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Reduction of Collagen Powder Size from Snakehead Fish Skin (Channa Striata) Using Desolvation Method

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Abstract: The size of collagen which was isolated from the skin of snakehead fish (Channa striata) is important in relation to the pharmaceutical applications of collagen, especially in collagen solubility and its dispersion systems for topical applications. In smaller sizes, even reaching nanometer sizes, it is hoped that it will improve the penetration of collagen through the skin when applied. The aims of this study is to obtain collagen microparticulates isolated from the skin of snakehead fish (Channa striata) in a smaller size using the desolvation method with variations in the ratio of collagen and ethanol (w/v) 1:1 and 1:2, stirring time 15, 30, 60 , 120 minutes, and the stirring speed of 3000, 6000, 12000 rpm. The collagen was characterized by its physicochemical properties including particle size, thermal properties, X-ray diffraction patterns, morphology with SEM and infrared spectra. The results of characterization of collagen after treatment with the ratio of the addition of collagen and ethanol 1:2, for 1 hour, and a stirring speed of 12000 rpm showed the smallest particle size was 24.746 µm. Morphological analysis showed random coils indicating the nature of collagen fibrils and no change in functional group analysis. Thermal analysis showed a decrease in the melting point peak from the thermogram results, as well as the intensity of the X-ray diffraction pattern which still shows the characteristics of collagen. The results showed that there was a reduction in the size of the new collagen particles down to the micrometer scale and there was no change in the structure of the collagen triple helix.

Keywords: Collagen; Desolvation method; Powder size; Snakehead fish skin.

Introduction

Collagen with a small particle size even up to nanometers can increase the effectiveness of its use. namely it can penetrate through hair follicles and skin pores, so that with a small collagen particle size it will easily penetrate the epidermis layer of the skin transfollicularly (Setyowati & Setyani, 2015). Commercialized collagen is usually obtained from animal skin, such as pig skin, cow skin, or chicken skin. The use of skin collagen has several problems, including views from religious aspects and health aspects such as biological contamination of TSE (Transmissible Spongiform Encephalopathy), BSE (Mad Cow Disease), FMD (Foot and Mouth Disease) and so on (Setyowati & Setyani, 2015). These problems require alternative sources of collagen that are suitable other than mammals. One alternative source of collagen is snakehead fish skin which is known to contain high protein compared to other fish protein levels. In addition, snakehead fish also contains around 16.57% collagen in its skin (Safithri et al., 2016), so it can be used as a source of collagen. It's just that collagen isolated from snakehead fish skin will produce collagen fibers. so that efforts are needed to obtain collagen in the form of small particles for pharmaceutical purposes (Jafari et al., 2020; Martien et al., 2012).

Efforts to reduce the size of collagen particles can be done biologically, physically and chemically (Devatha & Thalla, 2018). One method of reducing the size of collagen nanoparticles that is simple, easy to apply and affordable is the desolvation method using the bottom-

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up technique. This method is done by adding a desolvation agent such as ethanol to the protein. This desolvation agent will reduce the amount of water contained in the collagen molecule, and form intramolecular hydrogen bonds in collagen so that the collagen becomes dehydrated (Herson et al., 2023; Lastri & Putra, 2020). This method is accompanied by sizing using an ultra turrax tool to be more effective in producing nanoparticles. Ultra turrax has a working principle of providing friction between particles with a rotor-stator system. The speed of the rotor can pull the sample to the rotation axis and then spread out through the stator slot so that smaller and more uniform particles are obtained in terms of shape and size (Paroutoglou et al., 2021).

Particle size reduction can be affected by stirring time and speed. The longer the stirring time, the smaller the particle size, this is because more particles are broken down into smaller ones (Desmelati et al., 2020). Several studies have succeeded in reducing the size of collagen using the desolvation method. Among them are sea cucumber meat collagen with an average particle size of 285 nm at a stirring time of three hours and bamboo cone fish skin collagen with an average particle size of 146.71 nm with a stirring time of two hours (Desmelati et al., 2020; Suptijah et al., 2018). Collagen has properties that are susceptible to changes in its triple helix structure when given a treatment, but research on ensuring the characteristics of the collagen formed is still rare. Based on this, the author conducted a study on the reduction of the size and characterization of collagen from those isolated from the skin of snakehead fish (Channa striata) using the method of isolating snakehead fish skin collagen (Channa striata) which has been patented with the number IDP000073332.

Method

Materials

The materials used are collagen extracted from the skin of snakehead fish (Channa striata), glacial acetic acid pro analysis (PT. Smart Lab, Indonesia), sulfuric acid (PT. Brataco, Indonesia), sodium hydroxide (PT. Brataco, Indonesia), hexane (PT. Smart Lab, Indonesia), nitric acid (PT. Smart Lab, Indonesia), perchloric acid (PT. Smart Lab, Indonesia), hydrochloric acid (PT. Smart Lab, Indonesia), distilled water, 96% ethanol (PT. Smart Lab, Indonesia), standard buffer (Mettler Toledo, Switzerland), Kjeltabs, Sodium Dodecyl Sulphate solution (Vivantis, United States), acrylamide, ammonium persulfate, tetramethylethylenediamine, isopropanol, tris-HCl (Merck, Germany), glycerol (Vivantis, United States), beta-mercaptoethanol (Sigma-Aldrich, United States), polyacrylamide gel (Vivantis, United States), running buffer, separating gel, stacking gel (ThermoFisher Scientific, United States), Gangnam-Stain Prestained Protein Ladder (iNtRON Biotech, Korea), commassie blue staining (MP Biomedicals, California).

Isolation of Snakehead Fish Skin Collagen

Collagen was isolated from the skin of snakehead fish (Channa striata) and refers to a method previously patented by Nofita (IDP000073332), namely using a combination of acetic acid and fresh papaya latex (Carica papaya) without centrifugation (Rahmi, 2017)

Collagen Raw Material Examination

The examination of raw materials from collagen extracted from snakehead fish skin (Channa striata) with patent number IDP000073332 has been carried out based on the Indonesian National Standard. The examination of collagen raw materials carried out includes examination of collagen yield, protein band profile, pH value, water content, protein content, fat content, ash content, and heavy metal test. Then continued with collagen characterization which includes functional group analysis, particle size analysis, morphology analysis, thermal analysis and X-ray diffraction analysis.

Size Reduction of Snakehead Fish Skin Collagen Powder

Table 1. Optimization data for reducing the size of snakehead fish skin collagen powder

Ratio Collagen: ethanol 96% (b/v)	Time (minutes)	Speed (rpm)
1:1	15	300
		600
1:2		1200
1:1	30	300
		600
1:2		1200
1:1	60	300
		600
1:2		1200
1:1	120	300
		600
1:2		1200

The size reduction of snakehead fish skin collagen powder begins with optimization to obtain the best conditions in producing small-sized powder. Optimization is carried out by varying the ratio of collagen addition to ethanol (b/v), time and stirring speed (Table 1). The collagen after treatment is dried using an oven at a temperature of 35 ^{o}C (Desmelati et al., 2020). The results of the collagen powder optimization were then analyzed statistically using two-way analysis of variance (ANOVA) with SPSS statistics 25.

Characterization of Snakehead Fish Skin Collagen Powder Functional Group Analysis with Fourier Transform Infrared (FTIR)

The resulting powder was measured using the FTIR Attenuated Total Reflectance (ATR) technique with a wave number range of 4000-500 cm-1. A sample of less than 1 mg was placed on the surface of the ATR Crystal (ZnSe Crystal), then the FTIR spectrum was measured with a resolution of 2 cm-1 and a scanning speed of 64 scans/second (Lebon et al., 2016).

Particle Size Analysis with Particle Size Analyzer (PSA)

Particle measurements were carried out using the Shimadzu Sald-2300 tool using the Dynamic Light Scattering principle. Before the measurement, the sample was dispersed in 1 mL of distilled water, inserted into the PSA tool sample holder. Then the PSA tool will analyze and provide information on the particle size of the sample (Kolanus et al., 2019).

Morphological Analysis with Scanning Electron Microscopy (SEM)

The morphology of collagen extracted from snakehead fish skin can be observed using a Scanning Electron Microscopy (SEM) tool with an acceleration voltage of approximately 1.5 - 20 kV (Dachi et al., 2020).

Thermal Analysis with Differential Scanning Calorimetry (DSC)

Analysis to determine the denaturation temperature and melting temperature of the sample can be done using the Shimadzu DSC-60 Plus tool. A sample of 5-10 mg is placed on a closed aluminum plate, then heated from a temperature of 25-300oC at a heating rate of 10oC per min-1. Then the sample is measured with three repetitions (Zhang et al., 2021).

X-Ray Diffraction Analysis

X-Ray Diffraction analysis is carried out using a diffractometer. The sample is placed on a sample holder (glass) which is then flattened (Marelli et al., 2011). Measurements were carried out with Cu metal, Kα filter, 40 kV tube voltage, 40 mA tube current, and analysis in the range 2θ=5-50oC with a scan rate of 0.02o/second. Bragg equation $2d\sin\theta = \lambda$ (λ=0.154) (Cao et al., 2019).

Result and Discussion

Collagen Raw Material Examination

Collagen from snakehead fish skin isolation was obtained using a method that had been patented previously by Nofita, namely a combination of acetic acid and fresh papaya (Carica papaya) latex without centrifugation. The isolated collagen was then calculated for its yield and obtained at 18.05% (w/w). A protein or peptide band profile can be found from the visualization results with a qualitative method using SDS-PAGE (Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis). This method will separate proteins based on their molecular weight on the electrophoresis gel (Almada, 2018). The molecular weight of the protein is determined by comparing the protein ladder as a comparison on SDS-PAGE whose molecular weight is known.

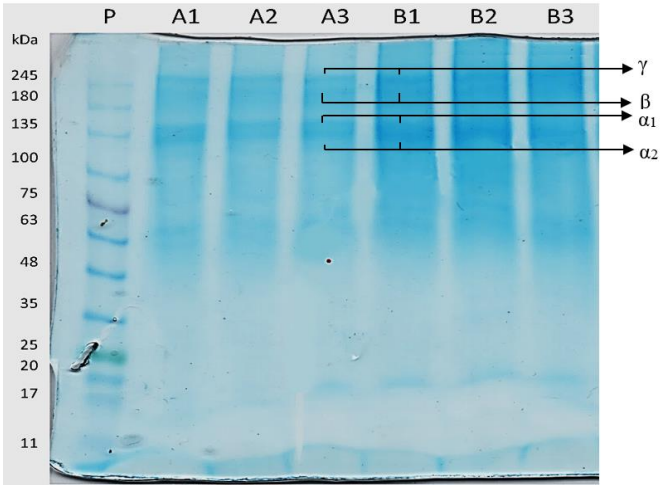


Figure 1. Results of analysis of collagen protein bands of snakehead fish skin (Channa striata) with SDS-PAGE

Description:
P: Gangnam-Stain Prestained Protein Ladder
A: Collagen sample 5 mg/ml
B: Collagen sample 10 mg/ml

The visualization results of snakehead fish skin collagen show separate protein bands at concentrations of 5 mg/ml and 10 mg/ml, namely at a molecular weight of around 135 kDa (Figure 1). The two separate bands are suspected to be the α1 and α2 chains which are one of the characteristics of type 1 collagen. In addition, there are β subunit chains (dimers) with a molecular weight below 245 kDa and γ (trimers) with a molecular weight above 245 kDa. The β subunit and γ subunit indicate the presence of cross-linking bonds in collagen. The β (intramolecular cross-linking) and γ (intermolecular cross-linking) subunits consist of collagen molecules that undergo cross-linking from the α chain forming dimers and trimers (Huma, 2018). The protein bands obtained are in accordance with Ramli's research where collagen still shows bands from type 1 collagen, which contains α, β, and γ components with each molecular weight ranging from 100-130, 200-250, and 264 kDa (Ramli et al., 2020).

Table 2. Results of examination of raw materials for snakehead fish skin collagen (Channa striata)

Analysis	Result	Condition
pH value	6.68 ± 0.01	6.5-8.0 (Panggabean et al., 2023)
Water content	6.012 ± 0.183% (w/w)	≤12% (w/w) (Panggabean et al., 2023)
Protein Content	92.410% (w/w)	≥75% (w/w) (Panggabean et al., 2023)
Fat Content	0.33 ± 0.052% (w/w)	As low as possible (Sari, 2021)
Ash Content	0.685 ± 0.838% (w/w)	≤1% (w/w) (Panggabean et al., 2023)
Heavy metal	Pb: 0.0015 mg/kg	Pb: ≤0.4 mg/kg (Panggabean et al., 2023)
	Cd: 0.095 mg/kg	Cd: ≤0.1 mg/kg (Panggabean et al., 2023)

In Table 2, it can be seen that the examination of the raw material of snakehead fish skin collagen has met the quality requirements of collagen raw materials according to the Indonesian National Standard (Carolina et al., 2024; Panggabean et al., 2023).

Optimization of Size Reduction of Snakehead Fish Skin Collagen Powder

Optimization data in Table 3 shows that all treatments experienced a decrease in particle size on a micrometer scale. The treatment of a collagen and ethanol ratio of 1:2 obtained a smaller particle size compared to the treatment of a collagen and ethanol ratio of 1:1. This shows that the decrease in particle size affects the increase in ethanol addition. The addition of ethanol to collagen functions to reduce the presence of water in collagen particles and causes protein dehydration (Lastri & Putra, 2020).

Optimization of the time and speed of stirring from 15 to 60 minutes shows that the particle size of the snakehead fish skin collagen is decreasing. This shows that the length of time of stirring collagen at high speed causes the collagen rods to be cut into shorter sizes. While at the optimization time of 120 minutes, the size of the collagen particles increased compared to the previous optimization size. This is because the increase in time and speed of stirring results in aggregation between particles which causes the particle size to become larger. Therefore, the best treatment based on the optimization results is the use of a collagen to ethanol ratio of 1:2, a speed of 12000 rpm with a stirring time of 1 hour resulting in a particle size of 24.746 µm.

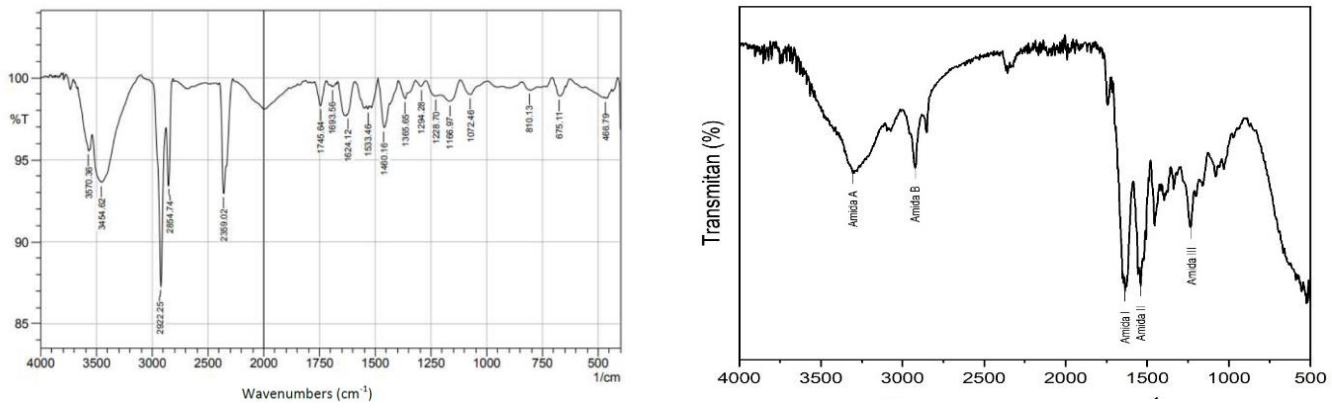
Table 3. Results of optimization of reducing the size of collagen from snakehead fish skin

Collagen and ethanol ratio 96% (w/v)	Time (minutes)	Particle size (µm) with stirring speed		
		3000 rpm	6000 rpm	12000 rpm
1:1	15	83.437	69.901	64.420
	30	79.411	64.630	55.010
	60	72.397	56.299	42.413
	120	75.152	60.212	53.176
1:2	15	73.510	57.324	49.228
	30	67.321	46.128	32.282
	60	59.612	38.190	24.746
	120	66.111	47.021	35.277

The optimization results obtained were analyzed statistically using a two-way ANOVA (Analysis of Variance) test with SPSS statistics 25. Normality and homogeneity tests were carried out first as a requirement for analysis with two-way ANOVA. The results of the normality and homogeneity tests obtained a significance value of >0.05, which means that the data is normally distributed. Based on the significance results of the two-way ANOVA, a sig value of 0.000 (sig <0.05) was obtained for the ratio of collagen to ethanol, time and stirring speed. This states that there is a significant difference in the particle size of the three treatments, so further testing is needed with the Tukey test. The Tukey test obtained a sig>0.05, which indicates that the time and speed of stirring have a significant difference in the particle size of the snakehead fish skin collagen (Channa striata) with the desolvation method.

Characterization of Snakehead Fish Skin Collagen Powder Functional Group Analysis with Fourier transform infrared (FTIR)

Changes in functional groups and secondary structures of collagen can be identified using Fourier transform infrared (FTIR). This analysis is carried out by monitoring the shift in wave numbers in the FTIR spectrum.



(a) (b)

Figure 2. FTIR spectrum (a) of snakehead fish skin collagen according to literature (Nurhayati et al., 2018) (b) raw material collagen

Table 4. Comparison of wave numbers of snakehead fish skin collagen according to literature, raw material collagen, and optimized collagen

Amide Types	Collagen standard range (cm-1)	Wavenumbers (cm-1)			Functional group
		Snakehead fish skin collagen	Collagen raw material	Optimized collagen	
Amide A	3550-3350	3544.62	3304.34	3291.34	Stretching N-H
Amide B	2935-2915	2922.25	2922.16	2926.44	Asymmetrical stretching CH_2
Amide I	1700-1600	1624.12	1635.84	1635.84	Stretching C=O
Amide II	1575-1480	1533.46	1541.12	1544.13	Stretching C-N
Amide III	1301-1229	1294.28	1236.37	1239.11	and Bending N-H

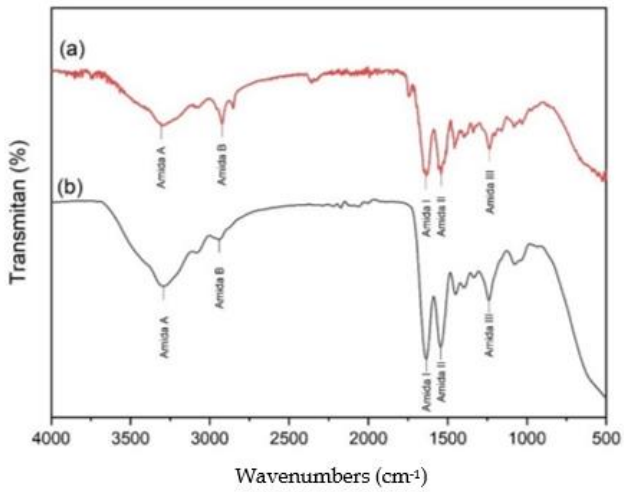


Figure 3. Spectrum (a) of raw material collagen (b) of optimized collagen

Based on the infrared spectrum obtained, the raw material collagen still shows the functional group characteristics of snakehead fish skin collagen, including amides A, B, I, II and III. All wave numbers in each band are still within the standard collagen range listed in Table 4. These results are in accordance with Ramli's research which obtained wave numbers of snakehead fish skin collagen including amide A at 3544.62 cm-1, amide B at 2922.25 cm-1, amide I at 1624.12 cm-1, amide II at 1533.46 cm-1, and amide III at 1294.28 cm-1. The FTIR spectrum of raw material collagen was then compared with the optimized collagen in Figure 3 with the wave numbers listed in Table 4.

Table 4 presents a comparison of the wave numbers of raw material collagen with optimized collagen from snakehead fish skin. The Amide A band indicates the stretching of the N-H group. In raw material collagen, the amide A band is still within the standard range, namely 3304.06 cm^{-1}, while in optimized collagen there is a slight shift to a lower wave number area, namely 3291.34 cm^{-1}. The shift or decrease in the amide A wave number to a lower frequency can be associated with the loss of water bonds between collagen molecules (intermolecular) and between intramolecular. If both of these molecular bonds are lost, it will cause damage to the triple helix conformation of collagen. In optimized collagen, the shift in Amide A only occurs when there is a loss of water bonds between molecules and has not lost the water bonds between intramolecular collagen, so that collagen is still in its triple helix structure (Rabotyagova et al., 2008).

Raw collagen and optimized collagen have amide B absorption areas indicating the presence of asymmetric CH2 groups. Amide I is a marker for spectroscopic analysis of the secondary structure of proteins that confirms the presence of carbonyl groups (C=O)

throughout the polypeptide (Rahmi, 2017). When snakehead fish skin collagen is denatured, the secondary structure of the protein in amide I, namely β sheet (1624-1642 cm^{-1}) will become α helix (1654-1658 cm^{-1}) which is a characteristic of gelatin (Duan et al., 2018; Liu et al., 2019; Liu et al., 2021). The amide I group in raw collagen (1635.64 cm^{-1}) and optimized collagen (1635.84 cm^{-1}) is still within the β sheet range indicating that collagen has not been denatured into gelatin. Amides II and III confirm the stretching of the CN group and bending of the NH. The intensity of Amide III can be related to the presence of a triple helix structure in collagen, namely through the intensity ratio between the peak of the amide III absorption region and the peak of the 1450 cm-1 region. The ratio value of the raw material collagen and the optimized collagen showed the same ratio value, namely 1.17. Both of these values are close to 1.0, which indicates that collagen still has a triple helix structure (Chuaychan et al., 2015; Kaewdang et al., 2014; Matmaroh et al., 2011). Based on this, it can be concluded that the raw material collagen and the optimized collagen obtained have not been denatured into gelatin.

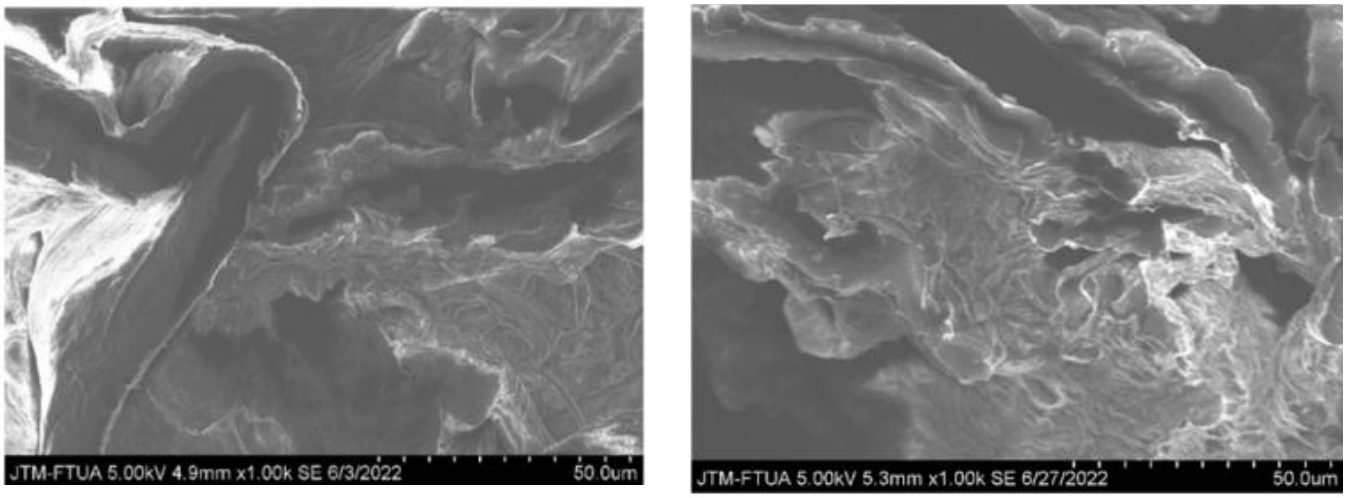
Particle size analysis with Particle Size Analyzer (PSA)

Particle size testing was carried out to determine the size and distribution of particles from the optimized collagen. The measurement results can be seen in Table 5.

Table 5. PSA results of optimized collagen

Measurement	Particle size (μm)	Average ± SD	Measurement	Particle size (μm)
	Repetition 1	Repetition 2	Repetition 3	
Optimized collagen	21.015	21.350	20.090	20.818 ± 0.653

The results of particle measurements with PSA obtained the size of the optimized collagen with an average of 20.818 μm. Based on this, it shows that there is a difference in particle size with respect to the ratio of collagen to ethanol, time and stirring speed. It can be concluded that the desolvation technique can reduce the particle size of snakehead fish skin collagen on a micrometer scale.



(a) (b)

Figure 4. SEM morphology (a) of raw material collagen (b) of optimized collagen

Morphological Analysis with Scanning Electron Microscopy (SEM)

Morphological analysis using SEM was carried out to visually determine the shape and appearance of the raw material collagen and the optimized collagen. The results of the SEM examination of the two samples are shown in Figure 4. It can be seen at a magnification of 1,000x that the raw material collagen shows thick random coils like coils indicating the nature of collagen fibers (Arumugam et al., 2018; Iswariya et al., 2018; Tan & Chang, 2018). While the optimized collagen shows a thin and clearer fibril structure compared to the raw material collagen indicating the presence of a triple helix structure bond (Chapman & Hulmes, 1984; Wess, 2005). This is in accordance with Arumugam's research which observed the morphology of sole fish skin collagen (Aseraggodes umbratilis), showing that the coil-like structure is a collagen fiber (Alves et al., 2023).

Differential Scanning Calorimetry (DSC) Analysis

In polymers, there are transition phases that can be identified by measuring their thermal properties using Differential Scanning Calorimetry (DSC). The DSC results are in the form of a thermogram curve that shows the temperature of the sample which can be below if there is an endothermic change and above if there is an exothermic change (Rahmi, 2017). The DSC thermogram of raw material collagen and optimized collagen is observed in Figure 5.

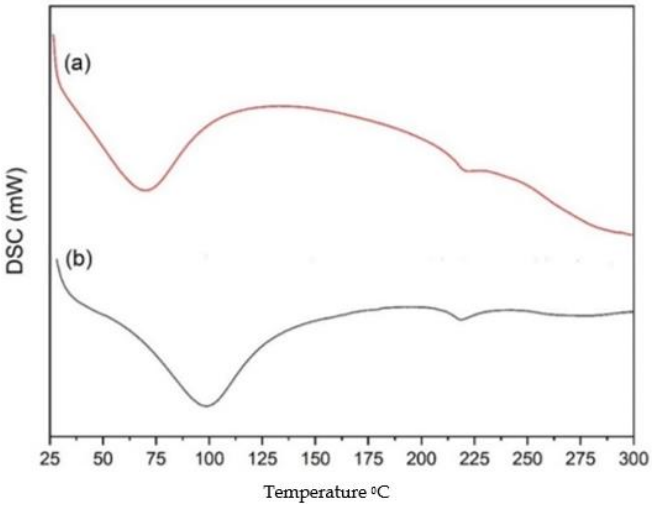


Figure 5. DSC thermogram (a) raw material collagen (b) optimized collagen

Based on the thermogram curve of raw material collagen and optimized collagen, two endothermic peaks were obtained which were not much different. Endothermic peak I is a glass/glass transition that occurs due to the breaking of hydrogen bonds (Asmiati et al., 2024; Maskur et al., 2024). The high denaturation temperature at the glass transition indicates more stable collagen (Hirota-Nakaoka & Goto, 1999; Mu et al., 2007; Pietrucha, 2005; Zhang et al., 2021). The glass transition temperature of raw material collagen is 70.07 °C and 98.71 °C for optimized collagen. The increase in temperature in optimized collagen can be associated with the effect of collagen dehydration, which is thought to be by replacing the water contained in collagen with organic solvents (ethanol). So that there is an increase in hydrophobic interactions through the formation of intramolecular hydrogen bonds in collagen (Shen et al., 2019; Shi et al., 2020). This is in accordance with the results of Shen's research which states that ethanol dehydration can increase the thermal stability (Td) of collagen (Shen et al., 2019).

Endothermic peak II is the maximum transition temperature (Tmax) indicating the melting peak of collagen (Badea et al., 2012, 2015), which is 222.16 °C in raw collagen and 218.62oC in optimized collagen. The high melting temperature of collagen indicates that the collagen produced is more resistant to heat (Wulandari & Suptijah, 2015).

X-Ray Diffraction (XRD) Analysis

X-ray diffraction (XRD) is often used to provide information on the triple helix structure of collagen (Göçer, 2022). The results of the XRD diffractogram of raw collagen and optimized collagen are shown in Figure 6.

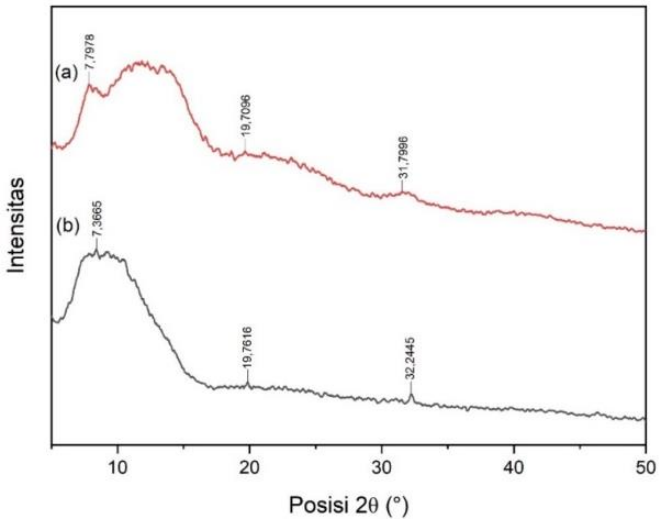


Figure 6. X-ray diffractogram (a) of raw material collagen (b) of optimized collagen

Each sample has two characteristic diffraction peaks (2θ) of collagen, namely 7.7978° and 19.7096° (raw collagen) and 7.3665° and 19.7616° (optimized collagen). The first peak seen around 8° is related to the triple helix structure of collagen and the distance between the molecular chains (Bak et al., 2018). While the second peak shows the distance between the collagen framework chains, at this peak an amorphous peak is seen at the diffraction peak around 16-25° (Laaziri et al., 1999)]. Figure 6 also shows a small diffraction peak at 31.7996° for raw collagen and 32.2445° for the optimized collagen. This implies that the two samples do not have a clear difference in the separation distance between amino acid residues around 30-35° (Ju et al., 2020; Zeugolis et al., 2008). The results of X-ray diffraction can be described by the Bragg equation $2d\sin\theta = \lambda$ (λ = 0.1540). The distance between the molecular chain and the collagen chain at the first and second peaks are respectively 1.1331 nm and 0.4498 nm (raw material collagen) and 1.1984 nm and 0.4488 nm (optimized collagen). While the distance between amino acid residues is known from the d value at the third peak, which is 0.2810 nm for raw material collagen and 0.2772 nm for optimized collagen.

Conclusion

Reduction of particle size of snakehead fish skin (Channa striata) using a new desolvation method to the micrometer scale and no changes in the triple helix structure of collagen. The reduction of the particle size of snakehead fish skin (Channa striata) using a new desolvation method successfully reached the micrometer scale without causing changes to the triple helix structure of collagen. These results indicate that the applied desolvation method is able to produce small collagen particles, which still maintain their structure and characteristics. This study opens up further opportunities for the development of this desolvation method to obtain even smaller collagen, even up to the nanometer scale.

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Author Contributions
This research consists of three authors i.e R. A., I. I., and R. N. All author members work together to carry out each stage of the research.

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Conflicts of Interest
The authors declare no conflict of interest.

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