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CHARACTERIZATION OF COLLAGEN EXTRACT FROM THE SKINS OF COMMERCIAL FRESHWATER FISH

Anida Aminudin[a] Shamsul Muhammad[a], Ruhil Hayati Hamdan[b], Rumaizi Shaari[b], Mariam Firdhaus Mad Nordin[c], Roselina Ahmad Saufi[d], Soon Jan Mei[a*]

[a]Faculty of Agrobased Industry, Universiti Malaysia Kelantan, Jeli Campus, Locked Bag No. 100, 17600 Jeli, Kelantan.
[b]Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, City Campus, Locked Bag No. 36, 16100 Kota Bharu, Kelantan.
[c]Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia, Semarak Campus, 54100 Kuala Lumpur
[d]Faculty Of Enterpreneurship And Bussiness, Universiti Malaysia Kelantan, Pengkalan Chepa, 16100 Kota Bharu, Kelantan

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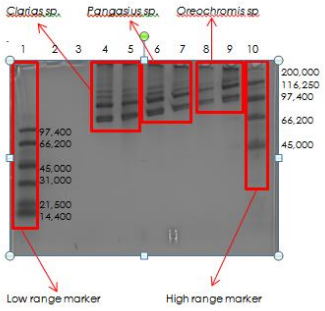
*Corresponding author
soon_jan@umk.edu.my

Graphical abstract



Abstract

Collagen was documented as a difficult and expensive protein to quantify due to its insoluble. It is particularly since solubility is a key functional property in a variety of applications such as healthcare and cosmetic products. The main aim of this study was to extract the collagens from the skins of commercial freshwater fish such as red tilapia *(Oreochromis niloticus*), catfish (*Clarias gariepinus*) and pangasius (*Pangasius pangasius)* together with characteristics defined for each type of collagen extraction. The extracted collagens were determined in their molecular weights by using SDS-PAGE and the structure of it was observed under SEM. The obtained molecular weight of all three commercial fish collagens is approximately >80kDa. The characteristics of each type of collagen were then defined by using appropriate analysis through this research.

Keywords: Collagen, application, characteristics, SDS-PAGE.

Abstrak

Kolagen telah dikategorikan sebagai protein sukar dan mahal untuk di kira kerana sifat ketidaklarutannya, di mana kelarutan adalah kunci utama yang penting dalam pelbagai aplikasi produk penjagaan kesihatan dan produk kosmetik. Tujuan utama kajian ini adalah untuk mengekstrak kolagen daripada kulit ikan air tawar yang komersial di Malaysia seperti ikan Tilapia Merah (*Oreochromis niloticus*), ikan keli (*Clarias gariepinus*) dan pangasius (*pangasius pangasius*) bersama-sama dengan penentuan ciri-ciri untuk setiap jenis kolagen. Kolagen ekstrak kemudiannya ditentukan berat molekularnya melalui SDS-PAGE . Semua jenis kolagen yang telah diekstrak tadi akan ditentukan berat molekul kemudiannya dengan menggunakan analisi SDS - PAGE . Berat molekul yang diperolehi daripada ketiga-tiga kolagen dari spesies ikan komersial adalah lebih kurang >80kDa . Ciri-ciri bagi setiap jenis kolagen kemudiannya ditakrifkan dengan menggunakan analisis yang sesuai di dalam kajian ini.

Kata kunci: Kolagen, aplikasi, ciri-ciri, SDS – PAGE

1.0 INTRODUCTION

Freshwater fish such as *Oreochromis sp.*, *Pangasius sp.*, and *Clarias sp.* are typically processed as frozen whole, frozen fillet and fresh fillet in factories. Due to environmental defense in shallow water, fresh water fish comprise of the high quantity of skin, scales and fins per unit mass. This type of fish also had grown in popularity in fish farming due to their good growth in ponds and their number of traits, such as culture rightness in cage or any other system in aquaculture [7].

Some fish processing companies were established as the by-products of fishery processing, around 170,000 tons were generated annually and for the processors, interest is growing in obtaining maximum financial gain from the processing wastes. So, there is enormous scope for utilization of these abundantly accessible fish processing wastes for extraction of value added product like collagen [9].

The solid waste from surimi processing, which may range from 50–70% of the original raw material [13] could also be the initial material for obtaining gelatin or collagen from under-utilized fish resources. More specifically, refinery discharge from the Pacific whiting surimi process, representing around 4-8% of whole fish, consisted of muscle (95%), skin (2.1%), bone (2.9%) and trace amounts of scale fragments [9].

Collagen is the main protein of connective tissue in animals and fish, where it is the most abundant protein in mammals [1]. Collagen constitutes up to 25-30% of the total protein content of the body and on the other hand, collagen constitutes 1-2% of muscle tissue besides contributing to the tune of 6% of the weight of strong and tendinous muscles. Even the people of early civilization discovered collagen's multiple utility value, such as waterproofing, adhesive, and decoration [18].

Collagen has become very useful in both bio medical and non-bio medical industries in this modern era, with an extended range of usage [3]. Collagen, due to its biocompatibility, stability and bioactivity has been widely used as a biomaterial, e.g. scaffold of cell and growth factors, wound dressing, soft tissue augmentation, and dietary supplement [2,16]. The most commonly used is bovine collagen that has been a common source for cosmetic applications nowadays. However, the source for collagen has to be reconsidered due to the risks of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) in recent years. Thus, more attention has been paid to isolate collagen from the byproducts of fish processing, such as skin,
scales and bones, which are used as low value feedstuff or just dumped as wastes [8].

Collagen extracted from by-products such as fish scales are biocomposites of highly ordered type I collagen fibers and hydroxyapatite Ca10(OH)2(PO4)6. In fact, previous studies showed that collagens extracted from fish scales of black drum, sheep's head sea bream, Red seabream, tilapia, sardine, Japanese seabass, skipjack tuna, ayu, yellow sea bream and horse mackerel mainly type I collagen with a lower denaturation temperatures than the collagen of porcine dermis.

In various parts of the world, different fish species are being consumed daily in large quantities. As a result, huge quantity of wastes, ranging from 50–70% of original raw materials are generated in fish shops and processing factories [10]. The solid waste from surimi processing, could also be the initial material for obtaining gelatin or collagen from under-utilized fish resources [6]). Serious environment pollution with offensive odors was caused by the improper disposal of the wastes from aquaculture industry such as scales, skins, feces and bones that were these sources have been mainly used as animal feedstuff or fertilizer [14].

Overall, this research aims to investigate the potential of collagen extracted from freshwater fish to be utilized as active ingredient in formulating cosmetic products. The problem with safety and efficacy of the cosmetic product will be explored throughout this research.

2.0 EXPERIMENTAL

The methods for extraction of collagen from *Oreochromis sp.*, *Clarias sp.* and *Pangasius sp.* skin consist of the following steps; skin preparation, removing non-collagenous tissue, solubilizing collagen, centrifuging and precipitating collagen and concentration measurement (all the preparation method was performed at 4˚C) [14].

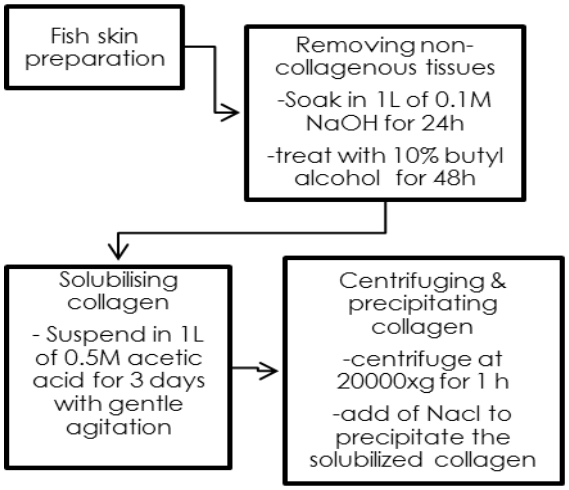


Figure 1 Extraction method flow for each type of fish skin

2.1 Fish Skin Preparation

Fish skin was obtained from the fish suppliers. Prior to use, the skin were descaled using sharp knife, cleaned and the remaining muscles removed. After which, 50 gram of skin were chopped into smaller pieces of approximately 0.5x0.5 cm [11].

2.2 Removing Non-Collagenous Tissues

1 litre of 0.1M of NaOH (pH 12) for 24h, Four gram of NaOH were mixed with 1 litre of distilled water. Then, the solution was placed on the skin and gently stirred for 5 minutes. After that, the resulting solution was placed at 4˚C for 24 hours.

2.3 Solublizing Collagen

The previously defatted skins were washed thoroughly for four times using cold distilled water. After which, the skin were suspended in 1 litre of 0.5M acetic acid for three days with gentle agitation at 4˚C. The main reason of this step is to solubilize the fibril collagen into tropocollagen which is the subunit of collagen fibril.

After 24 hours, the solution was filtered using filter paper (size 125mm). Then, the skins were washed thoroughly with cold distilled water until its pH become neutral. In this step, the skin was washed thoroughly for four times. After each wash, pH measurement was taken. In each wash, 600ml of cold distilled water was used.

After thoroughly washing, the skin was treated with 10% butyl alcohol solution for 48h to remove fat tissue from the skin. The skins were soaked in 500ml of 10% butyl alcohol solution for 24 hours at 4˚C. The solution was filtered after 24 hours and was resuspended into the remaining 500ml of previously prepared 10% butyl alcohol solution for another 24 hours inside the chiller.

2.4 Centrifuging And Precipitating Collagen

After three days of acetic acid suspension, the solution was filtered by using double filter papers which about 200 ml of solution been obtained. Then, the solution was centrifuged into a refrigerated centrifuge at speed of 20,000 times gravity at 4˚C for 1h. After which, 5ml of supernatant was collected and transferred in a tube. In order to precipitate the solubilized collagen, NaCl was added to the supernatant to get final concentration of 0.9M NaCl. After salting out the collagen, the solution was centrifuged again for another hour with 20,000 times gravity. Then the supernatant was disposed and the precipitate was collected [14].

2.5 Bradford Protein Assay

2.5.1 Bradford reagent

Reagent was prepared first by dissolved in 100mg coomassie Brilliant Blue G-250 in 50ml of 95% ethanol. Then, 100ml of 85% (w/v) of phosphoric acid was added.

2.5.2 Assay

Prior to measurement, the spectrometer was warmed up while the samples and standard are prepared. The unknown if necessary to obtain between 5 and 10 µg protein in at least one assay tube containing 100µl sample was diluted. An equal volume of 1M NaOH was added to each sample if desired and then been vortex. The standard containing a range of 5 to 100 µg protein albumin was used. 5ml dye reagent was prepared and added in about 10µL into each 100µL samples and incubated for 5 minutes. The absorbance was measured at 595nm.

2.6 SDS-PAGE Electrophoresis

Collagen samples was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% (w/v) running gels. Prior to analysis, collagen samples in sample buffer were neutralized, if necessary, with 2 M Tris and then heated at 100˚C for 2 min. Separation of the otl(I) and otl(III) chains will be by reduction with 2-mercaptoethanol during interrupted electrophoresis [4].

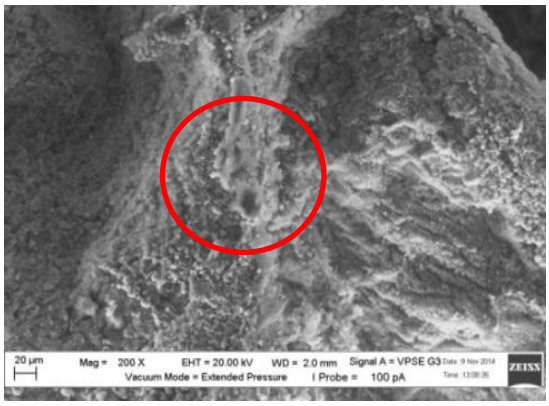
2.7 Scanning electron microscopy (SEM)

Some pieces of lyophilized collagen samples was first placed on the clean SEM plate stand and then were gold coated. The samples were then viewed under scanning electron microscope (INCAPentaFETx3 model no 8100).

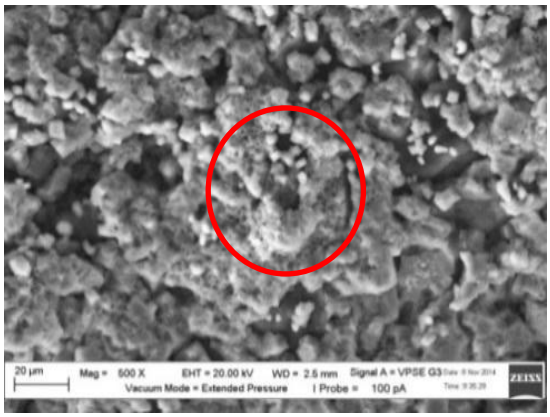
3.0 RESULTS AND DISCUSSIONS

Figure 2 Colorless jelly-like structure of collagen extracted from (a) *Clarias sp.*, (b) *Pangasius sp*. and (c) *Oreochromis sp*. before freeze-dry

Figure 3 Sponge-like white colored structure of crude collagen extracted from *Oreochromis sp*. after freeze-dried



(a)



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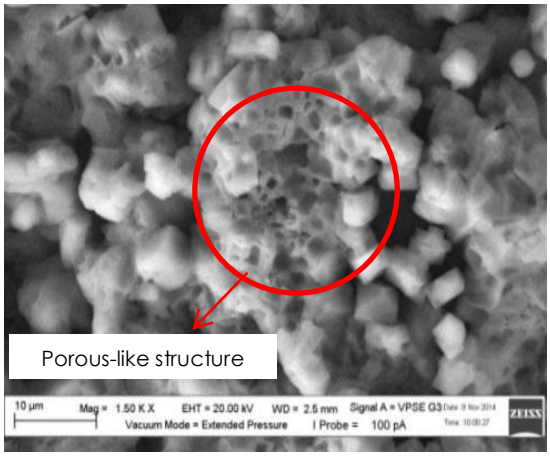

(c)

Figure 4 The porous-like structure of collagen (a): *Pangasius sp.*;(b); *Clarias sp.;* (c): *Oreochromis Sp* seen by SEM under 15000X-30000X magnification.

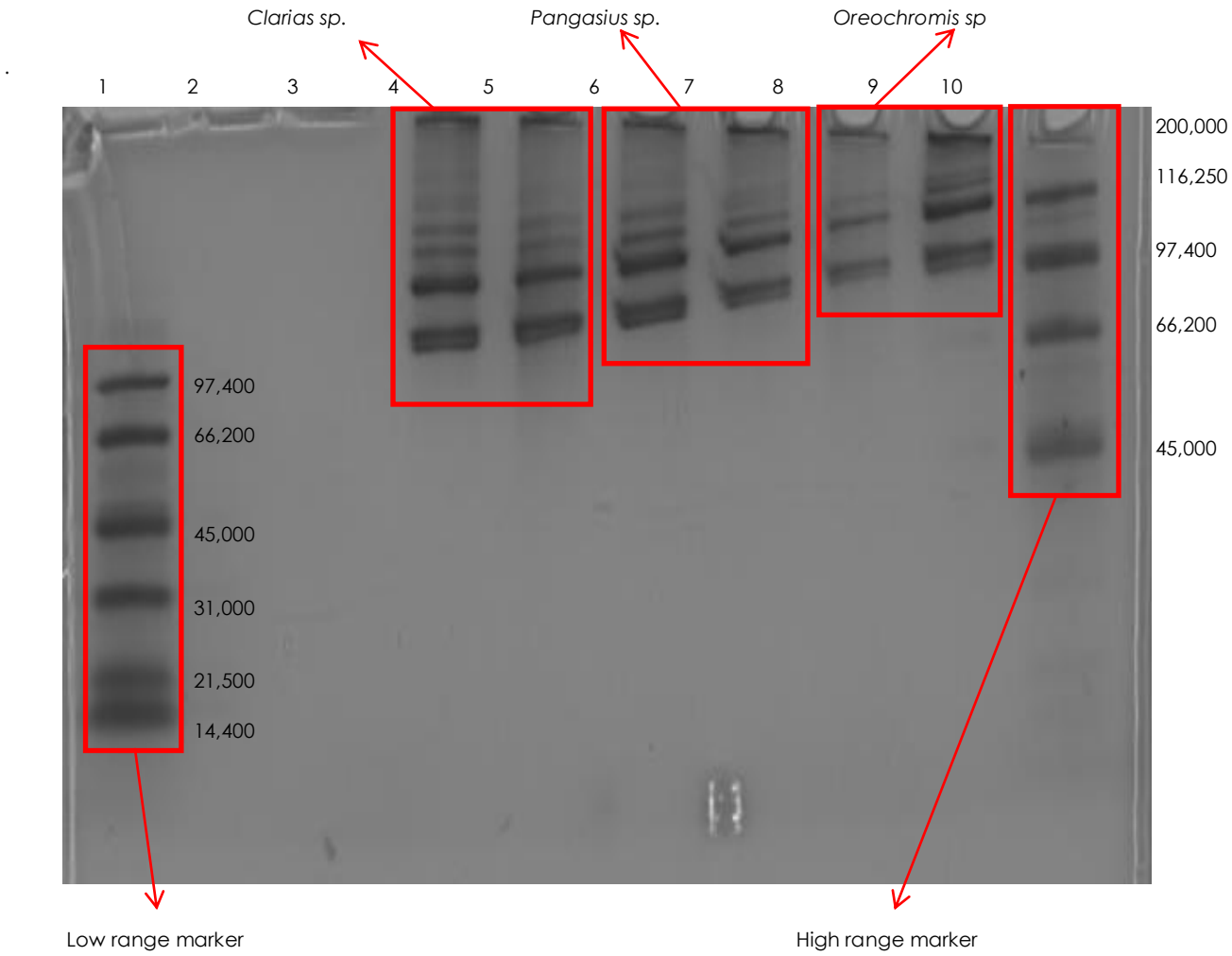

Figure 5 SDS-PAGE of collagen protein extracted from *Oreochromis sp. Pangasius sp.* and *Clarias sp.* with 5% stacking gel and 12% resolving gel; Lane 1: Low range marker; Lane 4 & 5: *Oreochromis sp*. (<97kDa);Lane 6 & 7: *Clarias sp.* (>116kDa);Lane 8 & 9: *Pangasius sp.* (97-116kDa);Lane 10: High range marker

The freeze-dried crude collagen extracted from the skins of *Oreochromis sp., Clarias sp.,* and *Pangasius sp.* formed the sponge-like structure (Figure 3). Before that, colorless jelly-like structure (Figure 2) was formed after being suspended in acetic acid. The efficiency of the collagen extraction process from the muscle of *Clarias sp.* can be influenced by many process factors such as acid concentration, extraction time, stirring speed, and liquid to solid ratio [17,19]. At constant acetic acid concentration and liquid to solid ratio used, higher stirring speed could improve the initial extraction rate.

Based on Figure 4(a-c), the crude collagen showed porous-like structure for all three different species of fish under the magnification of 15000X-30000X. These result showed that there are banded structures on collagen fibrils surface which confirmed that collagen extracted from the skin of freshwater fish like *Oreochromis sp., Clarias sp.,* and *Pangasius sp*. are bounded in fiber bundles. The collagen fibers are composed of specific amino acids like proline, hydroxyproline and glycine which are positioned to form a fibrous structure. This collagen fibres was supposed to support the internal structure of the skin [15]. Basically, the alpha-chains are wrapped around each other to make a triple helical conformation in the collagen molecule. The ratio of type III to type I collagen in humans increases with increasing age [5]. In aged skin, the density of collagen network appears to increases where this likely reflects a decrease in ground substance that would otherwise form spaces between the collagen. There is less room also for the skin to be stretched, so tensile strength increases as fibers become straighter in aged skin [11].

Based on Bradford analysis, the changes of brownish color of Bradford solution into blue color had indicated the existence of the protein extracted from the skin of the fish. All the skin extraction from three different fish had shown the color changes after run the Bradford analysis.

Based on the result of SDS-PAGE (Figure 5) for all three different protein collagens, the molecular size for

Oreochromis sp. was <97 kDa, *Clarias sp*.was >116kDa, and *Pangasius sp*. was around 97 -116kDa when compared to the standard marker. These results demonstrated that the molecular weight of protein collagen extracted from the skin of *Oreochromis sp*. was smallest when compared to *Clarias sp*. and *Pangasius sp*. protein collagen. This result also confirmed that from previous research, generally the molecular weight of protein collagen from freshwater fish was >80 kDa [8]. According to these results, the collagen extracted from the skins of *Oreochromis sp*. had the potential to be formulated in cosmetic products due to its lower molecular weight which makes it easily absorb by human skins.

4.0 CONCLUSION

From this research, we known that the molecular size of collagen extracted from the skin of tilapia had shown the lowest size which <96 kDa compared to the collagen from the skin of catfish and pangasius. The structure of collagen that had been observed through SEM was formed in the porous-like structure at the surface of crude collagen. This characterization of collagen from the skin of commercial freshwater fish had shown the potential to be used in cosmetic field. However, more investigation on the safety and efficacy should be done more to establish the use of this collagen in cosmetic products and others.

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