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Extraction and characterization of collagen from the skin of *Pterygoplichthys* spp. (armored catfish) from Chiapas

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Abstract

Because of its biocompatibility and safety, collagen is a valuable biomaterial that is used in various industries. While traditionally sourced from terrestrial mammals, collagen extracted from fish waste is a promising alternative due to its chemical properties and low cost. In Mexico, *Pterygoplichthys* spp. is considered an invasive species, inedible, and of limited commercial importance due to the low muscle content and skin covered with rigid bony plates. This study aimed to extract and characterize collagen from the skin of *Pterygoplichthys* spp. obtained from two communities in Chiapas, Mexico. Our results show that the extracted collagen is type 1, with a yield of 43.0% (dry weight). Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis revealed the presence of two alpha chains (α1, α2) and a beta and gamma component, consistent with type 1 collagen. Liquid chromatography coupled to mass spectrophotometry analysis identified peptide sequences homologous to those reported in other species. This study highlights the potential of *Pterygoplichthys* spp. skin collagen as a viable alternative to mammalian collagen. The efficiency of the extraction process and the identification of peptides resembling those of the other species underscore the feasibility of utilizing collagen from the skin of *Pterygoplichthys* spp. in industrial applications, offering a sustainable solution to environmental and economic challenges. Practical Application: Collagen extracted from the skin of *Pterygoplichthys* spp., an invasive fish species, can serve as a sustainable alternative to mammalian collagen, offering a cost-effective biomaterial with properties suitable for various industrial applications.

Keywords: collagen extraction; *Pterygoplichthys* spp.; liquid chromatography coupled to mass spectrophotometry; sodium dodecyl sulfate polyacrylamide gel electrophoretic profiling; type 1 collagen.

1 INTRODUCTION

Collagen, a fundamental protein comprising approximately 30% of the total proteins in the cell matrix, plays a crucial role in connective tissues such as blood vessels, corneas, bones, cartilage, tendons, and skin. It is widely used in various industries, including food, cosmetics, and drug formulation, particularly in regenerative medicine and cell biology, due to its biocompatibility, mechanical strength, and ability to support growth while preserving muscle rheological properties (Felician et al., 2018; Hashim et al., 2015; Herath et al., 2020; Nurubhasha et al., 2019).

Despite its versatility, the application of collagen is limited by sociocultural practices as its primary source from mammals raises concerns regarding additional health costs and the transmission of bovine and porcine diseases (Chen et al., 2019; Singh et al., 2011; Yu et al., 2018). A potential solution lies in utilizing collagen from fish waste, including bones, scales, and skin, which has gained attention for being a cheaper, higher yielding, and readily available source (Chen et al., 2019; Hashim et al., 2015; Herath et al., 2020; León-López et al., 2019; Zeng et al., 2009). Among the fish species considered for collagen extraction, *Pterygoplichthys* spp. (armored catfish, devil fish, or plecos) stands out as a promising source due to its abundance and low commercial value, attributed to its low muscle content and skin covered with bony plates (Ebenstein et al., 2015; Herath et al., 2020; Nurubhasha et al., 2019). In Mexico, *Pterygoplichthys* spp. is considered an invasive species that, due to its easy adaptability and wide distribution, has caused socioeconomic and environmental problems (Amador-del Ángel et al., 2016; Lorenzo-Márquez et al., 2016).

Previous studies have highlighted the complete discarding of *Pterygoplichthys* spp. by affected sectors, such as fishermen,

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in countries such as Mexico, India, and Indonesia, resulting in wasted protein-rich material (Ayala-Pérez et al., 2014; Herath et al., 2020; Nurubhasha et al., 2019).

The use of this species in products of low commercial value, such as fertilizers and animal feed, has been studied to control overpopulation caused by its invasion (Ayala-Pérez et al., 2015; Prihanto et al., 2021). Some studies have also reported using the skin for collagen extraction to generate value-added biomaterials (Ebenstein et al., 2015; Herath et al., 2020; Nurubhasha et al., 2019). The use of the skin of *Pterygoplichthys* spp. could be an attractive alternative to control the species' invasion in the aquatic systems of Chiapas, Mexico, reducing the impact that their presence has caused on biodiversity, such as silting of water bodies, erosion of riverbeds by their nests, turbidity generated by the resuspension of sediments while they feed, pollution produced by the decomposition of *Pterygoplichthys* spp. discarded by fishermen on the banks, as well as an increase in nutrients, especially nitrogen and phosphorous, originating from the excrement of these fish in rivers and lagoons (Amador-del Ángel et al., 2016; Ayala-Pérez et al., 2014; Capps & Flecker, 2013; Herath et al., 2020; Lorenzo-Márquez et al., 2016). The aim of this study was to extract and characterize collagen from the skin of *Pterygoplichthys* spp., compare the methods used by other authors, and generate a proposal for a strategy for further utilization in localities impacted by the fish's precariousness.

2 MATERIALS AND METHODS

A total of 50 specimens of *Pterygoplichthys* spp. were captured from the municipalities of La Flor de Chiapas (17.487189379699288, -91.82607435206123) and Rómulo Calzada (17.34261470132608, -93.55631205895111), Chiapas, Mexico. The fish were sacrificed and preserved on ice for transport to the laboratory and refrigerated at 4°C. The skins were manually removed, dried at 60°C for 24 h in a drying oven (Felisa, FE-133), then pulverized using a food processor (KitchenAid, KFP0919LCU), and sieved to a particle size of approximately 0.6 mm. The skin powder was stored at 4°C until further use (Fonseka & Radampola, 2022).

2.1 Skin conditioning

To remove noncollagen proteins, the powdered skin of *Pterygoplichthys* spp. was treated with a 0.1 M sodium hydroxide (NaOH) solution at a ratio of 1:10 (w/v) for 12 h at 4°C, with the solution changed every 3 h. The wastewater was then washed with Milli Q ultrapure water until reaching a pH of 7.0 (Singh et al., 2011; Zeng et al., 2012). Subsequently, the skin samples were subjected to lipid removal using 10% butanol for 12 h at a ratio of 1:10 (w/v) at 4°C, followed by three washes in ultrapure Milli Q water for 15 min (Herath et al., 2020; Singh et al., 2011; Zeng et al., 2012). All the reagents used were of high-performance liquid chromatography grade.

2.2 Extraction of collagen in acetic acid

Collagen extraction was performed according to the methodology described by Nurubhasha et al. (2019), with some modifications according to our working conditions. We used 0.5 M acetic acid (CH_3COOH) for 72 h at 4°C, with a mass/volume ratio of 1:20. At the end, the mixture was filtered through a food grade mesh, and the pH was adjusted to 8.0 with 1 N NaOH. Precipitation was achieved by adding 2 M sodium chloride (NaCl) and incubating for 12 h at 4 °C, following the procedure described by Vidal et al. (2020). The precipitated sample was centrifuged at 6,000 rpm for 15 min at 4°C. The precipitate was dialyzed through a 14 kDa molecular cutoff dialysis bag (Sigma-Aldrich, D527-100FT) with 0.1 M CH_3COOH for 48 h at 4°C with agitation at 4,000 rpm and solution change every 12 h. This process was repeated with deionized water under the same conditions as described above (Vidal et al., 2020; Yu et al., 2018). Subsequently, the supernatant was lyophilized using a Labconco, Freezone 18 and stored at 4°C (Herath et al., 2020; Singh et al., 2011; Zeng et al., 2012). The yield was calculated based on the ratio of the skin weight to the dry weight of the sample (Zeng et al., 2012) (Equation 1).

$$\text{Yield \%} = (\text{Weight (g) of lyophilized collagen/Weight (g) of dry skin used}) \times 100 \quad (1)$$

2.3 Quantification of soluble protein

The soluble protein content in the collagen obtained from the skin of *Pterygoplichthys* spp. was determined using the Bradford method (Bradford, 1976) for which the sample was prepared by dissolving 1 g of collagen in 0.5 M CH_3COOH. Solutions of 100 µL of distilled water and collagen solution were prepared in a ratio of 1:10, 1:100, and 1:1,000 (v/v), then mixed with 1,000 µL of Bradford reagent (Bio-Rad, Cat# 5000205) composed of Coomassie Brilliant Blue G-250 dissolved in 55% phosphoric acid and 15% methanol. After incubating for 5 min, it was analyzed at 595 nm in a UV-visible spectrophotometer (Thermo Fisher, Genesys 150). Bovine serum gamma globulin at a concentration of 1 mg/mL (Bio-Rad, Cat# 5000208) was used as a standard protein.

2.4 Collagen characterization

2.4.1 Sodium dodecyl sulfate polyacrylamide gel electrophoretic profiling (SDS-PAGE) and UV-visible spectroscopy

To identify the type and purity of the collagen, SDS-PAGE was performed using a Mini-PROTEAN Tetra Cell system (Bio-Rad), according to the Laemmli method with slight modifications (Laemmli, 1970). The concentrator gel had a concentration of 4%, and a separator gel was prepared at 8%. The collagen sample was solubilized in 0.1 M CH_3COOH, mixed with 2 mL of Tris–HCl sample buffer, and then heated at 95°C for 5 min. The mixture was centrifuged at 5,000 rpm for 1 min. Each well was loaded with a volume of 10 µg/µL sample. The gel ran at 100 V for 60 min. A type 1 collagen standard from rat tendon (Merck, Cat# 8-115) was used for protein identification. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 6.8% (v/v) glacial acetic acid for 5 h, then destained using 7.5% (v/v) glacial acetic acid and 5% (v/v) methanol (El-Rashidy et al., 2015; Nurubhasha et al., 2019; Vidal et al., 2020).

The UV-visible absorption spectrum was determined using a spectrophotometer (Thermo Fisher, Genesys 150) in the wavelength range of 190–400 nm at a scan rate of 1 nm/s. The collagen sample was solubilized in 0.5 M CH_3COOH at a 1:1 (m/v) ratio (Nazeer et al., 2014; Nurubhasha et al., 2019; Sampath Kumar & Nazeer, 2013).

2.4.2 Amino acid sequencing in collagen

The amino acid sequence of collagen was determined according to the method described by Chinh et al. (2019). For this purpose, after the SDS-PAGE process, the bands of interest obtained in the polyacrylamide gel were cut, washed, and analyzed by liquid chromatography coupled to mass spectrophotometry (LC-MS/MS). The results obtained were searched and compared in the protein database of the National Center for Biotechnology Information.

3 RESULTS AND DISCUSSION

3.1 Collagen extraction

We obtained a yield of 43.05 ± 0.05% of acetic acid soluble collagen (ASC) from the skin of *Pterygoplichthys* spp. on a dry weight basis (freeze-dried). The yield obtained under our operating conditions was higher than that reported by Herath et al. (2020), who obtained a yield of 26.20% from *Pterygoplichthys* spp. skin caught in Lake Tempe, South Sulawesi, Indonesia. Nurubhasha et al. (2019) reported a lower yield of 19.6% on wet weight basis from the skin of *Pterygoplichthys* spp. obtained from Kolleru Lake, Andhra Pradesh.

The difference in collagen extraction yield can be attributed to the slight modifications in the methodology used. For instance, our study employed a 12-h period for globular protein removal using a 0.1 M NaOH concentration and pH 11. In contrast, Herath et al. (2020) and Nurubhasha et al. (2019) conducted the extractions with an incubation period of 4 and 36 h using NaOH concentrations of 0.5 and 0.1 M, respectively. Furthermore, the collagen extraction time was 24 h (Nurubhasha et al., 2019); on the contrary, Herath et al. (2020) used a period of 72 h, like the one implemented in this study. However, it should be noted that the raw material/solvent ratio for this study was 1:20, whereas the aforementioned authors used a ratio of 1:10. Likewise, the difference in collagen yield could also be attributed to the unequal cross-linking of the collagen molecule. Highly cross-linked nonhelical terminal ends of the telopeptide region can decrease collagen solubility in the acid solvent (Abinaya & Gayathri, 2019; Zeng et al., 2012). Previous studies have shown that collagen is soluble in 0.5 M acetic acid (Abinaya & Gayathri, 2019; Kuwahara, 2021; Zeng et al., 2012). However, slight modifications in the experimental and preparative conditions of the methodology play a key role in the extraction performance of collagen, and factors such as pH, solvent concentrations (sodium hydroxide and acetic acid), particle size of the sample, weight/volume ratio, and interaction time (raw materials/solvent) influence the extraction process (Chen et al., 2016; Nurubhasha et al., 2019; Tan & Chang, 2018; Zeng et al., 2012). For example, pH can reduce collagen solubility by affecting the protein's charge density, which can alter electrostatic interactions and protein structure (Tan & Chang, 2018). Moreover, the solvent and raw material determine the yield by the effective collision between both components; this interaction influences the matter transfer processes. This reflects a higher or lower yield in the collagen extraction as determined by Lewis' theory of coalitions established in 1918. There is a critical concentration depending on the solvent/raw material ratio; increasing the ratio between the components will no longer benefit the process and the yield, as well as increasing the extraction time (Chen et al., 2016; Tan & Chang, 2018). This is due to the mechanism of action of the organic acid during protein extraction. The direct interaction of acetic acid with the hydrogen bridges present in the collagen triple helix separates the bonds between the alpha helix chains, causing the protein to solubilize in the medium (Tan & Chang, 2018; Zeng et al., 2012). This mechanism is affected if the solvent/raw material concentration is inadequate.

3.2 Quantification and identification of protein in collagen

The concentration of soluble protein in the collagen extracted from *Pterygoplichthys* spp. was 69.7 mg/mL, higher than that obtained by Nurubhasha et al. (2019), who reported a concentration of 22.56 mg/mL. The difference in concentration could be due to the experimental conditions such as collagen extraction time and raw material/solvent concentration similar to the dialysis process. The increase in protein concentration may be due to a lower loss of protein when dialysis was performed to remove NaCl ions for collagen precipitation. Figure 1A shows the electrophoretic pattern determined by protein mobility in SDS-PAGE. Line 1 corresponds to the molecular weight marker, line 2 to the type 1 collagen standard, and line 3 to the collagen extract obtained. The modeled gel shows alpha 1 (α1) and alpha 2 (α2) chains, weighing approximately 130 and 120 kDa, respectively, which form the collagen triple helix. Also, high-molecular-weight components were observed, called β chain with a molecular weight of 250 kDa, formed by two α1 chains in laminar form crosslinked by hydrogen bridges and γ chain (gamma) with a molecular weight higher than 250 kDa, formed by three chains α1 (Chen et al., 2016). Therefore, the ASC extracted in this study was classified as type 1 collagen. These results are similar to those reported by Nurubhasha et al. (2019), who extracted type 1 collagen from acetic acid *Pterygoplichthys* spp. skin. Furthermore, Singh et al. (2011) and Tan and Chang (2018) reported the extraction of type 1 collagen from the skin of different *Pterygoplichthys* spp. species and report bands with molecular weights of 100–130, 90–120, and 200–260 kDa corresponding to α1, α2, β, and γ chains, respectively.

The UV-visible characterization was analogous to that reported by Nurubhasha et al. (2019), confirming that the protein obtained is type 1 collagen, as well as coinciding with the electrophoresis analysis. Figure 1B shows the absorption spectrum in a spectral sweep from 190 to 400 nm of the collagen of the *Pterygoplichthys* spp. and the type 1 collagen standard. The similarity of absorbance in the sweep can be seen, with the maximum absorption length found at 236 and 235 nm, respectively. This absorption is mainly attributed to the n–n* bonds of the C = O, -COOH, and $CONH_2$ groups present in

the polypeptide chain of collagen (Abinaya & Gayathri, 2019; Chen et al., 2019). The ultraviolet region of proteins generates absorbance near 280 nm; this signal corresponds to aromatic amino acids such as tyrosine, phenylalanine, and tryptophan (Zeng et al., 2012). However, the collagen sample obtained in this study showed a low absorbance at this length as shown in Figure 1B. The inability to absorb at higher UV regions is related to tyrosine, phenylalanine, and tryptophan deficiency

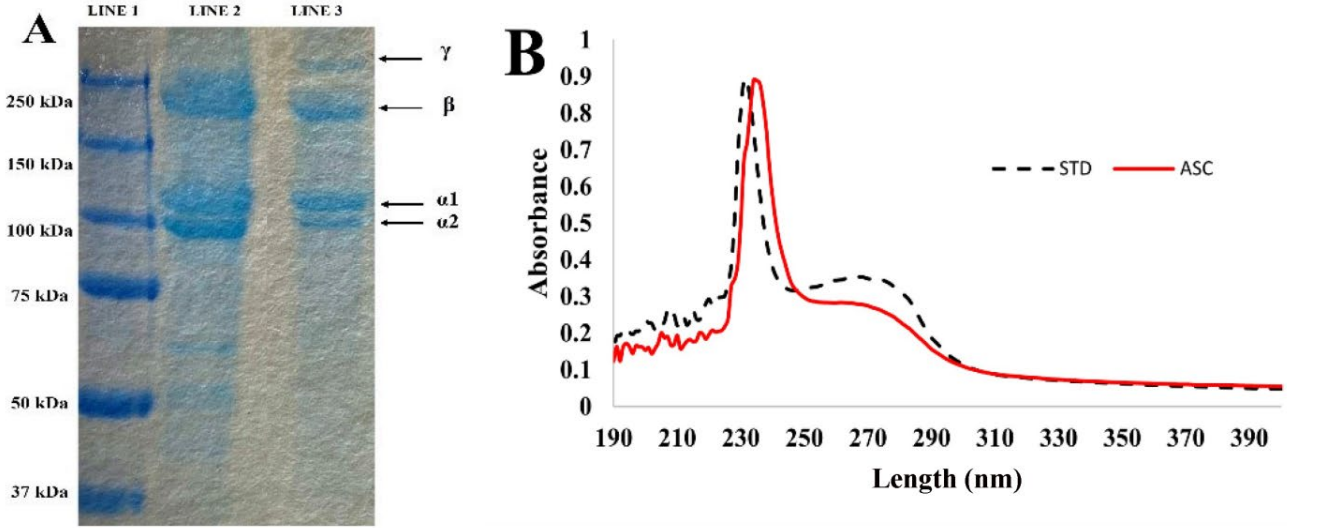

Figure 1. SDS-PAGE and UV-visible spectrophotometric analysis of ASC obtained from *Pterygoplichthys* spp. skin. (A) Line 1: molecular weight marker. Line 2: collagen type 1 standard (STD). Line 3: extracts from ASC. (B) UV-visible spectrum. Continuous red line: acid-soluble collagen from *Pterygoplichthys* spp. Dotted black line: collagen type 1 standard.

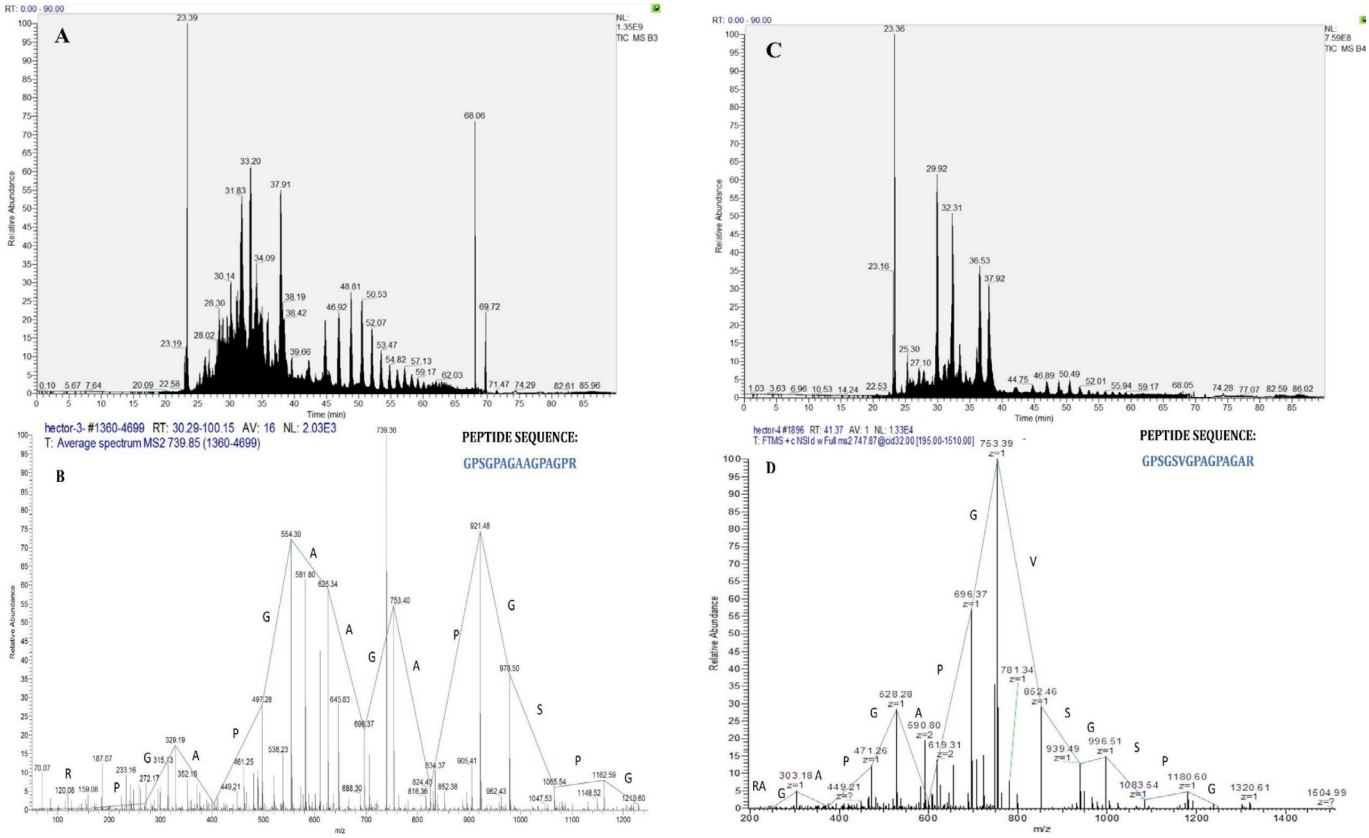

Figure 2. Sequencing of collagen peptides. (A) Chromatogram of total ions released from the α1 chain of *Pterygoplichthys* spp. after 18 h of enzymatic digestion. (B) MS/MS spectrum corresponding to ion 739.36. (C) Chromatogram of total ions released from the α2 chain. (D) MS/MS spectrum corresponding to the 747.87 ion released from the α2 chain.

in *Pterygoplichthys* spp. collagen (Abinaya & Gayathri, 2019; Nurubhasha et al., 2019; Zeng et al., 2012). Nurubhasha et al. (2019) reported analogous findings, obtaining collagen from the same species with a maximum absorbance at 235 nm.

3.3 Collagen identification (amino acid sequencing)

Peptide sequence identification by LC-MS/MS of collagen is shown in Figure 2. Figure 2A shows the total ion chromatogram of the peptide mixture released after the enzymatic digestion performed on the identified α1 band in SDS-PAGE (Figure 1A); this peptide mixture was useful to corroborate the type of collagen obtained (Kleinnijenhuis et al., 2022; Zhang et al., 2006). The mass spectrum was implemented to determine the m/z of the ions and their charge states (Figure 2B), where the ion with m/z 739.36 retained at minute 30.29 in the ion chromatogram was manually identified to avoid assignment errors as GPSGPAGAAGPAGPR (Kleinnijenhuis et al., 2022; Richter et al., 2020). A molecular weight of 1,477.72 Da was manually calculated for this peptide. The ion had a charge of +2, indicating that the peptide is charged differently (Chen et al., 2018), that is, there are peaks with m/z above 739.36 as seen in the MS/MS spectrum (Figure 2B), and the 921.48 peak was the most abundant peak generated from the cleavage of the Pro-Gly peptide bond. Both peaks 753.40 and 978.50 were of relatively high abundance and formed from the cleavage of Gly-Ala and Gly-Ser peptide bonds, respectively. The peak at m/z 1162.29 generated by cleavage of the Pro-Gly peptide bond had a relatively low abundance. The peptide (GPSGPAGAAGPAGPR) was found in the α1 chain of type 1 collagen of *Ictalurus punctatus* (Table 1). The digested mixture of the α2 chain identified in SDS-PAGE (Figure 1A) was analyzed using the same method as above; according to the mass spectrum (Figure 2D), the peptide GPSGSVGGPAGPAGPAGAR was identified with an m/z of 747. 8, a molecular weight of 1,542.80 Da, and a charge of +2, which was found in the α2 chain of type 1 collagen from *I. punctatus* (Table 1), and its retention peak was identified at minute 41.37 (Figure 2C).

SDS-PAGE was used to analyze the composition and molecular weight distribution of the collagen obtained. The enzymatic digestion of the chains identified in SDS-PAGE (Figure 1A) was performed, followed by analysis with LC-MS/MS for the amino acid sequences of collagen. Additionally, BLAST analysis was used to study the homology of the sequences, where the number of coincident peptides and the coverage of the collagen chain sequences were evaluated as the main parameters for their identification (Chen et al., 2017; Chinh et al., 2019; Huang et al., 2015; Zeng et al., 2012).

Two matching peptides were identified from the α1 chain of type 1 collagen reported in species such as *I. punctatus*, *Ictalurus furcatus*, *Oreochromis niloticus,* and *Cyprinus carpio* (Table 1), in which their coverage in the α1 chain sequence was 13% for each species, similar to that reported by Zeng et al. (2012). Furthermore, variations between peptide positions were found to be given by only one unit, that is, the GPAGPAGAAGPAGPR peptide was found at position 1,056–1,070 for *I. punctatus* and at position 1,054–1,068 for *O. niloticus*.

Harvey et al. (2018) mentioned that the ion found in MS/MS is known as a collagen precursor ion because the peptide repeats, that is, it can be found in the collagen of other fish species. However, Kleinnijenhuis et al. (2022) mentioned that the

Table 1. Summary of LC-MS/MS results based on those obtained by SDS-PAGE.

Experimental protein	Protein description	No. adhesion	Mass (Da)	Score	Number of peptides	Matched amino acid sequence
ASC alpha 1	Collagen type 1 alpha 1 (*Ictalurus punctatus*)	XP_017348849.2	137,314	3,487	2	604 GAPGAPGPA 612 1057 GPSGPAGAAGPAGPR 1071
	Collagen, type 1, alpha 1 (*Ictalurus furcatus*)	XP_053469073.1	137,260	3,478	2	603 GAPGAPGPA 611 1056 GPSGPAGAAGPAGPR 1070
	Collagen type 1 alpha 1 (*Oreochromis niloticus*)	NP_001266373.1	134,883	3,444	2	1054 GPSGPAGAAGPAGPR 1068 1027 GAPGAPGP 1034
	Collagen, type 1, alpha 1 (*Cyprinus carpio*)	XP_042576683.1	136,931	2,825	2	602 GAPGAPGPA 610 1055 GPSGPAGAAGPAGPR 1069
ASC alpha 2	Collagen type 1, alpha 2 (*Ictalurus punctatus*)	XP_047006669.2	126,437	3,757	2	479 PGNIGFPGPK 488 972 GPSGSVGPAGPAGAR 986 853 GPAGPPGA 860 305 PGVAGTPGF 313
	Collagen type 1, alpha 2 (*Ictalurus furcatus*)	XP_053468726.1	126,608	3,742	2	479 PGNIGFPGPK 488 972 GPSGSVGPAGPAGAR 986 853 GPAGPPGA 860 305 PGVAGTPGF 313
	Collagen type 1, alpha 2 (*Oreochromis niloticus*)	NP_001269826.1	124,065	2,137	4	478 GNIGFPGPK 486 877 G-VGEPGR 884 886 VGPAGPPGA 894 770 PPGLTGFP 777
	Collagen type 1, alpha 2 (*Cyprinus carpio*)	XP_042601316.1	127,304	1,967	4	479 PGNIGFPGPK 488 879 G-VGEPGR 886 815 VGPAGPPG 822 772 PPGLTGFP 779

content and variation of amino acids in the collagen sequence is different between one species and another; therefore, in other sequences, the change in amino acid could be found, for example, GSAGPAGAGAAGPAGPR and GPAGPAGAAGPAGPR, where S is the change (Huang et al., 2015). Furthermore, four peptides of the α2 chain of collagen type 1 reported in the species *I. punctatus*, *I. furcatus* and four peptides for the species *O. niloticus* and *C. carpio* were identified (Table 1); these results are superior to those reported by Chen et al. (2017) and Zeng et al. (2012). The sequence coverage of the collagen chain was 6%; it was found that the PGNIGFPGPK peptide has a variation of one unit between *I. punctatus* and *C. carpio*, while for the other peptides, the position variations were more than one unit (Chen et al., 2018; Huang et al., 2015; Kleinnijenhuis et al., 2022).

The results presented above revealed that the extracted collagen possessed a higher similarity to the species mentioned above in relation to the α1 and α2 chains of type 1 collagen. The relative molecular weights (α1: 136,656 Da; α2: 126,472 Da) are analogous to the SDS-PAGE results (α1: 130,000 Da; α2: 120,000 Da), indicating that the collagen extracted corresponds to a type 1 collagen (Chen et al., 2018). Furthermore, based on the percentage of protein sequence coverage, it can be confirmed that the amino acid sequence of the collagen extracted from the skin of *Pterygoplichthys* spp. was very similar to that of *I. punctatus*.

4 CONCLUSION

The results showed that the extraction process in 0.5 M acetic acid for 72 h at pH 2.5 implemented the skin of *Pterygoplichthys* spp. with a 0.1 M sodium hydroxide pretreatment for 6 h at pH 11.0 that allowed us to obtain a recovery yield of 43.05% of collagen. The collagen extracted from *Pterygoplichthys* spp. was characterized chemically and identified as type 1 collagen, confirmed by SDS-PAGE, UV-visible, and LC-MS/MS techniques. The presence of two alpha chains (α1 and α2) and β and γ components supports the typical structure of type 1 collagen. *Pterygoplichthys* spp. skin is a promising source of type 1 collagen, being a viable alternative for the use of mammalian-derived collagen used in the food, cosmetic, and biomedical industries. It is important to point out that future studies are required to assess its complete effectiveness in terms of extraction since this work constitutes a preliminary alternative for the exploitation of a fish that generates environmental and economic problems in the areas where it is present.

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