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Production and Characterization of Micro-Collagen from Carp Scales Waste (*Cyprinus carpio*)

ABSTRACT:
Carp (*Cyprinus carpio*) has the potential which is not only consumed from flesh as an edible portion but it is also able to be utilized from waste. One of waste is the scales of the carp known potentially contain of collagens. Micro-collagen has been extensively applied in various fields which were health and cosmetics. The problem to find the supply of collagens from non-halal animal sources and prone to infectious diseases is the fundamental consideration of this research to be undertaken in order to discover alternative sources of them. It was aimed at production and characterization of micro-collagen by utilizing carp scales waste. The stages of the proximate test, deproteinization, extraction, analysis, and characterization were series of processes to acquire collagen. The extraction results found that the yield of collagen extracted from carp scales waste was 8.62% with a yellowish-white color. Physical characterization of collagen obtained was pH of 6.59. The maximum of UV absorption at a wave length of 268nm was originated from the structure of collagen fibrils with amide bonds of A, B, I, II, and III. Furthermore, the characterization of micro-collagen showed a particle size distribution from the smallest particles which was 668 – 1581nm with the highest intensity at a particle size of 1146 nm according to PSA analysis and corresponding with the morphology of micro-collagen through visualization using SEM. It indicates that the carp scales waste have the potential to be used as an alternative source to find supply micro-collagen.

KEYWORDS: Carp (*Cyprinus carpio*), extraction, micro-collagen, scales waste.

INTRODUCTION:

Carp (*Cyprinus carpio*) is one of the leading freshwater aquaculture commodities in Indonesia. It was evidenced reported by review of Nugroho et al.[1] It stated that the contribution of freshwater fish cultivations reached around 75.71% which one of them was carp (production in 2014). It was projected to increase by 138% /year. In addition, Virgantari et al.[2] analyzed the dynamics of fish consumption patterns in Indonesia stated that the average consumption of carp attained 0.12kg/capita/year which was the second-largest consumption after tilapia according to the data of expenditure category (Rp/month).

The average edible portion of carp flesh are around 27.3 – 27.9% of the total body weight of 34.45 – 37.57g aged 12 weeks as protein and nutrition sources[3]. In term of processing, the unconsumed portion of carp which is discards of scales, bones, and internal organs contributes waste about 70 %[4,5].

Therefore, the utilization of carp scales waste can be an alternative and a solution as a source of collagen. Because it was reported that the scales of carp contained collagen about 41.3% (specific on the skin), 1.35% (on the scales), and the bone part (only 1.06%)[6]. It is also inseparable from the aspect to find out the demand for collagen which is still a challenge today. Because mammals both bovine and porcine are constantly dominating to produce collagen[7]. Reviewed from utilization and application, there are some problems which are diseases like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathies (TSE), foot and mouth disease (FMD) as well as mad cow disease on collagen extracted from bovine[8]. In addition, collagen extracted from porcine has some problems related to the religious restriction[9], especially for Muslims in Indonesia. Moreover, it also provides the infection risk of zoonotic diseases[10].

Biodiversity of aquatic resources both of freshwater and marine including fish, porifera, cnidarian, and mollusk offers a variety of collagen feedstocks[11]. So that various collagens which are types of I, II, III, and IV, can be explored from the aquatic resources[12]. Furthermore, the supporting of research and reviews that discussed the fish as a source of collagen, procedure schemes, extraction methods using various concentrations, types of solvents, and operating conditions of various variables, characterization methods as well as the application of collagen as biomaterial engineering have been comprehensively explained[13]. Based on the beneficial term, it is also reviewed that collagen from aquatic resources had high yields, low gelling, high absorption, low melting point, no religious or ethical restriction, and low biological

contaminant as well as low toxic[14].

Besides that, the application of extraction methods to obtain collagen from carp scales has been widely implemented and discussed. Both of methods acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) can be applied to extract collagen[15]. According to Chinh et al.[16] the yield of collagen extracted from carp scales waste using the ASC method was around 13.6% with a fibril-shaped collagen structure composed of functional groups amide A (absorption area at 3294 cm^{-1} or 3400 – 3440cm^{-1}), amide B (absorption area at 3076 cm^{-1} or 2935 – 2915cm^{-1}), amide I (absorption at 1689 cm^{-1} or 1600 – 1690cm^{-1}), amide II (absorption area 1542 cm^{-1} or 1480 – 1575cm^{-1}), and amide III (1249cm^{-1} or 1229 – 1301cm^{-1}). Furthermore, absorption characteristics of the UV-Vis spectrum at 220nm indicated peptide bonds, 280 nm from aromatic amino acids, 250 -270nm from the phenylalanine amino acid, 270 - 290nm from the tyrosine amino acid, 280 - 300nm from the tryptophan amino acid, and 192.07nm from collagen of type I. Comparison of collagen extraction methods conducted by Pal and Suresh[17] showed that the ASC method was more effective for extracting collagen from the scales of Indian's carp species such as catla (1.72%) and rohu (2.74%) than the PSC method with the yields (1.48% and 2.26%), respectively. Collagen extracted from silver carp scales (*Hypophthalmichthys molitrix*) using ASC and PSC methods obtained about 0.86% and 2.32%[18], while collagen extracted from bighead carp scales (*Hypophthalmichthys nobilis*) using PSC method contained 2.5 – 2.9%[19].

Reviewed from the application of collagen, the high of demanding for collagen in various fields such as cosmetics, surgery, dentistry, ophthalmology, continuous drug delivery, biotechnology, biomedical and industrial fields as hemostatic agent, blood vessel, heart valve, tendon and ligament, burn wound dressing, intradermal augmentation, and drug delivery system[20,21] become one of the challenges to find these needs not only in size of micro-collagen but also in size of nano-collagen[22]. Related to this case, it shows that the utilization of carp scales waste as a source of collagen has the potential to be further developed into micro-collagen. Because it has several advantages such as high absorption and high diffused into the skin during the regeneration process[23] as well as being able to improve physical stability and its chemical properties when it disperse in water[24]. So that the development of micro-collagen can be widely applied in the form products of hair care, oral care, skin care and beauty mask, mucous membrane care[25], dermal fillers, skin substitutes as well as facial product[26].

Therefore, the producing of micro-collagen extracted from carp scales waste is carried out which is not only an effort to reduce the residual waste from carp processing but also to generate micro-collagen products.

METHODS:

Instruments and Materials:

The following instruments were used to obtain physical characterization data: scanning electron microscopy (SEM) Zeiss EVO MA 10, ultraviolet-visible spectrophotometer (UV-Vis) Shimadzu UV-2450, Fourier- Transforms Infrared (FTIR) JASCO FT/IR-5300, and particle size analyzer (PSA) Horiba LA-960.

The main material was carp (*Cyprinus carpio*). Besides, the chemicals utilized were 0.30% NaOH (Merck, pellet pure), 0.5 M CH_3COOH (Merck, Emprove ® Essential), distilled water, and 96% ethanol (Merck, EMSURE ® Reag.).

Procedures:

Samples preparation and proximate test of carp scales:

Carp was carried out from fish traders at Way Halim Market, Bandar Lampung, Lampung, Indonesia. The carp scales waste was washed, weighed, and dried for further utilization.

The proximate test was executed to determine the moisture, ash, crude fat, and protein content (Kjeldahl) of carp scales which were analyzed according to the standard method AOAC (Association of Official Analytical Chemists) used by Pal and Suresh[17].

Collagen extraction:

Deproteinization process:

This process was modified from the method applied by Pal and Suresh[17]. First, carp scales were weighed 137grams, after that, they were deproteinized by using 0.30% NaOH for 72 hours with a ratio (fish scales sample/NaOH) of 1:8 (w/v) as a pre-treatment, and then washed.

Extraction process:

The extraction process was adapted from the method applied by Chinh et al.[16]. After the deproteinization process, carp scales were hydrolyzed by using 0.5M CH_3COOH for 48 hours with a ratio (fish scale sample/CH_3COOH) of 1:10 (w/v) and then washed. Next, the extraction of collagen from carp scales was carried out by using distilled water with a ratio of 1:2 (w/v) and a water bath at 45ºC for 2 hours. Then, the extract which was as wet collagen was dried by using a freeze dryer to obtain dry collagen.

The yield (%, w/w) of collagen from carp scale waste was calculated based on the dry weight basis by applying equation (1)[27].

$$\text{Yield (\%)} = \frac{\text{Dry collagen weight (g)}}{\text{Dry carp scales weight (g)}} \times 100\ \% \quad (1)$$

Collagen characterization:

pH determination:

pH determination were modified by applying the method from Huda et al.[27]. The collagen sample was added with distilled water with a ratio of 1:100 (w/v) until homogeneous. Then, the pH of collagen was measured by using a pH meter.

Visualization using SEM:

This method was modified from the procedures conducted by Chinh et al.[16]. Before the observation using SEM, the collagen powder was coated with Pt (platinum) by using a mini sputter coater at 18mA for 4 minutes. Then, the collagen morphology was visualized by utilizing SEM at 20 kV and magnified 2000x.

Analysis using UV-Vis spectrophotometer:

The analysis method was adapted from the procedures performed by Chinh et al.[16]. About 1mg of collagen sample was dissolved in 5mL of n-hexane, then analyzed at a wavelength of 190 – 380nm with an absorbance of 0 – 1.5.

Analysis using FTIR:

The determination of functional groups and chemical bonds of collagen using FTIR was carried out according to the method applied by Huda et al.[27]. First, 1mg of dry collagen was crushed and mixed with 100mg of potassium bromide (KBr). Then, the sample was formed into a pellet and measured with a wavelength range of 4000 – 500 cm^{-1}.

Micro-collagen preparation:

This procedure was adjusted from the method applied by Trilaksani et al.[28]. Firstly, the production of micro-collagen was undertaken by dissolving collagen in distilled water with a ratio of 1:2 (w/v). Next, the sizing process by using a sonicator was conducted at 1000rpm for 3 hours. Furthermore, the sample was dripped with 96% ethanol solution with a ratio of 1:1 (v/v) and homogenized to form micro-collagen.

Micro-collagen characterization:

Analysis of particle size using PSA:

This analysis was adjusted from the procedures conducted by Trilaksani et al.[28]. First, particle size and distribution of micro-collagen were measured using PSA. Next, the micro-collagen sample was carried out about 5mL, put into a PSA Kuvet, dropped on the identification lens, and fired with a nano wave laser beam.

Visualization using SEM:

This method was referred and modified from the procedures performed by Chinh et al.[16]. The first, Micro-collagen morphology was analyzed using SEM at 20 kV and magnified 2000x. Next, the morphology of micro-collagen and collagen were compared to identify the differences in structure and particle sizes.

RESULTS:

Sample preparation and proximate test of carp scales:

This process was carried out to prepare sample of carp scales and to determine the proximate composition (Kjeldahl) of carp scales which were analyzed according to the standard method of AOAC used by Pal and Suresh[17]. Firstly, wet carp scale waste which was washed and cleaned from dirt, obtained as much as 500 grams. The total of wet carp scales attained about 197 grams of dried carp scales as shown in Figure 1 (a-c).

In this study, the proximate composition of carp scales obtained the moisture, fat, ash, and protein content, as shown in Table 1.

Figure 1. Samples preparation of carp scales

Table 1. Proximate composition of carp scales

Carp species	Proximate composition (%, w/w)			
	Moisture[a]	Fat[b]	Ash[b]	Protein[b]
Cyprinus carpio	36	0.7	9.28	54.02

[a]Wet weight basis (wwb)

[b]Dry weight basis (dwb)

Collagen extraction from carp scales waste:

After deproteinization process of carp scales was conducted to remove non-collagenous proteins, minerals, fat and pigment as a pre-treatment[17], The extraction process which was adapted from Chinh et al.[16] was applied to obtain collagen (wet and dry). The results of collagen extraction from 197grams of dried carp scales obtained wet collagen with a yellowish-white color and 17grams of dry collagen, as shown in Figure 2 (a-d). The yield of collagen calculated using equation (1) was acquired at 8.62%.

Figure 2. The collagen extraction process of carp scales waste

Collagen characterization:

pH determination:

To identify pH of collagen, the pH measurement was carried out using method modified from Huda et al.[27], the pH of collagen was measured by using a pH meter which was obtained pH of 6.59.

Analysis using UV-Vis spectrophotometer:

The measurement of UV absorption on collagen samples extracted from carp scales waste was carried out by a wavelength range of 190-380 nm and an absorbance of 0 – 1.5. The UV spectrum, which was shown in Figure 3, obtained maximum absorption at a wavelength of 268.0 nm with an absorbance of 1.0724.

A maximum wavelength of 268 nm indicates the presence of a bathochromic shift from the benzene, especially for the K band (204nm) shifts to a wavelength of 268nm. The shift is not only originated from the benzene aromatic but it is also derived from alkyl substituents and functional groups. The wavelength (λ) which undergoes the bathochromic effect is the B band or K band around 270nm. It indicates the presence of carboxyl and hydroxy groups. The band intensity/ molar extension of the substituted aromatics increases with ε of 10 - 100 due to the effect of excitation from n – π* electrons with the maximum wavelength are about 270 nm.

Figure 3. The UV absorption spectrum of collagen

Analysis using FTIR:

The FTIR spectrum of collagen extracted from carp scales was shown in Figure 4 below. Vibrations of hydrogen-bonded N-H stretching in the absorption area at 3408.22 cm^{-1} indicated the presence of an amide A bond. The absorption areas at 3080.32 cm^{-1} and 2960.73 cm^{-1}, were presented with the stretching vibration of asymmetric CH_3 and asymmetric CH_2, originated from the amide B position, as well. Meanwhile, the absorption areas at 1653 cm^{-1} indicated the presence of an amide I bond from the stretching vibration of C=O, which was originated from COO^-. Besides, the absorption areas at 1546.91 cm^{-1} were produced from the amide II peak with bending vibration of N-H and stretching vibration of C-N including in the areas of 1454.13 cm^{-1}, 1404.18 cm^{-1}, and 1330.88 cm^{-1}, it also provided the bending vibration of an asymmetric CH_2 and appeared the vibration of COO^- symmetrical stretching. Furthermore, the vibration of the amide III bond, which appeared at 1247.94 cm^{-1}, was originated from the bending vibration of N-H with a couple of vibrations of C-N stretching. Moreover, the fingerprint areas provided vibrations of CO stretching at 1082.07 cm^{-1}, CO stretching/ bending from COH at 1033.85 cm^{-1}, =CH bending at 921.97 cm^{-1}, and CH_2 bending at 665.44 cm^{-1}.

Figure 4. FTIR spectrum of collagen

Micro-collagen preparation:

The micro-collagen productions were modified from the procedures applied by Trilaksani et al.[28]. Micro-collagen, which was formed from the collagen of carp scales waste, obtained a yellowish-white colour. Then, it was characterized by using PSA and SEM to identify the size and morphology of the particles. The process and results of micro-collagen production are displayed in Figure 5 (a-c).

Figure 5. The process of micro-collagen production

Micro-collagen characterization:

Analysis of particle size using PSA:

The results of measurements and analysis of micro-collagen using PSA (see Figure 6) obtained 668 nm with d10 (<10%) to 1581 nm with d90 (<90%). The average particle size with a distribution of d50 (<50%) (median) was 1181 nm. The highest distribution intensity of micro-collagen was a particle size of 1146 nm.

Figure 6. PSA spectrum of micro-collagen

Comparison of SEM observations between collagen and micro-collagen:

The surfaces of collagen and micro-collagen morphologies were observed by using SEM with 2000x magnification[28]. The morphological observation of the collagen showed a heterogeneous fibril shape, as shown in Figure 7a.

To provide a morphology comparison between collagen and micro-collagen, the micro-collagen was characterized by using SEM with the same magnification. The micro-collagen morphology, as shown in Figure 7b, displayed morphology with homogeneous particles in which the particle sizes confirmed on PSA analysis with the particle sizes of collagen as microparticle sizes.

Figure 7. The comparison of morphology at 2000x magnification

DISCUSSION:

This study was conducted to prepare and characterize of micro-collagen extracted from carp scales waste of Cyprinus carpio species. The proximate test was carried out as an initial step to determine the moisture, ash, fat, and protein content (Kjeldahl) in carp scales[29]. These values are very useful in determining the quality and purity of samples[30]. The procedure was adopted from the standard AOAC method as it was conducted by Pal and Suresh[17].

The proximate test (%, w/w) of carp scales in this study (see Table 1) was compared with the literature from Mahboob et

al.[31]. It showed that the moisture of the carp scales samples in this study was lower than the literature reported. While the protein content was significantly higher than it. The high protein content appeared from the amino acid composition which indicated the presence of collagen in the carp scales[32]. The fat content was relatively similar to the literature reported. But the ash content was higher quantity than it. Based on research[17] it stated that the high ash content was largely due to the presence of calcium-based salts which localized in two different layers of the scales : the upper osseous layer and the lower fibrillar plates. Furthermore, the differences in proximate composition are influenced by several factors like species of fish, their biological conditions, and the testing method.

The percentage yield (%, w/w) of collagen extracted from carp scales waste was calculated based on the dry weight basis (dwb)[27]. The extraction process in this study was modified from the method carried out by Chinh et al.[16]. The yield of collagen was higher than the literature[17], which it mentioned that the factors of the protein content were the fish types, its biological condition, the method, and its collagen structure. However, when the yield of collagen compared with the literature data[16], it was lower than it. Several factors were revealed which were extraction conditions such as pH, temperatures, and times[33]. Furthermore, when the yield of collagen in this study was compared with the literature data of other feedstock, like the yield of collagen extracted from silver carp[18], bighead carp[19], and Indian carp species of Catla and Rohu, the results showed that the yield was higher than it. The difference in collagen yield is affected by the chemical solution and the method type as the solubility factors in the extraction process. So that it becomes the cause of high or low collagen obtained[17].

The characteristic color of collagen provides added value, but it does not affect the color of the collagen product. Because the color of collagen is associated with the heme pigment of myoglobin and hemoglobin in the skin. The pH of collagen extracted from carp scales waste in this study was higher than the pH of collagen extracted from duck feet as discussed by previous research[27].

The peak of the UV-Vis absorption in this study was in the range of 250-270 nm which indicated the presence of phenylalanine and tyrosine[16]. In addition, the results of FTIR characterization showed vibrations which were majority originating from the amide group. In the amide A, amide B, and amide II, it reveals that the vibrations originate from protein molecules as reported by previous research[17]. The amide I group indicates the presence of a peptide bond arrangement from protein as reported by other research[19]. The absorption area of the amide III bond listed in the FTIR spectrum derives from the triple helix layer from the protein β-sheet as reported by previous research [17,19]. Vibrations derived from the amino acid of tyrosine are from beta-protein. The results correspond to the absorption of UV-Vis measurements and vibrations of CH_2 bending from proteins, lipids, and carbohydrates[17]. The majority of functional groups presented in collagen is amida functional groups, as reported by [16,28].

The morphology of collagen is identical with the literature as reported by previous research[16] which mentions a protein fibril presence of the collagen. The shape of the fibril structure in the collagen is also appropriate as reported by other research[18].

In general, the results of this characterization of micro-collagen are suitable with the standard of collagen microparticle sizes which is on a scale of >1000 nm (1 – 1000 µm) as a microparticles category[34,24,35,36]. The difference in particle size distribution in collagen especially as microparticles is influenced by several factors, namely the matrix composition, the evaporation process of organic solvents, and the mixing process [28].

The comparison of morphology between the collagen and the micro-collagen provided the difference in structure significantly. As shown in Figure 7a for collagen, the fibril structure visualized expressly. While Figure 7b for micro-collagen displayed smoothness particle sizes as confirmed by PSA measurements. These results are conformable with that reported by the previous research[22] that morphology structure in collagen has been transformed into microparticles (micro-collagen).

The particle sizes conversion of collagen into micro-collagen provides several advantages such as high absorption, high diffused into the skin during the regeneration process[37,38], and being able to increase physical stability and its chemical properties when it disperses in water[24]. Therefore, micro-collagen has the potential to be utilized both in the biomedical (health) and industrial fields as reported by the other research[21]. Moreover, the use of micro-collagen in the cosmetic field provides benefits like enhancing skin elasticity, skin moisture, and transepidermal water loss, moisturizing properties, regenerates skin, as well as forms a skin layer [25].

CONCLUSIONS:

Based on the results of this study, it can be concluded that carp scales waste has the potential to be exploited as an alternative source of collagen. It is evidenced by the yield of collagen about 8.62%, and the characteristic which is yellowish-white and pH of 6.59. Furthermore, the collagen characterization provides a fibril structure with the presence of chemical bonds dominated from amide groups of A, B, I, II, and III. The micro-collagen characterization presents a distribution of particle sizes from the smallest which is 668 – 1581 nm with the highest intensity of 1146 nm and homologous with the morphological structures. So, it confirms that the micro-collagen production has been successful.

Because the micro-collagen provides several advantages like high absorption, high diffused , and being able to increase physical stability as well as its chemical properties when it disperses in water[24], so that it has the potential to be utilized both in the biomedical (health) and cosmetic fields[21,25].

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CONFLICTS OF INTEREST:
There are no conflicts to declare.

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