An update review: several extraction methods for collagen isolation in vertebrate fish

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Abstract. Fish are vertebrate animals with a backbone that live in marine environments, presenting a potential source of collagen due to their high protein content. Collagen is a structural protein in the body's connective tissue, contributing 25 to 30% of the total body protein. Collagen sources in fish can be found in the skin, scales, and bones. The isolation of collagen from fish involves several stages, including separation and cleaning, size reduction, removal of non-collagenous components, collagen extraction, and precipitation. The extraction method utilized in collagen isolation impacts the quantity of collagen produced. The aim of this literature review is to identify extraction methods applicable for collagen isolation in vertebrate fish, serving as a reference for selecting extraction methods in future research. The updated review method involved collecting journal data from various literature sources, obtained from national and international journals discussing collagen extraction methods in vertebrate fish. Based on the update review, the isolation of collagen from vertebrate fish is carried out using several extraction methods, including acid soluble collagen extraction, pepsin soluble collagen, water soluble collagen, organic acid ultrasound-assisted, extrusion hydro extraction, deep eutectic solvent, and supercritical fluid extraction.

1 Introduction

Collagen is the primary protein found in animal connective tissues, accounting for approximately 25% to 30% of the total body protein. Collagen plays a significant role in various aspects of our daily lives, including biomedical applications, pharmaceuticals, the food industry, the pharmaceutical industry, and cosmetics. Collagen is naturally produced within the body by fibroblast cells. However, the body's ability to produce collagen decreases with age and due to factors such as poor lifestyle choices. Therefore, there is a need for external sources of collagen. Collagen can be extracted from various animal sources, with the current market primarily relying on collagen sourced from pigs and cows. However, this raises concerns about halal certification for pig-derived collagen and potential disease transmission for collagen sourced from cows. An alternative source of collagen is fish. Fish are cold-blooded vertebrate animals that inhabit aquatic environments. Common sources of fish collagen include bones, skin, and scales, which are byproducts of fish processing. The isolation of collagen from these byproducts involves several steps, including preparation,
extraction, and recovery. The extraction methods can vary depending on the marine source [1].

The aim of this review is to provide the latest information on collagen extraction methods applicable to vertebrate fish. The information presented in this review is gathered from both national and international journals that discuss collagen extraction from vertebrate fish, as well as various reference books.

1.1 Vertebrate Fish
Vertebrates are animals characterized by having an internal backbone or spinal column. Vertebrate animals encompass more than 85,000 species [2], and they are categorized into mammals, fish (pisces), reptiles, amphibians, and birds. Among vertebrate animals, fish constitute the largest group, with approximately 25,000 recorded species. Fish are cold-blooded creatures known for their distinctive features, including a backbone, gills, fins, an aquatic habitat, and the use of fins for maintaining balance in water [3]. Various types of fish, such as tilapia, catfish, cod, short-finned fish, and others, offer significant health benefits and can also serve as alternative sources of collagen.

1.2 Collagen
Collagen, derived from the Greek words "cola," meaning glue, and "genno," meaning birth, plays a crucial role in connecting cells to form the structural framework of tissues and organs within the body. Collagen molecules have a diameter of 1.5 nm, a length of 280 nm, and a molecular weight of 290,000 Daltons. The composition of collagen consists of three polypeptide chains, each containing over 1,000 amino acids within [4]. As a protein compound commonly found in vertebrate connective tissues such as skin, scales, and bones, collagen contributes to approximately 25-35% of the total protein content in the body. Collagen, being a long-chain protein, is composed of amino acids such as alanine, arginine, lysine, glycine, proline, and hydroxyproline. Its properties involve easy absorption by the body, non-toxicity, high water affinity, biocompatibility, biodegradability, relative stability, ease of shaping, and water solubility [5]. There are 28 types of collagen varying based on structural domains and suprastructural organization. However, 80-90% of collagen in the body consists of types I, II, and III. Type I collagen, the most abundant, can be found in bones, skin, tendons, and organs. Type II is present in cartilage, while type III is found in reticular fibers, blood, and skin [1].

Collagen type I functions as a membrane for tissue regeneration, antioxidant, anti-inflammatory, anti-aging, and wound healing. Collagen type II acts as a chondroprotective agent, reducing pain in osteoarthritis. Meanwhile, collagen type III plays a role in preventing diastasis recti, wound healing, and participates in bone formation [6-11]. Collagen plays a vital role in building the body's structure, including bones, teeth, joints, muscles, and skin. However, factors such as aging, exposure to ultraviolet radiation, smoking, and diabetes can lead to a decrease in collagen, resulting in skin aging, inflammation, slower wound healing, decreased muscle mass, and weakened cartilage (joint pain or osteoarthritis). Therefore, obtaining collagen externally is crucial for the body. Collagen has several pharmacological activities so it can be used as a raw material in pharmaceutical products such as cosmetics, biomedical products and supplements [12].

1.3 Collagen Isolation
Collagen from vertebrate fish can be isolated through several initial stages. The initial stages involve preparing the fish parts intended for isolation, including separation and cleaning, size reduction, and the removal of non-collagen components. Parts of the fish such as the skin and bones, which serve as collagen sources, are separated from the meat or other impurities and then cleaned with water. Subsequently, the sample is reduced in size or ground to facilitate
the purification process. The removal of non-collagen components such as fat, minerals, pigments, and odors is carried out using NaOH. After the initial or pre-treatment stages, the extraction phase is conducted using an extraction method. This is followed by collagen precipitation and drying to obtain collagen yield [13]

Tabel 1. Several extraction methods in vertebrate fish collagen isolation

<table>
<thead>
<tr>
<th>Extraction Methods</th>
<th>Fish Species</th>
<th>Source Collagen</th>
<th>Yields</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid soluble collagen (ASC)</td>
<td>Shortfin scad (Decapterus macrosoma)</td>
<td>Skin, Bone</td>
<td>3.35 ± 3.43%</td>
<td>[14]</td>
</tr>
<tr>
<td>Pepsin soluble collagen (PSC)</td>
<td>Shortfin scad (Decapterus macrosoma)</td>
<td>Skin, Bone</td>
<td>0.10 ± 0.13%</td>
<td></td>
</tr>
<tr>
<td>Hydro-extraction</td>
<td>Catfish (Pangasius sp.)</td>
<td>Skin</td>
<td>12.15%</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Alaska Pollock (Theragra chalcogramma)</td>
<td>Skin</td>
<td>3.13 ± 0.04%</td>
<td>[16]</td>
</tr>
<tr>
<td>Ultrasound-assisted (UA) extraction</td>
<td>Acetic acid</td>
<td>Sharpnose stingray (Dasyatis zugei)</td>
<td>Skin</td>
<td>11.49 ± 0.03%</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>Sharpnose stingray (Dasyatis zugei)</td>
<td>Skin</td>
<td>3.50 ± 0.01%</td>
</tr>
<tr>
<td>Deep eutectic solvent (DES)</td>
<td>Atlantic codfish (Gadus morhua)</td>
<td>Skin</td>
<td>5.2%</td>
<td>[18]</td>
</tr>
<tr>
<td>Supercritical fluid extraction (SFE)</td>
<td>Atlantic codfish (Gadus morhua)</td>
<td>Skin</td>
<td>13.8 ± 0.013%</td>
<td>[19]</td>
</tr>
</tbody>
</table>

2 Collagen extraction method in vertebrate fish

2.1 Acid soluble collagen

The use of acetic acid as a solvent in collagen extraction is promising in the extraction process [5]. This is because acetic acid can disrupt the salt bonds between molecules and Schiff bases, which causes the expansion and dissolution of collagen fibers. In addition, acetic acid has the ability to break the hydrogen bonds that initially stabilize the triple helix structure of collagen. As a result, the hydrolysis process is accelerated in the acid method. The procedure for extracting acid-soluble collagen (ASC) from the samples involved using a 0.5 M acetic acid solution at a ratio of 1 part sample to 10 parts solution (w/v) for a 24-hour period while continuously stirring. Afterward, the resulting extracts were subjected to centrifugation at 10,000 x g for 30 minutes at 4°C, leading to the separation of the supernatant. Subsequently, the remaining residues underwent a second extraction using 0.5 M acetic acid at the same 1:10 (w/v) ratio for a duration of 12 hours, followed by centrifugation at 10,000 x g for 30 minutes at 4°C. The two sets of supernatants were combined, and sodium chloride (NaCl) was added to reach a final supernatant concentration of 0.7 M to induce precipitation. Another round of centrifugation at 2,500 x g was performed to collect the precipitate. Finally, the precipitate was freeze-dried [20]. Based on research results, the collagen yield from shortfin scad skin and bone was 3.35 ± 3.43% [14].

2.2 Pepsin soluble collagen
Extraction using pepsin can increase collagen extraction results. However, this reaction can only act on nonhelical peptide chains, and usually does not affect helical peptide chains peptide chain. Therefore, this method will be retained part of the triple helix in collagen protein, so collagen still biologically active. The reactions that occur in this enzyme method are mild, do not corrode the equipment used and consume low energy. The procedure for extracting pepsin soluble collagen from the samples involved using of 0.5 M acetic acid with a pepsin concentration of 1.5% (w/w) for a duration of 30 hours at 4°C, with continuous stirring. After this stage, the resultant extract underwent centrifugation at 10,000 x g for 30 minutes at 4°C, separating the supernatant. Subsequently, the remaining residue underwent a second extraction using 0.5 M acetic acid with a pepsin concentration of 1.5% (w/w) for 12 hours before being subjected to centrifugation at 10,000 x g for 30 minutes at 4°C. The two batches of supernatants were combined, and sodium chloride (NaCl) was gradually added until the final supernatant concentration reached 0.7 M, facilitating the precipitation process. The supernatant then underwent another round of centrifugation at 2,500 x g to isolate the precipitate. Finally, the precipitate was freeze-dried. According to the research findings, the collagen yield from shortfin scad skin and bone was 0.10 ± 0.13% [14].

2.3 Hydro-extraction

The hydroextraction method is an isolation technique that uses water as a catalyst (acting as heat as a transfer medium) and temperature as a determining factor. This method has several advantages, such as shorter production time, lower production costs, high yields, and ease in controlling the amount of waste, which is in line with the principles of clean technology and sustainable production. The collagen produced in this way is water-soluble and safe for long-term consumption. The water extraction method involved first rinsing the sample with distilled water until the pH approached neutrality. Subsequently, the sample was extracted using water, specifically aquabides, at a 1:1 ratio (by weight/volume) at a temperature of 40°C for a duration of 2 hours. The extraction yielded water-soluble collagen, which was then dried using a freeze dryer to obtain collagen in powdered form. The heating process of the sample in warm water leads to the continuation of the disruption of hydrogen and covalent bonds that had previously occurred during the acetic acid soaking process. The choice of a temperature of 40°C is made with the aim of preventing the degradation of collagen into gelatin during the ongoing extraction. Based on the results of research that has been carried out, the yield of collagen from catfish skin was 12.15% [15] and Alaska pollock skin was 3.13 ± 0.04% [16].

2.4 Ultrasound-assisted extraction

Ultrasonic-assisted extraction (UAE) utilizes high-intensity sound waves surpassing the normal human hearing range (20 kHz) to expedite mass transfer, improve mixing, drying, homogenization, and extraction processes. Moreover, UAE has gained widespread use in collagen extraction due to its capacity to enhance yields and reduce processing time. The mechanism relies on wave propagation, creating zones of high and low pressure that are directly proportionate to the applied energy in the system. Typically, collagen extraction employs organic acid solutions such as acetic acid, citric acid, and lactic acid, as they demonstrate higher extraction efficiency compared to inorganic acids.

The extraction procedure commenced by immersing the pre-treated sample (100g) in a 0.5M acetic acid solution, maintaining a sample-to-acid solution ratio of 1:20 (w/v), for 30 minutes at 4°C. Subsequently, ultrasound treatment was administered using an ultrasound processor operating at 20 kHz and 500W for a duration of 30 minutes. To prevent overheating, the ultrasound operated in a pulse mode, featuring 5-second treatment intervals followed by rest periods. Following this, the resulting extract underwent centrifugation at 6,500×g for 15 minutes at 4°C, effectively separating the supernatant from the residue. The
residual material then underwent a second extraction using 0.5 M acetic acid at a sample-to-solution ratio of 1:10 (w/v), employing the same ultrasonic processor at 20 kHz and 500W for 30 minutes. Subsequently, another round of centrifugation was conducted at 6,500×g for 15 minutes at 4°C. The two batches of supernatants were amalgamated, and the solution was salted with NaCl to achieve a final concentration of 0.7 M. This mixture underwent centrifugation once more at 5000×g for 10 minutes at 4°C, resulting in a precipitate that was subsequently frozen overnight at -80°C before undergoing the drying process. These extraction steps were replicated using hydrochloric acid. According to the research findings, the ultrasound-assisted method with acetic acid yielded 11.49 ± 0.03%, while with hydrochloric acid, a yield of 3.50 ± 0.01% was obtained [17].

Higher collagen yields were achieved by extraction with acetic acid compared to hydrochloric acid, due to the better extraction ability of acetic acid. Acetic acid plays an important role in determining the pH of the extraction medium and controlling the collagen loading density. These factors influence the structure, solubility, and extraction of collagen [21]. The increase in collagen yield associated with acetic acid extraction can be further explained by the phenomenon of cavitation, which results in loss of pretreated skin. This loss facilitates more effective penetration of acetic acid through the skin, thereby increasing collagen extractability [19].

2.5 Deep eutectic solvent

Deep Eutectic Solvents (DES) and eutectic mixtures have been effectively employed for the dissolution and extraction of various bioactive compounds. DES typically consists of hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD), which, when combined, form robust hydrogen bond interactions, resulting in the creation of eutectic mixtures that often exhibit liquid properties at temperatures near room temperature. DES is utilized to regulate temperature during extraction, favoring lower temperatures to prevent thermal degradation of collagen. The DES method presents an environmentally friendly, efficient, and straightforward approach to collagen recovery. Collagen extraction was carried out using 0.5 M acetic acid as the control solvent, and the results were compared with those obtained using a Deep Eutectic Solvent (DES) solution in water. This DES comprised betaine (Bet), urea (U), cholinium chloride (CC) as hydrogen bond acceptors (HBA), and formic acid (FA), acetic acid (AA), propanoic acid (PA), and lactic acid (LA) as hydrogen bond donors (HBD). Parameters such as the solid-liquid ratio, pH, and DES concentration were further explored and optimized. The molar ratio of DES components used was typically 1:2, except for instances where U : FA (1 : 4) and U : AA (1 : 5) were employed. The preparation of DES involved several steps. Initially, the water content in the starting material was determined using a Metrohm 831 Karl Fisher coulometer. Subsequently, the two-component mixture (HBD and HBA) was precisely weighed and placed in a round-bottom flask. The mixture was then stirred in an oil bath at 60 ± 2°C with a stirring speed of 500 rpm until it became a homogeneous and transparent liquid. The pH of all aqueous solutions was measured at room temperature. All procedures were conducted at 4°C.

The collagen extraction process commenced with the pre-treatment of the sample, involving cleaning, washing, size reduction, and the removal of non-collagen proteins. This included immersing the skin in a 0.1 M sodium hydroxide (NaOH) solution at a solid-to-solvent ratio of 1:10 (w/v) for 24 hours, with periodic replacement of the NaOH solution every 5-6 hours. Subsequently, the sample was rinsed with cold distilled water until a neutral pH was achieved. Deproteinized samples were then defatted using a 10% (v/v) butyl alcohol solution at a solid-to-solvent ratio of 1:10 (w/v) for 48 hours, with solvent changes every 6-7 hours. Once again, the samples were washed with cold distilled water. Extraction was performed at 4°C for 48 hours using an optimized solid-liquid ratio of 1:10 (w/v) in either 0.5 M acetic acid or an aqueous DES solution, utilizing an orbital shaker. The resulting dense
solution was subjected to centrifugation to eliminate insoluble components. The supernatant was salted by adding NaCl to a final concentration of 0.9 M, followed by collagen precipitation by introducing NaCl (final concentration 2.6 M) at a neutral pH (0.05 M TrisHCl, pH 7.5). The resulting precipitate was obtained by centrifugation at 15,000 g for 1 hour and dialyzed against deionized water at 4°C for 72 hours. Finally, the collagen was dried through a process of lyophilization. The DES method is an environmentally friendly method for restoring collagen, efficient and simple. According to research results, the yield of Atlantic codfish skin was 5.2% [18].

2.6 Supercritical fluid extraction

The extraction of collagen using supercritical fluid technology involves employing acidified water under CO$_2$ pressure for the extraction process. Carbon dioxide is the preferred choice in this technology due to its advantageous properties, such as low toxicity, non-flammability, cost-effectiveness, high availability, stability, and environmental acceptability. Furthermore, carbon dioxide's suitability stems from its operational conditions at moderate temperatures and pressures. Additionally, carbon dioxide serves as a versatile acidifying agent, released from the aqueous medium post-extraction, resulting in a purified compound. The use of CO$_2$-acidified water entails a one-step extraction with mild operational conditions and eliminates the need for organic solvents.

The extraction process begins with a thorough cleaning of the sample using distilled water to eliminate any remaining impurities, followed by grinding the sample into small pieces. The weighed wet samples are then placed in a high-pressure vessel (30 cm$^3$) containing distilled water (1 g per 20 ml). The vessel is heated to 37°C, and the system is pressurized with carbon dioxide up to 50 bar. The extraction is conducted in batches, utilizing an intermittent mode without external exchanges, for 3 hours. Subsequently, the high-pressure vessel is depressurized efficiently, and the extract is obtained through a double filtration process, initially with filter paper and then with a 0.45 μm syringe filter. The resulting acidic water-treated collagen (AWC) is frozen, subjected to freeze-drying, and stored at room temperature for future use. According to the research findings, the collagen yield from Atlantic codfish skin was 13.8 ± 0.013%. The method operates under mild conditions and employs acidified and pressurized water. The obtained results were higher compared to the conventional acetate-based methodology [22].

3 Collagen yield

Yield is the percentage of collagen obtained from the initial raw material. The yield shows the portion of raw materials that can be utilized and functions as an important parameter for assessing the economic value and efficiency of a material. The percentage yield calculation is obtained by the ratio of the dry weight of the isolated collagen to the weight of the raw material before isolation. The calculation of the % yield value utilizes the AOAC method with the formula [23]:

$$\text{Collagen yield (\%)} = \frac{\text{Dry weight of collagen}}{\text{Weight of raw materials}} \times 100\%$$

The percent yield differs between various vertebrate fish species using different extraction methods. This variation is caused by several factors such as differences in protein content in fish, pretreatment conditions [24], temperature, time, and extraction method [15]. Fish with high protein content will yield a higher collagen yield compared to those with low protein content. The initial processing of raw materials using a basic solution has proven to be more efficient than an acidic solution in separating non-collagen proteins, resulting in increased collagen production. Temperature and duration in the collagen isolation process also significantly influence the amount of collagen produced. An increase in temperature
above 40°C can lead to the denaturation of collagen into gelatin. Additionally, the duration of soaking the raw materials during the pretreatment process also contributes to the solubility of collagen in the basic solution [15, 24].

4 Conclusions

Based on the journal review above, we can conclude that vertebrate fish can be used as a source of collagen which comes from fish waste in the form of skin, bones and scales. Collagen isolation from vertebrate fish can use different methods depending on the collagen source, in the form of acid soluble collagen extraction, pepsin soluble collagen, hydro-extraction, organic acid ultrasound-assisted, deep eutectic solvent, and supercritical fluid extraction. The extraction method that produces the highest collagen yield is supercritical fluid extraction, where this method uses CO2 pressure and is more efficient. Collagen yield results produced using different methods have differences because they are influenced by protein content, pre-treatment, temperature and time.

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References

15. P. Suptijah, D. Indriani, S. E. Wardoyo, Jurnal Sains Natural, 8, 8–23 (2018).