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Effects of Acid Pre-Treatment on Removal of Organic and Inorganic Substances from Black Tilapia Bones and Scales

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Abstract

Fish bones and scales are good sources of gelatin. However, high mineral content in bones and scales often lead to low quality gelatin with high percentage of ash. Therefore, pre-treatment with acid prior to gelatin extraction is necessary. Black tilapia (Oreochromis niloticus) bones and scales were subjected to Hydrochloric (HCl) acid of 0.6 M to 1.4 M for 0 to 32 hr. The suitable pre-treatment conditions were determined using percentage of ash removed and loss of protein from the samples. Results obtained indicate, 99.86 % and 99.84 % of ash can be removed from bones and scales, with 1.4 M HCl (32 hr) and 1.0 M HCl (10 hr), respectively. Further analysis with UV-Vis revealed that, excessive protein breakdown starts to occur at 1.0 M (32 hr) and 0.6 M (12 hr), in bones and scales, respectively. The data suggest using pre-treatment conditions lower than the above to avoid protein loss which may lead to low gelatin yield.

1. Introduction

Gelatin is one of the most commonly available types of biopolymers that falls under the animal protein category [1]. Some common characteristics of gelatin are brittle, colorless, translucent, and flavorless. The good gelling, foaming and emulsifying properties of gelatin contribute to a wide range of applications in the food, medical, pharmaceutical, cosmetic, and photographic industries. Gelatin is also edible, safe and used as a surfactant in food products [2-3].

Over the years, the global demand for gelatin has been snowballing, with porcine skin accounting for the highest output, followed by bovine hides and bones, as well as other sources [4]. Though having a wide range of useful applications, pessimism, and strong concern with regard the usage of porcine and bovine gelatins persists among consumers. This is mainly associated with religious sentiments and the risk of transmitting pathogenic vectors due to the outbreak of Bovine Spongiform Encephalopathy (BSE) [5]. Hence, these issues have revived the interest in extracting gelatin from non-mammalian sources such as fish [6-8].

Fish wastes such as skin, bones and scales from both cold and warm water species are the major raw materials used in the production of fish gelatin. However, the lower yield of fish gelatins compared to mammalian gelatins limits its usage [9]. Fish bones and scales are bio composites, composed of connective tissue protein, hydroxyapatite, type I collagen fibers, lipid, trace elements, and water [10-12]. The major constituents in fish bones are hydroxyapatite, which accounts 65 to 70 % of the total weight. The amount of hydroxyapatite in fish scales is about 38 to 46 % with a small percentage of calcium carbonate (CaCO3) in them [13-14].

The presence of impurities such as ash and fat may also affect the viscosity of gelatin [15]. Ash refers to any inorganic substances (minerals) in gelatin that remain after a heating process (600 °C). The recommended ash content for a high-quality gelatin is lower than 2.6 % [16]. Generally, raw materials with a high level of inorganic substances produce gelatin with high ash content [17]. However, pre-treatment process prior to gelatin extraction can reduce the inorganic substances in the raw materials and increases the yield [18].

In order to extract high quality gelatins, researchers had been utilizing various pre-treatment methods to demineralize the raw materials prior to gelatin extraction [18-20]. Pre-treatment with alkalis or acids are the methods that had been carried out all this while as a measure to reduce the ash content in gelatins. However, many attempts had been failures as the percentage of ash was usually determined at the final stage after the gelatin had been produced.

A previous study done by Taheri et al. [19] shows, extraction of gelatin from the skin and bones of lizardfish yields gelatin with high ash content in bone (11.17 %) compared to skin (1.98 %). They attributed this to the low concentration of acid used or a short period of demineralization during pre-treatment of the bones. They also suggested the forthcoming studies to measure the ash content of bones after pre-treatment process prior to gelatin extraction as means to reduce the ash content of gelatin.

Therefore, in this study, the ash content of the bones and scales was minimized by pre-treatment process prior to gelatin extraction. Ash content analysis was conducted upon demineralized bones and scales to identify suitable pre-treatment conditions (HCl acid concentrations and treatment time) that are able to remove maximum number of inorganic materials from the samples. Loss of protein (collagenous and non-collagenous) from the bones and scales due to demineralization process were also observed.

2. Materials and Methods

2.1 Raw Materials

Frozen fish wastes including bones and skins of Black Tilapia (Oreochromis niloticus) were bought from a fish filleting factory (Trapia Malaysia Sdn. Bhd). Upon arrival, scales were detached from its skin using a scale remover, while excessive flesh sticking to the bones were removed via manual scrapping method. Bones were segmented into small pieces (± 2 cm). Cleaned bones and scales were washed thoroughly with running tap water and distilled water before being stored in freezer till further use.

2.2 Acid Pre-treatment Process

Bones and scales were soaked separately in different concentrations (0.6 M to 1.4 M) of Hydrochloric (HCl) acid (Brand QRec, Grade AR, 37 %) for 4 to 32 hr and 4 to 12 hr, respectively. Pre-treatment temperature was 5 °C and the sample to acid solution ratio used was 1:8 w/v. Treated bones and scales were neutralized by washing them thoroughly in running tap water and distilled water.

2.3 Proximate Analysis

Proximate composition in the raw bones and scales were analyzed by measuring moisture, ash, protein and fat contents based on AOAC (2000) official methods. The moisture content was measured via digital moisture analyzer (Model Mx-50, AnD Company United). All results were recorded in triplicate.

2.4 Energy Dispersive X-ray Spectroscopy (EDX)

Elemental analyses were done using an attached energy dispersive spectrometer (Model EDAX, Apollo X, U.S.A.). Raw bones and scales were cleaned and dried prior to testing.

2.5 Ash Content Analysis

The percentage of ash in the raw materials and gelatin were determined according to AOAC official methods 942.05 [21]. Sample (2 g) was weighed into porcelain crucible and placed in a furnace at 600 °C for 2 hr. The initial weight of the empty crucible was measured prior to the heating process. Upon completion of the heating process, the crucible was transferred directly to desiccator, cooled, and weighed immediately.

2.6 Protein Analysis

Loss of protein from tilapia bones and scales during demineralization was determined based on Biuret method via UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). Remaining HCl acid solutions (2 ml) after pretreatment were placed into test tubes separately. Subsequently, 4 ml of Biuret reagent (Sigma-Aldrich, St. Louis, Mo., U.S.A.) were added into each test tube and the solutions were mixed well. After 5 min, the color

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changes in the samples were observed and recorded. The percentage of protein in the samples was then determined using UV-Vis spectrophotometer at 540 nm. Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, Mo., U.S.A.) was used as a standard protein in the assay.

3. Results and Discussion

3.1 Proximate Analysis

The moisture content of initial untreated tilapia bones and scales is 36.23 ± 0.23 % and 26.04 ± 0.82 % (wet weight), respectively. Nonetheless of equal method of preparation used, the moisture content in bones is 28.13 % higher than the scales, which could be related to the complex surface morphology of bones that allows it to capture more moisture during washing and cleaning process. Meanwhile, the bigger surface area and smaller thickness of scales permits the moisture to dry faster, leading to lower moisture content. However, according to Norziah et al. [22], the moisture level in the raw materials have inconsequential impact on the final gelatin produced as the samples will go through drying process.

Meanwhile, ash content in raw tilapia bones and scales is found to be 30.90 ± 0.85 % and 29.93 ± 0.75 % (wet weight), respectively. Results obtained are much higher than some other fish bones and scales reported in the literature such as bones of lizardfish (25.06 ± 3.08 %) [19], catfish (12.62 ± 0.73 %) [20], and rohu scales (11.60 %) [12]. The higher percentage of ash in tilapia bones and scales indicates a greater amount of minerals in them [23].

The protein content of raw tilapia bones is 19.05 ± 0.25 %, while scales is 37.72 ± 0.32 %. In comparison to the bones, the percentage of protein in scales is nearly two times higher. The high amount of protein in the raw scales suggests higher yield of gelatin that could be extracted from them. In addition, it has been observed that the presence of fat in bones covers up to 13.58 ± 0.10 %, while it is only 2.94 ± 0.04 % in scales. The high fat content in tilapia bones increases the possibility of producing gelatin with high fat as well.

3.2 Elemental Analysis

Fish bones and scales are composed of both organic and inorganic substances. Apart from collagen and proteins, bones and scales are mainly regarded as rich in minerals such as calcium and phosporus. EDX analysis (area analysis) was conducted upon black tilapia bones and scales as a mean to identify the types of minerals present in them. Graph in Fig. 1 shows the different types of elements in terms of mass percentage present in tilapia bones and scales based on EDX analysis. Results obtained revealed that both samples contain carbon (C), oxygen (O), calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), and Zink (Zn).

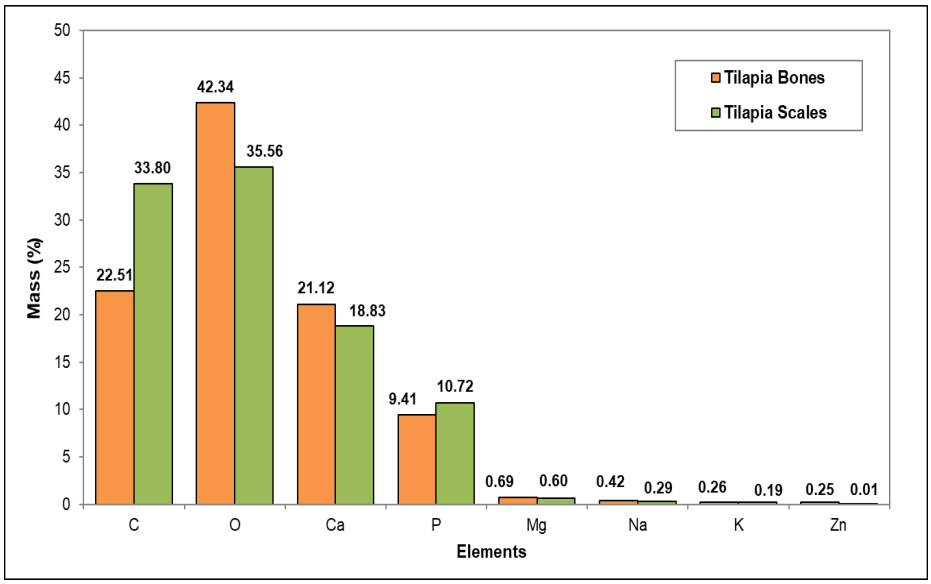

Fig 1 *Types of elements present in tilapia bones and scales*

The major elements found in bones are C and O, which is about 22.51 % and 42.34 %, respectively. Presence of C and O are common in organic materials as they are generated naturally within the body depending on the surrounding. Apart from C and O, Ca and P were found to be the next major elements in the bones, accounting 21.12 % and 9.41 %, respectively. This is because Ca and P plays a major role in forming the principal crystalline

material in bones. Ca and P are essential for the skeletal formation of the fish and vary in amount depending on the types of fish.

Other elements such as Mg, Na, K, and Zn were also detected in the bones in smaller amounts accounting about 0.69 %, 0.42 %, 0.26 %, and 0.25 %, respectively. These minerals are derived by the fish depending on its diet and environment. Halver and Hardy [24] stated that freshwater fishes accumulate minerals such as Mg, Zn, and Cu from both water and dietary sources, which are essential in skeletal tissue metabolism and osmoregulation of the fish.

Like bones, C, O, Ca, and P were also detected as the major elements in scales accounting about 33.80 %, 35.56 %, 18.83 %, and 10.72 %, respectively. Presence of Ca and P in scales act as a requisite elements in building resilient scales to protect the fish body from its surroundings. Lin et al. [25] conducted EDX analysis upon arapaima gigas scales and found that the calcium content in the external layer is twice higher than the internal layer of the scales. Meanwhile, Mg, Na, K, and Zn were also traced in the scales which are about 0.60 %, 0.29 %, 0.19 %, and 0.01 %, respectively. These traced elements in scales were derived by the fish from its surroundings and hold analogous function as in the bones.

Based on the results, it is apparent that the total amount of minerals in the bones (32.15 %) is slightly higher than the scales (30.64 %). This is in according to the results obtained from the ash content analysis upon raw bones and scales. The ash level in bones is also higher than the scales, indicating higher mineral content in bones. The higher the mineral levels, the harder is the bone. Extraction of gelatin from bones and scales requires pre-treatment process (removal of impurities and minerals) in order to produce high quality gelatin. Hence, based on the results obtained, it can be predicted that the tilapia bones need a more severe pre-treatment process compared to the scales to demineralize them.

3.3 Ash Content Analysis

The percentage of ash in untreated bones is found to be 30.90 ± 0.85 %. Subsequent pre-treatment with 0.2 M HCl for 12 hr reduced the ash level up to 18.93 % only. Henceforth, higher concentrations of HCl acid (0.6 to 1.4 M) and longer treatment time (16 to 32 hr) were used to facilitate the ash removal process. Fig. 2 (a) and Fig. 2 (b) show the percentage of ash in the demineralized bones as affected by HCl concentration and treatment time, respectively. Referring Fig. 2 (a), the percentage of ash in bones reduces when HCl concentration increases. Bones treated with 0.6 M HCl for 16 hr possess 17.65 ± 0.26 % of ash. Further increasing the HCl concentration to 1.4 M reduces the percentage of ash to 2.20 ± 0.02 %. Like acid concentration, increasing the treatment time also reduces the ash content in the bones (Fig. 2 (b)). Bones treated with 1.2 M HCl for 16 hr possess 8.29 ± 0.32 % of ash. Increasing the treatment time to 32 hr reduces the percentage of ash up to 0.97 ± 0.03 %.

Meanwhile for the scales, HCl concentration of 0.2 to 1.0 M and treatment time of 4 to 12 hr were used to demineralize them. Fig. 3 (a) and Fig. 3 (b) show the effects of HCl concentration and treatment time on the ash content of the scales, respectively. The initial untreated tilapia scales exhibit 29.93 ± 0.75 % of ash. Demineralization with 0.2 M HCl for 4 hr successfully reduced more than half of the ash content in the raw scales leaving behind 14.55 ± 0.14 %. Like bones, the ash content in the scales reduces when the HCl concentration and treatment time increases. For instance, increasing the treatment time to 12 hr lessens the ash level up to 0.23 ± 0.02 %. HCl acid generally reacts with the minerals in the bones and scales such as tricalcium phosphate [Ca3(PO4)2] to form monocalcium phosphate [Ca(H2PO4)2] and calcium chloride (CaCl2). Meanwhile, HCl also reacts with calcium carbonate (CaCO3) to produce calcium chloride, carbon dioxide (CO2) and water (H2O). The monocalcium phosphate and calcium chloride are soluble in the leaching solution, while carbon dioxide is evolved as gas.

Hence, increasing the HCl concentrations encourages more reaction to occur, allowing higher amount of Calcium salts to dissolve. Meanwhile, longer treatment duration gives time for the bones and scales to absorb HCl acid completely, permitting chemical reaction to occur. Results obtained are according to Figueiredo et al. [26], who stated that the demineralization rate for cortical bones increases when the HCl acid concentrations and treatment time increased. They also added that this rate is not directly proportional to the HCl concentration and treatment time. Based on Fig. 2 and Fig. 3, the effects of acid concentrations appear to be more significant in removing the ash presence in the samples compared to pre-treatment time. Though different pre-treatment time also plays a role in reducing the ash content, the effects are minimal. Larger amount of ash was removed using higher concentration of acid at shorter treatment time. For example, 50.0 % of ash was removed from the bones when the HCl concentration is increased from 0.6 to 1.4 M at 16 hr of treatment time. On the other hand, 20.88 % of ash only was removed when the treatment time is increased from 16 to 32 hr at 0.6 M HCl.

As an overview, it is crucial to highlight that bones required harsher treatment conditions to demineralize them compared to the scales. Near-complete demineralization in bones was achieved with 1.4 M HCl and 32 hr, where 99.86 % of ash was removed. Meanwhile, scales only needed 1.0 M HCl and 10 hr of treatment to remove 99.84 % of ash. The harsher treatment conditions required to demineralize bones was an indicative of the

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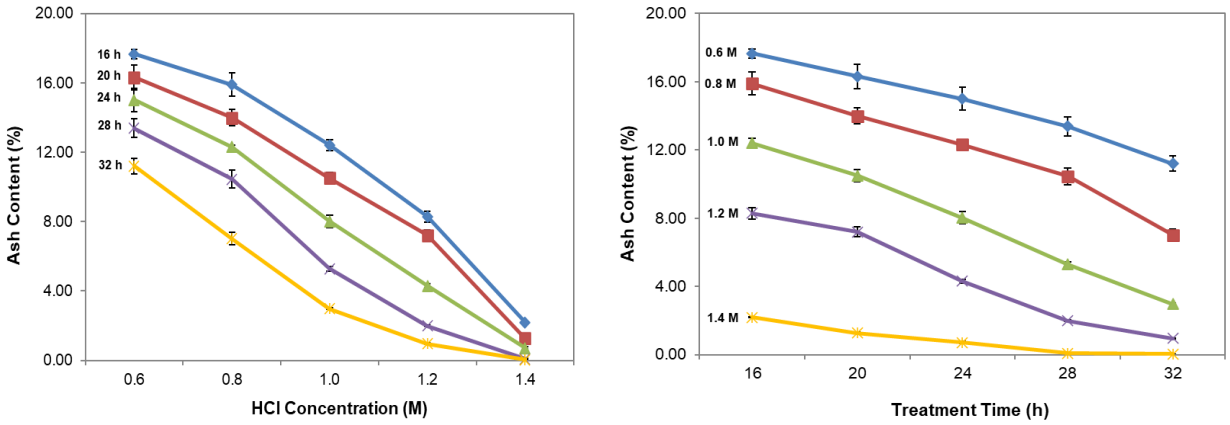
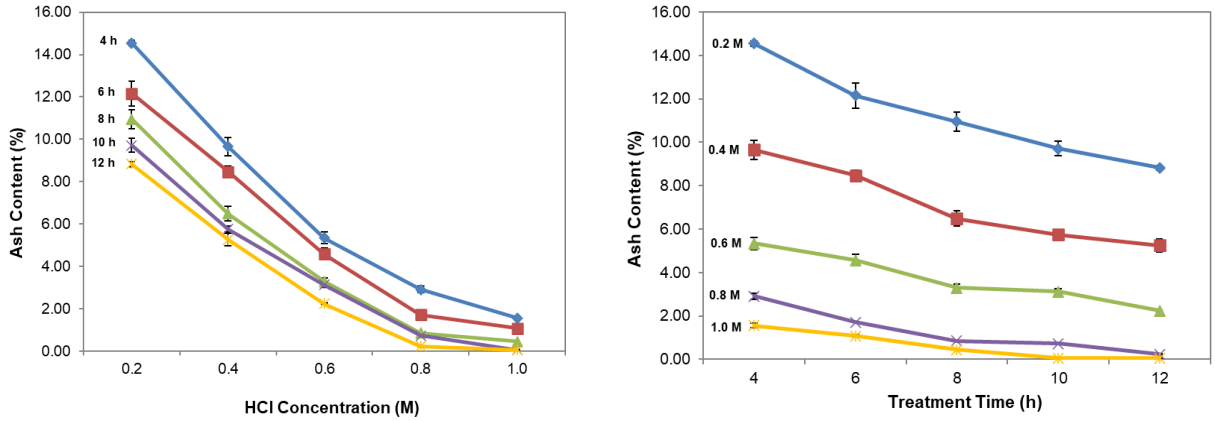
presence of large amounts of minerals in them originally. Contrarily, lower amount of minerals and larger surface area in scales allows it to release more ash even in milder treatment condition.



(a) **(b)**

Fig. 2 *Effects of (a) HCl acid concentrations; (b) Treatment time on percentage of ash in tilapia bones*



(a) **(b)**

Fig. 3 *Effects of (a) HCl acid concentrations; (b) Treatment time on percentage of ash content in tilapia scales*

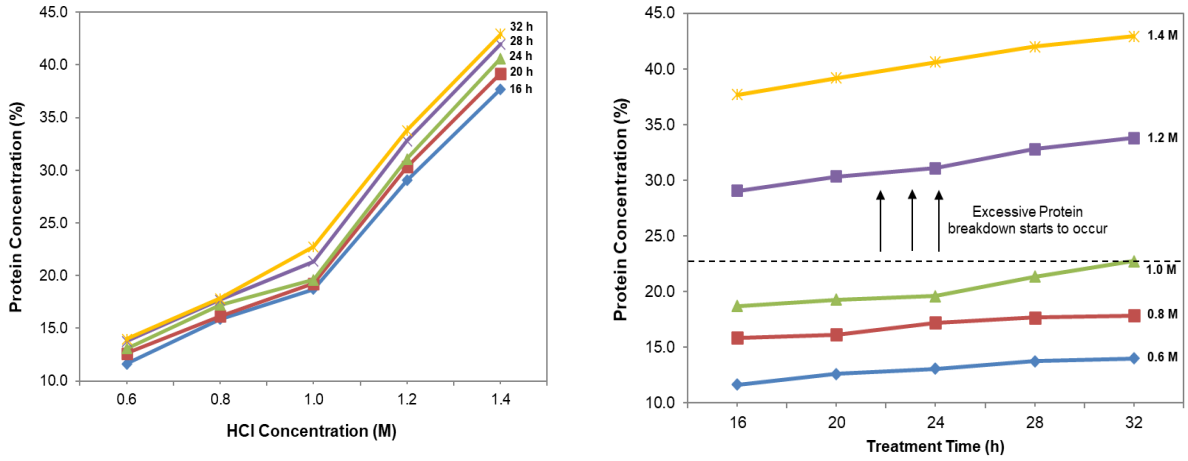
3.4 Protein Loss Due to Demineralization Process

Graph in Fig. 4 (a) and Fig. 4 (b) represent the percentage of protein loss from the bones, as affected by HCl acid concentrations and treatment time, respectively. In general, the average protein loss from bones was in the range of 11.63 ± 0.00 % to 42.92 ± 0.01 % for samples treated with 0.6 to 1.4 M HCl for 16 to 32 hr. Based on Fig. 4 (a), it can be concluded that the percentage of protein loss increased when the HCl concentrations increased. Pre-treatment with 0.6 to 1.4 M HCl (16 hr) promotes the protein loss from 11.63 ± 0.00 % to 37.68 ± 0.01 %. Similarly, the percentage of protein loss from bones also increases with the pre-treatment time as can be seen in Fig. 4 (b). For instance, bones treated with 0.8 M HCl experienced an increase in protein loss from 15.86 ± 0.02 % to 17.83 ± 0.00 %, when the treatment time is increased from 16 to 32 hr, respectively.

As for the scales, the average protein loss was in the range of 6.66 ± 0.01 % to 36.21 ± 0.00 % when treated with 0.2 to 1.0 M HCl for 4 to 12 hr. Graph in Fig. 5 (a) and Fig. 5 (b) show the percentage of protein loss from the scales, as affected by HCl acid concentrations and treatment time, respectively. Similar to bones, the percentage of protein loss in scales increases when the HCl concentrations and pre-treatment time increase. The protein loss went from 6.66 ± 0.01 % to 30.57 ± 0.01 % when the samples are treated with 0.2 to 1.0 M HCl for 16 hr. Meanwhile, increasing the treatment time from 4 to 12 hr at 0.2 M HCl also stimulates the loss of protein from 6.66 ± 0.01 % to 7.42 ± 0.01 %.

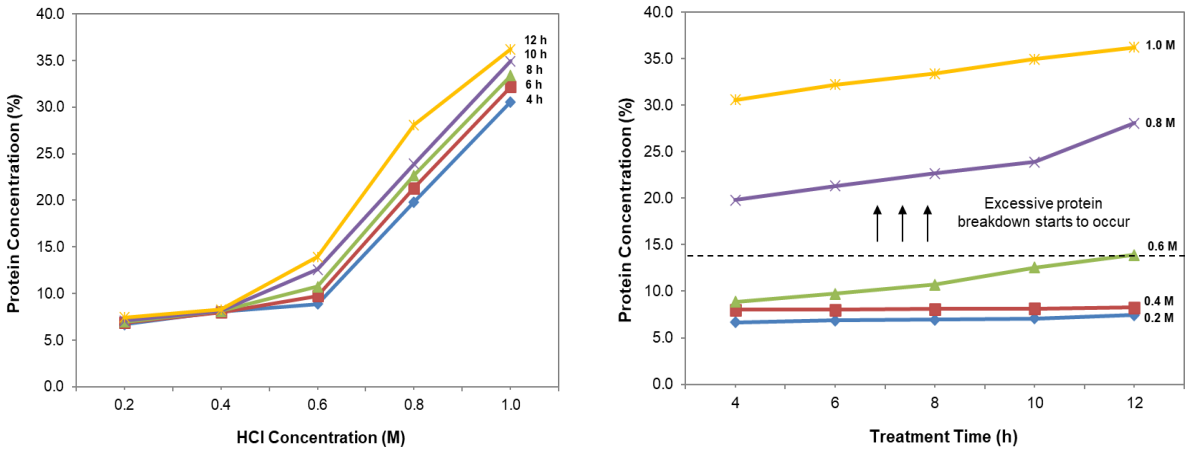
The increase in protein loss can be associated with hydrolysis of protein due to severe treatment conditions. Generally, apart from removing minerals and impurities, acid pre-treatment will destabilize the triple helical structure of collagen by breaking the non-covalent bonds. Thus, providing adequate swelling and solubilization for the collagen as means to prepare itself for gelatin conversion through thermal extraction. However, excessive

pre-treatment processes, such as overdose of acid concentration and prolonged treatment time have the tendency to further destroy the swelled collagen molecules easily.



(a) **(b)**

Fig.4 *Effects of (a) HCl acid concentrations; (b) Treatment time on percentage of protein loss from tilapia bones*



(a) **(b)**

Fig. 5 *Effects of (a) HCl acid concentrations; (b) Treatment time on percentage of protein loss from tilapia scales*

HCl, as a strong electrolytic acid is anticipated to have a greater number of reactive hydrogen ions, hence, increasing its concentration may lead to over-hydrolysis of collagen, leading to protein loss from the bones and scales. Results obtained are in accordance with Niu et al. [27] who reported that the yield of tilapia skin gelatin decreased as HCl concentrations increased. They related this to the large number of reactive hydrogen ions in HCl acids causing excessive hydrolysis of protein to occur.

Based on Fig. 4 (b), it can be observed that excessive protein breakdown or extreme hydrolysis of protein in bones occurred after 1.0 M (32 h). At this state, the bones lost a total of 22.74 % of protein only. However, further increasing the HCl concentration to 1.2 M (16 h) caused rapid loss of protein (29.05 %). This denotes pre-treatment with 1.0 M HCl for 32 hr to be the maximum possible treatment conditions for bones. Meanwhile in scales, the maximum possible treatment condition for scales was found to be 0.6 M HCl (12 h), at where protein loss is only 13.92 %. Increasing the HCl concentrations to 0.8 M (4 h) and above promotes excessive protein breakdown, where 19.82 % of protein leached from the scales.

Excessive protein breakdown can be attributed to the extreme conditions causing degradation of high molecular weight components in the collagen molecules. The significant decrease in high molecular weight portions may lead to extreme loss of collagenous protein. Niu et al. [27] stated that pre-treatment with relatively high concentrations of citric acid and HCl lead to the decrease in β-chains and molecules with molecular weight higher than 200 kDa. In general, it has been observed that the rate of protein loss in scales is faster than the

bones. This can be correlated to the physical structure of scales, which is less complex compared to the bones. The thinner structure of scales enables them to absorb acid solutions more rapidly, which leads to higher loss of protein.

4. Conclusion

As an overview, it can be signified that pre-treatment with 1.0 M for 32 hr and 0.6 M for 12 hr to be the maximum possible treatment conditions for bones and scales, respectively. At this condition, bones possess 2.97 % of ash with 22.74 % of protein loss. On the other hand, scales possess 2.24 % of ash with 13.92 % of protein loss. Pre-treatment with HCl acid prior to gelatin extraction not only helped in removing ash content, but it also removed some lipids and other impurities from the samples.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of the paper.

Author Contribution

The authors confirm contribution to the paper as follows: ***study conception, design, data collection and draft manuscript preparation:*** *Kanageswary Sockalingam;* ***analysis and interpretation of results:*** *Kanageswary Sockalingam, Hassan Zuhudi Abdullah, Maizlinda Izwana Idris. All authors reviewed the results and approved the final version of the manuscript.*

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