Membrane fractionation of gelatins extracted from skin of yellowfin tuna (Thunnus albacares): effect on molecular sizes and gelling properties of fractions

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ABSTRACT
Membrane separation technologies allow the implementation of environmentally friendly and cost-effective processes to separate fine particles and molecules. Fractionation process of proteins from tuna skin gelatin was performed in four consecutive filtration steps using four membranes with pore diameters of 800 nm, 100 nm, 50 nm, and 20 nm. The retentates obtained from all membranes exhibited a similar protein size distribution. Gel strength and gelling and melting temperatures did not differ significantly among the tuna skin gelatin and the four retentates (p > .05). The permeate from the 20 nm pore diameter membrane exhibited significantly lower gelling properties than the other fractions (p < .05). The retentates of the four membranes presented gel strength values between 228 and 440 g. Gelling and melting temperatures from retentates and tuna skin gelatin ranged from 10.0 to 13.4°C and 18.0 to 20.6°C, respectively.

Fraccionamiento por membrana de las gelatinas extraídas de la piel del atún de aleta amarilla (Thunnus albacares): efecto sobre los tamaños moleculares y las propiedades gelificantes de las fracciones

RESUMEN
Las tecnologías de separación que emplean membranas permiten implementar procesos ecológicos y rentables para separar partículas finas y moléculas. Para este estudio se realizó el fraccionamiento de las proteínas de la gelatina de piel de atún en cuatro pasos de filtración consecutivos, utilizando cuatro membranas con diámetros de poro de 800 nm, 100 nm, 50 nm y 20 nm. Las proteínas retenidas por las cuatro membranas presentan una distribución de tamaño similar. La fuerza del gel y las temperaturas de gelificación y fusión no difirieron significativamente entre la gelatina de piel de atún y los cuatro retenidos (p > .05). El permeado de la membrana de 20 nm de diámetro de poro exhibió propiedades de gelificación significativamente inferiores a las de las otras fracciones (p < .05). Los retenidos de las cuatro membranas registraron valores de fuerza de gel de entre 228 y 440 g. Las temperaturas de gelificación y fusión de los retenidos y de la gelatina de piel de atún oscilaron entre 10.0 y 13.4°C y entre 18.0 y 20.6°C, respectivamente.

1. Introduction
In recent decades, total fish production increased around the world due to the growing demand for fish and seafood (Bruno et al., 2019; FAO, 2020; Ozogul et al., 2021). The expansion of seafood production has led to increasing quantities of by-products, which can account for 30 to 70% of the total weight of fish (Bruno et al., 2019; Desai et al., 2022; FAO, 2020; Vázquez et al., 2019). Fish by-products are mainly composed of heads, viscera, skin, fins, scales and bones. Historically, these non-edible products, when not discarded, were used as animal feed for aquaculture, or used as fertilizers or in fish silage (Bruno et al., 2019; FAO, 2020; Ozogul et al., 2021). However, these discards and by-products have gained attention in recent years, for their potential to be processed into added-value products since they are an important source of bioactive compounds, including amino acids, proteins, peptides, enzymes, gelatin, collagen, omega-3 long-chain polyunsaturated fatty acids, chitin, vitamins and minerals (Bruno et al., 2019; FAO, 2020; Ozogul et al., 2021; Simat, 2021).

In most fish, protein is the major component on a dry mass basis, therefore by-products of fish processing can be considered as raw materials for the preparation of high-protein ingredients. In particular, they can be used for the production of food grade gelatin, since fish skins, bones and fins typically contain large amounts of collagen (Karayannakidis & Zotos, 2016; Sae-Leaw et al., 2017). Gelatin is a proteinous macromolecule obtained from collagen by thermal denaturation. Collagen is widely used in food, pharmaceutical, biomedical, leather, cosmetics and tissue-engineering industries due to its structural and functional characteristics (Bruno et al., 2019; Karayannakidis & Zotos, 2016; Yang et al., 2019). Collagen and gelatin are obtained mainly from bovine and porcine sources and are extensively used globally. Nevertheless, the use of gelatin from mammalian species is restricted in some countries and communities by socio-cultural and health-related concerns, including religion (Judaism, Hinduism and Islam do not allow the consumption of pork- or non-religiously slaughtered cow-related products), adherence to vegetarian diets and...
lifestyles, and concerns regarding transmission of pathogenic agents (prions related to bovine spongiform encephalopathy). Consequently, gelatin from seafood by-products has emerged as a valuable alternative (Bruno et al., 2019; Karayannakis & Zotos, 2016; Lv et al., 2019; Şimat, 2021).

Fish gelatin can be used in a wide range of applications, particularly in biomedical and food applications, due to its unique functional properties, which include survival to enzymatic digestion in the gastrointestinal tract, good absorption capacity and excellent film-forming properties (Lv et al., 2019; Ozogul et al., 2021; Şimat, 2021). The functional properties of gelatin can be classified in two categories: gelling properties (gel strength, viscosity, and gelling and melting points), and properties related to the surface behavior of gelatin (such as foaming, emulsification and film formation) (Karayannakis & Zotos, 2016; Lin et al., 2017).

Regarding surface properties, gelatin’s ability to stabilize emulsions is highly valuable due to its advantages of high emulsification, nontoxicity, low cost and biocompatibility (Feng et al., 2021; O’Sullivan et al., 2016; Tang et al., 2020). Recent investigations have focused their attention on fish gelatin, particularly from fish skin, as a valuable alternative for stabilizing emulsions. These studies have demonstrated that fish gelatin has a wide application prospective in the gelatin industry (Feng et al., 2021; Huang et al., 2020; O’Sullivan et al., 2016; Tan et al., 2020; Tang et al., 2020; Vall-llosera et al., 2021; Xu et al., 2021; Yang et al., 2022). Among gelling properties, gel strength and gelling and melting temperatures are three of the most important properties used to characterize commercial gelatins and to determine their potential uses and applications (Lin et al., 2017; M. Yang et al., 2022). These properties can be affected by different factors, including the nature of the raw material and the source of the gelatin (species, anatomical part and the water temperature), the amino acid composition, the average molecular weight of the gelatin hydrolysate and the molecular weight distribution of the peptides in the gelatin extract and the processing and extraction temperatures (Kumar et al., 2018; Lin et al., 2017; M. Yang et al., 2022).

There are different methods for the extraction of gelatin from fish processing by-products depending on sources of raw material. However, every process consists of two basic steps: a pre-treatment or conditioning step to remove non-collagenous proteins and materials, and a collagen extraction step to obtain gelatin (Karayannakis & Zotos, 2016; Kumar et al., 2018; Mirzapour-Kouhdašt et al., 2021). The non-collagenous proteins are removed by treating with an acid or alkaline salt solution or by enzymatic treatment (Karayannakis & Zotos, 2016; Kumar et al., 2018; Mirzapour-Kouhdašt et al., 2021; Pal & Suresh, 2016). The collagen extraction is a thermal hydrolysis step usually carried out with hot water for a defined period of time. Conditions should be optimized in each case (Karayannakis & Zotos, 2016; Kumar et al., 2018; Mirzapour-Kouhdašt et al., 2021; Pal & Suresh, 2016).

Production of proteins with the desired molecular sizes is a challenging process, therefore, separation techniques must be properly selected and designed to achieve effective fractionation and purification of desired protein fractions (Abejón et al., 2018; Roslan et al., 2018). Membrane separation technologies are effective for the separation of fine particles and dissolved molecules. They represent a great alternative to traditional separation techniques that allow the implementation of environmentally friendly and cost-effective processes. These technologies have been successfully applied in efficient and ecological processes for the extraction, concentration, purification and fractionation of important biomolecules from waste and by-products (Bruno et al., 2019; Roslan et al., 2017, 2018). Regarding aquatic by-products, commonly published studies have focused on the fractionation of peptides and protein hydrolysates from various sources. Nevertheless, membrane fractionation of macromolecules such as proteins is unusual and has been poorly explored (Abejón et al., 2018; Bruno et al., 2019; Desai et al., 2022; Gao et al., 2021; Roslan et al., 2017, 2018; Saïdi et al., 2013, 2014; Villamil et al., 2017).

Considering that proteins have a wide prospective application and could be used as functional ingredients in numerous industries, and that application of membrane separation technologies to obtain protein fractions from aquatic by-products is not fully explored, this study aims to evaluate the use of these technologies for fractionation of proteins from tuna skin gelatin derived from industrial fish processing by-products to obtain fractions with different gelling properties. The fractionation process was performed in four consecutive filtration steps using four membranes with pore diameters of 800 nm, 100 nm, 50 nm and 20 nm, one for each filtering step. Gel strength and gelling and melting temperatures were defined as response variables, and permeate flux was monitored during each filtration step.

2. Materials and methods
2.1. Materials
Two independent lots of Yellowfin tuna (T. albacares) skin provided by TUNATUN S.A. (Alajuela, Costa Rica) were mixed to obtain a single, homogeneous, 76 kg lot which was used throughout the study. Tuna skin was chopped (approximately 1.5 cm² pieces), mixed, packed in bags, and kept frozen (−20 °C) until used.

2.2. Gelatin extraction and enzyme treatment
Gelatin was extracted from tuna skin as previously described (Montero & Acosta, 2020) with the addition of an enzymatic treatment with the commercial protease Novo-Pro D® produced by Novozymes® and donated by Trisan Food & Tech (San José, Costa Rica). This enzyme is a serine endo-protease that hydrolyzes internal peptide bonds, allowing the production of protein chains with different molecular sizes.

The extracted gel was weighed and heated to 60°C, then the Novo-Pro D® enzyme was added at a concentration of 38 µL of enzyme per kg of tuna gel and the mixture was stirred manually at 60 °C for 20 min. The gel was then heated to 85°C and maintained at this temperature for 15 min to inactivate the enzyme.

A 1 kg sample of enzyme treated gel was freeze-dried on a lyophilizer (Sublimator 2 × 3x3, Zirbus technology GmbH, Germany) and used to determine gel strength and gelling and melting temperatures of proteins as previously described (Montero & Acosta, 2020). SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) was performed to determine the molecular weight profile of the extracted proteins.
2.3. Equipment

Fractionation of proteins from tuna skin gelatin was performed in four consecutive filtration steps using four membranes, one for each step. At each batch step, the corresponding membrane was connected to a microfiltration/ultrafiltration pilot unit equipped with a centrifugal pump to provide tangential flow, a pneumatic pump to feed the system and provide pressure, and a heat exchanger for temperature control (Figure 1). Membralox® (Pall, USA) tubular ceramic membranes (microfiltration: α-alumina; ultrafiltration: zirconia) were used with a pore diameter adequate for each process (microfiltration: 800 nm; ultrafiltration: 100, 50, 20 nm). Membranes were 1.02 m long, with 19 channels 4 mm in diameter and a filtration surface area of 0.24 m².

2.4. Experimental design and statistical analysis

An unrestricted randomized design was used to evaluate the effect of the membrane filtration fractionation process on gelatin characteristics, including gel strength and gelling and melting temperatures of proteins derived from different treatments. Experiments were carried out in triplicate.

Significant differences between treatments were determined by one way ANOVA (α = 0.05), using JMP 8 software (SAS, USA). When significant differences were detected, Tukey’s test was performed (α = 0.05) using JMP 8 software (SAS, USA).

2.5. Fractionation of proteins from tuna skin liquor

Proteins from tuna skin gelatin were fractionated by molecular weight to obtain products with distinct functional characteristics. The fractionation process was performed in four consecutive filtration steps using the system described in section 2.3. All filtration steps were carried out at 60°C, with a 6.1 m/s cross flow rate and a 124.1 kPa (1.24 bar) transmembrane pressure. Permeate flux and retentate temperature were measured during each filtration stage. Inlet and outlet pressure were monitored to ensure that transmembrane pressure was stable throughout the process.

In the first filtration step, enzyme-treated tuna gelatin was filtered with an 800 nm pore diameter membrane, using the concentration configuration (without removing the retentate, increasing volume reduction ratio). Subsequent filtration steps were also carried out at increasing VRR, changing the membrane module on each step and using membranes of 100 nm, 50 nm and 20 nm pore diameters, using as feed the permeate obtained in the filtrations of 800 nm, 100 nm and 5 nm, respectively (Figure 2).

At the end of each filtration step, 1 kg samples of retentates and permeates were refrigerated at 4°C for 18 h to generate a gel. The gels were crushed and placed on trays to be frozen and freeze-dried using a lyophilizer (Sublimator 2 × 3x3, Zirbus technology GmbH, Germany). Dried proteins from each fraction were weighed and gel strength and gelling and melting temperatures (°C) were determined as described by Gómez-Guillén et al. (2002) with some minor modifications (Montero & Acosta, 2020). The molecular
weight profile of the extracted proteins was determined by SDS-PAGE using the method as described by Laemmli (1970), with minor modifications (Montero & Acosta, 2020). All analyses were performed as previously described by Montero and Acosta (2020).

2.6. Membrane cleaning protocol

After filtration processes, membranes were cleaned following a standardized procedure with three basic cleaning phases: one aqueous (softened water, 60 °C), one alkaline (0.05% NaOCl and 1% m/m NaOH, 60 °C) and one acid (1% m/m HNO₃, 60 °C), for both microfiltration and ultrafiltration membranes.

At the end of the cleaning protocol, membrane hydraulic permeability was determined using softened water at 25 °C and 124.1 kPa (1.24 bar) transmembrane pressure. If permeability was 20% below original membrane permeability, the previously described protocol was repeated until permeability value was adequate.

3. Results and discussion

3.1. Effect of membrane pore diameter on permeate flux

The experimental parameters for each filtration step and the effect of membrane pore diameter on permeate flux are presented in Table 1. Additional data on permeate flux as a function of VFR for each filtration step are provided as Supplementary Materials. The permeate flux values obtained with ultrafiltration membranes (100, 50 and 20 nm) where within a previously reported range (5–500 L/h·m²) (Maroulis & Saravacos, 2003). However, the process should be optimized for greater productivity at each filtration step. For example, transmembrane pressure could be increased. This study used pressure values lower than 2 bar (200 kPa), but transmembrane pressures higher than 9 bar (900 kPa) have been reported in ultrafiltration processes (Maroulis & Saravacos, 2003; Tewari, 2015). Another option could be to increase the cross-flow rate.

3.2. Fractionation of proteins from tuna skin gelatin/liquor

Four membranes were used in four consecutive filtration steps to separate the tuna skin liquor used as feed into five fractions: retentates from the four membranes with pore diameters of 800 nm (R800), 100 nm (R100), 50 nm (R50) and 20 nm (R20), and the permeate from the 20 nm pore diameter membrane (P20). The gelling properties of the tuna skin liquor and the five fractions are shown in Table 2.

Table 1. Experimental parameters, volume reduction ratio (VRR), and range of permeate flux for each filtration step of the fractionation process using four membranes (temperature: 60°C; cross flow rate: 6.1 m/s; transmembrane pressure: 1.24 bar (124.1 kPa)).

| Parameter                              | Microfiltration 800 nm | Ultrafiltration 100 nm | Ultrafiltration 50 nm | Ultrafiltration 20 nm |
|----------------------------------------|------------------------|------------------------|-----------------------|-----------------------|
| Mass of feed (kg)                      | 35.2 ± 0.9             | 28.3 ± 0.7             | 23.1 ± 0.1            | 16 ± 3                |
| Mass of permeate (kg)                  | 31.7 ± 0.8             | 24.8 ± 0.6             | 19.7 ± 0.2            | 12 ± 3                |
| Mass of retentate (kg)                 | 3.5 ± 0.1              | 3.5 ± 0.1              | 3.5 ± 0.1             | 3.5 ± 0.1             |
| Time of filtration (min)               | 6.7 ± 0.7              | 38 ± 12                | 46 ± 7                | 100 ± 23              |
| Volume reduction ratio (VRR)           | 10.0 ± 0.4             | 8.0 ± 0.1              | 6.6 ± 0.03            | 4.6 ± 0.9             |
| Range of permeate flux (kg/h·m²)       | 1002 – 1635            | 93 – 425               | 75 – 263              | 10 – 51               |

Medias de los resultados observados ± intervalo de confianza del 95% (n = 3).

Table 2. Gelling properties of the tuna skin liquor (feed) and the five fractions obtained in the filtration process using four membranes.

| Treatment               | Gelling temperature (°C) | Melting temperature (°C) | Gel strength (g) |
|-------------------------|--------------------------|--------------------------|------------------|
| Tuna skin liquor (feed) | 11 ± 1                   | 19 ± 1                   | 228 ± 130        |
| Retentate 800 nm        | 11 ± 1                   | 19 ± 1                   | 242 ± 93         |
| Retentate 100 nm        | 12.7 ± 0.7               | 19.9 ± 0.7               | 440 ± 130        |
| Retentate 50 nm         | 12 ± 1                   | 19 ± 1                   | 326 ± 89         |
| Retentate 20 nm         | 11.3 ± 0.4               | 18.6 ± 0.6               | 296 ± 38         |
| Permeate 20 nm          | –1.6 ± 0.7               | 5.3 ± 0.5                | 5.1 ± 0.68       |

Medias de los resultados observados ± intervalo de confianza del 95% (n = 3). Los valores con letras diferentes en la misma columna son significativamente diferentes (p ≤ 0.05).

4. Conclusion

The results obtained in this study confirm the feasibility of using ultrafiltration as a tool for the fractionation of tuna skin liquor. The use of different membrane pore diameters allowed to separate tuna skin liquor into fractions, with significant variations in the gelling properties of the fractions obtained. These variations could be attributed to the differences in the size of the proteins and the gelatin components retained in each fraction. The results of this study can be used as a basis for future work on the optimization of the fractionation process, aiming at obtaining fractions with specific gelling properties for different applications. Further studies are needed to investigate the functional properties of the fractions obtained and their potential uses in the food industry.
The gel strength of the retentates of the four membranes ranged from 228 to 440 g. These values were lower than those obtained in a previous study (Montero & Acosta, 2020). The lower values were due to the smaller protein size caused by the enzymatic treatment. Previous studies have shown gel strength values for aquatic gelatin in the 100–300 g range. This wide range can be attributed to variations in methods of measurement and to differences in proline and hydroxyproline contents. Higher molecular weights and α/β subunit contents of gelatins may produce higher gel strengths (Kumar et al., 2018; Lin et al., 2017; Yang et al., 2022).

The tuna skin liquor feed and retentate fractions showed a similar protein size distribution according to SDS-PAGE (Figure 3). These fractions contained proteins with molecular weights greater than 75 kDa and 100 kDa, characteristic sizes of the α/β-gelatin chains important for better gelling properties. Techno-functional properties of proteins depend on protein structure and especially on molecular weight, hydrophobicity and secondary and tertiary conformation. Gelling properties are correlated with the size of gelatin proteins; higher values for gelling properties are obtained with larger protein sizes (Darine et al., 2010), as seen in the results of the fractionation process.

Molecules greater than 75 kDa were partially retained by the 100 nm and 50 nm pore diameter membranes and were present both in the retentate of these membranes and in the retentate of subsequent filtrations. Meanwhile, proteins greater than 75 kDa were completely retained by the 20 nm pore diameter membrane. This result indicates that the molecular weight cut-off of this membrane is smaller than the size of these fractions.

The significantly lower gelling properties of the permeate from the 20 nm pore diameter membrane can be attributed to lower molecular weight of the proteins in the permeate, due to high retention by the membrane of molecules in the 50–75 kDa range and total retention of proteins with molecular weights greater than 75 kDa. The fraction containing proteins larger than 75 kDa is comprised of the α/β chains present in the fish liquor. Gelling properties (gel strength, melting and gelling temperatures) are positively correlated with proportion of α and β chains and negatively correlated with lower molecular weights (Kumar et al., 2018; Lin et al., 2017; Yang et al., 2022). Thus, the permeate was expected to have low values for gel strength and gelling and melting temperatures.

The usefulness of the membrane system depends on the desired degree of separation and on the speed of separation (Pina et al., 2015). The 20 nm pore diameter membrane allowed the separation of larger proteins, and could potentially be used alone to fractionate the fish liquor. Nevertheless, the effect of the previous filtration steps on fluxes in the UF at 20 nm should be evaluated, since the partial retention of larger molecules could increase fouling of the membrane and decrease the permeate flux. The successful application of membrane techniques depends both on the appropriate separation of specific molecules and on minimizing the effects of fouling on permeate flux (Kuca & Szaniawska, 2009).

Given that the 20 nm pore diameter membrane allows separation of larger proteins, this membrane could be used in industrial processes where there is a great diversity of protein sizes. A diafiltration step to separate and purify (by diafiltration) larger molecules could be incorporated to increase the gelling properties of gelatin. Diafiltration is an effective membrane process used to achieve high purity of biomolecules, including proteins, by increasing the concentration of the desired product and decreasing the concentration of salts, unwanted microsolutes and impurities in solution (Aspiyanto, 2015; Conidi et al., 2022; Paulen & Fikar, 2016). Previous studies have shown the applicability of diafiltration for the concentration and desalination of gelatin (Paulen & Fikar, 2016; Simon et al., 2002).

4. Conclusion

Membrane separation technology for fractionation of proteins from tuna skin gel was evaluated and fractions with distinct gelling properties were obtained. The process was performed in four consecutive filtration steps using four membranes. Retentates from the four membranes had a similar size distribution and non-significant differences in gelling properties (gel strength, gelling and melting temperatures). The permeate from the 20 nm pore diameter membrane exhibited the lowest values for gelling properties.

Figure 3. SDS-PAGE electrophoresis of gelatins from tuna skin liquor feed, retentates (R800; R100; R50; R20) and final permeate (P20) derived from fractionation process using four membranes. STD: standard.

Figura 3. Electroforesis SDS-PAGE de las gelatinas de la alimentación del licor de piel de atún, de los retenidos (R800; R100; R50; R20) y del permeado final (P20) derivados del proceso defraccionamiento utilizando cuatro membranas. STD: estándar.
and the size distribution showed that proteins with molecular weights greater than 75 kDa were not present. This confirms that gelling properties depend on the size of the chains of proteins. The results obtained in this study show the technical feasibility of employing membrane separation technologies to obtain proteins with various gelling properties from tuna skin that could be used as functional ingredients in numerous industries and applications. Further studies are recommended to evaluate the type and content of amino acids of the obtained gelatins and the proportion of proline and hydroxyproline and its relation to gelling properties. Properties of tuna skin gelatin should be compared with those of gelatins obtained from other fishery by-products. Furthermore, the incorporation of a diafiltration step to separate and purify (by diafiltration) larger molecules could be considered to increase the gelling properties of gelatin.

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