INTRODUCTION

Gelatin is a functional protein obtained from partial denaturation of collagen, the most abundant structural protein in both vertebrates and invertebrates. Gelatin and gelatin-derived products are widely used in the food, pharmaceutical, photographic, and cosmetic industries due to their unique nutritional and functional properties (Kouhdasht, Moosavi-Nasab, & Aminlari, 2018; Liu, Nikoo, Boran, Zhou, & Regenstein, 2015; Mirzapour-Kouhdasht, Sabzipour, Taghizadeh, & Moosavi-Nasab, 2019). Different sources are used for commercial gelatin production for various applications, albeit, the most used source is skin and bones of porcine and bovine. Due to growing global concern on many diseases such as encephalopathy and religious issues for Muslims, Jews, and Hindus in using porcine and bovine-derived products. Fishery by-products are a good source of gelatin with a lack of abovementioned concerns. On the other hand, more than 50 percent of fish processing materials are useless by-products for seafood processing industries. Using this source

The effects of enzymatically aided acid-swelling process on gelatin extracted from fish by-products

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Abstract
The objective of this study was to investigate the effects of the enzymatic aided acid-swelling process on gelatin obtained from fish by-products. For this purpose, gelatin was extracted by an acidic swelling procedure in the presence of protease extracted from Rainbow trout pyloric caeca. The yield of gelatin extraction and the most important physicochemical characteristics of the fish gelatin samples were investigated and compared with those of commercial bovine gelatin (CBG). The yields of gelatin from Epinephelus coioides skin (ESG) either with or without crude protease from pyloric caeca (15 units/g alkaline treated) were 14.98% and 50.89%, respectively. The yields of gelatin from Cyprinus carpio scales (CSG) with crude protease from pyloric caeca (15 units/g) were 49.97%. The gel strength of the CSG (259.66 g) was significantly higher than that of CBG (228.30 g) and ESG (187.75 g). Similarly, the gelling and melting points, foaming capacity and stability, and the SDS-PAGE pattern of gelatins were compared. The electrophoretic pattern confirmed the results of gel strength which was due to the narrower alpha and beta bands in fish skin and commercial bovine gelatins than that of fish scales gelatin. The results of this research showed that the production of high-quality gelatin can be achieved by the enzymatically aided acid-swelling procedure from fish scales and skin.

KEYWORDS
enzymatically extraction, fish gelatin, fish protease, pyloric caeca, scales and skin
could be a brilliant way to get rid of environmental problems and also issues related to mammalian gelatin (Senaratne, Park, & Kim, 2006). Using enzymes can efficiently improve the yield of gelatin extraction (Balti et al., 2011). In research conducted by Bougatef et al. (2012), the effect of the acid-swelling process on the yield and quality of gelatin from smooth hound (Mustelus mustelus) skin. Acid swelling process either with or without smooth hound visceral protease (at the concentration of 15 Units/g) was investigated. The results indicated that the addition of protease led to an increase in the yield of gelatin production. Different researches have investigated the effects of different enzymatic reactions on the physicochemical characteristics of gelatin obtained from various sources including fish, bovine, and porcine (Ahmad et al., 2020; Gaspar-Pintilieescu et al., 2019; Kouhdasht et al., 2018). In a study performed by Ma, Zeng, Ma, Yang, and Zhao (2019), a novel single-step enzymatic procedure was suggested to extract the gelatin from porcine bones by which the 3 to 8 weeks hydrolysis of ossein was reduced to 3 hr. However, there is still a gap between the enzymatic procedures and gelatin extracted from different fish species. That is why this research has focused on the determination in the differences of gelatins of various fish species. On the other hand, in this research, because the major by-products of each species were different from the other one, so the by-product of fishes was not the same. The physicochemical characteristics of fish gelatin related to the species and tissues applied for gelatin production (S.-H. Cho, Jahncke, Chin, & Eun, 2006), so this study aimed to prepare gelatin from different by-products of various fishes using an enzymatically aided acid-swelling process to compare the gelatin properties.

2 | MATERIAL AND METHODS

2.1 | Materials

Fish by-products provided from marine products distribution center in Shiraz, Iran. Ladder of SDS-PAGE analysis with the range of 10 to 200 kDa was purchased from Sigma Aldrich Co. Other chemicals and reagents were of analytical reagent grade.

2.2 | Extraction of the enzyme

Fish viscera protease extract was performed as described by (Phanturat, Benjakul, Visesanguan, & Roytrakul, 2010). Fish was washed with distilled water, and internal parts were separated to achieve pyloric caeca from which protease meant to be extracted and frozen immediately. The pyloric caeca (10 g) were mixed in 50 mM Tris buffer (pH 8) containing 10 mM CaCl2 and homogenized at 4°C for 30 min. Subsequently, the homogenate mixture was centrifuged at 11,000 × g for 30 min at 4°C. The supernatant was passed through Whatman filter paper No. 2 for separating the fat content. The per -

2.3 | Enzymatically gelatin production from skins

This experiment was performed as described by (Kouhdasht et al., 2018) with slight modifications. The skins (100 g) were soaked in 0.05 M NaOH at the ratio of 1:10 (w/v) and stirred at room temperature for 2 hr to remove noncollagenous proteins. The alkaline solution was changed every 30 min. Afterward, the skins were rinsed three times with distilled water. The skins were then soaked in 0.2M acetic acid at the ratio of 1:10 (w/v) for deamination. Subsequently, the neutralized using distilled water and freeze dried (IFD-5012, Dena Vacuum, Iran). The dried powder was mixed with distilled water (1:10) and the pH was adjusted at 7.5 to add the enzyme at 0–15 units/g. The mixture was stirred for 48 hr at 25°C. The mixture was then heated at 60°C for 4h with continuous stirring followed by centrifugation at 10,000×g for 30 min to remove insoluble material. The supernatant was filtered with Whatman filter NO.2 and freeze dried. The resulting powder was stored at 4°C as fish skin gelatin for further experiments.

2.4 | Enzymatically gelatin production from scales

Scales were cut into 1cm² pieces and washed with 5% (w/v) NaCl by stirring the solution for 24 hr to remove impurities on the surface and then rinsed with distilled water. All the steps for gelatin production were performed as described in section 2.3.

2.5 | Hydroxyproline content and yield of extraction

The hydroxyproline content in gelatin samples was measured according to Kouhdasht et al. (2018). The gelatin sample (100 mg) was hydrolyzed by 6N HCl (5 ml) for 12 hr at 110°C and filtered through a Whatman filter NO.4. An aliquot was transferred into a glass tube then 300 µl isopropanol and 600 µl oxidant solution (acetate/citrate buffer, pH 6.0, and 7% (w/v) chloramine T) were added to the mixture and vortexed thoroughly. After 5 min incubation at
room temperature, Ehrlich’s reagent (4 ml) was added and stirred for 30 min at 60 °C. The absorbance of the mixture was measured at 660 nm using a UV-vis spectrophotometer (Perkin-Elmer UV-VIS-NIR). The hydroxyproline content (mg/g) was measured using L-hydroxyproline standard curve.

The extraction yield was calculated using the amount of hydroxyproline by Equation (1).

\[
\text{Extraction yield (\%) = \frac{[\text{Vs(ml)} \times \text{HPs(g/ml)}]}{[\text{Wr(g)} \times \text{HPr(g/g)}]} \times 100.}
\]  

Where the Vs is the volume of supernatant, HPs is the hydroxyproline content of supernatant, Wr is the weight of raw material, and finally, the HPr is the hydroxyproline content of raw material.

### 2.6 | Proximate analysis and pH determination

The protein content of the samples was measured by a Kjeldahl method as described by (Howitz, 2000). The conversion factor of 5.4 was used (Kouhdasht et al., 2018). The moisture content was determined according to the AOAC methods number 927.05. Concisely, samples were heated after weighting (wet weight) to allow for the release of moisture. Subsequently, the samples were cooled in a desiccator following a weighting step (dry weight). The moisture content (%) was determined by calculating the difference between wet and dry weight. The ash content was determined according to the AOAC methods number 942.05. Summarily, gelatin samples (1g) were dissolved in water, methanol, and trichloroacetic acid at the ratio of 5:4:1. A solution containing methanol, water, and acetic acid at the same ratio was used to destain the gels. The molecular weight of the marker was ranged from 10 to 200 kDa.

### 2.7 | Color

This parameter was detected based on (Afshari-Jouybari & Farahnaky, 2011) method with minor modifications. The image samples were captured in a black plastic box (20 x 20 x 30 cm) with Natural light Source (K = 6,500) using a digital camera (Canon Power shot A630 with 8 Mega Pixels). The distance of the camera from the samples was 25 cm. The L*, a*, and b* values samples were measured by filter/blur/average command in Photoshop software.

### 2.8 | Molecular weight distribution pattern

Molecular weight distribution is an important characteristic that can be determined using the electrophoresis method. SDS-PAGE was performed based on modifications of Mirzapour-Kouhdasht et al. (2018) on the method of (Laemmli, 1970). Briefly, samples were added to the loading buffer containing 2% SDS, 5% mercaptoethanol, and 0.002% bromophenol blue at a ratio of 1:4. After heat denaturation at 90 °C, samples were run in a mini SDS-PAGE apparatus (Bio-Rad Laboratories). Separating was performed on 7.5% resolving and 4% stacking gels at a fixed voltage (100V). Staining of the gels was performed in a 0.1% coomassie brilliant blue R-250 solution dissolved in water, methanol, and trichloroacetic acid at the ratio of 5:4:1. A solution containing methanol, water, and acetic acid at the same ratio was used to destain the gels. The molecular weight of the marker was ranged from 10 to 200 kDa.

### 2.9 | Gelling point determination

The gelling temperature and time were determined as described by (Muyonga, Cole, & Duodu, 2004) with modification. A gelatin solution (10% w/v) was prepared in a glass tube and transferred to a water bath with a temperature of 40°C. Subsequently, the temperature of the water was cooled by adding cold water (2°C) every 15s, while a thermometer was in the solution. The thermometer was lifted out every 15s after adding cold water. The time and temperature were recorded until the gelatin solution was no longer dripped from the head of the thermometer.

### 2.10 | Melting point determination

The melting temperature and time were determined as described by (Muyonga et al., 2004). Gelatin solutions (6.67% w/v) were prepared in 15 ml test tubes and held at 7°C for 16–18 hr, after which they were transferred into a water bath (10°C) and inverted so that the headspace was at the bottom. The water bath was warmed gradually (about 1°C per min) by adding warm (∼45°C) water at intervals of about 60 s. The temperature at which the gel melted, to allow the gas in the headspace to start moving up was recorded as the melting point.

### 2.11 | Foaming attributes

Foam forming capacity (FC) and foam stability (FS) of gelatin samples were determined according to Cho et al. (2004) method with little changes. Gelatin solution at the concentration of 2% (w/v) in distilled water was heated at 60°C until a clear solution achieved. Afterward, the solution was left at room temperature for 20 min following homogenization at 15,000 rpm for 5 min. The solution was immediately transferred into a 100 ml cylinder to record the volume of the solution at 0, 30, and 60 min after homogenization. The FC and FS were calculated using the Equations (2) and (3):

\[
\text{FC(\%) = } \frac{V_t - V_0}{V_0} \times 100. 
\]

\[
\text{FS(\%) = } \frac{V_t - V_0}{V_0} \times 100. 
\]
Where VT, V0, and Vt were the total volume after homogenizing, the volume before homogenizing, and the total volume after remaining times (30 and 60 min), respectively.

### 2.12 Gel strength

This analysis was performed based on Balti et al. (2011) with minor modifications (Balti et al., 2011). Gelatin solution (6.67% w/v) was heated at 60°C for 30 min to dissolve the gelatin then cooled down at 10°C in for about 16–8 hr. Eventually, the gelatin bloom (g) was measured using a texture analyzer (TA.XT2, Mason Technology, Ireland) of which load cell and penetration speed were 5 KN and 2 cm/min, respectively. The probe was flat faced with 1.27 cm in diameter. The experiment was performed in triplicate.

### 2.13 Statistical analysis

All tests were performed in triplicate, and the results were analyzed by a completely randomized design using SPSS version 21.0 software and reported as mean ± SD. The one-way and two-way analyses of variance (ANOVA) were performed with the Tukey test to determine the significance among the mean comparisons at the probability level of 95%. Design expert ver.7.0.0, the response surface method (RSM) with a central composite design (CCD) was used to optimize the activity of the extracted enzyme.

### 3 RESULTS AND DISCUSSION

#### 3.1 Enzyme activity

The extracted enzyme showed the maximum activity (0.247 U/mg) at the temperature and pH of 25°C and 7.50, respectively. The predicted enzyme activity measured by suggested conditions was 0.250 U/mg which was not significantly different from the result of the real experiment (174.26 U/mg).

#### 3.2 Hydroxyproline content determination

The hydroxyproline content of ESG and CSG at the different levels of the enzyme are shown in Table 1. The results revealed that the enzyme concentration had a direct relation with hydroxyproline content. The maximum amounts of hydroxyproline (9.63 and 9.21 mg/g for ESG and CSG, respectively) were obtained by adding 15 U/g enzyme which indicated 3.4 and 5.26 times increase in proportion with nonenzymatic gelatin extraction for ESG and CSG, respectively. It could be concluded that the enzyme treatment had a higher effect on gelatin extraction from scales than that of skin. As it was expected from the results of hydroxyproline content, the maximum yield (50.89%) was obtained by adding enzyme at the
concentration of 15 U/g from the skin but the highest ratio (4.55 times increase in proportion with nonenzymatic gelatin extraction) was related to the gelatin extracted from scales at the same enzyme concentration. Several researches have reported the hydroxyproline content of extracted gelatin. In a study, the hydroxyproline content of 9.91 mg/g was obtained at the enzyme concentration of 15 U/g (Balti et al., 2011). The maximum hydroxyproline content of gelatin extracted from Cyprinus carpio (common carp) scales using a microbial alkaline protease in our previous study was 8.70 at the 25 U/g enzyme concentration (Kouhdasht et al., 2018). The covalent bonds in collagen structure can stabilize it against heating and acid treatment (Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006), consequently, either the chemical (alkaline or acidic) reaction (Ma et al., 2018) or enzymatically aided reaction could help to increase the yield of extraction based on the results of hydroxyproline content. The different yields of gelatin extraction among diverse fish species may be due to the various extraction methods, raw material, and the variable collagen content of different tissues including skin, scales, bones, and fins. The gelatin samples extracted with the enzyme at the concentration of 15 U/g were chosen for further experiments and comparing with the CBG.

3.3 | Proximate analysis and pH determination

The results of this experiment are shown in Table 2. The protein content of dried scales was estimated to be 33.96% which shows the potential to get a high yield of gelatin extraction. The ash and moisture contents for scales were 45.16% and 13.17%, respectively. A study has demonstrated that the protein content and nonorganic material of sea beam scales were 51.2% and 47.3%, respectively (Fahmi et al., 2004). Another research on deep-sea redfish reported the protein and ash contents of 56.9% and 39.4%, respectively (Wang et al., 2004). Another research on deep-sea redfish reported the protein content and nonorganic material with various amounts of nonorganic material and impurities which may be due to several factors including the sources of raw material with various amounts of nonorganic material and impurities which did not remove during the extraction. The previous study on gelatin extracted from common carp scale gelatin showed the higher L* in fish scales gelatin than that of commercial gelatin but the b* of commercial gelatin was higher (Kouhdasht et al., 2018).

3.4 | Color

The color of gelatin samples is shown in Table 2. Generally, gelatin is an amber yellow color colloidal protein. The CBG was significantly lighter (higher L*) and yellow (higher b*) in color than CSG and ESG which may be due to several factors including the sources of raw material with various amounts of nonorganic material and impurities which did not remove during the extraction. The previous study on gelatin extracted from common carp scale gelatin showed the higher L* in fish scales gelatin than that of commercial gelatin but the b* of commercial gelatin was higher (Kouhdasht et al., 2018).

3.5 | Molecular weight distribution

The electrophoretic pattern of ESG, CSG, and CBG is shown in Figure 1. The functional characteristics of gelatin are affected by the molecular weight distribution, structure, and the subunits components which are illustrated by the SDS-PAGE pattern (Balti et al., 2011; Yuliani, Awalsasi, & Jannah, 2019). The proportion of higher molecular weight components and also alpha and beta chains in CSG was higher than other samples, and these components were in minimum amounts in ESG. These results are in accordance with the gelatin gel strength and also gelling and melting points. The CSG showed thicker α1, α2, and β chains. Some lower molecular weight components were also observed in ESG pattern which could be a result of partial hydrolysis of gelatin structure into polypeptides with lower molecular weight. Jongjareonrak et al. (2006) reported some components with the molecular weight lower than alpha chains in Bigeye snapper skin gelatin which was due to the partial hydrolysis of the gelatin as a consequence of using protease. Another reason

| Sample | Protein (%) | Moisture (%) | Ash (%) | pH     | L*     | a*     | b*     |
|--------|-------------|--------------|---------|--------|--------|--------|--------|
| Skin   | 45.04 ± 0.99b | 49.33 ± 0.99a | 3.28 ± 0.70b | 7.55 ± 0.01a | -      | -      | -      |
| Scale  | 33.96 ± 0.89c | 13.17 ± 0.93b | 45.16 ± 0.97c | 7.68 ± 0.53c | -      | -      | -      |
| ESG    | 88.62 ± 0.79a | 70.3 ± 0.75c | 0.74 ± 0.65d | 7.20 ± 0.40e | 40.98 ± 0.65b | -16.73 ± 0.26a | 21.55 ± 0.01b |
| CSG    | 85.39 ± 0.92b | 8.76 ± 0.80c | 2.46 ± 0.40e | 7.61 ± 0.28f | 33.80 ± 0.22h | -16.90 ± 0.49g | 22.00 ± 0.15b |
| CBG    | 91.70 ± 0.84a | 7.47 ± 0.65c | 0.31 ± 0.07d | 5.80 ± 0.11e | 53.17 ± 0.75f | -16.43 ± 0.71e | 28.37 ± 0.84f |

Note: Data reported as mean ± SD. The different lowercase letters in columns show the significant differences (p value < .05).
for appearing these low molecular weight components may be the heat deterioration during the gelatin extraction (Gómez-Guillén et al., 2002). The low molecular weight components in the gelatin lead to the lower gel strength, melting point, and gelling temperature and increasing the gelling time (Kouhdasht et al., 2018; Muyonga et al., 2004; Normand, Muller, Ravey, & Parker, 2000).

3.6 | Gelling point determination

The gelling temperature and time of gelatin samples are shown in Table 3. The gelling temperature of ESG (19.96°C) and CSG (19.73°C) was statistically lower than that of CBG (23.56°C). This could be due to the difference among their amino acid composition especially proline and hydroxyproline which are responsible to construct hydrogen bonds with water molecules leading to a three-dimensional gel (Fernandez-Diaz, Montero, & Gómez-Guillén, 2003; Muyonga et al., 2004). Several researches have considered the gelling temperature of the gelatin (Ninan, Jose, & Abubacker, 2011; Ninan, Zynudheen, & Joseph, 2011) in which the outcomes were remarkably closed to the results of this study. The gelling time of ESG (109.00 s) and CSG (97.23 s) were also showed an accommodation with a former study in which showed that the gelling time for Rohu and Common carp skin gelatin samples were 106 s and 103 s, respectively (Ninan, Jose, & Abubacker, 2011; Ninan, Zynudheen, & Joseph, 2011; Sha, Hu, Ye, Xu, & Tu, 2019) in which the outcomes were remarkably closed to the results of this study. The gelling time of ESG (109.00 s) and CSG (97.23 s) were also showed an accommodation with a former study in which showed that the gelling time for Rohu and Common carp skin gelatin samples were 106 s and 103 s, respectively (Ninan, Jose, et al., 2011). The ESG gelling time was significantly higher than CSG and CBG (96.67 s). Low molecular weight components in ESG which are approved in the electrophoretic pattern (Figure 1), could be a reason for higher gelling time.

3.7 | Melting point determination

As a thermo-reversible gel, gelatin begins to melt when the temperature rises above a specific point called the gel melting point (Arnesen & Gildberg, 2006). The melting point for fish gelatin is reported to be ranged from 11 to 28 °C (Karim & Bhat, 2009). The results of this experiment are shown in Table 3. The melting temperature of CSG (23.56°C) and ESG (22.96°C) was significantly lower than that of CBG (29.53°C). Generally, the melting temperature of gelatin derived from the skin of warm-blooded animals and warm-water fish is higher than that of cold-water fish gelatin (Gilsenan & Ross-Murphy, 2000).

The melting time of ESG (117.00 s) was statistically lower than that of CSG (129.00 s) and CBG (133.80 s). Several reasons have been demonstrated to be effective on the melting point of gelatin including the content of proline and hydroxyproline (Haug, Draget, & Smidsrød, 2004) and molecular weight of polypeptides (Fernandez-Diaz et al., 2003). The greater the atomic bonding strength, the higher the melting point will be (Al-Hassan, 2020).

3.8 | Foaming attributes

The results of foaming attributes are represented in Table 3. The foaming capacity of CBG (129.67%) was statistically higher than that of ESG and CSG (91.00% and 78.83%, respectively). The results of foaming stability showed that the stability in all samples was decreased during the time. The foaming stability of CSG (36.17%) after 60 min was dramatically lower than that of ESG and CBG (41.63% and 93.80%, respectively). Foaming attributes of proteins is depended on the source, chemical composition, conformation in water/oil interfacial, and molecular weight of proteins (Kouhdasht et al., 2018; van der Ven, Gruppen, de Bont, & Voragen, 2002).

3.9 | Gel strength

The gel strength of ESG, CSG, and CBG are presented in Table 3. The gel strength is one of the most important rheological properties of gelatin which is used in the industries. The appropriate gel strength for food industries is ranged from 100 to 300 grams (Kouhdasht et al., 2018). In the present study, the gel strength of CSG (187.75 g) was significantly higher than those of ESG and CBG (259.66 g and 228.30 g, respectively). The gel strength of gelatin is a function of complex interactions by amino acids and the proportion of alpha chains and also the beta chain in the structure (S. Cho et al., 2004). In another research, the direct relationship between the alpha chains or high molecular weighted components and the gel strength of gelatin was determined (Shi, Chen, Wang, & Peng, 2002) which proved the results of our study as it could be deducted from the electrophoretic pattern (Figure 1) and the results of gel strength. Several studies including (Al-Hassan, 2020; Kouhdasht et al., 2018; Mirzapour-Kouhdasht et al., 2019; Wautteau & Noomhorm, 2009;
Zhang, Xu, & Wang, 2011) have reported different gel strength amounts for fish by-products gelatin which was produced by various protocols. In all cases, the gel strength had a reverse relation with the extraction temperature. This could be due to the loss of hydrogen bonds among imino acids as a result of higher temperatures. However, in this study, as the temperature of the CSG and ESG production was the same, what most important is the difference between structures as confirmed by the results of the molecular weight distribution.

4 | CONCLUSION

The preliminary hypothesis of this study was that using enzymatic procedures can improve the yield and physicochemical characteristics of the fish gelatin. The results revealed that the first idea came to true. The yield of gelatin extraction was enhanced by using crude protease from pyloric caeca (15 units/g alkaline treated). The proximate analysis of ESG and CSG showed acceptable properties for application in food industries and even other applications in comparison with CBG. The rheological and functional characteristics of ESG and CSG including gel strength, gelling and melting points, and foaming capacity and stability, and the SDS-PAGE pattern as well, indicated that these gelatins are appropriate compared with the CBG. Eventually, this study proved that the CBG could be replaced by ESG and CSG obtained by the enzymatically aided acid-swelling procedure. It is highly recommended for future researches to investigate the effects of different thermal methods in combination with an enzymatic method to enhance the rheological properties of fish gelatin.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This study does not involve any human or animal testing.

INFORMED CONTENT

Written informed consent was obtained from all study participants.

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