Effect of acid treatment on extraction yield and gel strength of gelatin from whiptail stingray (*Dasyatis brevis*) skin

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Abstract Chemical properties of fish gelatins differ from those of conventional mammalian sources, representing an attractive technological alternative for the food industry. Ray filleting generates a considerable amount of skin waste that can be used as a collagen source for gelatin extraction. Thus, this research evaluated the HCl and CH₃COOH effect, at 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, and 0.2 M, on extraction yield, molecular weight distribution, and gel strength (GS) of whiptail stingray (*Dasyatis brevis*) skin gelatins. Results showed differences ($P < 0.05$) between acid type and concentration used. CH₃COOH (0.15 M) gave the highest extraction yield (7.0% vs. 5.5% at 0.15 M HCl) and GS (653 ± 71 g vs. 619.5 ± 82 g at 0.2 M HCl). Gelatin electrophoretic profile from CH₃COOH revealed α -/ β -components and high molecular weight (> 200 kDa) polymers. Ray gelatin GS was higher than commercial bovine gelatin, suggesting its possible use for technological food applications.

Keywords Fish skin collagen · Fish gelatin · Acid treatment · Gel strength · Extraction yield

Introduction

Stingrays, elasmobranch cartilaginous fish related to sharks, are common species living in coastal tropical and subtropical marine waters. In Mexico, common rays found in the Gulf of California are *Rhinobatos productus*, *Gymnura* spp., *Rhinoptera steindachneri*, *Rhinobatos glaucostigma*, *Narcine entemedor* and *Dasyatis brevis* (whiptail stingray) (Bizzarro et al., 2009). These specimens are mainly commercialized as fresh fillets, discarding a considerable amount of waste material, such as viscera, cartilage, liver, and skin, during the filleting process. However, these byproducts can be recovered and treated accordingly, to generate valuable products. Eagle ray (*Myliobatis tobi-jei*), red stingray (*Dasyatis akari*) and yantai stingray (*Dasyatis laevis*) skins have been used to obtain acid-soluble collagens with a high denaturation temperature, among other unique attributes, thereby increasing their potential to be used as a substitute for mammalian collagens (Bae et al., 2008). Indeed, marine collagen is a functional protein with powerful applications in the pharmaceutical and food industries (Bama et al., 2010; Kim and Park, 2004). So far, fish gelatin is the most versatile form of marine collagen with industrial applications.

Crude collagen must be pretreated before it can be converted into a suitable compound for its extraction. This procedure is commonly conducted by heating the sample in water at temperatures above 45 °C (Gómez-Guillén et al., 2011; Haug and Draget, 2009). Usually, a chemical pretreatment helps to attain an adequate swelling and solubilization of collagen during heating; this is promoted through the disruption of non-covalent bonds, resulting in the unfolding of protein structures (Haug and Draget, 2009). Thus, the conversion rate of collagen to gelatin depends on the severity of these chemical pretreatments

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(alkali or acid) and the warm-water extraction conditions (temperature and extraction time) (Gómez-Guillén et al., 2011).

Two gelatins are manufactured commercially, type A and type B. Type A is derived from acid pretreatment extraction, whereas, type B originates from alkali pretreatment extraction (Wang et al., 2014). It is well known that fish gelatins have different physicochemical properties than their mammal and terrestrial counterparts, so they could be used to impart new characteristics in gelatin-based products (Gómez-Estaca et al., 2009; Gómez-Guillén et al., 2009; Haug et al., 2004; Karim and Bhat, 2008). The quality of gelatin is defined as a function of factors associated with its microbiology safety, appearance, odor, color, and flavor. However, for technological applications, analyses measuring its physicochemical, rheological, and functional properties become relevant to ensure its quality (Schrieber and Gareis, 2007). Hence, this study evaluated the effect of two acid pretreatments on the extraction yield and gel strength (GS) of gelatins extracted from whiptail ray (*D. brevis*) skin.

Materials and methods

Raw material

Whiptail stingray (*D. brevis*) specimens were harvested from off the coast of Bahía de Kino (Sonora, Mexico) and immediately transported on ice to the seafood laboratory, for further processing. Once in the laboratory, ray specimens were measured (fin length), weighed, and washed with cold water; manual skinning of fins was carried out at room temperature (25 °C). The skins were then cut into small pieces of 1 × 2 cm and stored in polyethylene bags at − 20 °C before analysis.

Proximate analysis

The compositional analysis of the skin was determined according to the Association of Official Analytical Chemists (AOAC) standards, for water (method 950.46), crude protein (method 984.13), using 5.4 as the protein conversion factor (Boran and Regenstein, 2009), lipid (method 960.39), and ash (method 938.08) contents (AOAC, 2000).

Skin preparation and acid pretreatments

The skins were thawed, washed with water, weighed (20 g) and then placed in a 250 mL Erlenmeyer flask. Non-collagenous proteins were removed by washing skins with 0.1 M NaOH, 1:6 (w/v) ratio, at constant stirring (125 rpm) for 1 h. After draining off the NaOH solution, the skins

were squeezed for 5 min in a funnel covered with a cotton mesh. This washing procedure was repeated twice. Afterward, the ray skins were treated with either HCl or acetic acid (CH₃COOH) solutions, at 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, and 0.2 M, at 1:6 (w/v) ratio, under constant stirring (125 rpm) for 1 h. The acid solution was eliminated by draining the skins, as mentioned above. This procedure was repeated twice. All acid solutions were stored at 4 °C before use (Zhou and Regenstein, 2005).

Gelatin extraction

After the ray skin acid pretreatments, gelatin was extracted using deionized water (1:6, w/v) at 60 °C for 3 h, and the resultant gelatin solution was filtered through a funnel covered with a cotton mesh. Recovered gelatin solutions were left to cool to room temperature before the total volume was measured. Finally, the gelatin solutions were freeze-dried, for subsequent analysis (Zhou and Regenstein, 2005).

Protein yield

The protein content in gelatin solutions was estimated by the Biuret method (Gornall et al., 1949), using bovine serum albumin as the protein reference standard. The protein yield (%) was calculated as shown below:

$$\text{Protein yield (\%)} = \frac{A \times V}{20\text{g}} \times 100$$

where *A* = protein concentration (g/mL); *V* = volume of solution (mL).

pH measurement

Gelatin pH was measured as described by the Gelatin Manufacturers Institute of America (GMIA, 2013), with some modifications. Gelatin solutions were prepared by dissolving 0.05 mg of lyophilized gelatin in 5 mL of deionized water at 60 °C. The pH was measured in gelatin solutions at 35 °C using a 240 model Corning digital potentiometer (Corning Science Product, New York, NY, USA).

Gel strength (GS)

Gelatin GS was measured as detailed by Kittiphattanabawon et al. (2010a) and following the international guidelines (GMIA, 2013). Gelatins were dissolved in deionized water at 60 °C to obtain a final protein content of 6.67% (w/v), transferred to a 3.0 × 2.5 cm (diameter × height) circular cast, and incubated at 4 °C for 18 h before analysis. The GS was measured using a TMS-Pro texture

analyzer (Texture Lab Pro, Food Technology Corp., Virginia, VA, USA) equipped with a 12.5 cylindrical flat-faced plunger, which was operated using a 40 N load cell and a cross-head speed of 1 mm/s. Commercial bovine gelatin (Sigma–Aldrich, St. Louis, MO, USA) was used as the control.

Electrophoresis analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted based on Laemmli (1970). Discontinuous gels with 7.5% and 4.0% (resolving and stacking gels, respectively) were used. Solubilized protein samples (5 mg/mL) were mixed at a 1:1 (v/v) ratio with SDS–PAGE sample buffer (4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.125 M Tris–HCl, pH 6.8) and heated in a water bath at 65 °C for 15 min. Then, 25 μ g of protein per lane was loaded onto the gel. Electrophoresis was performed at 160 V, using a Mini Protean[®] 3 Cell Multi-Casting Chamber (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresed gels were stained with a Coomassie brilliant blue R-250 solution, and destained in a methanol:water:acetic acid (5:4:1, v/v/v) solution.

Statistical analysis

Data analysis was performed using a 2×7 factorial design, in which acid pretreatment (two types) and concentration (seven values) were used as factors. The data were analyzed using analysis of variance (ANOVA) and adjusted to a general linear model (GLM), using NCSS 2007 (Kaysville, UT) software. A Tukey–Kramer test was used when differences ($P < 0.05$) were detected between means. The experiment was repeated three times ($n = 3$).

Results and discussion

Proximate composition of whiptail stingray skin

The proximate composition of whiptail stingray (*D. brevis*) skin showed $63.7 \pm 4.4\%$, $29.0 \pm 1.6\%$, $5.6 \pm 0.5\%$, and $0.2 \pm 0.0\%$ for moisture, crude protein, ash, and lipids, respectively. This composition is similar to values reported on other elasmobranchs, such as thornback ray (*Raja clavata*) (Lassoued et al., 2014), blacktip shark (*Carcharhinus limbatus*) (Kittiphattanabawon et al., 2010b), and bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon et al., 2010a) skins, as well as skins from other fish species, such as grey triggerfish (*Balistes capriscus*) (Jellouli et al., 2011) and Nile perch (*Lates niloticus*) (Muyonga et al., 2004). However, the proximate composition of fish skin can vary between/within species since it

is determined by the influence of several factors, such as size, age, gender, food consumption, and seasonal variations (Huss, 1988). Thus, this variation in composition among species influences the collagen extraction.

Extraction yield of gelatins

Gelatin is a biopolymer derived from collagen, the most abundant connective tissue protein. Some intrinsic aspects, such as source, the age of the animal, and type of collagen, will influence the properties of gelatins (Gilsenan and Ross-Murphy, 2000; Muyonga et al., 2004). Since gelatin is derived from denatured collagen, its properties depend not only on the species or tissues from which it is extracted, but also on the extraction conditions used, such as pH, temperature, and chemical pretreatment of the raw material (Gómez-Guillén and Montero, 2001; Tabarestani et al., 2010). Acid pretreatment helps to improve the extraction yield of gelatins, through the disruption and destabilization of hydrogen bonds in the collagen triple helix (Schrieber and Gareis, 2007; Zeng et al., 2010). The present study revealed that gelatin extraction yields obtained after skin pretreatment with HCl and CH_3COOH were 2.8–5.5% and 4.1–7.0%, respectively. The highest yields of extraction were achieved using 0.15 M of either HCl or CH_3COOH (Fig. 1); however, CH_3COOH provided the highest yield. Further acid addition affected the extraction of collagen (in both pretreatments), probably due to undesirable acid hydrolysis, leading to the formation of low molecular weight peptides that could be lost by lixiviation during the ray skin washing steps (Jamilah and Harvinder, 2002; Zeng et al., 2010). Comparable results were reported by Jellouli

Fig. 1 Acid type and concentration effect on the extraction yield of gelatin from whiptail stingray (*Dasyatis brevis*) skin. Bars are standard deviations ($n = 2$)

et al. (2011), obtaining an extraction yield of 5.67% gelatin from grey triggerfish (*B. capricus*) skin, after consecutive alkaline (0.2 M NaOH) and acid (0.05 M CH_3COOH) pretreatments. A similar acid extraction behavior was found by See et al. (2015), in which weak organic acids (acetic and citric) extracted more gelatin from African catfish (*Clarias gariepinus*) skin than a strong acid (sulfuric).

The variation in gelatin yields among species is due to the differences in their skin conformation, besides the extraction conditions utilized. In the present study, the difference in gelatin extraction yields between the two acid pretreatments can be related to the dissociation constants (pK_a) of the acids used. In this respect, HCl is a strong acid, which is completely dissociated, whereas, the weak CH_3COOH tends to attain equilibrium between CH_3COO^- and H^+ chemical species in aqueous solution. However, despite using equimolar concentrations of the acids, the ionized protons (H^+) that went into solution were different, which, in turn, promoted a different effect on whiptail stingray (*D. brevis*) skin during the gelatin extraction pretreatment. Thus, the final pHs were different (Fig. 2) in all extractions, leading to the yield differences.

The experimental results indicated an inverse association between the increment of acid concentration (up to 0.1 M HCl and 0.15 M CH_3COOH) and the ultimate pH values of gelatins (Fig. 2). However, increasing the acid concentrations caused an increment in the pH values of gelatins, suggesting a possible buffering effect of the peptides generated by the collagen hydrolysis (see the electrophoretic analysis).

Fig. 2 Acid type and concentration effect on the final pH of gelatin extracts from whiptail stingray (*Dasyatis brevis*) skin. Bars are standard deviations ($n = 2$)

Fig. 3 Correlation analysis between gelatin extraction yield from whiptail stingray (*Dasyatis brevis*) skin and pH of gelatin extracts. Bars are standard deviations ($n = 2$)

Figure 3 shows the association between the gelatin's yield and pH, displaying negative correlation coefficients (r values) of -0.55 and -0.77 for HCl and CH_3COOH pretreatments, respectively. The gelatin extraction yields were remarkably improved by lowering the pH values. It is known that the swelling, hydration, and extraction of type A gelatins are favored at low pH (See et al., 2015), as also observed in the present study.

Gel strength (GS)

Fish gelatins offer different technological properties, such as viscosity and gelation temperatures than mammalian gelatins. These properties can be an opportunity for novel industrial applications (Choi and Regenstien, 2000; Leuenberger, 1991). The bloom or GS is one of the most common physical parameters used to differentiate gelatins, secure quality, and select their possible technological applications (Boran and Regenstien, 2010). The present study used the method developed by the GMIA (2013) to prepare gelatins. The GS of gelatins extracted from whiptail stingray (*D. brevis*) skin pretreated using the various acid concentrations, is shown in Fig. 4. The statistical analysis identified significant differences ($P < 0.05$) between the GS of gelatins extracted with the two acid pretreatments at various concentrations. In general, gelatin extracted with CH_3COOH showed higher GS values than those with HCl, especially at 0.15 M, at which the maximal GS of all tested samples was obtained (653 ± 71 g, $P < 0.05$). Meanwhile, gelatin extracted with HCl was maximal (619 ± 82 g, $P > 0.05$) at 0.2 M. Frequently, high-quality gelatins (with high GS values) are preferred in

Fig. 4 Acid type and concentration effect on the gel strength of gelatin from whiptail stingray (*Dasyatis brevis*) skin. Bars are standard deviations ($n = 2$)

gelatin-based food formulations. Thus, the high GS values of *D. brevis* gelatin could make it attractive to the food industry.

Similarly, gelatin from Alaska pollock skin extracted with weak organic acids (acetic and citric acids) produced higher GS values than the one obtained with sulfuric acid (strong acid) (Zhou and Regenstien, 2005). One interesting observation in the present study is that the *D. brevis* skin gelatins extracted with both acids displayed higher GS values ($P > 0.05$) than the commercial bovine gelatin (618 ± 19.1 g) used as a reference. Thus, whiptail stingray (*D. brevis*) skin can be considered as a potential technological alternative to be used in the food industry for the

production of gelling-based products. Besides, for further studies, the 0.15 M CH₃COOH pretreatment can be used as a fixed parameter in an optimization process of gelatin extraction from this species. The differences in GS values of the two acid gelatins can be associated with the amino acid profile of each collagen source, as well as with the particular distribution of the α -, β -, and γ -components, oligomers, and low molecular weight fragments, present in the manufactured product (see the electrophoretic analysis).

Molecular weight distribution of gelatins

The electrophoretic analysis helps to identify the protein integrity and recovery of the different gelatin components (α , β , and γ), due to the variation in extraction conditions (Gómez-Guillén et al., 2002; Kittiphattanabawon et al., 2010a; Tabarestani et al., 2010). Typical electrophoretic profiles of gelatins include a mixture of α -chains (one polymer chain), β -chain (two α -chains covalently cross-linked), and γ -chains (three covalently cross-linked α -chains) (Karim and Bhat, 2009). The molecular weight distribution of gelatins obtained after whiptail stingray skin pretreatment with HCl and CH₃COOH at different concentrations is shown in Fig. 5. Regardless of the type of acid used, the gelatin electrophoretic profile showed two representative protein bands, with molecular weights of 200 and 125 kDa, and a less dense band at 100 kDa, corresponding to β -components, and α_1 - and α_2 -chains, respectively (Jellouli et al., 2011; Kittiphattanabawon et al., 2010a; Schrieber and Gareis, 2007). Furthermore, HCl promoted the collagen hydrolysis, presenting protein bands with diverse molecular weights (57–84 kDa),

Fig. 5 SDS-PAGE of gelatins from whiptail stingray (*Dasyatis brevis*) skin obtained with different acid type and molar concentration (M). C1: 0.01, C2: 0.025, C3: 0.05, C4: 0.075, C5: 0.1, C6: 0.15, C7:

0.2 M; MM: Molecular weight standards. α (1 and 2) and β represent gelatin protein chains

especially when concentrations over 0.05 M were used. In general, CH₃COOH showed denser 125 and 100 kDa (α 1 and α 2, respectively) bands than HCl, indicating its advantage for protein extraction. Since α -chains play an important role in gelatin GS, the results agree with the higher GS shown by gelatin samples extracted with CH₃COOH compared to HCl (Fig. 4) (Schrieber and Gareis, 2007).

In agreement with other electrophoretic profiles from fish gelatins, α - and β -components contained in whiptail stingray skin gelatins were characterized as type I collagen, similar to grey triggerfish (Jellouli et al., 2011), Alaska pollock (Zhou and Regenstein, 2005; Zhou et al., 2006), bigeye snapper (Binsi et al., 2009), sole, megrim, cod, hake, and squid gelatins (Gómez-Guillén et al., 2002).

Thus, whiptail stingray (*D. brevis*) skin gelatin extraction was dependent on the acid concentration used. This parameter greatly influenced the yield and GS of the final product. Both acid pretreatments used in the present study allowed to obtain gelatins in comparable yields to those reported for other fish skins treated under similar extraction conditions. However, acetic acid (CH₃COOH) showed the best results. Based on the findings, whiptail stingray (*D. brevis*) skin gelatin can be considered as a potential technological alternative to be used in the food industry for gelling-based products. Such utilization can reduce skin discards to the environment.

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