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Extraction and Characterization of Collagen from Cattle Horns for Potential Wound Healing

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ABSTRACT

The need for bandages and gauze for wound dressing and healing is inevitable due to the high increase in accidents. Most of the material used for the production of bandages and gauzes for wound healing are from non-renewable synthetic material. The sustainability of non-renewable resources is at jeopardy due to the health and associated environmental impact. Consequently, the use of naturally occurring and biodegradable material is a promising solution to the sustainability challenges of synthetic material. In this paper, the extraction of collagen films from cattle horns for potential wound healing is reported. Collagen was extracted from cattle horns by acid soluble method. The maximum yield of collagen was 12% (on dry weight basis). The physicochemical characterization was carried out to confirm the purity and structural integrity of the extracted collagen. Based on the SDS-PAGE analysis, the estimated molecular weight of the α chain and β chain were approximately 135 kDa and 245 kDa respectively. UV/Vis absorption peak at 220 nm indicated that the collagen produced was type I collagen. FTIR spectra showed regions of amides A, B, I, II, and III were 3424, 2932, 1640, 1564, and 1230 cm^{-1} respectively. The results of this study indicate that, cattle could be used as an alternative source for collagen extraction. However, further study is required to optimize the conditions for collagen extraction from cattle horns including acid concentration, pH, time and temperature in order to increase yield.

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INTRODUCTION

Collagen is a long, fibrous structural protein found mostly in connective tissue like cartilage, bones, tendons, ligaments, and skin of animals (Yulianti and Rukmana, 2018). It accounts for approximately 25-30% of the total protein in animals (Schmidt *et al.*, 2021). In the body, collagen exists in the form of fibers and plays a role in forming cell structures in the extracellular matrix to maintain tissue shape. Collagen is important for the generation and support of body tissues; hence it enhances wound healing. It also gives the skin strength and elasticity, replacing dead skin cells (Upasen *et al.*, 2019). Collagen has surface binding sites for cells, and is an excellent substrate

for cell attachment. Collagen in various forms is considered as the most extensively used biomaterial for biomedical applications (Lee *et al.*, 2001). Due to its biological characteristics such as biodegradability and weak antigenicity, excellent biocompatibility and safety have made collagen the most important resource in medical applications such as wound healing (Lee *et al.*, 2001). 2001). Furthermore, its versatile processing enables the manufacture of a wide variety of tissue-engineered scaffolds in a number of different forms, such as hydrogels, sponges, and fibrous mats (Dong and Lv, 2016).

In general, 28 different types of collagen have been identified, each with its amino acid sequence, structure and properties (Cao *et al.*, 2020; Dong and Lv, 2016). Type I collagen is the most abundant, occupying about 90% of the organic mass of bones and skin (Araújo *et al.*, 2018) and is the most studied type of collagen due to its fibrillar structure. Other common types of collagen are type II collagen, the main component of animal cartilage and type III collagen, which is found in bones and digestive systems of animals. Other types of collagen are only present in minimal quantities, mainly in specific organs such as the basement membranes, cornea, heart muscle, lungs and internal mucosa (Schmidt *et al.*, 2016).

In particular, the best animal source for collagen extraction is represented by terrestrial mammals, such as cattle, pigs and sheep due to the high sequence homology with human collagen (Salvatore *et al.*, 2021). Collagen extracted from cattle has many advantages over other potential sources, such as having higher denaturation temperatures compared to marine sources (Schmidt *et al.*, 2016). Numerous studies have reported the extraction and characterization of collagen from sources such as marine (Bhuimbar *et al.*, 2019; Li *et al.*, 2020; Ramanathan *et al.*, 2014), porcine (Wang *et al.*, 2020) and chicken (Cliche *et al.*, 2003; Potti and Fahad, 2017) for wound healing. However, the application of marine sources collagen is limited because of its lower hydroxyproline content which results in low denaturation temperature and greater degradation rate, while porcine products are prohibited by Muslims and Jewish communities (Salvatore *et al.*, 2021). Extraction of collagen from cattle bones and skin have been reported (Göçer, 2022; Noorzai, 2020). However, there is limited literature on the extraction of collagen from cattle horns. Since animal horns such as cattle are not edible, they are treated as wastes. Therefore, a study on the extraction and characterization of collagen from horns is of interest as it can add value to cattle products. This paper investigates the extraction and characterization of collagen from cattle horns for potential wound healing.

MATERIAL AND METHODS

Material

The fresh cattle horns were collected from Kogwa Ranch, Dodoma were packed in plastic bags, frozen to avoid exudate leakage and rotten of the flesh side of the horn. The frozen samples were transported to the University of Dar es Salaam. Sodium hydroxide (97% purity), acetic acid (99.5% purity), acetone (99.98% purity), sodium chloride (99.5% purity) and tris HCl were purchased from Sigma Aldrich, Germany.

Analytical equipment

High Performance Liquid Chromatography (1260 Affinity II Agilent, Germany). UV-V is spectrophotometer (SPECORD 210 PLUS-223F1376), FTIR.

Sample preparation

The horn samples were washed with clean water before cutting into smaller pieces using hacksaw. Then a keratin out layer was peeled out then the sample was crushed into small hammering. Then the hammered sample was deboned and homogenized by using a grinder and subsequently kept frozen until extraction.

Sample pretreatment

The pulverized horn samples were pretreated using the method described by Wang *et al.* (2008). Samples were mixed with 0.1 M NaOH at a sample/alkali solution ratio of 1:10 (w/v) to remove noncollagenous proteins. The mixture was stirred for 6 h with magnetic stirrer while changing sodium hydroxide after every 2 h. After the reaction time and the removal of the supernatant, the sample was washed with distilled water until the pH became neutral. The deproteinized sample was mixed with acetone at a sample/solvent ratio of 1:15 for 24 h to remove fats and then the defatted material was washed three times with distilled water while the acetone was changed after every 8 h.

Extraction and isolation of collagen

Molecular collagen was isolated from cattle horns by acid extraction using acetic acid as per a previously developed method (Wang *et al.*, 2009) with minor modification. The pretreated samples of cattle horns were soaked in 0.5 M acetic acid at a sample/solvent ratio of 1:10 (w/v) at 25 ˚C for 24 h with continuous agitation. Then the acid soluble collagen was separated from the insoluble residue by centrifugation at 4000 rpm for 40 min. The extract was then filtered using cotton cloth. Collagen in the filtrate was precipitated by adding 2.6 M sodium chloride solution in the presence of 0.05 M tris-HCl (pH 7.5). The resulting deposits were recovered by centrifugation at 4000 rpm for 40 min. The deposits were dissolved in 0.5 M acetic acid and then dialyzed using 0.1 M acetic acid and distilled water sequentially using dialysis membrane (MwCO 10-14 kDa). Then, the extracted collagen was freeze dried and weighed. The percentage yield of the dry product was calculated based on the weight of pretreated sample.

Characterization

The molecular weight of the extracted collagen was determined by the Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The amino acid composition in collagen was determined using High Performance Liquid Chromatography (1260 Affinity II Agilent, Germany). The functional groups of the collagen were identified by Fourier transform infrared (FTIR). About 2 mg of collagen was mixed with approximately 100 mg of potassium bromide (KBr). All spectra were collected by transmission mode at 2 cm^{-1} interval and in the wavelength range of 4000–400 cm^{-1} wavenumbers in a dry atmosphere at room temperature. The purity of the extracted collagen was determined by using UV spectrophotometer (SPECORD 210 PLUS-223F1376) at room temperature in the 200 to 400 nm range and a scan speed of 50 nm per min.

RESULTS AND DISCUSSION

Appearance and yield of extracted collagen

The extracted acid soluble collagen product was a white solid material with the yield of 12%. The yield obtain in this study is higher compared to the yields obtained from other parts of the cattle such as cattle bones and hide using similar extraction method. For example, (Cao *et al.*, 2020) extracted collagen from cattle bones using acetic acid and the yield was 4.3% whereas, (Noorzai, 2020) reported only 3.8% from cattle hide. This may suggest that cattle horns contain more collagen than bon and hide. However, the difference in yield may be attributed by other factors such as extraction conditions and age of the cattle age. This suggests that the current study requires further investigation by comparison of amount of collagen extracted from different parts of the same cattle using similar extraction conditions.

UV-Vis absorption spectrum

UV-Vis scanning is one of the means to assess collagen purity, since its triple helical structure has a single main absorption peak near 230 nm (Wakjira *et al.*, 2022). The findings from the UV-Visible spectrometric analysis indicate that the acid soluble collagen exhibited a maximum absorbance of about 220 nm and a wide peak positioned at 268 nm (Figure 1). The peak at 220 nm may be related to the C=O, COOH, and $CONH_2$ groups in polypeptide chains of collagen. While the absorption peak at 268 nm corresponds to tyrosine and phenylalanine peak (Song *et al.*, 2021). According to Cui *et al.* (2007), collagen fibrils exhibited maximum absorbance at 220 nm was identical to type I collagen. The broad absorption peak at 268 nm shows that the extracted collagen contained very little tyrosine and phenylalanine (Rizk and Mostafa, 2016). The UV-Vis spectrum of the current collagen is in agreement with the UV-Vis spectrum reported for collagen extracted from other animals such as sea cucumber (Cui *et al.*, 2007; Saallah *et al.*, 2021), Caspian Sea (Gharagheshlagh *et al.*, 2020) and buffalo skin (Rizk and Mostafa, 2016).

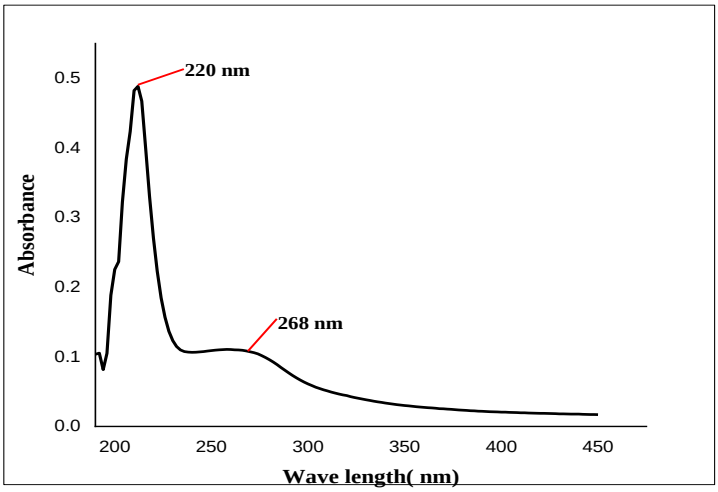

Figure 1: UV-Vis spectrum of acid-soluble collagen from cattle horns.

FTIR spectroscopy

Examination of the FTIR spectrum of the extracted collagen Figure 2, indicates the absorption peaks of amide A (3424 cm^{-1}), amide B (2932 cm^{-1}), amide I (1640 cm^{-1}), amide II (1564 cm^{-1}) and amide III (1230 cm^{-1}). These peaks have been previously reported as a signature of the triple helix configuration of Type I collagen (Boskey and Pleshko Camacho, 2007; Chen *et al.*, 2008). The amide A indicates the presence of hydrogen bonding, probably with a carbonyl group of the peptide chain, corresponds to free N-H stretching vibration. When an NH group interacts with an H bond in a collagen peptide chain, the position begins to shift to lower frequencies. A free N-H stretching vibration occurs in the range of 3400-3440 cm^{-1} (Ramanathan *et al.*, 2014; Wang *et al.*, 2014) and the peak observed for amide A in this study is within this range. Similarly, the amide B peak position which was approximately 2932 cm^{-1} is associated with the asymmetrical stretching of CH_2 (Song *et al.*, 2021). The amide I peak which is a crucial component in determining the protein's secondary structure was generally observed at 1640 cm^{-1}, which is within the previously reported range of 1600 to 1700 cm^{-1} (Wu *et al.*, 2019). Similarly, the amide II peak observed at 1564 cm^{-1} was due to the N-H bending vibrations together with C-N stretching vibrations. Similar peak was previously reported in the literature (Rizk and Mostafa, 2016). The peak observed at around 1450 cm^{-1} corresponds to pyrrolidine ring vibration of proline and hydroxyproline (Pal *et al.*, 2015). The

absorption peak at 1230 cm^{-1} was due to amide III, which is an indication of the helical organization structure in the collagen. Furthermore the absorption ratio of 1 between Amide III and 1454 cm^{-1} peak confirms the triple helical structure of collagen suggesting that the collagen did not denature during extraction. Hence, FTIR analysis reveals the presence of all peaks anticipated from collagen structure, as well as helical collagen configurations (Liu *et al.*, 2015).

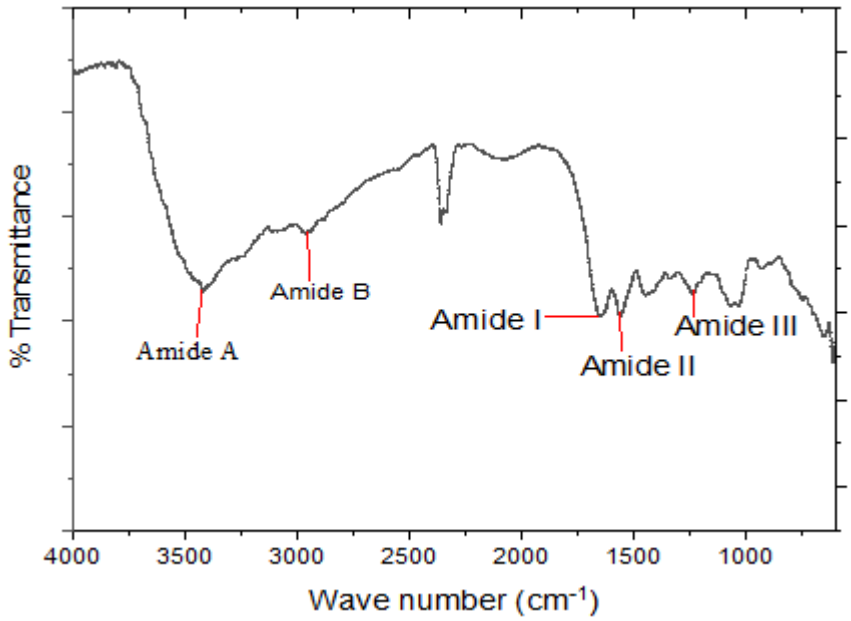

Figure 2: FTIR spectrum of acid soluble collagen extracted from cattle horn.

Molecular weight of collagen

The molecular weights of the α and β chains were estimated using a high molecular weight protein marker as a reference (1), Figure 3. Findings on the molecular weight of the extracted collagen (2 and 3), Figure 3 indicated that the SDS-PAGE patterns were composed of α chains (α_1 and α_2) and one β chain with an approximate molecular weight of 130 kDa and 250 kDa respectively. Subunit fragments with molecular weight lower than α-chains are barely evident in the electrophoretogram of the acid soluble collagen implying that the collagen extracted is not further degraded (Ran and Wang, 2014). Furthermore, electrophoresis patterns revealed the absence of non-collagenous protein bands, signifying that the extracted collagen was pure and its structure was well conserved during pretreatment and extraction. The existence of the β-chain band in relatively high densities, indicating the presence of intermolecular cross-links (Pang, 2016). The current results are similar to the previously reported findings (Cao *et al.*, 2020; Jafari *et al.*, 2020; Ju *et al.*, 2020) hence, confirming that the collagen obtained was type I.

Amino acid composition

Further findings on the amino acid analysis indicate that out of the 13 amino acids identified by HPLC, glycine composition was 37.37%. Normally, a typical collagen is composed of a glycine-proline-hydroxyproline sequence whereby the composition of glycine dominates the chain (Nurilmala *et al.*, 2019; Wahyuningsih *et al.*, 2018). Therefore, the amount of glycine obtained in this study confirms the extracted product is collagen.

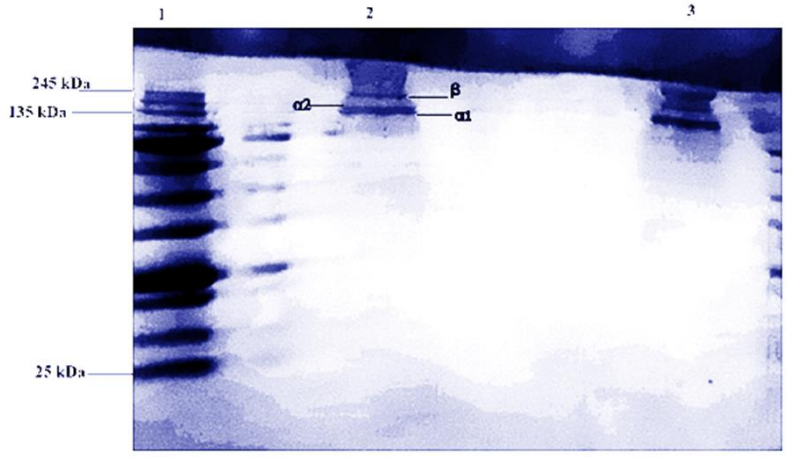

Figure 3: SDS-PAGE patterns of acid-soluble collagen from cattle horns: 1-protein marker (standard); 2 and 3 extracted acid soluble collagen.

CONCLUSIONS AND RECOMMENDATION

Collagen was successfully extracted from cattle horns using the acid soluble extraction method. The yields obtained were relatively higher compared to the previously reported yields from other parts of cattle. This implies that cattle horns can be used as an alternative source of collagen. However, further studies on optimization of extraction conditions and confirm the yield are recommended. The extracted collagen was characterized in terms of amino acid composition, molecular weight, and purity, confirming the presence of type I collagen. This implies that, the extracted collagen can be used for wound healing. Further study is recommended for electrospinning of the extracted collaged to confirm if it can be made into fibrous fabric or non-fibrous membrane for easy usage.

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