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IDENTIFICATION OF COLLAGEN IN SOLID WASTE KEROPOK LEKOR

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ABSTRACT

Collagen is a protein made up of amino-acids and comprising approximately 20% to 30% of total body protein content. In Terengganu, the by-product of the 'Keropok Lekor' which primarily from the fish was dispose and the purpose of this study is to utilize those waste. The primary objective of this study is to determine whether collagen can be extracted from 'Keropok Lekor' and its solid waste. The 'Keropok Lekor', waste skin, bone, fins and scale were treated with sodium hydroxide, Ethylenediaminetetraacetic acid and butanol. Then, the samples were extracted using acetic acid for few weeks using acid soluble collagen (ASC) extraction. From this research, collagens were successfully extracted from 'Keropok Lekor' and its solid waste. The collagens extracted were light in colour, viscous properties and acidic odour. These studies also identify collagen using Thin Layer Chromatography (TLC), determining the yield of extracted collagen and characterization of collagen using FTIR. Based on the result, the retardation factor of TLC shows the possible content of amino acid for each sample. The yield of precipitate collagen obtain form skin, bone and scale waste are 16.00 %, 23.00% and 7.33% and the other collagen sample does not precipitated properly. The characteristic of Amide A, Amide B, Amide I, Amide II and Amide III is determined using FTIR and shows similarities from previous studies.

Keywords: collagen, solid waste, keropok lekor, acid soluble collagen extraction.

INTRODUCTION

Collagen is a protein made up of amino-acids, which are in turn built of carbon, oxygen and hydrogen. Collagen comprises specific amino acids like Glycine, Proline, Hydroxyproline and Arginine. Collagen is the major insoluble fibrous protein in the extracellular matrix and in connective tissue. In fact, it is the single most abundant protein in the animal kingdom. There are at least 16 types of collagen, but 80 - 90 percent of the collagen in the body consists of types I, II, and III (Lodish *et al*., 2000). Collagen can be found in skins, bones, cartilage, tendons, ligaments, blood vessels, cornea, and all other organs of vertebrates. Collagen type I possesses a triple helical structure, in which an inter chain hydrogen bonding between glycine and amide group in an adjacent chain is a key factor in stabilising the collagen triple helix (Dai and Etzkorn, 2009). Collagen type I is widely used in food industries, cosmetic, pharmaceutical, biomedical, and tissue engineering due to its excellent biocompatibility and biodegradability (Liu *et al.*, 2010).

'Keropok Lekor' or fish sausage is a popular and highly relished fish product in Malaysia. It is widely sold in the local market and usually produced on a daily basis to fulfil the market demand. Generally, 'Keropok Lekor' can be categorized as a type of snack which is made of flavored with fish. It is originally produce in Terengganu and now has been widely commercialized to entire country (malaysiavacationguide.com, 2014).

The production used fish meat as main material and others part of fish such as bone, fin, fish head and tail was removed as a waste. The type of fish used in 'Keropok Lekor' production is 'ikan selayang' (Trachurus trachurus), 'ikan tamban' (Sardinella albella) and 'ikan cencaru' (Megalaspis cordyla) was mixed for meat isolation process in production of 'Keropok Lekor'.

'Keropok Lekor' processing wastes not only affect the surrounding area directly, but can also spoil a wider coastal zone and different ecosystems. The wastes not only cause pollution but also it emit defensive odor (Nagai *et al*., 2000). However, there is considerable potential for gaining more value from 'keropok lekor' wastes. The wastes are rich in valuable minerals, enzymes, pigments and flavours that are required by many industries including food, agriculture, aquaculture and pharmaceuticals.Therefore, this paper will present identification of collagen in solid waste *keropok lekor* by using acid soluble collagen extraction in order to optimise utilisation of the waste.

In this study, the 'Keropok Lekor' and its solid waste was obtained in Kampung Tebakang and 'Keropok Lekor' Bukit Tok Beng BTB 2209 in Terengganu. The samples were washed to remove blood and other contaminant. Then it was prepared using Sodium Hydroxide, EDTA-2NA, and Butanol to remove unnecessary component. The 10g of each sample were extracted using Acetic acid to obtain the acid soluble collagen (ASC). During extraction and preparation the sample was keep in 4°C temperature. The samples ware centrifuged to separate the collagen mixture and precipitated the collagen using NaCl.

The extracted collagen samples were analysed

using 2 different analyses. The yield of collagen obtain were calculated. The FTIR analysis was conducted to study the composition of collagen and compared with other previous studies on collagen from difference species. The TLC analysis was carried out to determine the possible amino acid content based on the retention factor of standard amino acid.

MATERIALS AND METHOD

Basically the procedure is divided into three parts; first is sample preparation, the sample are prepared by using different solution. Second is extraction, the sample are extract by using solution for few days and the other part is sample analysis. Analysis that has been done is spectroscopic characterization, and mechanical. The extraction of collagen was done according to the method by Nagai *et.al* (2008) with slight modification.

Materials

The chemical used in this study for preparation and extraction of collagen from fins, scale, skin, bone, and 'keropok lekor' were butanol, sodium hydroxide, ethylenediaminetetraacetic disodium salt, acetic acid, sodium chloride and lastly distilled water supplied by Chemical Engineering Laboratory UiTM, Terengganu.

Sample Preparation

The sample was taken from 'Keropok Lekor' manufacturing at kampung tebakang in Terengganu. Solid waste of 'Keropok Lekor' manufacturing consists of fins, scale, skin, bone and 'Keropok Lekor' sample was taken from 'Keropok Lekor' Bukit Tok Beng BTB 2209. Fins, scale, skin, bone and 'Keropok Lekor' was separated, cut into small pieces and wash. Then samples were packed into polyethlyne bags and kept at -20^{0}C until used. The storage time is no longer than three month. All the preparative procedures were performed at room temperature. All the samples weight to 100g of sample using electronic balance (Mettler Toledo) before removing non-collagenous protein process.

Collagen preparation

The prepared skin, scale, bone, fins and 'Keropok Lekor' was treated with NaOH (PH 12) for 6 hours with continuous stirring for removing Non-Collagenous Protein. After that, the sample was wash with distilled water until obtain neutral ph. While for demineralization process, for sample bone and scale demineralization process are needed. Demineralization of fish scales was carried out using 0.5 M EDTA-2Na solution (pH 7.4) for 24 h at the ratio of 1:10 (w/v). The sample solution mixture was kept in 4^{0}C. After 24 h, the sample was wash with distilled water until obtain neutral ph. Later process of removing fat is done before the extraction of collagen begin.

Yield of Collagen

The concentration of extracted collagen was calculated by taking a glass Petri dish and measuring its weight. Then, a small amount of extracted collagen was placed on the dish. Then the collagen was dried until all remaining solution to evaporate using hair dryer. The difference between the two measurements of the weight was collected. Finally, the concentration of collagen was measured by dividing the weight of collagen on the volume of the solution.

Analysis

Determination of amino acids using thin layer chromatography (TLC)

While the plate is being prepared for chromatographic analysis it should be kept on a piece of filter paper. Start the analysis by insert the chromatographic plate through the chamber and cover it. The solvent was travelled up the plate until 7-10 mm from the lid. The the plate was remove the paper from elution chamber and place it on a sheet of filter paper. After 2-3 minutes mark the eluent front with pencil after the plate is dry. Mark the moving spot and calculate Retention factor.

Fourier Transform Infrared (FTIR) Spectroscopy

The precipitate samples were dried and were subjected to FTIR analysis. The FTIR spectra were obtained from discs containing 10 mg dried sample. The spectra were obtained over the range of 4000-650 cm-1 at a 4 cm-1 resolution.

RESULTS AND DISCUSSIONS

Table-1. Yield and Properties of Collagen.

Sample	Sample weight	Collagen precipitation weight	Yield of collagen / Collagen percentage	Physical condition solvent-collagen under 4°C temperature
Keropok	15g	-	-	Slightly viscous
Skin	15g	2.3g	16%	Highly viscous
Fins	15g	-	-	viscous
Scale	15g	1.1g	7.33%	Slightly viscous
Bone	15g	3.5g	23%	viscous

In recent years, a trial has been aggressively performed to extract the collagen from the aquatic organisms to utilize it in industry and the collagens obtained from these organisms have been used in the food, cosmetic, and medical fields. In fact, no. of jurnals have reported the yields of collagens from marine organisms as follows: collagen from fins, scales, skins, bones and swim bladders of bighead carp which obtain yield of collagen 5.1%, 2.7%, 60.3%, 2.9%, 50.9% (liu et al., 2012), fish waste material skin, bone, and fin 51.4%, 49.8% and 50.1% (Nagai et al., 2000), The Skins of the Surf Smelt 24.0% (Nagai, 2010), and the scale of spotted golden goatfish 1.20% (Matmaroh, 2011).

In this research, 'keropok lekor' and fins precipitate of collagen was hardly seen. It was estimated the yield of dry collagen was around ~1-3 % from 15g of sample. However the skin sample can produce around 16% of 15g sample collagen and the scale around 7.33%. The highest collagen yield obtains from bone sample. It was 23% from 15g of sample.

Table-2. Thin Layer Chromatography (TLC) Analysis.

sample	Distance travelled			
	By spot (cm)	By solvent (cm)	Retention factor	Amino acid possibilities
Skin	3.3	10.3	0.32	L-Glutamic acid
fins	2.4	10.3	0.23	L-Cysteine acid
Scale	1.3	10.3	0.12	L-Arginine
Keropok lekor	0.8	10.3	0.07	L-Lysine
Bone	1.3	10.3	0.13	L-Arginine
Fish oil	6.5	10.3	0.63	L-Leucine

Accoding to Ramakrishnan, (2013) each Retention factor represent the spacific amino acid like L-Leucine, L-Arginine and more. However in this experiment each spot observe to be hardly seen. Thus, this can be assume that amino acid spot were undergo the capillary action representing the specific amino acid possibilities.

From the table, the skin collagen sample represent the Retention factor of 0.32 show the possibilities of containing amino acid in highest of L-Glutamic acid. The fin collagen sample with 0.23 Retention factor has the highest L-Cysteine acid of amino acid. Both scale and bone have the Retention factor of 0.12 and 0.13 respectively which may contain the highest L-Arginine and the 'keropok lekor' sample may contain highest in L-Lysine with the Retention factor of 0.07. The fish oil was only reference for the capillary action on amino acid which have the highest Retention factor and containing highest in L-leusine.

Fourier Transform Infrared Spectra (FTIR) On Skin Collagen Sample

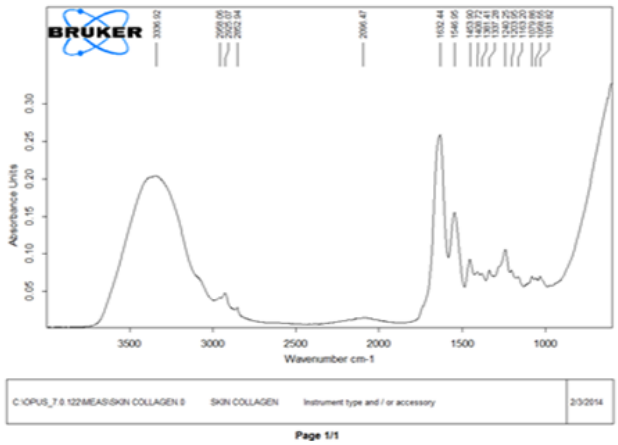

Figure-1. FTIR result for skin collagen sample.

FTIR spectra of skin ASC exhibited the characteristic peaks of Amide I, II, III as well as amide A and B. The absorption characteristics of Amide A, commonly associated with N-H stretching vibration, occurs in the wavenumber range 3400-3440 cm-1 (Sai and Babu, 2001). The absorption peaks of skin ASC were found at 3336.92 cm-1, respectively. When NH group is involved with H bond in peptide chain, the position starts to shift to lower frequencies. Amide B peaks of skin ASC were found at 2958.06 cm1, representing asymmetrical stretch of CH2 (Nagai et al., 2010). The amide I band with its characteristic frequencies (1600-1700 cm-1), mainly associated with the stretching vibrations of the carbonyl group along the polypeptide backbone (nagai et al, 2010). The amide I band of this collagen was detected at 1632.44 cm-1. This observation confirmed that the formation of hydrogen bond between N–H stretch (X position) and C=O (Gly) of the fourth residue is responsible for introducing into triple helix (Zanaboni, Rossi, Onana, and Tenni, 2000).

Amide II is generally responsible for the combination of the NH in-plane bend and the CN stretching vibration (nagai, 2010). Amide II of ASC was found at wavenumber (1546.95 cm-1). Amide III bands were found at wavenumber of 1240.28 cm-1 for ASC. The Amide III peak is complex with intermolecular interactions in collagen, consisting of components from C-N stretching and N-H in plane bending from amide linkages, as well as absorptions arising from wagging vibrations from CH2 groups from the glycine backbone and proline side-chains indicating that hydrogen bonds were involved in ASC and PSC. Additionally, the absorption peaks around 1451-1450 cm-1 were also found in ASC. This considerably corresponded to pyrrolidine ring vibration of proline and hydroxyproline as described by Muyonga et al. (2004). The present of amide A, amide B, amide I, amide II, and amide III prove the presence of collagen according past research.

CONCLUSIONS

This research is considered success as the entire objective is achieved. Objective that been archives are identification of collagen in keropok lekor, scale, fins, bone, and skin, extraction and comparison the amount collagen and identification of the composition of the extract by using FTIR.

The thin layer chromatography (TLC) result of Retardation factor of each sample were compared with the standard amino acid from previous studies (Ramakrishnan, 2013) and it was approximately resemble the retardation factor of standard amino acid. However, without ninhydrin the reaction between the amino acid and ninhydrin producing Ruhemann's purple cannot occur. It is assume that the sample result on TLC analysis have approximately highest content of amino acid (type of amino acid) from the result gain.

From FTIR result, each of samples shows the peak of absorbance unit and the wavenumber which is similar to previous studies that have been conducted from different type of collagen species which mainly characterised as type I collagen. The skin collagen has the most similar characteristic of type 1 collagen among others. Thus, the following analysis shows that the collagen can be extracted from ‘Keropok Lekor’ and its solid waste and shows the characteristic of a collagen.

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