Isolation and Characterization of Collagen Extracted from Fish Scales and Applied as Anti-TNFα Protein

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ABSTRACT

Background: In the recent years, the focus on taking out biologically active molecules from animals' byproducts, especially from marine organisms have been increased. These sources play a major role in extracting collagen to be used as a biomaterial for medical applications and in the food industry. However, collagen-producing firms can be found all around the world, but not all of them kind 100% pure collagen; instead, they make gelatin hydrolyzed collagen. These businesses lack additional collagen development. Therefore, extracting collagen from fish scales as a potential raw material and using it to study high-value applications might potentially produce an economic potential for anti-inflammatory protein.

Methodology: Isolation and characterization methods were undertaken when fish scales (450gm) were procured from a local market, then washed with distilled water several times to remove any dirt, dust, and unwanted materials from the scales. Then, they were soaked with (0.2 M) of $NaOH_2$ for 12 hours to get rid of non-collagen proteins. For demineralization purposes, they were immersed with (0.5 M) of HCl for 12 hours. Later, for digestion to be carried out (0.5 M) of acetic acid was added and drenched for about 36 hours with stirring performed at 4 ºC. after the digestions step, the samples were filtered. Moreover, collagen was detected and characterized by the size exclusion chromatography (SEC) assay. Furthermore, the extracted collagen was used in vivo to detect fish-tumor necrosis factor (TNFα) to notice the effect of extracted collagen as an anti-inflammatory protein as a part of the medical application method.

Results: The SEC assay revealed that Col-I is purified and separated as soluble monomeric protein (53 min) fractions which correspond to a molecular weight of roughly 300 kDa. Also, the result indicated a mild significant decrease of collagen on TNFα (down-regulation).

Conclusion: In summary, we conducted to use collagen for biomedical and pharmaceutical purposes as an anti-inflammatory.

Keywords: Fish scale, TNFα, Collagen, Biomedical, Chemical action, Inflammatory agents

INTRODUCTION

Collagen is representing one of the most biopolymers in the extracellular matrix of multicellular organisms, accounting for 30% of the total proteins found in the animal body including bone, skin, tendon, ligaments, and the cornea[1]. All collagen molecule is comprised of trimeric polypeptide chains present as a triple helix of repeating Gly-X-Y triplet chain held together by hydrogen bonds, in which X is usually proline and Y is usually hydroxyproline amino acids[2-5]. The presence of these chains allows for diversity in collagen structure, where it ranged to be 96% for collagen I and less than 10% for collagen XII[5]. There are over 28 different forms of collagen, and all these types have a similar structure. However, they might vary in the length and the type of the amino acids present, and they might combine noncollagenous domains along with the actual collagen domains[6,7]. Because it is the most prevalent protein in the body, it has the potential to perform a wide range of tasks in the body. For example, tissue scaffolding and repairing, cell adhesion and migration, angiogenesis, and tissue morphogenesis[8]. Moreover, collagen showed some features related to its ability for gelation and create texture thickened gel with a high capacity for water binding. Besides, its surface showed features related to an emulsion, foam formation, and the capability to penetrate a lipid-free interface[9]. The collagen structure with its exceptional amino acid configuration gives it the properties of antioxidative and antihypertensive, antimicrobial activity, and immunomodulatory activity[10]. Therefore, its importance is derived from having a biocompatible, biodegradable, and weak antigenicity and immunogenicity in comparison with other types of biopolymers, for instance, gelatin and albumin[11,12]. Therefore, understanding the physiological structure and mechanical integrity of native collagen helps us in further enhancing the extraction procedure and artificial collagenous synthesis for biomedical and pharmaceutical applications, food manufacturing, as well as for cosmetics[10,13].

There has been a slew of researchers in recent years who have concentrated on extracting biologically active molecules from animals' byproducts such as those from cows, pigs, and marine species and using these materials in a variety of applications for human usage. Meanwhile, the extraction of collagen from cattle, on the other hand, raises concerns about different diseases for instance mad cow disease and bovine spongiform encephalopathy (BSE)[14]. Thus, the attention toward marine collagen increased, since it possesses a low risk of unknown pathogens[15,16]. Furthermore, collagen extracted from porcine is forbidden among the Islamic and Jewish populations for religious convictions similarly Hindus do not consume bovine-related materials[17,18]. Moreover, marine animals are easily gained in high yield with low cost, making them a good candidate for collagen extraction. For instance, Type I collagen in most marine animals, Type II collagen in fish cartilage and jellyfish, and type IV collagen in marine sponges such as Chondrosia reniformis[19]. Similarly, Shalaby

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and the other co-authors have investigated how isolated fish collagen can play an important part in the application of wound healing. Their results showed inhibitory activity in contradiction to all the bacteria that have been tested, and it exhibited better-wound contraction[20]. The discovery that collagen peptides isolated from fish scales include short peptides molecules, which allow for easy absorption by the body, has recently been revealed. These collagen peptides might be utilized as supplements to treat arthritis, atherosclerosis, and other signs of age[21].

Collagen extraction can be accomplished either from chemical hydrolysis or enzymatic hydrolysis; acetic acid is the most often utilized acid for extraction, and pepsin enzymes are also employed to conquer specific protein concentrations[22]. Some pretreatment is required to break the strong covalent and intermolecular cross-links that make the collagen hard to dissolve even in boiling water to make the process less complex[22]. Regardless of the types of marine sources, there are general techniques are used to attain collagen. Preparation of the samples by reducing their sizes either by cutting or mincing to simplify the consequent chemical (pre) treatment process required for removing noncollagenous proteins and fat. For the pretreatment, one of the essential components used to prepare the samples are sodium hydroxide (NaOH), oxygen peroxide, ethylenediaminetetraacetic acid (EDTA), and HCl for demineralization purposes[23].

In this study, we used fish scales as a potential raw material to isolate collagen from these marine by-products and characterize the extracted material to be considered an auspicious source for medical and industrial applications.

MATERIALS AND METHODS

Sample Preparation: Fish scales (450gm) were procured from a local market, then washed with distilled water several times to remove any dirt, dust, and unwanted materials from the scales. Then, they were soaked with (0.2 M) of NaOH for 12 hours to get rid of non-collagen proteins. For demineralization purposes, they were immersed with (0.5 M) of HCl for 12 hours. Later, for digestion to be carried out (0.5 M) of acetic acid was added and drenched for about 36 hours with stirring performed at 4 ºC. after the digestions step, the samples were filtered.

Purification of Col-I protein

- **Size exclusion chromatography (SEC) assay :** To purify and separate Col-I protein, an ÄKTA FPLC™ system (GE Health care) and a HiLoad 16/600 Superdex 200 pg prepacked column (GE Health Care) were used (SEC). The column firstly was pre-equilibrated with loading buffer (100 mM NaCl, 20 mM KCl and 20 mM Tris-HCl pH 8.5). Before using the SEC assay, we run standard proteins (Bio-Rad) of different molecular weights ranging from (1,350 to 670,000 Da) for column calibration based on the following manufacturer's instructions figure 1.

Then, we loaded Col-I protein onto a HiLoad 16/600 Superdex 200 pg prepacked column (GE Health Care) and separated using loading buffer (100 mM NaCl, 20 mM KCl and 20 mM Tris-HCl pH 8.5) on ÄKTA FPLC™ purifier system (GE Health care).

Collagen as anti-inflammatory

- **Collagen -TNFα gene (in vivo-method):** We used to fish- tumor necrosis factor (TNFα) to detect the effect of extracted collagen as an anti-inflammatory protein as a part of the medical application method. It is the Sandwich-ELISA method that is employed in this ELISA kit. It contains a Microelisa strip plate that has been pre-coated with a TNF-specific antibody, which is included in the kit. Micro ELISA strip plate wells are occupied with standards

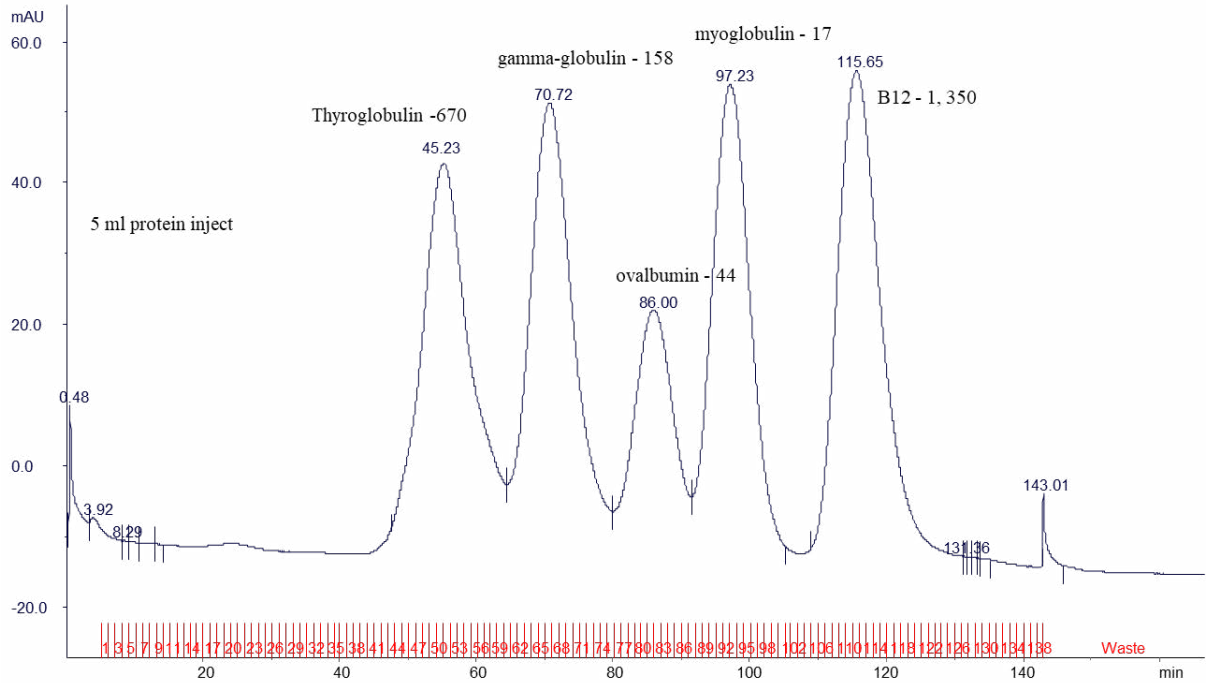

Figure 1: Standard proteins (Bio-Rad) Thyroglobulin (bovine) 670,000, gamma-globulin (bovine) 158,000, Ovalbumin (chicken) 44,000, Myoglobin (horse) 17,000 and Vitamin B12 1,350 were loaded onto an ÄKTA FPLC™ system (GE Health care) and a HiLoad 16/600 Superdex 200 pg prepacked column (GE Health Care)

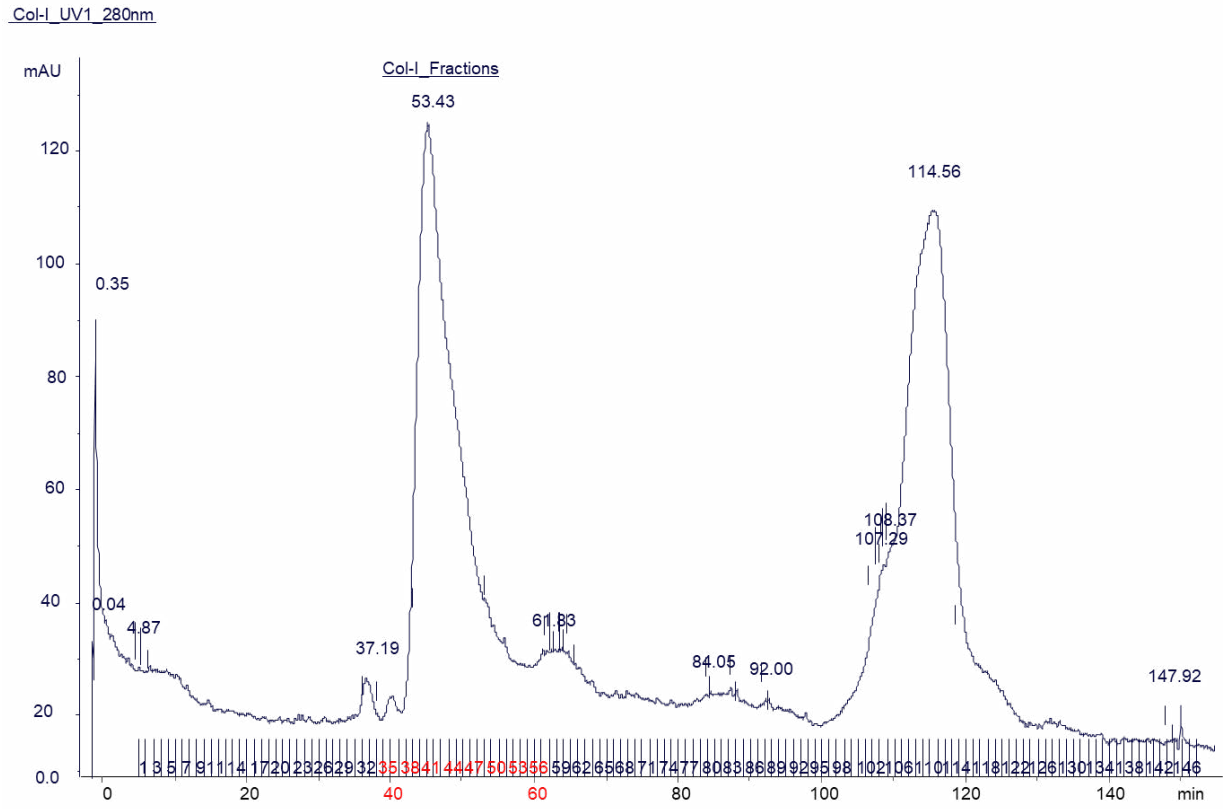

Figure 2: Size exclusion chromatography (SEC) analysis shows the single peak of soluble monomeric protein Col-I (53 min) fractions

or samples, which are subsequently mixed with the relevant antibody to obtain the desired results. An antibody specific for TNF- conjugated with Horseradish Peroxidase HRP- is then to each Microelisa strip plate wells and incubated for additional 30 minutes. The components that aren't required are removed by rinsing them away. The TMB substrate solution is injected into each well. In this experiment, only the wells containing TNF- and HRP conjugated TNF-antibodies would show blue before becoming yellow once the stop solution is introduced into the mixture. Spectrophotometry is used to determine the optical density (OD). The Kit was bought from (Sun Long Biotech Co., LTD-Hongkong) and followed the method in the kit sheet with some modifications (100µl from collagen).

Statistical analysis of data

According to the results, we noticed mild significant ($P \leq 0.05$) changes can be detected when we used collagen as anti-TNFα depression (mild down-regulation tumor necrosis gene) (T-test analysis)

RESULT AND DISCUSSION

The Col-I protein was loaded on an SEC column (HiLoad 16/600 Superdex 200 per grade) as shown in (Figure 2). The Col-I elution peak at 53 minutes with pure protein fractions is collected and concentrated using 3,000 Da cut-off filters (EMD Millipore) by centrifugation at 4000 x g. The SEC assay revealed that Col-I is purified and separated as a soluble monomeric protein which corresponds to a molecular weight of roughly 300 kDa.

TNFα- calculation

For the purpose of plotting the concentration of fish TNF-α standard the corresponding reading OD, where the log scale (x-axis) and the log scale (y-axis) are both utilized to express the result. The concentration of Fish TNF- in the sample might be calculated through plotting the sample optical density on the y-axis. Multiplying with the dilution factor yields the original concentration. Intra-assay accuracy 3 specimens with low, intermediate, and high levels of Fish TNFα were each examined 20 times on a single plate. TNF-α-producing fish were examined on three distinct plates, each with eight duplicates. SD/ meanX100Fish, CV%12.

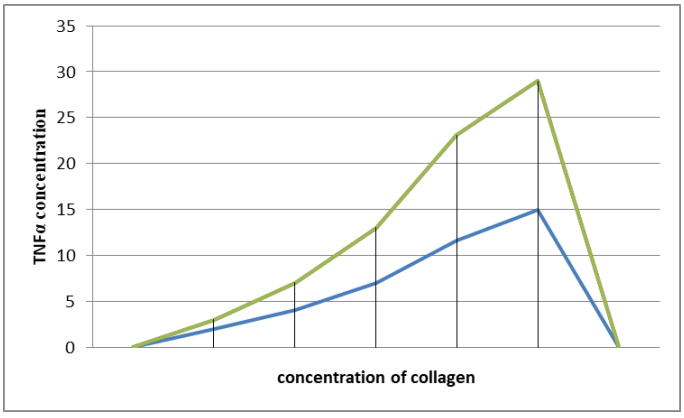

Figure 3: Show the relationship (extracted collagen –TNFα gene) in vivo experiment

There are mild tumor necrosis factor concentrations changes when increase collagen level. The properties of collagen as an anti-inflammatory protein might be responsible for the action of collagen. The condensation of the conjugated antibody was significantly higher in the inflamed paw of mice with collagen antibody-induced arthritis (CAIA) than in the non-arthritis hind paw. CAIA was more prevalent in

one hind paw than in other organs for example the liver or the kidney. Healthy mice's paws likewise did not acquire the conjugated antibodies. Furthermore, following delivery of the CBP-conjugated anti-TNF antibody, it was demonstrated that anti-TNF antibody localization to the arthritic paw is significantly greater than that found with the unmodified form[9,24]. Collagen-conjugated anti-TNF antibody was more effective than the unmodified antibody at preventing inflammatory arthritis development in animals with CAIA. As compared to control IgG, a conjugated anti-TNF antibody decrease arthritis severity even more significantly when administrated intravenously to mice. The conjugated antibody significantly reduced joint tissue breakdown and neutrophil and macrophage penetration into the paws in comparison with the unmodified antibody. Since collagens are plentiful in the subcutaneous tissues, injection of anti-TNF antibodies might be difficult. CBP-conjugated antibody gathered in the arthritic paw and was more effectual than the unmodified antibody at preventing arthritis. Due to the mild avidity of CBP-conjugate medicines for collagens, the antibody was able to infiltrate from the subcutaneous tissue into the circulation system and subsequently concentrated in the inflammatory area[25]. ECM and lymphatic drainage may quickly remove molecules from inflamed tissue, making it difficult to direct drugs to inflammatory regions. Most tissues contain collagen, especially vascular and inflammatory tissues. Also, it was observed that the anti-TNF antibody conjugated to CBP adhered to collagen and had the same anti-TNF receptor blocking action as an unmodified antibody. It stuck to collagen, particularly around blood vessels, in rheumatoid arthritis and osteoarthritis patients, tendon, and cartilage[25,26].

CONCLUSION

As a consequence of to study results collagen extracted from fish scale by a biochemical method characterized by size exclusion chromatography (SEC) analysis can use in pharmaceutical drugs as an anti-TNFα gene in high concentration (in vivo lab experimental) and that is need more supporting research in this field.

Authorship Contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, acquisition, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes.

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Competing Interest: None

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