Laemmli, 1970). Samples were suspended in buffer containing 10% β-mercaptoethanol; loaded onto resolving gel (8%) and stacking gel (5%) at 100 v using electrophoresis unit.

### Amino acid composition

Percentage of amino acid of acid hydrolyzed collagen was determined using standard protocol (Yan et al., 2008). Sample was loaded onto high performance liquid chromatography (HPLC; Agilent 1100) connected to Zorbax 80 A C<sub>18</sub> column at 40 °C with detection at 338 and 262 nm.

### Denaturation temperature

Collagen samples were prepared by dissolving in 0.1 M (CH<sub>3</sub>COOH) (0.1%) at 10 °C and loaded onto Ostwald's viscometer. Temperature was increased for both the samples stepwise from 10 to 40 °C by dipping the viscometer

in water bath. Fractional viscosity was estimated by using the formula:

$$\text{Fractional viscosity} = \frac{(\text{Measured viscosity} - \text{min. viscosity})}{(\text{Max. viscosity} - \text{Min. viscosity})}$$

### Influence of pH and salt on solubility of ppASC and ppPSC

Samples were dissolved in 0.5 M acetic acid at a concentration of 4 mg/mL and pH was adjusted between 2 to 12. The mixture was centrifuged at  $8000\times g$  for 15 min at 6 °C and supernatant was collected to determine the protein concentration by Kjeldahl method (AOAC, 1991). Similar concentration of samples was taken in fresh test tubes and equal volume of NaCl with various concentrations (0 to 5%) was mixed and centrifuged at  $8000\times g$  for 15 min at 6 °C. The protein concentration in the supernatant was estimated by the Kjeldahl method (AOAC, 1991).

### Zeta potential

Samples (ppASC and ppPSC) were dissolved in 0.05 M acetic acid (0.4 mg/mL) and stored at 6 °C for 2 days. Isoelectric point (pI) of both the samples tested on a Zeta potential analyzer (SZ-100, HORIBA scientific, Japan) after adjusting the pH of sample between 2 to 12.

### Collagen supplemented in fresh cheese

Standardized buffalo milk (6% fat) was divided into two groups and 3% collagen was added to one group after heating the milk to 80 °C for 10 min and then cooled to 70 °C. Both the samples were coagulated using 2% citric acid by continuous agitation until clear whey was separated and carefully removed. Leftover was pressed for 20 min by applying physical pressure and then immersed in cool water and transferred onto a wooden plank to get loose of water (Kumar et al., 2008). Physico-chemical properties of the fresh cheese were determined by the standard methods of Association of Analytical Chemists (Felicio et al., 2016). Sensory analysis of the samples were evaluated by a committee constituting of 5 experienced judges using 9 point hedonic scale (scale of 1 refers to disliked extremely and 9 refers to liked extremely by the judge) (Felicio et al., 2016).

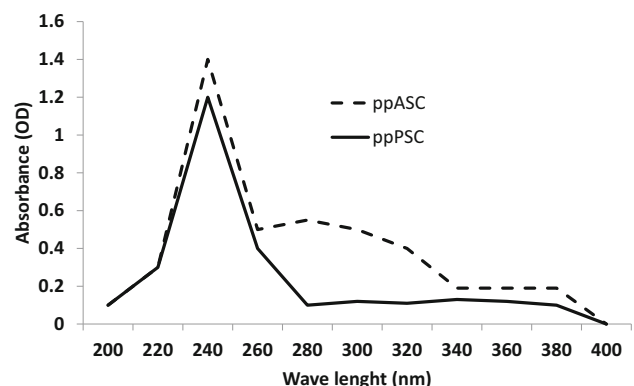
## Results and discussion

### Extraction of ppASC and ppPSC

Yield of ppASC and ppPSC were found to be 19.6 and 23.8% on wet weight basis, which was found to be better and higher than black drum (Ogawa et al., 2004) and threadfin bream (Nalinanon et al., 2011). In general yield of collagen is attributed to the species of fish, its age along with body parts used, habit and habitat (Rigby, 1968); and the key role is played by the experimental conditions and preparative methods (Chen et al., 2016a; 2016b). Usually, acetic acid within a range of 0.1 to 0.5 M was used to extract collagen (Liu et al., 2015), but the yield of ppASC was comparatively low than the ppPSC due to the complexity of molecule with high density cross links intertwined in tissue through interaction of aldehydes with lysine and hydroxylysine (Chen et al., 2016a; 2016b). These amino acids hold the telopeptide helical region of tropocollagen and are responsible for the integrity of the molecule. Sufficient time, appropriate concentration of acetic acid or suitable enzyme is required to break these bonds and allows the protein to solubilise. A range of proteolytic enzymes such as collagenases, pepsin and trypsin were used to cleave peptides in helical region and thereby improve the amount of collagen without disturbing triple helical structure of the protein (Supthatharaprateep et al., 2011). But based on the cost efficacy and its efficiency in breaking and release of hydrophilic amide and carboxyl groups in collagen; pepsin was used in the present study and it has yielded 23.8%, which was comparatively high than the ppASC.

### Spectral analysis of ppASC and ppPSC

As shown in Fig. 1, maximum absorbance for extracted protein was recorded at 235 nm for both acid and pepsin solubilised collagen samples. In general, proteins show



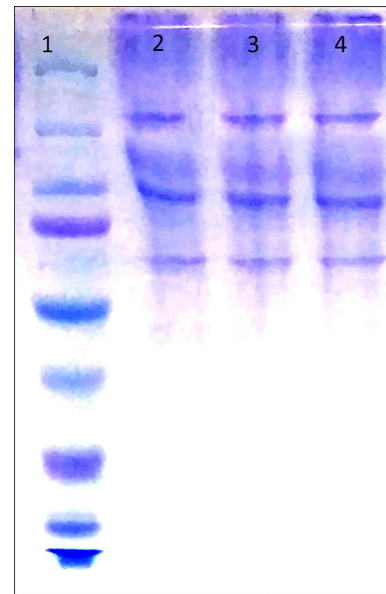
**Fig. 1** UV-Vis spectral analysis of ppASC and ppPSC

maximum absorbance i.e.,  $\lambda$  max at 280 nm due to the influential participation of tryptophan (Duan et al., 2009). But collagen is an exceptional protein that has  $\lambda$  max within a range of 210–240 nm because it is mostly made of glycine followed by proline/ hydroxyproline and lacks tryptophan (Kittiphattanabawon et al., 2010; Yan et al., 2008).

FTIR spectral analysis of extracted collagen from skin of *P. pardalis* showed characteristic peaks as shown in Fig. 2. Secondary structure of collagen usually has an N-H stretching peak at  $3321\text{ cm}^{-1}$  because of the standard frequency of the bonding (Yan et al., 2008). ASC and PSC samples had clear presence of amide I peak within the range of  $1615$  to  $1680\text{ cm}^{-1}$  and a very lower frequency of amide II in between  $1530$  to  $1600\text{ cm}^{-1}$  which may be due to the availability of hydrogen bonds that holds the triple helix of collagen. Both the spectral analysis has confirmed that extracted protein is type I collagen based on the available literature.

### Electrophoretic determination

Electrophoresis of collagen from skin of *P. pardalis* showed three specific bands conforming their molecular weight of 205 kDa ( $\beta$  chain) and two  $\alpha$  chains with 116 kDa ( $\alpha 1$  and  $\alpha 2$ ) respectively. The lane L2 represents the high molecular weight protein marker, while L1 consists of standard calf skin collagen, lanes L2 and L3 were loaded with ppASC and ppPSC. Both ppASC and ppPSC showed similar electrophoretic patterns as standard type I collagen with disulfide bonds (Noitup et al., 2008), there by conforming that extracted protein as type I collagen (Fig. 3). Comparatively high intensity of  $\alpha$ -bands were found in standard type I collagen than the extracted ASC



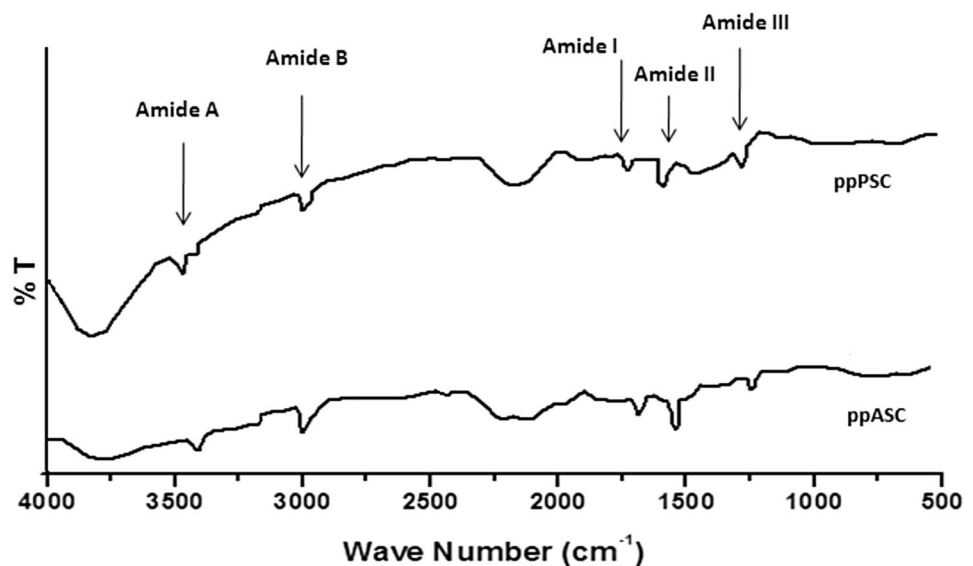
**Fig. 3** Electrophoretic analysis of ppASC and PPPSC. Lane 1—Protein marker; lane 2—Standard type I collagen; lane 3—ppASC and lane 4—ppPSC

and PSC, which is very similar to that reported by Kumar and Rani (2017) and Zhang et al. (2016).

### Amino acid composition

Amino acid analysis (Table 1) of the ppASC and ppPSC showed similar composition of amino acids with very slight variations. A total of 16 amino acids were identified using standard HPLC protocol, among which glycine was dominant in both the samples with 28.3% and 27.9%. This percentage was very close to the percentage of mammalian collagen (30%) and many other marine and fresh water fish

**Fig. 2** FTIR spectral analysis of ppASC and ppPSC



**Table 1** Amino acid composition

Amino acid	Abbreviation	ppASC	ppPSC
Hydroxyproline	Hyp	16.3	16.8
Aspartic acid	Asp	1.4	5.8
Threonine <sup>a</sup>	Thr	5.2	1.8
Serine	Ser	5.4	4.4
Glutamic acid	Glu	7.9	7.6
Proline	Pro	14.6	14.3
Glycine	Gly	28.3	27.9
Alanine	Ala	2.8	6.3
Valine <sup>a</sup>	Val	3.4	1.8
Methionine <sup>a</sup>	Met	0.8	1.4
Isoleucine <sup>a</sup>	Ile	2.4	1.6
Leucine <sup>a</sup>	Leu	3.8	1.9
Tyrosine	Tyr	1.2	0.4
Phenylalanine <sup>a</sup>	Phe	1.7	2.1
Lysine <sup>a</sup>	Lys	3.2	2.6
Histidine	His	0.7	0.9
Arginine	Arg	0.9	2.4
Total		100	100
Imino acids		30.9	31.1

<sup>a</sup>Essential amino acids

collagen. As a matter of fact collagen was known to have a characteristic Gly-x-y sequence, where alternatively x and y were repeated with proline and hydroxylproline. These two amino acids usually were meant to be 1/6 of the total composition and collagen extracted from *P. pardalis* showed 14.6 and 16.3% for ASC and 14.3 and 16.8 for PSC. Amino acids (Proline and Hydroxylproline) are key players in upholding the veracity of the collagen structure and their presence in extracted protein may be the main reason in sustaining the triple helix. Since the obtained amino acid composition was matching to other mammalian and aquatic organisms, the extracted collagen can be definitely used as an alternative to existing commercial collagen. ppASC and ppPSC had no cysteine and also contains low concentrations of tyrosine (1.2 and 0.4), histidine (0.7 and 0.9), and methionine (0.8 and 1.4). These marginal variations in percentage of amino acids between ppASC and ppPSC may be linked to missing of small pieces in telopeptide regions after protease activity (Matmaroh et al., 2011).

### Denaturation temperature (dT)

Denaturation temperature of ppASC and ppPSC were analyzed by exposing the samples at various temperatures and measuring their viscosity using viscometer (Table 2). Extracted collagen in both the methods was found to be denatured at 25 °C which was quite lower than the porcine

collagen (37 °C) and slightly close to fresh water fishes which are within a range of 26.9 to 19.9 °C respectively (Kimura et al., 1988).

### Solubility of collagen

ppASC and ppPSC extracted using 0.5 M acetic acid at pH 2.5 were used for determining the influence of pH and NaCl concentration related to protein solubility (Table 2). Both the samples showed highest solubility in acidic region (1–4) and started decreasing from pH 5. Least solubility was observed at pH 6 which may be due to the isoelectric point (pI) of the protein, because it is a known fact that proteins after reaching pI, the total net charge becomes zero leading to precipitation (Jongjareonrak et al., 2005).

Influence of NaCl on relative solubility of ppASC and ppPSC extracted using 0.5 M acetic acid was assessed from 0 to 7% (Table 2). NaCl at a concentration of 0–3% stayed consistent with very slight variations for both the samples. On a comparative note PSC showed better solubility than ASC, it was due to the slight changes in structure, properties and composition of trimers ( $\alpha$ ,  $\beta$  and  $\gamma$  chains) (Chen et al., 2016a; 2016b). A slight steep was observed from 4% and slowly the solubility reduced with improving in percentage of NaCl. This may be due to the increase in ionic strength of NaCl in water which must have contributed to the decrease in collagen solubility.

### Zeta potential

Acid and pepsin soluble collagen were tested to identify their zeta potential response at different pH (Table 2). Sample behaviour at acidic pH (2–6) and basic pH (7–11) ranges were studied and found that isoelectric point (pI) was 6.39 and 6.41 respectively. Usually collagen attains net zero charge at pH range of 6–7, where the +ve charges becomes equal to the –ve charge golden carp (Ali et al., 2018). Isoelectric point of pepsin solubilised collagen was always greater than that of acid solubilised collagen because of the elimination of certain amino acids in the non-helical region or may be due to the availability of amino acids with charge viz., Asp, Lys and Glu (Benjakul et al., 2010).

### Collagen supplemented in fresh cheese

Collagen is one of the most important animal proteins responsible for elasticity and flexibility of tissues. During aging, the ability to produce collagen and the strength of the existing collagen decreases leading to the reduction of thickness, suppleness and resilience in tissues. To overcome these situations, collagen supplements in various forms such as dietary supplements, capsules, milk powders,

**Table 2** Denaturation temperature, Influence of pH and salt on solubility and Zeta potential of ppASC and ppPSC

Effect of temperature			Effect of pH			Effect of NaCl (W/V)			Zeta potential		
°C	ppASC	ppPSC	pH	ppASC	ppPSC	NaCl	ppASC	ppPSC	pH	ppASC	ppPSC
10	1.20 ± 0.03	1.50 ± 0.04	0	79.6 ± 0.9	82.3 ± 1.3	0	96.0 ± 2.7	97.1 ± 2.1	2	27.3 ± 1.9	29.8 ± 1.9
15	1.10 ± 0.01	1.20 ± 0.03	2	91.6 ± 0.7	94.5 ± 1.03	1	89.1 ± 1.4	95.6 ± 1.5	4	8.5 ± 1.2	10.2 ± 1.2
20	0.90 ± 0.06	1.00 ± 0.07	4	86.2 ± 1.1	83.6 ± 2.1	2	84.2 ± 1.2	91.3 ± 1.3	6	0.39 ± 0.03	0.41 ± 0.7
25	0.20 ± 0.9	0.20 ± 0.02	6	19.9 ± 1.6	24.5 ± 1.07	3	81.5 ± 1.1	86.9 ± 0.8	8	- 2.1 ± 0.1	- 1.9 ± 0.3
30	0.11 ± 0.03	0.15 ± 0.07	8	19.1 ± 1.0	21.3 ± 1.05	4	48.2 ± 0.9	43.1 ± 1.1	10	- 4.6 ± 0.5	- 3.5 ± 0.9
35	0.09 ± 0.04	0.10 ± 0.01	10	18.6 ± 1.5	20.0 ± 0.9	5	42.3 ± 1.5	41.6 ± 1.7	12	- 6.1 ± 0.7	- 5.8 ± 0.8
40	0.05 ± 0.01	0.08 ± 0.03	12	19.1 ± 1.2	19.9 ± 0.7	6	41.6 ± 1.7	40.9 ± 1.6			

Results are represented as mean ± SD (n = 3)

**Table 3** Physico-chemical analysis of fresh cheese enriched with collagen from *Pterygoplichthys pardalis*

	Moisture (%)	Protein (%)	Ash (%)	Lactose (%)	Fat (%)	Sensory score
Test	48.44 ± 0.53	22.56 ± 0.64	3.12 ± 0.24	2.46 ± 0.04	23.84 ± 0.56	7.2 ± 0.4
Control	45.80 ± 0.39	19.20 ± 0.84	3.74 ± 0.1	2.82 ± 0.06	26.25 ± 0.24	8.1 ± 0.2

Results are represented as mean ± SD (n = 3)

capsules, creams etc are available in market. Considering these options collagen was incorporated with paneer made out of buffalo milk to enhance the protein percentage and weight of the product which in turn helps in supplying high protein content to consumers in less quantity. Proximate analysis of the test sample (collagen + paneer) and control sample (only paneer) revealed that, test samples had high moisture and protein contents as shown in Table 3. On the other hand, organoleptic analysis revealed that the addition of collagen to paneer has not altered the taste, odor or over all acceptability (Hashim et al., 2015; Seda and Sibel, 2015) but only slight changes were observed in hardness and adhesiveness of the test samples.

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