ORIGINAL ARTICLE

Particle size analysis of yellowfin tuna *(Thunnus albacares)* skin collagen powder using papain-soluble collagen method with varying NaCl concentrations: an experimental laboratory

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ABSTRACT

Introduction: The skin of yellowfin tuna (Thunnus albacares) contains high protein, which could potentially be a halal collagen product. Collagen extraction using the papain-soluble collagen method has the advantage of producing a higher collagen yield compared to the acid method. Particle size, one of the physical properties of collagen, plays a crucial role in its efficacy in dentistry. This study aims to analyze the particle size of collagen powder synthesized from *Thunnus albacares* skin using the papain-soluble collagen method, with varying concentrations of NaCl. **Methods:** Type of research is an experimental laboratory. *Thunnus albacares* skin was synthesized by chopping, cleaning, and soaking in a 0.1 M NaOH solution. The extraction process used the enzyme papain and 0.5 M of acetic acid. Samples were divided into four groups, each with different concentrations of NaCL: 0, no NaCL;, 0.9 M NaCl, 1.3 M NaCl, and 1.7 M NaCl. After centrifugation, the samples were freeze-dried. The particle size of collagen powder was measured using a Particle Size Analyzer test tool. The data collected was then analyzed using the Mann-Whitney test. **Results:** Particle size distributions are as follows: K group (3.36 nm), P1 (1.842 nm), P2 (3.36 nm), and P3 (10.12 nm). There is a significant difference in groups K-P1 and P3, P1-P2 and P3, and P2-P3 ($p<0.05$). However, there is no significant difference in groups K-P2 ($p>0.05$). **Conclusion:** Particle size of this research produced nano-sized collagen powder, with the lowest particle size observed in the 0.9 M NaCl group, measuring at 1.842 nm. The particle size increased in the group without NaCl and in the 1.3 M NaCl group at 3.36 nm, and reached the highest value in the 1.7 M NaCl group at 10.12 nm.

KEYWORDS

tuna, collagen, particle size

INTRODUCTION

The majority of collagen requirements in Indonesia are currently met through imports.[1]In 2015, this country imported 4109 tons of gelatin and collagen.[2] Collagen is the most abundant protein that binds the entire human body. It is a protein found in skin, bones, muscles, and tendons, serving as a scaffold to provide strength and

structure.3 Collagen has three polypeptide chains (triple-helical) that each contain one or more amino acid repeats with a structure (Gly-X-Y), where X and Y can be composed of many amino acids.[4]

The process of extracting collagen relies on the source material, aiming to eliminate all non-collagen components and obtain collagen as the end product. Typically, the recovery procedure includes pre-treating the source tissue, extracting collagen, and then purifying the extracted collagen.[5] There are three main methods for extracting collagen : neutral salt-soluble collagen, acid-soluble collagen (ASC), and pepsin-soluble collagen (PSC).[6] The neutral salt-soluble collagen method is used less frequently.[5] The PSC method uses pepsin enzyme derived from pigs, which, however, may raise concerns due to the recent swine flu outbreak, and the necessity to consider the dietary restrictions of the Muslim community, particularly regarding the use of pepsin porcin for consumption. Collagen can also be extracted using proteases, known as papain-soluble collagen (PaSC). This method is known to have higher collagen solubility than the PSC method, and produce higher collagen yields than the ASC method. The papain enzyme used in the PaSC method comes from papaya fruit and sap, making it permissible for consumption according to halal dietary guidelines.[7]

Collagen can be isolated from animal sources, especially vetebrata.[8] Collagen from external sources finds extensive applications in the food industry, biomaterial development, and pharmaceutical settings. Collagen is used in dentistry for a variety of purposes, such as employing collagen blocks or sponges for hemostatic materials, creating oral wound dressings, expediting wound healing, covering graft and extraction sites, and using collagen membranes in periodontal and implant treatments.[9] Collagen is employed in tissue engineering applications due to its favorable biocompatibility, biodegradability, and low antigenicity. It serves as an effective scaffold for bone regeneration as it can be naturally absorbed by the body and exhibits excellent adhesion to cells.[10] In 2020, Indonesia produced up to 515 thousand tons of tuna .[13] Among the various species of tuna, the Yellowfin tuna (*Thunnus albacares*) is the most commonly harvested types.[14] Tuna is typically sold in its fresh form (refrigerated), fillets, and canned products. The bones and skin are the byproducts/remnant/waste materials obtained from these tuna products. Yet, their use remains suboptimal in certain industries. As a result, the skin waste of tuna is often simply disposed, causing the formation of unused solid waste.[15]

Particle size is one of the important factors in ensuring the effectiveness of collagen applications in the biomedical and cosmetic fields.[16] Drug delivery systems utilizing smaller particles provide enhanced drug effectiveness, prolonged drug half-lives, improved solubility for hydrophobic drugs, and controlled, sustained release of drugs specifically in infected regions. Nanoparticles, which are responsive to stimuli, regulate drug biodistribution and mitigate drug toxicity. The size of nanoparticles in collagen is in the form of granules or very small particles between 1 and100 nm.[17] Recreating the composition of the natural extracellular matrix (ECM) in tissue involves building a three-dimensional (3D) cellular scaffold with a suitable mechanical strength. This approach facilitates the observation of cellular activities and enables the delivery of bioactive agents. To achieve satisfactory results, it is necessary to employ a nanoscale strategy rather than a macroscopic approach.[18]

Size significantly affects the surface area of nanomaterials involved in interactions within biological environments. Consequently, size represents a crucial physicochemical attribute influencing the cellular response and the fate of nanomaterials in vivo. Cellular responses encompass cytotoxicity, penetration of biological barriers, and immune reactions. For instance, smaller nanomaterials exhibit a higher surface area-to-volume ratio, thereby enhancing their reactivity. Furthermore, reduction in size is recognized to enhance vascular permeability positively.[19]

Several studies have applied nanomaterials in alveolar bone regeneration. Nano-hydroxyapatite (nHA) and collagen are extensively studied biomaterials for alveolar bone regeneration. Hydroxyapatite, a natural mineral found predominantly

in bone tissue, serves as the primary component. Nano-hydroxyapatite (nHA) offers a higher surface area-to-volume ratio compared to conventional hydroxyapatite, enhancing its suitability as a bone graft substitute. Collagen, a crucial component of the extracellular matrix in various tissues, including alveolar bone, provides structural support, facilitates cell-biomaterial interactions, and promotes cell adhesion, thereby influencing cellular behavior. These properties make collagen a desirable biomaterial for alveolar bone regeneration. Both nHA and collagen exhibit excellent osteoconductive properties, supporting the formation of new bone tissue by serving as scaffolds for bone growth. They are also noted for their good biocompatibility and mechanical strength.[19]

Collagen extraction is generally precipitated using NaCl in the presence of *tris(hydroxymethyl)aminomethane* at pH 7.5 with an adjusted salt concentration to maximize dirt removal and optimize collagen refinement.[20] Generally, increasing high concentrations of NaCl affects protein aggregation in the salting-out process, resulting in an increase in the particle size.[21] This study aims to determine the effect of variations in NaCl concentrations (0.9 M, 1.3 M, and 1.7 M) on particle size during the synthesis of collagen powder from yellowfin tuna (*Thunnus albacares*) skin, using the papain-soluble collagen method. Understanding these effects is essential for optimizing collagen extraction and improving the quality of the final product.

To date, no previous studies have specifically examined the particle size distribution of collagen powder synthesized from *Thunnus albacares* skin using the papains Soluble collagen method with varying NaCl concentrations.This study aims to analyze the particle size of collagen powder synthesized from *Thunnus albacares* skin using the papain-soluble collagen method, with various concentrations of NaCl.

METHODS

Type of research is an experimental laboratory. The tools used in this study were scissors, cutting boards, beakers, measuring cups, enlemeyers, glass stirring rods glass jars, basins, refrigerators at 4 °C and 10 °C, freeze dryers, centrifuges at 6000 rpm, analytical scales, 10 mL conical tubes, filter cloths, specimen containers, 5L jerry cans, PSA (Particle Size Analyzer) test equipment. The materials used in this study included yellowfin tuna skin, NaOH 0.1 M, CH_3COOH (acetic acid) 0.5 M, NaCl (0.9 M 1.3 M, and 1.7 M), Aquabidest, dialysis bag, papain enzyme 10.000 U/g, pH indicator paper, water, a hand scoon.

The yellowfin tuna (*Thunnus albacares*) skin was separated from the meat and thoroughly washed using water. After the washing process, the skin sample was chopped into small pieces with scissors to facilitate the extraction process. The weight of the skin after washing was recorded as 310.32 grams. The sample was then soaked in a 0.1 M NaOH solution for 24 hours, with the solution being changed every 8 hours (at a temperature of 10 °C) with skin comparison and a 1:10 solution. Following the 24-hour soaking, the sample was rinsed with Aquabidest to achieve a pH of 7 (neutral).

In hydrolysis and extraction stage, the sample was soaked in a solution of 0.5 M CH_3COOH (acetic acid) at a 1:10 ratio with a sample to acetic acid, along with 10.000 U/g of papain enzyme (270.249 gr). This mixture was stirred by hand for 3 days to ensure that the enzyme was well mixed in the sample. Following this, the collagen extract was filtered using a filter cloth to obtain the filtrate. In the precipitation process, the collected filtrate was taken and divided into 4 groups: K, P1, P2, and P3; except the K group, which was not treated with NaCL, each was treated with 0.9 M, 1.3 M, and 1.7 M of NaCL concentrations, respectively. The precipitation stage was conducted at a temperature of 4 °C for 24 hour. The filtrate was then pipetted into a 10 mL conical tube and subjected to centrifugation at a speed of 6000 rpm for 1 hour to collect the pellets. These pellets were then transferred into a 14 kDa dialysis bag and placed in an Aquabidest solution for a-24 hour dialysis process. This step was designed to to remove residual salts and acids accumulated during the extraction and precipitation stages, as well as to purify the

collagen by eliminating any undesirable small molecules. The dialyzed samples were subsequently frozen at 4 °C. Afterward, the frozen samples from each group were transferred into freeze-drying tubes and subjected to freeze-drying at -80 °C for three days, resulting in the production of collagen powder.

The Particle Size Analyzer (PSA) instrument, in conjunction with Biobase software, conducted particle size characterization. The process involved dispensing up to 10 mL of the sample into a cuvette, which was then inserted into the sample holder for analysis using a particle size analyzer (PSA). Image displayed on a computer screen visualized the resulting particle size distribution, covering a measurement range from micrometers to nanometer scale.

The PSA test results included multiple particle size measurements from each sample. For this research, the particle sizes considered were those from the top 4 volume peaks, which reflected the particle size distribution within the sample.

The collected data was then statistically analyzed using SPSS 25. The normality test was performed using the Shapiro-Wilk test and the homogeneity test using Levene's test. In this study, our data successfully passed the normality test ($p>0.05$) but doesn't fulfill the homogeneity requirement ($p>0.05$). Therefore, the statistical analysis in this study involved a non-parametric test using Kruskal Wallis, followed by a Mann-Whitney test with difference significancy ($p<0.05$).

RESULTS

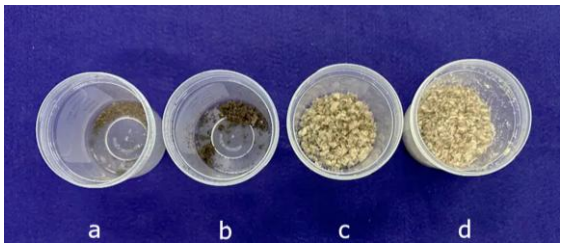

Figure 1. Synthesis of thunnus albacares collagen powder: a. 0 NaCl (K), b. 0.9 NaCl (P1), c. 1.3 NaCl (P2), d. 1.7 NaCl (P3)

Figure 1 illustrates the variations in collagen powder outcomes obtained from this study. The group dissolved in 1.7% NaCl exhibited a lighter, nearly white color compared to the other groups. According to the conducted research, the mean outcomes and standard deviation of the particle size for collagen powder from yellowfin tuna (*Thunnus albacares*) skin, obtained through the papain-soluble collagen method with different NaCl concentrations. This information is presented in the following table.

Table 1. Average value and standard deviation of collagen powder particle size from yellowfin tuna skin, with variations in nacl concentration

Group	Replication	Mean ± Std. Deviation (nm)
0 NaCl (K)	5	3.36 ± 0.47826
0.9 NaCl (P1)	5	1.842 ± 0.2934
1.3 NaCl (P2)	5	3.36 ± 0.47826
1.7 NaCl (P3)	5	10.12 ± 1.59354

K: Control Group (Synthesis of *Thunnus albacares* fish skin collagen powder using papain-soluble collagen method without or with 0 NaCl administration); P1: Synthesis of *Thunnus albacares* fish skin collagen powder using the papain-soluble collagen method with a 0.9 M NaCl 0.9concentration; P2: the Synthesis of *Thunnus albacares* fish skin collagen powder using the papain soluble collagen method with a 1.3 M NaCl concentration 1.3). P3: Synthesis of *Thunnus albacares* fish skin collagen powder using the papain-soluble collagen method with a 1.7 M NaCl concentration of 1.7.

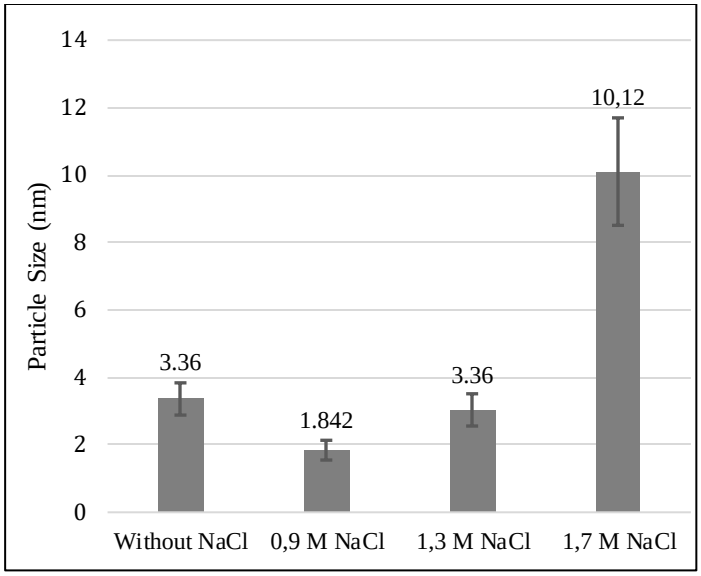

Figure 2. Diagram of average and standard deviation of particle size of collagen powder from yellowfin tuna skin with variations in nacl concentration.

Based on Table 1 and Figure 1, the lowest mean and standard deviation values are shown in group P1 (1.842 ± 0.2934). In the groups K and P2, it was observed that their average values exceed those of Group P1 (3.36 ± 0.47826). Conversely, Group P3 obtained the highest average (10.12 ± 1.59354). The Shapiro-Wilk normality test revealed that all treatment groups show a significance value of $p > 0.05$, indicating that the data is normally distributed. Additionally, the Levene test for homogeneity, with a significance value of 0.01 ($p<0.05$), confirmed that the particle size data of yellowfin tuna skin collagen powder is not homogenous. Therefore, hypothesis testing can proceed using non-parametric tests, such as the Kruskal-Wallis test, followed by a Mann-Whitney test for further analysis .

Table 2. Kruskal-Wallis statistical test

	Particle size	Asym. Sig
0 NaCl	3.36 nm	0.001
0.9 M NaCl	1.842 nm	
1.3 M NaCl	3.36 nm	
1.7 M NaCl	10.12 nm	

The Kruskal-Wallis test yielded a significance value of 0.001 ($p<0.05$) as presented in Table 2, indicating differences among all treatment groups.

Table 3. Post Hoc mann-whitney test

Group	0 NaCl (K)	0.9 M NaCl (P1)	1.3 M NaCl (P2)	1.7 M NaCl (P3)
0 NaCl (K)		0.009*	1.000	0.009*
0.9 M NaCl (P1)			0.009*	0.009*
1.3 M NaCl (P2)				0.009*
1.7 M NaCl (P3)				

The results of the Post Hoc Mann-Whitney test, as presented in Table 3, reveal a significance level of $p<0.05$ between the control group and groups P1 and P3, as well as between group P1 and groups P2 and P3, and also between groups P2 and P3, indicating significant differences. Conversely, a significance level of $p<0.05$ was observed in the comparison between the control group and group P2, suggesting no significant difference between them.

DISCUSSION

In this study, particle measurements were carried out using the PSA (Particle Size Analyzer) tool to determine the particle size distribution of each group. The size of the particle plays an important role, especially in the biomedical field. Particle size affects the effectiveness (efficacy) of drug delivery. This is because small particle sizes can easily enter the body through various routes and can also reach the most sensitive organs.[22] Utilizing nano-sized particles offers a promising approach to preventing, reducing the duration of treatment, or eliminating oral cavity issues such as like dental caries, periodontal disease, peri-implantitis, oral candidiasis, hyposalivation, and more. These nano-sized particles can also be integrated into dental materials, such as PMMA and glass ionomer cement, thereby enhancing the properties of prosthetics and dental restorations.[23]

During the precipitation process, which involves the use of 0.9 M NaCl solution, stirring is performed with a stirring rod to facilitate the formation of fine fibers. The prolonged duration of the stirring process increases the intensity of solvent molecules interacting with the collagen, resulting in a reduction in the particle size. Consequently, the average particle size of group P1 becomes smaller than group K collagen powder. In group K there is no NaCl solution used, therefore, no stirring treatment was involved in the process, which makes the average particle size larger than in group P1. This finding is similar to the results of the study by Syafrijal et al., (2018), who demonstrated that the particle size of collagen from sand sea cucumber (*Holothuria scabra*) decreased with increasing stirring time. However, the difference in our study is that we used collagen derived from yellowfin tuna skin.[16]

There is a significant difference between group K (without NaCl) and P3 (NaCl 1.7 M), where the average particle size of group K is 3.36 nm smaller than that of group P3, which is 10.12 nm. NaCl affects the formation of particle sizes in collagen. High concentrations of NaCl increases the ionization forces of salts compared to proteins, allowing NaCL to remove the ideal amount to stabilize collagen fibers and promote proteins aggregation, thereby increasing the size of the resulting particles.[21,24] Therefore, the P3 group of tuna skin collagen powder, which received a NaCl concentration of 1.7 M, produced a larger particle size than the K group, which did not receive NaCl. Thus far, there have been no specific studies similar to ours that investigate the effect of 0.9 M, 1.3 M, and 1.7 M NaCl concentrations and without NaCl on the particle size of yellowfin tuna skin collagen powder using the papain soluble collagen method.

The results showed that in group K (without NaCl) and group P2 (NaCl 1.3 M), there was no significant difference, and the average particle size of both was 3.36 nm. This happens because the effects of acetic acid, NaCl and stirring time also affect the particle size results. During the synthesis of collagen powder, the same treatment was carried out, which involved soaking samples in a 0.5 M acetic acid solution. The collagen extracted using this method tended to exhibit a relatively larger particle size compared to that obtained from natural acids.[25] Another factor that can interfere with the results of particle size is the duration of stirring. The prolonged stirring process will produce a smaller particle size due to an increase in the intensity of solvent molecules interacting with collagen, thereby reducing the particle size.[16]

When adding NaCl in the precipitation stage, stirring is conducted on samples from P2 group, which have been treated with a 1.3 M NaCl solution. This high concentration of NaCl in the P2 group can increase the particle size, but the stirring process can also reduce the particle size. Therefore, there is no significant difference in the average particle size of the P2 group (treated with NaCl at 1.3 M) compared to the K group (without NaCl). The increased particle size of group K (without NaCl) is related to the presence of 0.5 M acetic acid and the absence of a stirring process. Consequently, the effect of the average particle size of group K is no difference from that of the P2 group.

The study's results revealed a significant difference between the three groups: P1 (NaCl 0.9 M), P2 (NaCl 1.3 M), and P3 (NaCl 1.7 M). In the P1 and P2 groups, a significant difference was observed in the average particle size, with P2 demonstrating a larger average particle size of 3.36 nm, compared to P1 with an average particle size of 1.842 nm. This difference can be attributed to the higher concentration of NaCl affecting the ionic conditions during the salting-out process. Increased ionization levels can cause proteins to aggregate, which ultimately contribute to an increase in particle size. Furthermore, at higher salt concentrations, an increased amount of more water binds to stabilize collagen fibers, further significantly increasing particle size.[21]

Similarly, the P1 group has significant differences with the P3 group. The average particle size within the P3 group showed an increase of 10.12 nm compared to the P1 group, which had an average particle size of 1.842 nm. This is in accordance with the theory that higher NaCl concentrations have an impact on increasing particle size.

There is a significant difference between the P2 group (NaCl 1.3 M) and the P3 group (NaCl 1.7 M). It is shown by the average particle size of the P3 group of 10.12 nm; however, the average particle size of the P2 group of 3.36 nm. This significant difference in particle size occurs because a higher concentration of NaCl also leads to an increased size of the particle. The high concentration of NaCl affects ionic conditions during the salting-out process. higher ionization levels can also aggregate the proteins, which in turn, increasing the particle size.[21,24]

Other factors that can affect particle size include pH, temperature, and stirring duration.[26,27] Increased pH can decrease the size of nanoparticles.[17] Rising temperature leads to a reduction in viscosity, signifying a decline in resistance to fluid flow. Elevated temperatures will break the bonds between molecules in a solution, resulting in the formation of smaller particles.[28] A longer stirring time is more effective in decomposing compound particles, thereby increasing the surface area of the compound and reducing the content of water, fat, and ash.[27] The ideal particle size for a 3D biomaterial product, utilizing nanoscale technology, is collagen ranging between 1 and 100 nm. This size range offers a substantial surface area to volume ratio, facilitating the products to effectively penetrate into the wound area.[29]

Based on the results of research on the synthesis of collagen powder from *Thunnus albacares* fish skin using the papain-soluble collagen (PaSC) method, the average particle size observed in groups without NaCl administration and those with NaCl administration of 0.9 M, 1.3 M and 1.7 M were 3.36 nm, 1.842 nm, 3.36 nm, and 10.12 nm, respectively. These results differ from those reported by Kusa et al. (2022), who reported the particle size of yellowfin tuna collagen, measured using a binocular microscope at a magnification of 40x10, ranging from 73.8 nm to 693.72 nm. This inconsistency can occur due to a different extraction method employed in this study, specifically the papain-soluble collagen method, in contrast to Kusa et al., (2022), who used the hydro-extraction method for collagen extraction. Additionally, the selection of particle size analysis tool used in this study can also affects the results of collagen powder derived from tuna skin. In this instance, a Particle Size Analyzer (PSA) was utilized, which employs light diffraction principles to calculate particle size distribution. In contrast, a binocular microscope uses optical magnification to enlarge and observe particles.[30] Collagen nanoparticles, with a size smaller than 200 nm and larger than 5 nm, make it a potential drug delivery method to enter cells via endocytosis. [26]

Due to electrostatic interactions, collagen may easily attach to cell membranes as cationic nanoparticles.[26] This is consistent with the results of the research on the treatment group, which used NaCl at a concentration of 1.7 M and achieved an average particle size of 10.12 nm. Therefore, taking into account the concentration of NaCl used in the synthesis of collagen powder, it is expected that this will produce good collagen powder in a biomaterial product. A reliable characteristic of high-quality collagen powder is its colorless and odorless nature, with a white appearance. In this research, the treatment groups with NaCl

concentrations of 1.3 M and 1.7 M closely resembled these characteristics, exhibiting a visually yellowish-white color. The color of the produced collagen powder is influenced by factors such as the fish species, the initial color of the fish's skin, extraction conditions, and the drying process.[31-32]

The criteria for a material suitable as an intermediate biomaterial include possessing biocompatible properties, a well-defined pore structure, and bioresorbability. Consequently, further research is required. Biocompatibility refers to a biomaterial's ability to perform its intended function in medical therapy without causing any adverse local or systemic effects in patients receiving such treatment. Nanostructured materials have the potential to enhance both biocompatibility and mechanical properties in medical devices.

Additionally, the combination of drugs with nanostructured polymers can regulate the rate of drug release during administration. Porosity plays a significant role in network engineering. Porous biomedical materials find application in various fields such as tissue regeneration, carrier systems, controlled drug release, wound dressings, and hemostatic agents.[33]

Sustainable biomass and innovative biotechnologies are already transforming human life and offering new directions for research in marine and food sciences, chemical engineering, biotechnology, and pharmaceutical sciences. Collagen, with its diverse applications and critical importance in tissue engineering, is a vital biopolymer for human health and well-being. Due to the rising demand for collagen, there is an urgent need to find sustainable and cost-effective production methods that reduce/minimize animal use. Consequently, marine biomass is emerging as a promising source of collagen.

Therefore, acquiring a deeper understanding of the physicochemical and biological properties of marine collagen, in addition to its extraction and purification methods, would greatly contribute to addressing these challenges. It is imperative, however, to underscore the necessity for further comprehensive research in this area. Moreover, it's essential to establish connections between particle size and other test parameters due to their interdependency. The extraction conditions need to be optimized to produce the highest quality collagen powder suitable for clinical applications.

CONCLUSION

This research produced nano- sized collagen powder, with the lowest particle size observed in the 0.9 M NaCl group, measuring at 1.842 nm. The particle size increased in the group without NaCl and in the 1.3 M NaCl group at 3.36 nm, and reached the highest value in the 1.7 M NaCl group at 10.12 nm. The findings of this study indicate that the particle size of all groups fall within the accepted range for nanoparticle size suitable for collagen material in tissue engineering applications. However, a more comprehensive study is required to determine the optimal concentration of NaCl for biomaterial purposes. The implications of this research highlight the importance of fine-tuning NaCl concentration to effectively control the particle size of collagen powder. This control is crucial for enhancing the performance and applicability of collagen in medical fields.

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of data sets analyzed or generated during the research.
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