RESEARCH ARTICLE

Extraction and characterization of type 1 collagen from the skin and scales of *Heterotis niloticus* and *Lates niloticus*

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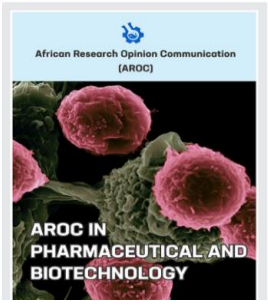

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ABSTRACT

The study is aimed to extract and characterize collagens from the skin and scale of two selected Nigerian freshwater fish species (*Heterotis niloticus* and *Lates niloticus*) using either pepsin (PSC) or acid-soluble (ASC) extraction. The collagen was extracted using 0.5M acetic acid and pepsin. The collagen yield was determined and characterized by SDS PAGE, and FTIR. Collagen extraction yields varied with the extraction process; the yield was significantly higher in the skin (5.08±0.34–33.97±1.78 %) than in the scale (1.76–8.05 %). The absorption peaks of the extracted collagen using acetic acid and pepsin show that only ASC of skin (3344.27 cm^{-1}) and scale (3495.85 cm^{-1}) of *H. niloticus* shows the peaks characteristic of Amide A, while Amide B peaks of collagen extracted from the skin and scale *of H. niloticus* and *L. niloticus* were found at 2974.46 cm^{-1} and 2925.7 cm^{-1}, representing an asymmetrical stretch of CH_2. Similarly, ASC on the skin (1558.36 cm^{-1}) and scale (1576.46 cm^{-1}) of *H. niloticus* shows the absorption peak characteristics of amide II. ASC on the skin of *H. niloticus* (1671.05 cm^{-1}), PSC on scale of *H. niloticus* (1658.55 cm^{-1}), and on scale of *H. niloticus* (1678.65 cm^{-1}) shows absorption peaks in range characteristic of amide 1. There were no differences in the skin and scale collagen profiles among the two fish species when characterized by SDS-PAGE. Our data revealed that the skin and scale of *Lates niloticus* and *Heterotis niloticus* could be a good alternative source of high-quality collagen for industries.

Keywords: Collagen; Skin; Scales; Characterization; *Lates niloticus; Heterotis niloticus*

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1.0 Introduction

Structural proteins such as collagens are mostly found abundantly in the extracellular matrix of several connective tissues which comprises about 30% of the entire proteins in the vertebrate's body including bones, ligaments, tendons and cartilage [1, 2].

Collagen plays a crucial function to assist and protect the body when dispersed in a wide range of vertebrate connective tissues [3]. Collagen also has emulsification properties, gel qua lity, low viscosity, biological compatibility, water int ake and saturation effects as biological natural ass ets that can be widely used in pharmaceutical, food beauty care products and biomedical materials [4]. In addition, several biological activities of collagen including anti-hypertensive, antioxidant, and antimicrobial properties have been reported [5-9]. Fish scales and skins are known to contain biocomposites with highly ordered type 1 collagen fibres and hydroxyapatite [10, 11]. Although there are many reports about collagen from the skin of marine organisms, there are few studies of fish scales like the studies of Kimura et al. [12] and those of Shao et al. [13].

Collagens from these sources are mainly the type I collagen with lower denaturation temperature than the collagen from porcine dermis [14]. *H. niloticus* and *L. niloticus* are freshwater species

widely distributed most especially in the Nigeria rivers. These are important food sources within the northern region of Nigeria due to their delicacy [15]. However, a large amount of these fish scales are great waste and they contain a large percentage of protein. Fish collagen can be produced from the discarded portion of the fish offal waste, such as the skin, scales, and fins, which are rich collagen sources [16]. Interestingly, type I collagen can be observed in all connective tissues, such as the skin and bones. In this study, we aimed to extract and characterize collagen from the skin and scale of *Lates niloticus* and *Heterotis niloticus* fishes sampled from a fish market, Minna, Niger State, Nigeria.

2.0 Materials and Methods

2.1 Experimental fish materials

Fish waste scale samples as well as skin of *Lates niloticus and Heterotis niloticus* were obtained and collected separately from the indigenous fish commercial market in Minna, Niger State, Nigeria. The samples were transported to the Laboratory under chilled condition, washed and used immediately. All chemical reagents used were analytical grades.

2.2 Extraction of collagen

2.2.1 Pretreatment

According to the method previously described by Nagai and Suzuki [17]. Collected wastes (Scales and Skin) of the fish species were cleansed using water, then chopped and further soaked in NaOH of 0.1M concentration (1:10 w/v) for 3 days while the scales were soaked for 5 days in the same solution. The solution was replaced each 24 hours and the samples kept at refrigerator temperature (4-6°C). This was done to expel non-collagenous proteins from the sample and furthermore to expand their surface area. After the third day the skin were washed with cold water until a neutral or faintly alkaline pH (between 7 and 7.2) was obtained. Deproteinised skin was immersed in 10% Butanol (1:10 w/v) for 48 h, to defat the samples and the solution changed once per 24 h. The deproteinised scales were decalcified in 0.5M ethylenediaminetetraacetic acid (EDTA) (1:10 w/v) which was replaced in every 24 h. The extract was thoroughly washed using cold water until a neutral or slightly alkaline pH (between 7 and 7.2) was obtained.

2.2.2 Extraction of Acid Soluble Collagen (ASC)

ASC was removed according to the technique of Li *et al*. (2013) with slight modifications. All procedures were done at 4°C with nonstop stirring. Both defatted and decalcified samples were soaked for 24 hours in acetic acid of 0.5M concentration (1:15 w/v). The blend was filtered utilizing a two-layer cheese fabric and then the residue was re-extracted using the same solvent, then filtered after 24 hours. The extracts were combined after filtration was performed. The extracts were then precipitated using cold acetone (5:1 v/v). It was further incubated for 1 h and then centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant was decanted and the pellet was dried by freezing. This is known as Acid Soluble Collagen (ASC).

2.2.3 Extraction of Pepsin Soluble Collagen (PSC)

The residue obtained after each extraction by ASC was made used in the extraction of PSC as previously described by Singh *et al.* [18 with little modification. Each residue was immersed in acetic acid (1:15 w/v) of 0.5 M concentration and pepsin (20 U/g residues) was added. The mixture was stirred continuously for 48 hours at 4°C, and further filtered using a two layered cheese cloth. Cold acetone was used to precipitate the filtrate the same way as described for ASC. The dialysate was dried by freezing and was called PSC.

2.2.4 Determination of Collagen Yield

Yield of collagen (dry weight) from the skin was determined using the formula below:

$$\%\text{Yield} = \frac{\text{(Weight of separated collagen in grams)}}{\text{(Weight of test after pretreatment in grams)}} \times 100$$

2.3 Characterization of the extracted collagens

2.3.1 Fourier Transform-Infra Red Spectrum Analysis (FTIR)

The functional group of the samples of collagen dried by freezing (ASC and PSC) of the fish species were assessed using Fourier transform infrared (FTIR) spectroscopy (Biored FT-IR 40 display, USA) as described by Muyonga *et al.* [19]. Ten (10 mg) of each sample was blended with 100 mg of KBr and fastens into a salt disc of 10 mm width for reading spectrum promoted by the use of KBr for pelleted types of samples. The range of ASC and PSC was

recorded, and effective picks were gotten and were compared with that of standard collagen.

2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples were analyzed by electrophoretic gel using discontinuous buffer system using 7.5% SDS-PAGE. The electrophoretic samples of the collagen tests were performed according to the technique previously described by Li *et al.* [20]. Each sample of collagen was mixed in acetic acid of 0.1M concentration with a 5 times sample buffer (0.5M Tris-HCl containing 4% (w/v) SDS, pH 6.8, 10% (v/v) β-mercaptoethanol and 20% (v/v) glycerol). Before loading, the samples were heated (93°C-95°C) for 4 minutes and then loaded onto 7.5% SDS-PAGE and a constant current of 70V/gel was used to run the gel using a Compact-PAGE device. The proteins were visualized using Coomassie Brilliant Blue (R-250). Molecular markers with high molecular weights (212kDa to 66.4kDa) were applied to approximate the molecular size of proteins. Collagen type I, type II, type III and type V were used as standards.

2.4 Statistical Analysis

The data obtained were analyzed using statistical examination utilizing a statistical package known as (SPSS) version 21. Comparisons were carried out by analysis of variance, ANOVA ($P<0.05$). Means differences were separated using Tukey's test.

3.0 Results

3.1 Percentage yield of collagen extracted

The yield of collagen extracted from the skin and scale *of H. niloticus* and *L. niloticus* was record presented in Table 1. The yield of collagen obtained from *L. niloticus* varied from 1.76% to 27.85%, while the collagen yield of *H. niloticus* varied from 3.78% to 33.97%. The highest collagen yield obtained from ASC transcend from 6.11±0.65% - 33.97±1.78% when compared to that of PSC, from 1.76±0.13% - 8.74±0.34%. Similarly, the collagen yield from the two method of extraction were significantly ($p<0.05$) different in skin (5.08±0.34 % - 33.97±1.78%) than the scale (1.76 %-8.05%).

Table 1: Percentage collagen yield from scales and skin of *Heterotis niloticus* and *Lates niloticus* fishes extracted by pepsin and acid methods.

Sample identification	Yield of collagen dry basis (%)
ASC HS	33.97±1.78 ^{c}
ASC LS	27.85±1.02 ^{c}
ASC LSC	6.11±0.65 ^{ab}
ASCHSC	8.05±0.95 ^{b}
PSC HS	8.74±0.34 ^{b}
PSC HSC	3.78±0.18 ^{a}
PSC LS	5.08±0.34 ^{ab}
PSC LSC	1.76±0.13^{a}

Data are Mean ± SEM of triplicate determination. Columns with different superscript are significantly ($p<0.05$) difference. PSC: Pepsin Soluble Collagen; ASC: Acid Soluble Collagen; HS: *Heterotis niloticus* Skin; HSC: Scale of *Heterotis niloticus*; LS: *Lates niloticus* Skin; LSC: *Lates niloticus* scale

3.2 Fourier Transforms Infrared (FTIR) Spectroscopy

FTIR spectra of collagen extracted from the skin and scale *of Heterotis niloticus* and *Lates niloticus* demonstrated the distinct highpoints of amides A, B, I, II as well as III were recorded and presented in supplementary material (Figure 1, Table 2). The peak regions of significant elongating FTIR frequencies of the extracted collagen are shown in Table 2: ASC HS and ASC HSC shows the absorption peak of 3344.27cm^{-1} and 3495.85cm^{-1} respectively characteristics of amide A. Similarly, ASC HS (1558.36cm^{-1}) and ASC HSC (1576.46cm^{-1}) shows the absorption peak (characteristics of amide II. Characteristics peak ranges of amide B were seen in ASC LS (2974.46cm^{-1}) and PSC HS (2925.7cm^{-1}). ASC HS (1671.05cm^{-1}), PSC HSC (1658.55cm^{-1}) and ASC HSC (1678.65cm^{-1}) shows absorption peaks in range characteristic of amide 1. Another peak representing carbohydrate moiety (1,100-1,005cm^{-1}) was seen on ASC LS, PSC HSC and ASC HSC.

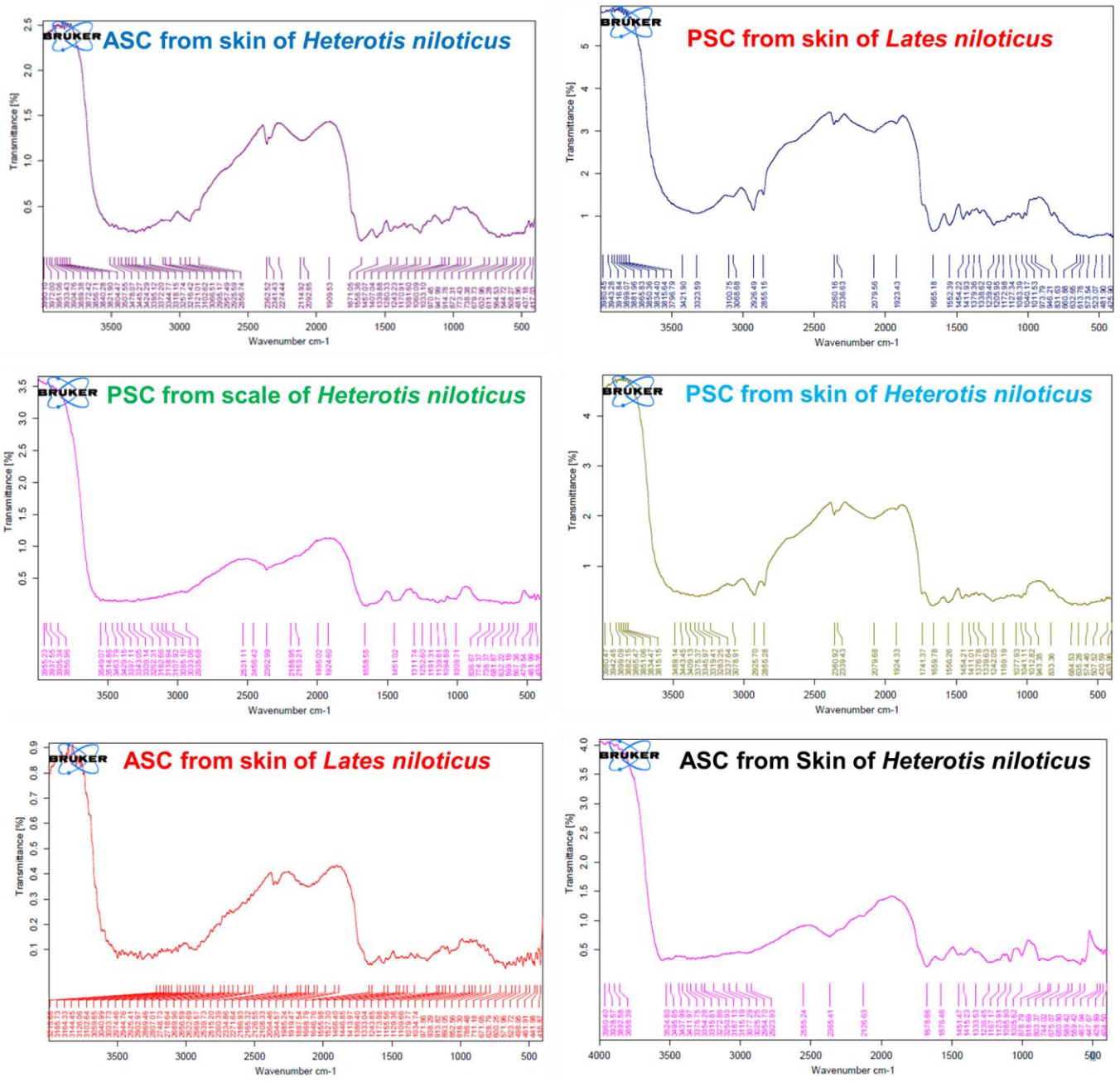

Figure 1: FTIR spectra of pepsin (PSC) or acid-soluble (ASC) collagen isolated from the skin and scale of *Heterotis niloticus* and *Lates niloticus*

Table 2: Important stretching FT-IR frequencies of isolated collagen

Region	Assignment	Peak_wavenumber range (/cm)	ASC_LS	PSC_HS	ASC_HS	PSC_HSC	ASC_HSC
Amide A	NH stretch coupled with hydrogen bond	3400 ~ 3440/cm			3344.27		3495.85
Amide B	CH2 asymmetrical stretch	2900- 2926/cm	297446	2925.7			
Amide I	C=O stretch/hydrogen bond coupled with COO-	1600 ~ 1700/cm			1671.05	1658.55	1678.65
Amide II	NH bend coupled with CN stretch	1,480-1,350/cm			1558.36		1576.46
Amide III	NH bend coupled with CN stretch	1,300-1,180 cm					1243.23
carbohydrate moieties		1,100-1,005 cm	1034.47			1009.71	1005.87

PSC: Pepsin Soluble Collagen; ASC: Acid Soluble Collagen; HS: *Heterotis niloticus* Skin; HSC: Scale of *Heterotis niloticus*; LS: *Lates niloticus* Skin; LSC: *Lates niloticus* scale

3.3 Molecular weight of the Extracted Collagen

The SDS-PAGE gel profile of the band obtained from collagen of ASC and PSC from skin and scale of *Heterotis niloticus* and *Lates niloticus* angles are portrayed in Figure 2. A marker with known molecular weights varied from 20 to 245kDa was run in the well (M). The bands gotten through SDS-PAGE indicated two groups in lane 1 speaking to PSC LS with molecular weight of 25 and 63kDa, individually. Lane 3 standing for ASC HS recorded five bands with the molecular weight of 25, 63, 75, 100 and 135kDa separately. Lane 4 stands for to ASC HSC recorded three bands with the molecular weight of 63, 135 and 180kDa separately. Lane 5 stands for PSC HS recorded four bands with the molecular weight of 25, 63, 100 and 135kDa separately. Lane 6 stands for PSC HSC recorded three bands with the molecular weight of 100, 135 and 180kDa individually. Lane 7 stands for PSC LS recorded three bands with the molecular weight of 63, 135 and 180kDa separately while lane 8 stands for AS LS in lane 8 depicted three bands with the molecular weight of 63, 135 and 180kDa individually.

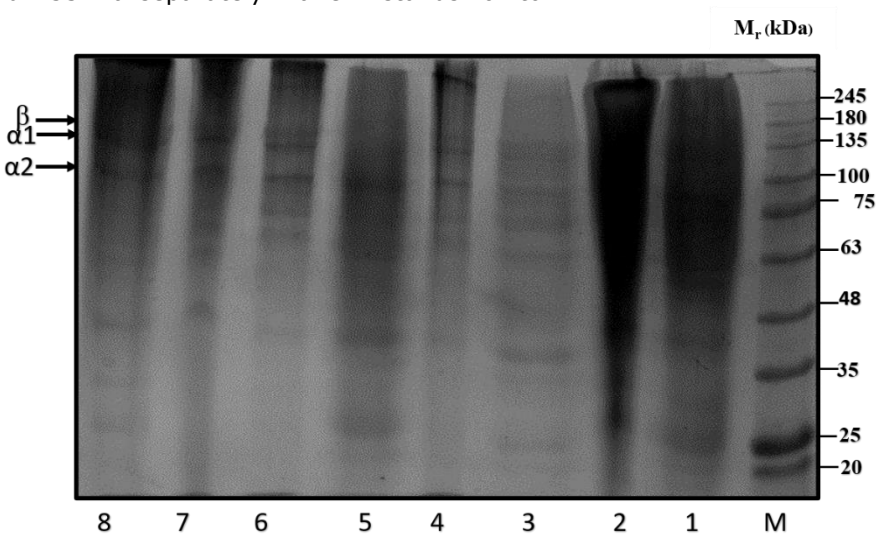

Figure 2: The SDS-PAGE gel profile of the bands obtained for ASC and PSC collagen from scales and skin of *Heterotis niloticus* and *Lates niloticus* fishes. M denote the molecular weight marker from 20kDa to 245kDa, PSC LS (lane 1) ASC HS (lane 3), ASC HSC (lane 4), PSC HS (lane 5), PSC HSC (lane 6), PSC LS (lane 7) and ASC LS(lane8).

4.0 Discussion

Pepsin, as well as acetic acids, were used to extract collagen and the yield was expressed on dry weight basis. The results obtained in this study revealed that the collagen yield varies with the method of extraction used. The skin of both species was almost solubilized completely by 0.5M acetic acid, thus the high yield of ASC than the PSC method. These findings did not correspond to Jongjaeonrak *et al.* [21] who recorded partial solubilization of big eye snapper skins in 0.5M acetic acids. The collagen yield ranging from 35% to 62.9% from various parts of marine animals has been reported in previous studies [22-30].

Some researchers have recently reported the yields of collagen from fish skin as follows: grass carp pepsin-soluble collagen (46.6%) [31], deep-sea redfish acid-solubilized (47.5%) and pepsin-solubilized collagens (92.2%) [32], and channel catfish acid-soluble (25.8%) and pepsin-soluble collagens (38.4%) [33] respectively. On the other hand, the acid-soluble collagen from the bones of the surf smelt was only slightly extracted and the yield was only 0.8% on a dry weight basis.

Cross-links formed as a result of the reaction of an aldehyde with hydroxylysine and lysine at the telopeptide helical sites could be the results for the low yield in PSC [34]. The cross-linked molecules at the telopeptide region were most likely cleaved, with further limited acid digestion which brought about an increase in collagen extraction efficacy [35]. Extraction using acid produced higher collagen yield from snakehead fish scale than extraction using pepsin [36]. *C. punctatum* produced 9.38% for ASC and 8.86% for PSC (wet weight basis) [37, 38]. The cross-linked molecules in the telopeptide region were cleaved through further limited pepsin digestion thus resulting in further extraction. Pepsin was able to cleave specifically at the telopeptide region of collagen from the skin of bigeye snapper [39].

The collagen yield in this study was higher than collagen yield from silky fowl feet (7.31 %, dry basis) [40], and bird feet collagen (16.79%, dry basis) using papain enzyme [41]. The low yield of collagen from the scales could be a result of a high amount of cross-links at the telopeptide region as well as other intermolecular cross-links, which could lead to low solubility in acid [31].

The use of FTIR spectroscopy has been applied to discover changes in the structure of collagen. The features are seen in amide A absorption mostly has to do with N-H stretching vibration which occurs in range 3400-3440cm^{-1} wavenumbers [42]. The absorption peaks of the extracted collagen using acetic acid and pepsin show that only ASC of skin (3344.27cm^{-1}) and scale (3495.85 cm^{-1}) of *H. niloticus* shows the peaks characteristic of Amide A. According to Wang *et al*. [43], when the N-H group is involved with H-bond in peptide chain, the position of Amide A peak starts to shift to lower frequencies, thus this indicates that more NH groups of the skin of *H. niloticus* were involved in hydrogen bonding than in scale [43].

Amide B peaks of collagen extracted from the skin and scale *of H. niloticus* and *L.s niloticus* were found at 2974.46cm^{-1} and 2925.7cm^{-1}, representing an asymmetrical stretch of CH_2. Similar absorption peaks between collagens suggested that *Heterotis niloticus* and *Lates niloticus* collagens complex with hydrogen bonding between free N-H stretch attached with hydrogen in polypeptide chain [19]. It was found that the amide I band, with characteristic frequencies in the range from 1600 to 1700cm^{-1} was mainly associated with the stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone and was a sensitive marker of the peptide secondary structure [44].

The absorption peaks of collagens characteristic of Amide I were found only for ASC HS, PSC HSC, ASC HSC in the range of 1658.55$cm^{-1}$and 1678.65cm^{-1}. These results implied the collagen extracted from *Heterotis niloticus* still preserved native conformation during purification processes. The peaks of amides I of ASC (1671.05cm^{-1} and 1678.65cm^{-1}) was at a higher frequency than those PSC (1658.55cm^{-1}). These indicated that ASC had a higher degree of molecular order than PSC, since the shift of these peaks to higher frequencies was associated with an increase in the molecular order [41]. However, Shanmugam *et al*. [45] reported that the peaks of amides I and II of PSC (1655 and 1548cm^{-1}, respectively) from the outer skin of *Sepiella inermis* were at a higher frequency than those of ASC (1644cm^{-1}). The amide III band (1220–1320cm^{-1}) is associated with N-H deformation and C-N stretching vibrations. In addition, absorption peaks around 1,100-1,005cm^{-1} were also found. This considerably corresponded to carbohydrate moieties as described by Muyonga *et al*. [19].

The smallest molecular mass to elicit hypertensive activity in collagen hydrolysates appeared to be between 900 and 1,900 Da. In particular, 1,000 Da which is the molecular weight distribution has shown to be a determinant [46]. Collagen hydrolysates from fish skin have a molecular weight distribution ranged from 300 to 1,500 Da, and most of the peptides were 1,200 Da [47]. There were no differences in the skin and scale collagen profiles among the two fish species when characterized by SDS-PAGE (Figure 2). For the same collagen, ASC or PSC, similar protein patterns were observed between collagens from the skin and scales *Heterotis niloticus* and *Lates niloticus.* The results suggested that no disulfide bonds were found in those collagens. These findings were similar to those reported by Ogawa *et al*. [48] and Yung *et al*. [49].

The isolated collagen may be type I, which was previously observed by Kimura *et al*. [12], who reported that the carp skin, scale, and bone collagen was the type I based on its electrophoretic mobility. Therefore, the results indicated that type 1 collagen is a major component of collagen from scales and skin of *Heterotis niloticus* and *Lates niloticus* fishes. However, among subunits of the SDS-PAGE band, low molecular weight protein fragments were observed in all treatments. These could however indicate the presence of some impurities like haemoglobin or enzyme in the collagen [50].

5.0 Conclusion

Type I collagen was retrieved out of scale as well as skin of *Lates niloticus* as well as *Heterotis niloticus* using two different methods, and it was affirmed by various analytical techniques. The result suggests that collagen could be gotten adequately from handling the misuse of *Lates niloticus* and *Heterotis niloticus* by the ASC and PSC method, as an alternative to collagen from animal source. The result of the study showed the existence of helical arrangements of collagen thus can be concluded that the skin and scale as waste from the *Lates niloticus* and *Heterotis niloticus* could become an additional and alternative possible non-conventional source of collagen for pharmaceutical and other industries.

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