

Swim Bladder of Farmed *Totoaba macdonaldi*: A Source of Value-Added Collagen

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Research Article

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Abstract

Purpose

Finding strategies to use swim bladder of farmed totoaba (*Totoaba macdonaldi*) is of utmost need to reduce waste. Fish swim bladders are rich in collagen; hence, extracting collagen is a promising alternative with benefits for aquaculture of totoaba and the environment.

Methods

The elemental biochemical composition of totoaba swim bladders, including proximate composition and amino acid composition were determined. Acid-enzyme solubilisation (PSC) was used to extract collagen from swim bladders and its characteristics were analyzed. The alcalase and papain were used for the preparation of collagen hydrolysates.

Results

Swim bladders contained 95% protein, 2.4% fat, and 0.8% ash (dry basis). The essential amino acids content was low, but the functional amino acids content was high. The PSC yield was high, 68% (dry weight). The amino acid composition profile, electrophoretic pattern, and structural integrity analyses of the isolated collagen suggested it is typical type-I collagen with high purity. The denaturalization temperature was 34.5 °C, probably attributable to the imino acid content (205 residues/1000 residues). Papain-hydrolysates (<3 kDa) of this collagen exhibited higher radical scavenging activity than Alcalase-hydrolysates.

Conclusions

Swim bladder from farmed totoaba is an ideal raw material for producing high-quality type-I collagen and a viable alternative to conventional collagen sources.

Statement of Novelty

To our knowledge, this paper is the first to examine the composition and characteristics of collagen of swim bladder from *Totoaba macdonaldi*. Although the processing currently wastes bladders, this study showed that they could be a potential source for producing high-quality type-I collagen.

Introduction

Totoaba (*Totoaba macdonaldi*, Sciaenidae) is a large croaker (up to 2 m long, and 100 kg weight) native to the upper Gulf of California (UGC), Mexico. Once among the most valuable fisheries in the region, *T. macdonaldi* is currently listed as a critically endangered species according to Mexican law (NOM-059-SEMARNAT-2010) and is included in the CITES Appendix I [1]. Originally developed for ecological

purposes (conservation and re-stocking), totoaba culture has attracted the attention of investors due to the high value of its meat [2].

The totoaba aquaculture industry in Mexico was established eleven years ago, and now consists of seven licensed producers registered as Wildlife Conservation Management Units (Unidad de Manejo Ambiental - UMA) for commercial production of totoaba (≈ 5 kg and 3-years-old) for human consumption [2]. Current market demand is approximately 20 tons per week and is expected to increase annually (UMA-Acuario Oceanico, personal communication). Processing totoaba for meat results in by-products such as swim bladders, the market potential of which is unknown. The dried swim bladder of adult wild totoaba (known as *maw*) has characteristics similar to the swim bladder of wild Chinese bahaba (*Bahaba taipingensis*). Both are considered highly nutritious and valuable food in Southeast Asia and China, and they are widely used as tonic foods in traditional Chinese medicine to improve brain function, treat insomnia and dizziness, and support postnatal recovery [3, 4]. The value of totoaba *maw* in Asian markets is based on bladder size and thickness, and fish age, meaning *maws* from small totoaba would not be highly valued [4, 5]. However, by-products from the processing of totoaba for meat may contain beneficial compounds (i.e., collagen from the swim bladder) with potential market value. Isolating these would help in processing waste and add value to totoaba production.

Collagen is a dominant fibrous protein in connective tissue such as skin, cartilage, bone, and other animal organs [6]. Type I collagen is widely used in the food, cosmetics, pharmaceutical, and tissue engineering industries [7]. Livestock (cattle and pigs) are the primary sources of commercial collagen. However, factors such as fear of disease transmission (zoonoses), as well as the high cost of pure collagen, drive a search for new and safer collagen sources [8, 9]. Aquatic environments are seen as a promising alternative source of collagen because collagen isolated from marine and freshwater fish exhibits weak antigenicity, reduced risk of disease transmission, among other advantages [7]. Collagen has been isolated mostly from fish skin and occasionally from swim bladders. The swim bladder of some fish species is a valuable source of bioactive compounds, mainly collagen [8]. There are various reports on preparation methods for collagen extraction from several marine and freshwater fish species and the biochemical characteristics of the extracted collagen [10–14]. All these collagens are Type I, although significant interspecies variation exists in collagen biochemical properties, including amino acid composition and thermal stability (denaturation temperature).

Collagen extraction method efficiency is critical. Extraction with acidic solutions produces low yields of acid soluble collagen (ASC). Pepsin has been used during the extraction process to increase collagen yield (called pepsin soluble collagen, PSC) and decrease toxicity from telopeptides [15]. For instance, ASC and PSC were extracted from the swim bladder of miiuy croaker (*Miichthys miiuy*) [12] with yields of 1.3% (ASC) and 8.3% (PSC), and catla (*Catla catla*) [8] with yields of 22.2% (ASC) and 62.3% (PSC). Due to its higher yield, the PSC method was used in the present study. Recent studies show that collagen hydrolysates from the swim bladder of the croceine croaker (*Pseudosciaena crocea*) and miiuy croaker exhibit anti-fatigue, anti-amnesic, and antioxidant activities [16, 17]. However, no research has been done

to date on the essential biochemical composition of the totoaba swim bladder or the properties of its collagen.

The objective of the present study was to isolate collagen from the swim bladder of totoaba and evaluate its composition and properties. Swim bladders were extracted from 3-year-old farmed totoaba, analyzed their biochemical composition, pepsin-soluble collagen isolated from them, and evaluated their physicochemical properties. Tests were also done of DPPH radical-scavenging activity of the collagen and collagen hydrolysates.

Materials And Methods

Chemicals

Pepsin from porcine stomach mucosa, dialysis membrane (14 kDa MWCO), DPPH (2, 2-diphenyl-1-picrylhydrazyl), and type I collagen standard solution from calfskin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alcalase® 2.4 L (proteinase from *Bacillus licheniformis*) was donated by Novozymes (Mexico City, Mexico). Papain enzyme from *Carica papaya* (30,000 U/mg) and Amicon ultrafiltration tubes (3 kDa MWCO) were purchased from Merck Corporation (Burlington, MA, USA). The protein marker and bovine serum albumin standard (2 mg mL⁻¹) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Solvents for amino acid analysis were HPLC grade (T.J. Baker, Chemicals, PA, USA). All other chemicals used in this investigation were analytical grade and used as received.

Swim Bladder Collection and Preparation

Totoabas were provided by the UMA (UBP) of the Facultad de Ciencias Marinas (FCM), Universidad Autónoma de Baja California (UABC), Mexico, where this study was performed. A total of 24 fish (average body weight = 2.57 ± 0.264 kg; 3-years-old) were randomly sampled from three, eight thousand-liter tanks (i.e. 8 fish/tank). The tanks were supplied with continuous recirculated seawater at a 1.6 L min flow rate. During cultivation the physical and chemical water parameters were monitored twice daily to maintain recommended conditions for totoaba culture. Temperature was controlled at 27 ± 1°C with thermo-control of chillers. Salinity was measured with a refractometer and maintained at an average of 35 ± 0.5‰. Photoperiod was set at a 12:12 light:dark ratio. Oxygen concentration was kept higher than 6 mg L⁻¹. Before feeding, total ammonia-nitrogen (NH₄-N) and total nitrite-nitrogen (NO-N) were measured daily with colorimetric test kits (Aquarium Pharmaceutical, Mars, PA, USA), and maintained below 0.2 and 0.1 mg L⁻¹, respectively.

Fish were sedated using clove oil solution (40 mg L⁻¹) and euthanized by pithing to avoid distress and suffering following applicable national animal welfare guidelines (NOM-033-ZOO-1995). The fish were dissected to manually remove the swim bladder, which was transported under refrigeration (3 ± 1°C) to the Aquaculture Nutrition Laboratory. Processing byproducts represented 53% of total fish weight, and the swim bladder accounted for approximately 11% of the total byproduct. Blood vessels and residual fat

attached to the swim bladder were removed manually, the bladders cleaned, and cut into small pieces (0.4 kg/bag). These pieces were used in the proximate analysis and collagen extraction.

Proximate Analysis

Swim bladder moisture, protein, and ash contents were quantified using established methods [18]. Moisture content was measured by weight difference after drying (105°C for 12 h), and ash content by combustion in a furnace at 550°C for 12 h. Total lipids were measured using a modification of the Folch extraction method, replacing chloroform with less toxic dichloromethane [19]. Total nitrogen content was measured with the Kjeldahl method in a Vapodest 450 analyzer (Gerhardt Analytical Systems Co., Königswinter, Germany). Crude protein was calculated using a 6.25 conversion factor.

Amino Acids Analysis

Swim bladder amino acid composition was analyzed with the PicoTag method (Waters Corp., Milford, MA, USA). Samples were hydrolyzed with 6 N hydrochloric acid and 0.1% phenol and incubated in a nitrogen atmosphere at 110°C for 22 h. After hydrolysis, samples and standards were derivatized with phenyl isothiocyanate (PITC) reagent and reconstituted in a sodium phosphate buffer (5 mM, pH 7.4) containing 5% (v/v) acetonitrile. The derivatives were analyzed by reverse-phase chromatography (RP-UHPLC) in an Ultimate 3000 UHPLC system (Thermo Scientific) with Chromeleon software 7.2 (Chromatography Data System). Five μL of the samples were injected into a Pico-Tag[®] C18 column (3.9 mm \times 150 mm, 4 μm and 60 \AA). Separation of the amino acids was done with a binary gradient using AccQ-Tag Eluent as mobile phase A (Waters Inc.) and aqueous acetonitrile as phase B (60% (v/v) in water) in the following gradient mode: 0.0% B at 0.0 min; 46% B for 10 min; then 100% B at 10.5 min; 100% B at 12 min; and returning to 0.0% B at 12.5 min. Total run time was 22 min with a 1.0 mL min flow rate. Column temperature was set at 38°C and UV detection done at 254 nm, which was used for calculation. Amino acid identification and quantitation was performed using a standard amino acid mixture as reference.

Swim Bladder Collagen Isolation and Purification

Pre-Treatment

Collagen extraction was done using the protocol described previously [11, 20]. All pre-treatment steps were done at 4°C under gentle continuous stirring for 12 h. The swim bladders were pre-treated to remove non-collagenous proteins, pigments, fats, and other impurities. Swim bladders were submerged in 0.1 M NaOH kept at a 1:20 ratio (w/v), then washed with cold distilled water until all the alkaline solution was eliminated. The tissue was degreased with 10% (v/v) *n*-butanol at a 1:20 (w/v) sample/solvent ratio. Both the alkaline wash and degreasing steps were performed by changing solutions at 4 h intervals.

Collagen Extraction and Purification

The pre-treated swim bladders were digested in 0.5 M acetic acid containing 2% pepsin (w/w) at a 1:40 (w/v) tissue/solution ratio. The mixture was continuously stirred at 4°C for 24 h. After digestion, the

viscous extract was filtered with two layers of cheesecloth and precipitated by adding NaCl to a 1.2 M final concentration. The precipitate was collected by centrifuging at $16,000 \times g$ at 4°C for 20 min using a Megafuge 16R centrifuge (Thermo Scientific Co., Waltham, MA, USA), and the resulting pellets dissolved in 0.5 M acetic acid. This solution was purified using a dialysis membrane (14 kDa molecular weight cut-off) against distilled water for 72 h with a change of solution every 4 h. The resulting collagen was lyophilized (Free Zone 2.5L, Labconco Corp., Kansas, MO, USA) and stored at -20°C until further analysis.

Collagen yield was calculated based on wet and dry weight of the raw material before and after processing, using Eq. (1).

$$\text{Yield (\%)} = \left[\frac{W_c \text{ (g)}}{W_d \text{ (g)}} \right] \times 100 \quad (1)$$

where W_c is the weight of the lyophilized collagen and W_d is the dry weight of the initial swim bladder prior to pre-treatments.

Collagen Characterization

Amino Acid Composition

Amino acid composition of the lyophilized collagen was analyzed as described in section of Amino Acids Analysis. Amino acid quantification was expressed as the number of residues per 1000 total residues. Hydroxylation of proline (Pro) and lysine (Lys) was calculated from the amino acid composition using Eq. (2 and 3):

$$\text{Degree of Pro hydroxylation (\%)} = \left[\frac{\text{Hydroxyproline content}}{\text{Hydroxyproline content} + \text{Proline content}} \right] \times 100 \quad (2)$$

$$\text{Degree of Lys hydroxylation (\%)} = \left[\frac{\text{Hydroxylysine content}}{\text{Hydroxylysine content} + \text{Lysine content}} \right] \times 100 \quad (3)$$

Electrophoretic Pattern

Collagen molecular weight (MW) was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method [21]. Briefly, the lyophilized collagen was dissolved in 0.1 M acetic acid and mixed at a 1:2 (v/v) ratio with sample buffer (0.5 M Tris-HCl, pH 6.8, containing 5% SDS, 20% glycerol, 5% β -ME and 0.2% bromophenol blue). The mixed solution was incubated at 95°C for 5 min. A 10 μL sample was processed with discontinuous polyacrylamide gel electrophoresis (7.5% separator and 4% stacking). A molecular weight protein marker was used to estimate collagen MW. The electrophoresis analysis was run at a 25mA constant current voltage, using a Mini-Protean apparatus (Bio-Rad Laboratories, UK). Protein bands were stained using Coomassie Brilliant Blue R250 solution.

Ultraviolet Measurements

Spectra measurement was done using a Multiskan GO spectrophotometer (Thermo Scientific). The lyophilized collagen was dissolved in 0.1 M acetic acid at a 0.1 mg mL⁻¹ concentration, under continuous stirring at 4 °C for 12 h. The sample solution was placed in a quartz cell with a 10 mm path length. The UV spectrum was measured at wavelengths between 200-450 nm. The baseline was set with 0.1 M acetic acid, and control standards (bovine serum albumin and collagen type I from calf skin) run under the same conditions.

Fourier Transform Infrared (FTIR) Analysis of Functional Groups

The FTIR spectra for collagen were measured using a Thermo Nicolet Nexus 670 FTIR spectrometer (Thermo Scientific). Lyophilized collagen (5 mg) was mixed with 100 mg dried potassium bromide (KBr) and compressed under dry conditions. A salt disc was inserted into the sample holder and scanned 40 times from 4000-400 cm⁻¹ with a resolution of 2 cm⁻¹ and compared to a background spectrum recorded from the empty cell at room temperature. The results were plotted between absorbance and wave number (cm⁻¹).

X-Ray Diffraction (XRD) and Circular Dichroism (CD)

The collagen crystal structures were determined using an X-ray diffraction instrument (Bruker D8 Advance DaVinci, Germany) equipped with CuK α radiation ($\lambda = 1.5406 \text{ \AA}$), 40 kV tube voltage and 40 mA current. The scans were recorded in the 2θ (2 theta) range between 3 and 60°, at 0.02°/s steps. The CD spectrum was recorded to quantify preservation of the collagen secondary structure. The collagen solution was placed in a quartz cell (10 mm) and CD spectra measured using a spectrometer (J-1500, JASCO, Tokyo, Japan). The lyophilized collagen was dissolved in 0.1 M acetic acid at a 0.1 mg mL⁻¹ concentration and continuously stirred at 4°C for 24 h. Collagen solutions were then placed in a quartz cell with a 10 mm path length. The CD spectra were recorded between 180 and 240 nm at 4°C at a 50 nm/min scan speed with a 0.1 nm interval. Collagen denaturation temperature (T_d) was measured by running a rotatory angle at a fixed 222 nm wavelength, within a 10-50°C temperature range at a 1°C/min heating rate.

Protein Solubility

Solubility was measured following an established method [22]. Briefly, lyophilized collagen was dissolved in 0.1 M acetic acid to a final concentration of 3 mg mL⁻¹. The mixture was stirred for 3 h at 4°C and centrifuged at 15,000 × g for 15 min (Megafuge 16R, Thermo Scientific). Supernatant pH was adjusted (1M NaOH or HCl) to obtain a final pH ranging from 2-10 (final volume 5 mL) and centrifuged at 15,000 × g for 15 min at 4°C. Supernatant protein concentration was determined based on the Bradford method. Bovine serum albumin (BSA) was used as the standard and control. Relative solubility was calculated by comparison with the solubility obtained at the pH exhibiting the highest solubility.

Zeta Potential

Lyophilized collagen was dissolved in 0.1 M acetic acid to a final concentration of 0.1 mg mL⁻¹ and the mixtures continuously stirred at 4°C for 24 h. Collagen solution pH was adjusted to a 2-10 range using NaOH and HCl (1M). One milliliter collagen solution was transferred to a capillary cell, and collagen Zeta (ζ) potential measured using a Zeta potential analyzer (Zetasizer Nano ZS90, Malvern Instr., UK). The isoelectric point was identified.

Collagen Hydrolysate

Enzymatic Hydrolysis

The collagen hydrolysate was prepared by first dissolving the collagen in ultrapure water (1:30, w/v), and denaturing it at 50°C for 10 min in a water bath. Hydrolysis conditions were 50°C and pH 8 for Alcalase[®], and 50°C and pH 7 for papain. Hydrolysis was initiated by adding protease to the mixture at a 2% (w/w) E/S ratio. Enzymatic hydrolysis was done in a water bath at 50°C for 5 h. After incubation, the enzymes were inactivated by heating the sample to 95°C for 10 min, and the undigested collagen precipitated by centrifuging at 10,000 × g for 10 min at 4°C. The supernatant of both hydrolysates was collected and ultrafiltered in an Amicon ultrafiltration unit (Merck Inc., Burlington, MA, USA) with a 3 kDa molecular weight cut-off (MWCO). The ultrafiltered fraction (<3 kDa) was collected, lyophilized, and labelled as Alcalase[®] (HCA) or papain (HCP) collagen hydrolysate.

Peptide Chromatographic Profile

Five microliters of each hydrolysate (3.2 mg mL⁻¹) were injected into a BEH300 C18 (5 μm 4.6 × 250 mm column, Waters Inc.) attached to an Ultimate 3000 UHPLC system (Thermo Scientific). Peptides were eluted using water as mobile phase A and acetonitrile as mobile phase B, following a gradient method of 0 to 100% phase B over 35 min with a 1 mL min flow. Column temperature during the run was set at 30°C. The peptides were analyzed at 215 nm.

DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging method was applied according to Lee [23]. Briefly, samples were dissolved in deionized water at 3.2 mg mL⁻¹, and a 50 μL sample mixed with 50 μL 0.120 mM DPPH in a 96-well microplate. This solution was mixed vigorously and left to stand at room temperature in darkness for 30 min. Sample absorbance was measured at 517 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific). Ascorbic acid was used as the reference. Percentage DPPH radical scavenging activity was calculated using Eq. (4):

$$\text{DPPH scavenging activity (\%)} = [(A_0 - A_{b1})/A_0] \times 100 \quad (4)$$

Statistical Analysis

Data were expressed as the mean \pm standard deviation of three replicates. All statistical analyses were run with the STATISTICA software (Version 12, TIBCO Software Inc., Palo Alto, CA, USA).

Results And Discussion

Swim Bladder Biochemical Composition

The swim bladder represented 2% of total body weight in totoaba and contained $67.43 \pm 1.24\%$ moisture. Crude protein content (dry weight) was $95.72 \pm 1.07\%$, lipids were $2.46 \pm 0.18\%$, and ash was $0.88 \pm 0.06\%$. Moisture content was low compared to other species: 75.20% in bighead carp (*Hypophthalmichthys nobilis*) [11]; 83.33% in yellowfin tuna (*Thunnus albacares*) [10]; 78.83% in miiuy croaker [12]; and 82.8% in catla [8]. These differences may be attributed to variations in swim bladder water content during tissue processing and storage, as well as biological factors. The low total lipids and minerals contents of the totoaba swim bladder are comparable to those of the miiuy croaker [12]. Protein content was higher than reported for catla (83.0%) [8] and miiuy croaker (90.55%) [12].

Proximate composition in fish can depend on many factors, including seasonal variations in feeding behavior, age, and habitat. Nineteen amino acids were identified in the totoaba swim bladders and the amino acids profile showed collagen to be the predominant protein, which coincides with the swim bladders of other fish species [24]. Glycine was the most abundant amino acid, followed by alanine, proline, arginine, glutamic acid, hydroxyproline, and aspartic acid, which represented 85% of total amino acids (AA). Its amino acids composition showed the totoaba swim bladder to be nutritionally poor since it contained only 12% essential AA compared to 53% conditionally essential and 35% non-essential (35%) amino acids. However, it is rich in functional AA (71%), such as glycine, glutamic acid, aspartic acid, proline, alanine, and arginine, all of which participate in and regulate key metabolic pathways [25]. It also contains high levels of hydrophobic amino acids which are frequently found in antioxidant peptides [26]. Overall, totoaba swim bladder had high protein and low lipids contents, suggesting its potential use in collagen extraction, as a source of functional AA, or a substrate for bioactive peptide production.

Collagen Yield

Hydrolysis time for PSC extraction can be as long as 72 h [11, 27], so four extraction times were used in the present study (20, 24, 32, and 72 h) (Fig. 1a). During the extraction process, swim bladder collagen fibers were solubilized entirely in acetic acid upon proteolysis with pepsin (24 h) with good collagen yields ($68.18 \pm 1.62\%$, dwb). According to previous studies, intermolecular cross-links in the telopeptide region and triple helices formed via condensation of aldehyde groups cause a decrease in collagen solubility [10], and the pepsin cleaves specifically on the telopeptide region, leading to isolated tropocollagen molecules. For this reason, pepsin is the principal protease used for increasing collagen extraction efficiency and reducing the collagen antigenicity caused by telopeptides [14, 15]. Thus, the PSC yields from totoaba swim bladder are comparable to those of Gulf corvina (*Cynoscion othonopterus*) (69%) [20] and significantly higher than PSC yields from other species: miiuy croaker (8%), yellowfin tuna (12%), giant croaker (15%) (*Nibea japonica*), bester sturgeon (38%) (*Huso x Acipenser ruthenus*), catfish

(40%) (*Tachysurus maculatus*), rohu (47%) (*Labeo rohita*), bighead carp (59%) and catla (61%) [8, 10–12, 14, 15, 22, 28]. Interestingly, collagen yield from totoaba and other fish species is lower than the 85.3% (dwb) ASC yield reported for seabass (*Lates calcarifer*) swim bladder [29], suggesting that seabass swim bladder may have less cross-linked collagen fibers. These differences in collagen yield are probably due to extraction conditions, swim bladder firmness (i.e., degree of cross-linking), animal age, nutrition, and development conditions (wild or farmed). Protein content in the totoaba swim bladder collagen (TSBC) was $96.34 \pm 1.19\%$, ash content was $0.83 \pm 0.09\%$, and no fat was detected, which is consistent with collagen from miiuy croaker [12].

Collagen Characterization

Amino Acid Composition

All collagens have a general (Gly-X-Y)_n sequence in their polypeptide chains, so glycine can be expected to be the main amino acid [6]. This agrees with the present results in that TSBC glycine content was 309 /1000 residues, followed by alanine (132 /1000 residues) and proline (122 /1000 residues) (Table 1). Low levels of tyrosine, histidine, isoleucine, hydroxylysine, and methionine were observed, and cysteine was not detected, which is reported for collagens [12,25,26]. Aromatic amino acids, mainly tyrosine, are generally found in low concentrations in PSC [22]. Compared to PSC isolated from Gulf corvina and miiuy croaker swim bladders (family, Sciaenidae) [12, 20], the TSBC had higher levels of aspartic acid, glutamic acid, proline, and alanine but lower levels of valine, threonine, isoleucine and leucine (Table 1). This variation in amino acid content could be due various factors, such as fish species biology (health state and age), environment (water temperature and feeding), and habitat (wild or farmed). Imino acid (proline and hydroxyproline) content in the TSBC was 205 /1000 residues (Table 1), which is consistent with miiuy croaker swim bladder collagen [12]. Imino acid content has been reported to positively affect collagen structural stability because the pyrrolidine ring imposes restrictions on polypeptide chain conformation, thus strengthening the triple helix structure [30, 31]. The degree of hydroxylation of proline (41%) and lysine (16%) also influences collagen self-assembly and stabilization [32].

Table 1. Amino acid composition of swim bladder (composition percentage) and swim bladder collagen from farmed totoaba (residues/1000 residues).

Amino Acids	Swim Bladder	TSBC ¹	Gulf corvina PSC ²	PSC Miiuy croaker ³
Asp	5.13 ± 0.05	52 ± 1.48	38	39
Glu	9.61 ± 0.14	101 ± 1.95	79	85
Hyp	5.43 ± 0.05	83 ± 1.43	81	88
Ser	2.02 ± 0.08	23 ± 0.69	34	28
Gly	29.19 ± 0.31	309 ± 3.15	303	334
His	0.47 ± 0.02	5 ± 0.34	7	8
Arg	11.58 ± 0.37	59 ± 4.47	70	55
Thr	1.76 ± 0.06	13 ± 2.62	15	22
Ala	12.26 ± 0.13	132 ± 1.27	118	95
Pro	12.10 ± 0.13	122 ± 1.33	106	112
Tyr	0.47 ± 0.03	2 ± 0.19	2	2
Val	1.58 ± 0.04	16 ± 0.30	20	33
Met	1.36 ± 0.03	7 ± 0.47	9	5
Cys	0.03 ± 0.01	Not detected		0.4
Ile	0.63 ± 0.01	5 ± 0.22	8	13
Leu	1.94 ± 0.05	20 ± 0.08	31	27
Hyl	0.28 ± 0.02	5 ± 0.22	9	6
Phe	1.61 ± 0.03	19 ± 0.54	26	23
Lys	2.52 ± 0.04	31 ± 1.63	44	24
Imino acid ³		205	187	199.5
Degree of Hydroxylation (%)				
Pro		40.65		
Lys		14.43		

TSBC¹: totoaba swim bladder collagen. Pepsin-soluble collagen (PSC) from Gulf corvina (*C. othonopterus*)² [20] and miiuy croaker (*M. miiuy*)³ [12]. Imino acid: Proline + Hydroxyproline.

Protein Patterns

Electrophoretic analyses of the TSBC showed it to be composed mainly of two different α chains ($\alpha 1$ and $\alpha 2$), in a 2:1 proportion, and a β chain (Fig. 1b). High molecular weight bands were also observed which correspond to β -chains (dimers) and γ -chains (trimer). This suggests that the TSBC is type I collagen, consisting of heterotrimer ($[\alpha 1(I)]_2\alpha 2(I)$) chains. Swim bladder from other fish species has been reported to contain type I collagen [10,12,30]. Using the GelAnalyzer software, the apparent molecular weights of the TSBC $\alpha 1$ (142 kDa) and $\alpha 2$ chains (134 kDa) were calculated based on migration distance. The extraction process was clearly effective because the collagen preserved its native structure. Moreover, no low molecular weight (<100 kDa) components were observed, suggesting the pepsin cleaved specifically to the telopeptide region, as previously reported [33].

UV-Vis and FTIR Spectroscopy

Maximum absorption for collagen is near 230 nm, due to the peptide bond (R-CONH-R, amide group) of the polypeptide chains [12]. The UV-vis spectrum is therefore an essential parameter for detecting purified collagen [12]. In this spectrum the TSBC exhibited a maximum absorption peak at 228 nm (Fig. 2a), which was similar to collagen from calf skin, grass carp (*Ctenopharyngodon idella*) [34], and miiuy croaker [12]. As expected, neither the TSBC nor the bovine serum albumin (BSA) reference exhibited a peak at 280 nm; in the TSBC this was due to its low aromatic amino acid (tyrosine and phenylalanine) content (Table 1). This result indicates efficient non-collagen protein elimination, and consequent high TSBC purity.

In the FTIR spectra, TSBC showed characteristic bands of amide A, B, I, II, and III (Fig. 2b). Amide absorption bands A and B, which correspond to the stretching vibration of group N-H and asymmetric stretching of CH_2 , were observed in wave numbers 3280 and 3071 cm^{-1} , respectively. Amide I (C=O stretching), amide II (N-H bending and C-N stretching), and amide III (C-N stretching and N-H bending) appeared in frequencies 1629, 1543, and 1237 cm^{-1} , respectively. The absorption ratio between amide III and the 1454 cm^{-1} wavelength was 1.05, indicating preservation of the collagen's triple helix structure. These results coincide with those reported for collagens isolated from other fish species [8, 10–12, 14, 34].

Structural Integrity

The TSBC x-ray diffraction (XRD) spectrum exhibited peaks at 7.7° and 20.02° (Fig. 2c). The former was sharp and corresponded to the triple helix arrangement and distance between molecular chains; the latter was broad and corresponded to the distance between the amino acid residues along the helix [35]. Both peaks were consistent with the characteristic diffraction pattern of the collagen triple helical structure [36]. The circular dichroism (CD) analysis showed the TSBC to have a weak positive absorption peak at 222 nm and a negative one at 197 nm with a crossing point (zero rotation) at 215 nm (Fig. 3a). This CD spectrum pattern is characteristic of the collagen triple helix structure and consistent with previous reports [28]; the 222 nm peak disappears after thermal denaturation [28, 37]. The results confirm the helix

structure of TSBC remained in its native form, and therefore that the isolation process did not affect its molecular integrity.

Thermal Behavior

Measurements of CD molar ellipticity (θ) as a function of temperature have been used to determine denaturation temperature (T_d) [28]. The present CD (222) values decreased by approximately 34.5°C, indicating decomposition of the collagen triple helix structure (Fig. 3b). Specifically, the intramolecular hydrogen bonds that stabilized the secondary structure of the collagen broke, leading to collapse of the triple helix into a random coil [38]. The present results were similar to those reported for collagen isolated from yellowfin tuna swim bladder (33.9°C) [10] and Gulf corvina (32.5°C) [20]. The T_d of TSBC was higher than for collagen from a cold-water fish such as cod (29.6°C) [37], and for a temperate water fish such as miiuy croaker (26.7°C) [12]. Swim bladder collagen from marine fish remains thermostable below 35°C whereas in freshwater fish the threshold is higher: 38°C in grass carp [33] and 39.38°C in catla [8]. Indeed, PSC isolated from the swim bladder of the freshwater fish rohu [14] retains thermal stability at up to 42.16°C, higher than pork skin collagen (37°C) and similar to calfskin collagen [35]. Collagen thermal behavior depends heavily on imino acid content [22, 30], as well as species optimum physiological temperature, which is closely related to its habitat [31, 39]. For the farmed totoaba from UMA, average water temperature is $27 \pm 1^\circ\text{C}$, while under natural conditions surface temperatures in the upper Gulf of California, Mexico, can range from 16 to 31°C on the surface and 13 to 19°C in deep waters (100 to 200 m) [40]. The present TSBC thermal stability result (34.5°C) is probably linked to water temperature in its natural habitat. Possible use of a collagen depends heavily on its thermal stability [41], and the fact that the studied TSBC has thermal stability close to that of terrestrial mammal collagen makes it a promising alternative.

Protein Solubility and Zeta Potential

Acid pH (2.0-4.0) caused higher solubility in the TSBC, but this parameter decreased from pH 5.0-6.0, resulting in protein precipitation (Fig. 3c). Collagen solubility was lowest at around pH 6, but increased slightly in the pH 7.0–10.0 range. This may be due to increased repulsion of collagen molecules as the negative charge increases [22]. Similar results have been reported for PSC from the swim bladder of grass carp [34], miiuy croaker [12], Gulf corvina [20] and giant croaker [22]. Zeta potential is a key marker of colloidal dispersion stability and varies in response to pH [12]. As pH increased in the TSBC suspension, the zeta potential progressively decreased from +27 mV (pH 2) to less than -24 mV at pH 10 (Fig. 3d). At a high magnitude of potential (positive or negative) a solution will resist aggregation, whereas low potential tends to formation of aggregates. For TSBC the zero surface net charge occurred at pH 5.4, this is considered the isoelectric point (pI) and is consistent with the protein solubility results (Fig. 3c). Since the pI occurred at an acid pH, it may be associated with higher contents of glutamic acid and aspartic acid rather than of basic amino acids, such as histidine, lysine, and arginine (Table 2). The pI value was lower than reported for swim bladder collagen from miiuy croaker (6.85) [12] but similar to

that of yellowfin tuna (5.93) [10]. In collagen, the pI is generally closely linked to amino acid composition distribution on its surface.

Collagen Hydrolysate

Totoaba swim bladder has putative positive therapeutic effects in traditional Chinese medicine [5]. Peptides and collagen from croaker swim bladders have been shown to remove free radicals [12,17,20,23]. The peptide profiles of the hydrolysates produced from the TSBC using Alcalase[®] (HCA) and papain (HCP) showed the HCA to have more hydrophilic peptides than the HCP (Fig. 4a). In contrast, the HCP had more hydrophobic peptides when eluted from 10 to 20 minutes. Protein hydrolysates with antioxidant activity frequently contain mainly hydrophobic amino acids, which play a significant role in free radical elimination [26]. Based on this and the present peptide profiles, the HCP was expected to exhibit higher antioxidant activity than the HCA.

The DPPH radical scavenging assay is a popular and efficient way of predicting antioxidant activity since the DPPH radical is more stable than hydroxyl and superoxide radicals [17]. Using the DPPH assay, the antioxidant activity of ultrafiltered fractions (<3 kDa) of the TSBC hydrolysates was tested at 3.2 mg mL⁻¹. Antioxidant activity was 37% higher ($p<0.05$) with the HCP than the HCA, although ascorbic acid far exceeded both (Fig. 4b). This contrasts with the antioxidant activity results of a study of hydrolysates from the swim bladder of croceine croaker and miiuy croaker in which, at 15-25 mg protein mL⁻¹, the Alcalase[®] hydrolysate had significantly higher activity than hydrolysates prepared with papain, pepsin, neutrase and trypsin [26]. Of note is that, after ultrafiltration, a lower concentration of HCA and HCP (3.2 mg mL⁻¹) produced higher antioxidant activity than in the above study. Overall, the present antioxidant activity indicates that this parameter depends strongly on the enzyme used for hydrolysis, suggesting further research is needed to isolate active peptides and clarify their antioxidant activity.

Conclusions

To our knowledge, this study is the first report on the elemental biochemical composition, isolation, and characteristics of collagen from the swim bladder of farmed totoaba (*T. macdonaldi*). Totoaba swim bladder has high protein and low lipid contents, suggesting it as a possible food supplement with health benefits. Collagen yield was high (68%). The amino acid composition and protein pattern were typical of type-I collagen ($[\alpha1(I)]2\alpha2(I)$). Structural integrity analyses confirmed that the extraction process used here preserved the collagen native triple helix structure with high purity. The extracted collagen exhibited good thermal stability (34.5°C), which correlated with its imino acid content. Collagen hydrolysate antioxidant activities were influenced by the enzyme employed, highlighting the need for further research on antioxidant peptide purification and identification. The present results constitute a baseline for future studies on the production of bioactive peptides and biomedical applications for this collagen. The results can also help to promote the use of the swim bladder from farmed totoaba as an alternative to conventional collagen sources or as a functional food, which will reduce by-product generation and provide added value to the culture of totoaba.

Declarations

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Ethical approval

Fish were handled and treated following the technical specifications for the production, care and use of laboratory animals decreed in the Official Mexican Regulation (NOM-062-ZOO-1999) and according to the ethics statement of the Autonomous University of Baja California (UABC), based on international guidelines. All procedures and experimentation conducted with organisms produced at the UMA (DGVS-CR-IN-1084-B.C./09) are annually reported and evaluated by the General Office of Wildlife (Dirección General de Vida Silvestre - DGVS).

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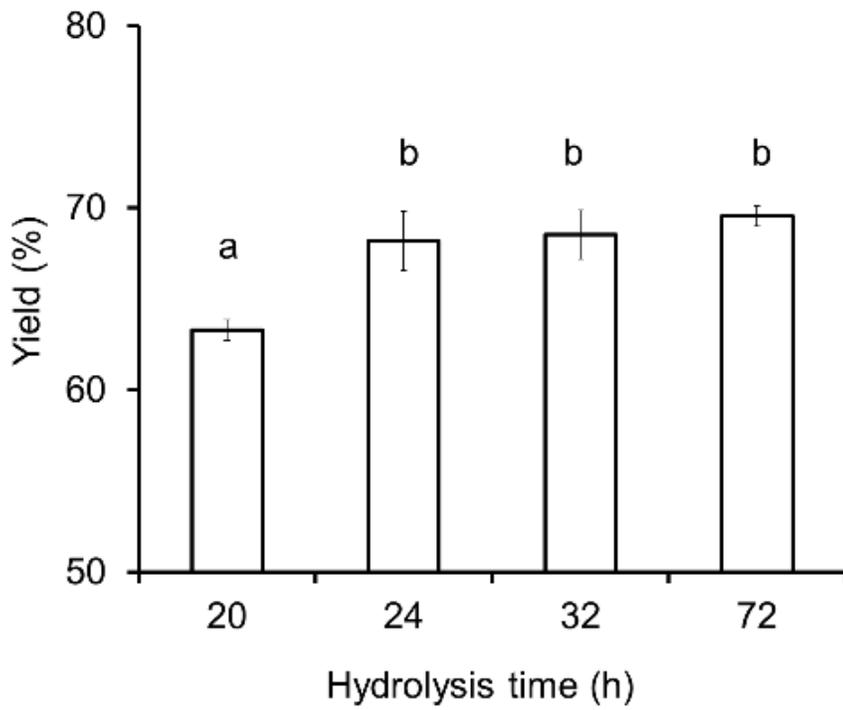
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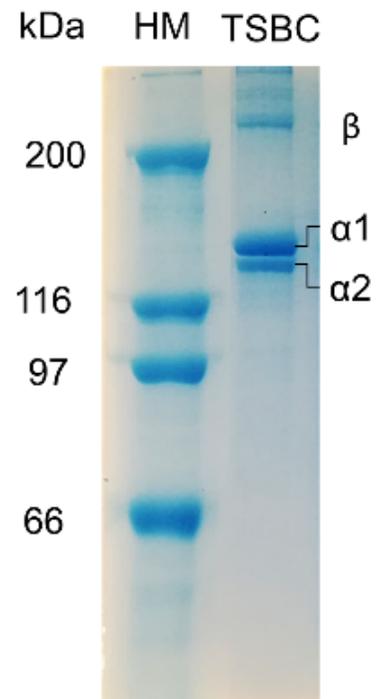
Tables

Table 2 is not available with this version.

Figures



(a)



(b)

Figure 1

(a) Effect of hydrolysis time on pepsin-soluble collagen yield; (b) SDS-PAGE patterns of collagen from the swim bladder of farmed totoaba (TSBC). HM: high molecular weight marker.

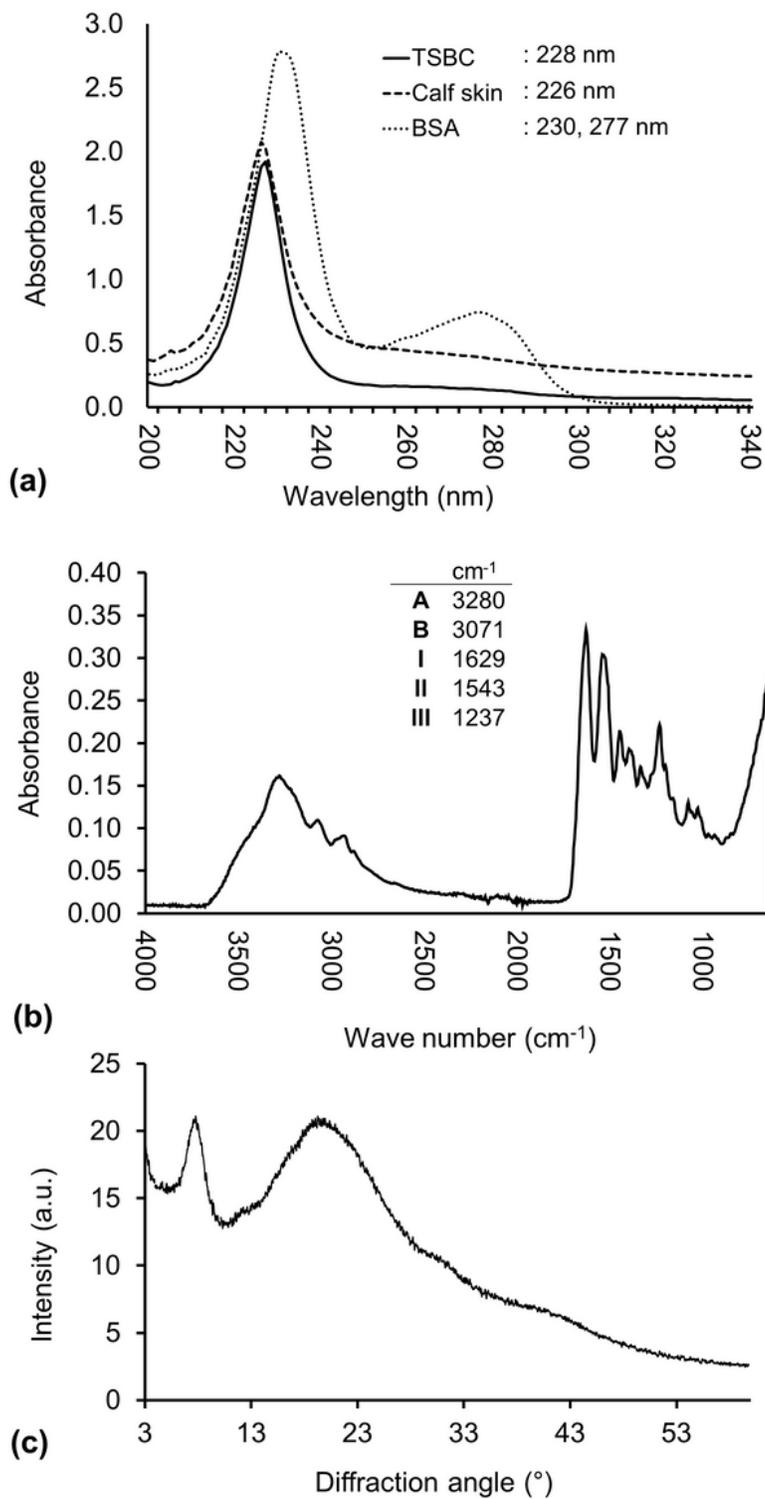


Figure 2

(a) Ultraviolet-visible spectra (UV/Vis); (b) Fourier-transform infrared spectra (FTIR); and (c) X-ray diffraction spectra (XRD) of collagen from the swim bladder of farmed totoaba (TSBC).

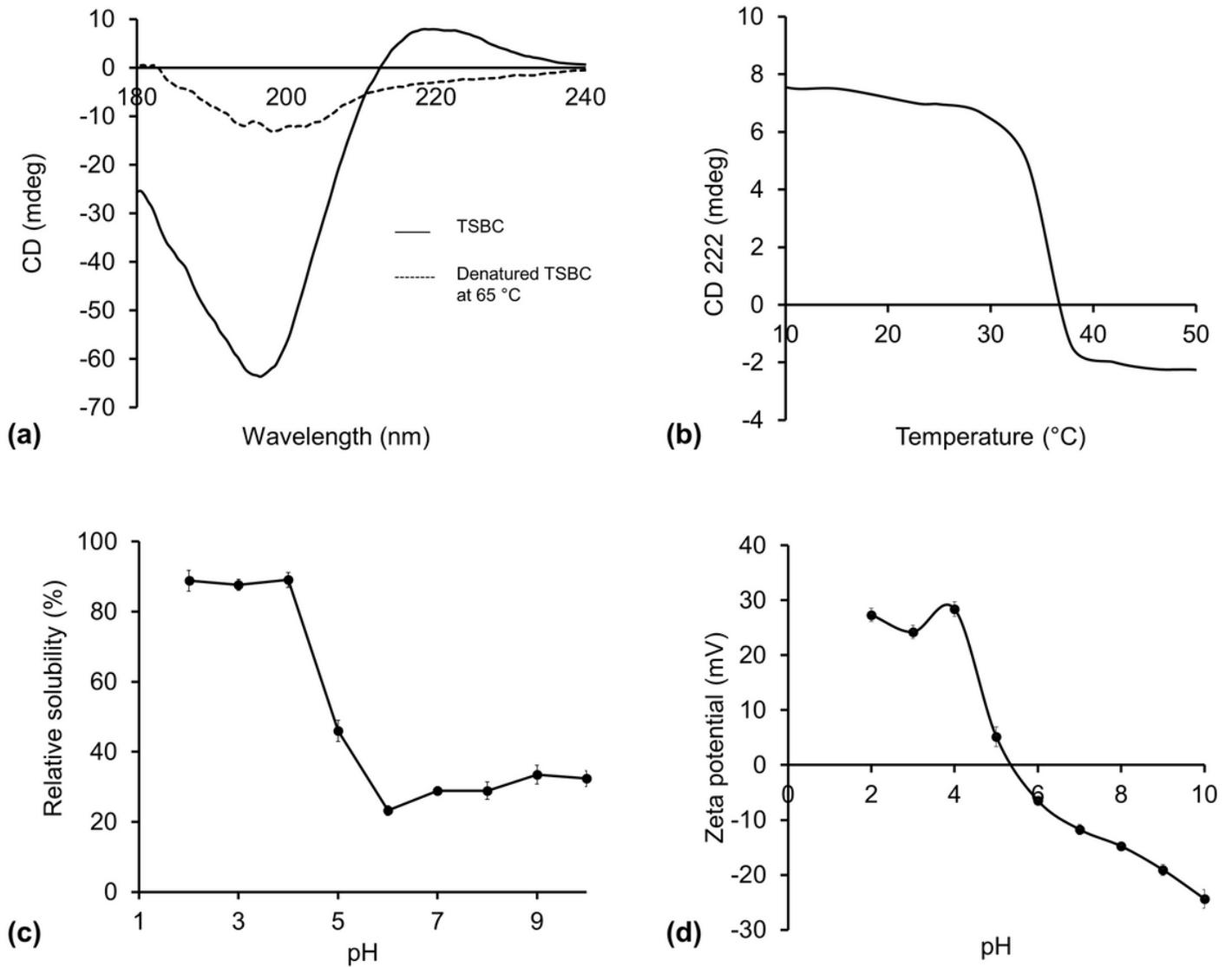


Figure 3

(a) Circular dichroism (CD) spectra; (b) temperature effect on CD spectra at 221 nm; (c) solubility; and (d) zeta potential of collagen from swim bladder from farmed totoaba (TSBC).

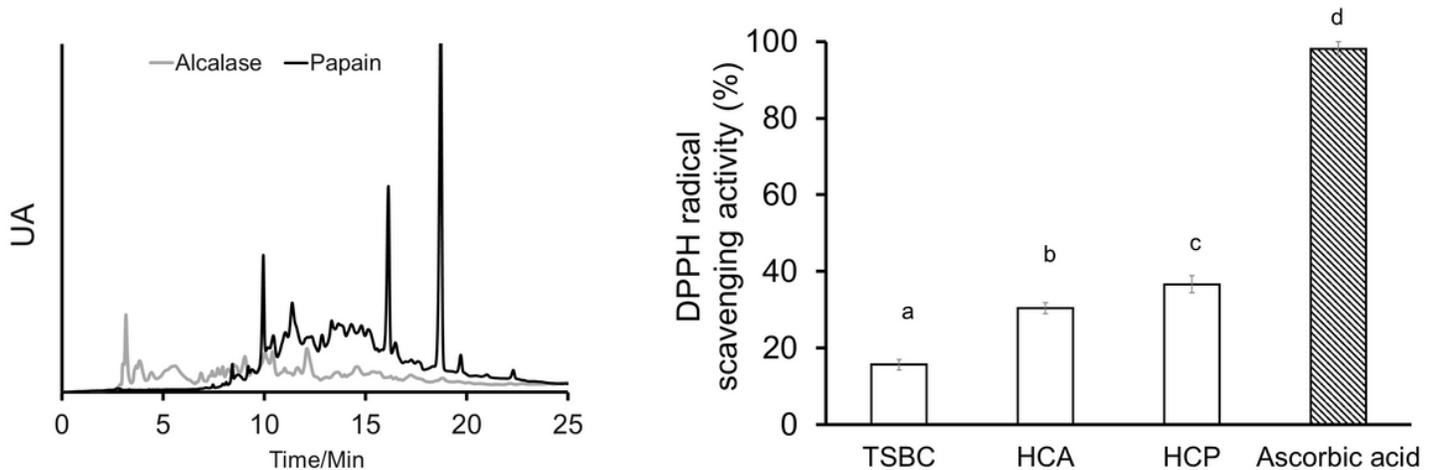


Figure 4

(a) HPLC chromatograms of TSBC hydrolysates produced using Alcalase® (HCA) and papain (HCP); (b) DPPH radical-scavenging assay of collagen hydrolysate (3.2 mg mL⁻¹) from swim bladder of farmed totoaba (TSBC).

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