© 2017 IJSRSET | Volume 3 | Issue 3 | Print ISSN: 2395-1990 | Online ISSN : 2394-4099
Themed Section: Engineering and Technology

Amino acid Composition of Gelatin Extracted from the Scales of Different Marine Fish Species in Kenya

Ann Wangari Mureithi[*1], John Mmari Onyari[1], Wycliffe Chisutia Wanyonyi[2], Francis Jackim Mulaa[3]

[1]Department of Chemistry, University of Nairobi, Nairobi, Kenya
[2]Department of Physical Sciences, University of Kabianga, Kericho, Kenya.
[3]Department of Biochemistry, University of Nairobi, Nairobi, Kenya

ABSTRACT

Gelatin in this study was extracted by an enzymatic process from the scales of three marine fish species; *Lutjanus sebea* (Red snapper), *Lethrinus harak* (Black spot emperor) and *Scalus ghobban* (Blue barred parrot fish). Concentration of bacteria for mass production of enzyme was done in a fermentation medium using a bio reactor. Scales were hydrolyzed at 50^0C and the pH maintained at 12. Complete hydrolysis took between 20 and 23 days for all species. The yield for the dried gelatin was between 28.2% and 41.4% for the marine fish scales under study. Fourier transform infrared spectra showed the presence of amide bands and two other additional absorption bands, indicating the presence of amide bonds for all the three species. The amino acid composition analysis for the gelatin of three species was then done showing the presence of 16 amino acids. Glycine was the most abundant for all the three species with about 35% followed by Alanine both adding up to around 50% of the total amino acid compositions. The amount of Proline was high for red snapper at over 14.2% compared to 11.1% and 11.6% for blue barred parrot fish and black spot emperor respectively.

Keywords: Gelatin, Enzymatic Process, Marine Fish Scales, Amino Acid Composition.

I. INTRODUCTION

Gelatin is a protein that does not occur naturally but is obtained by thermal denaturation of collagen (Gomez-Guillen *et al.,* 2002). Collagen is a key structural protein in animals including humans (Shoulders and Raines, 2009). This thermal denaturation is a hydrolysis process that involves breaking of covalent and hydrogen bonds in the chains of collagen's triple helix structure, in turn converting the insoluble collagen to soluble gelatin (Kim *et al.*, 2014).

The extraction of gelatin can be done chemically using acid or alkaline solutions or by use of enzymes. In an enzymatic process, alkaline protease is the best enzyme for gelatin extraction (Jiang, 2012). The protease enzymes are largely produced by bacteria, namely *Bacillus spp*, and are mostly extracellular, with the concentration being done in a fermentation media (Sevinc and Demirkan, 2011). *Bacillus cereus* stain wwcp is specifically said to exhibit good protease activity (Wanyonyi *et al.,* 2014). The extent of the bacterial multiplication and the eventual enzyme production is dependent on the nutrients present especially the carbon and nitrogen sources and the physical factors such as inoculums concentration, temperature, pH and incubation time (Lakshmi *et al.,* 2014).

The gelatin that is available in the market is mainly obtained from pig (porcine) skin and cattle (bovine) hide and bones (Ahmad and Benjakul, 2011). However, there has been health concerns (Zakaria and Bakar, 2015) and the religious restrictions ((Ardekani *et al.,* 2013) facing this mammalian sourced gelatin, leading to the recent entry of non mammalian sources, that is, fish gelatin (fish skin and bones) and poultry gelatin (feathers and feet) (Kim *et al.*, 2014). Fish skin and bone have been the main focus as sources of gelatin, with most of the extraction being done by chemical means.

This study involved the extraction of gelatin from the scales of three marine fish species; *Lutjanus sebea* (Red

snapper), *Lethrinus harak* (Black spot emperor) and *Scalus ghobban* (Blue barred parrot fish) selected in relation to abundance on the Kenyan coast. The extraction was done using enzyme.

II. METHODS AND MATERIAL

2.1 Scales collection and preparation.

The marine fish scales from the three species were purchased separately from sea food markets in Nairobi, Kenya. The fish were obtained from Malindi, along the Kenyan coast. Species identification was done at the Nairobi National Museum, Nairobi, Kenya with the marine fish being identified as Lutjanus sebea (Red snapper), Lethrinus harak (Black spot emperor) and Scalus ghobban (Blue barred parrot fish). The scales were washed separately using tap water to remove dirt and meat present and later sun dried. The dry scales were then packed in clean dry plastic bags and stored under room temperature awaiting the hydrolysis process.

2.2 Enzyme production

The bacteria (Bacillus cereus stain wwcp1) initially isolated from the mud waters from Lake Bogoria, Kenya (Wanyonyi et al., 2014) and re-plated was used for enzyme production. The enzyme production was done using a bioreactor (R'ALF plus duet fermenter, 3.7L). Media for enzyme production was prepared containing 0.25% casein, 5% glucose, K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, urea and yeast extract in distilled water. The pH of the media was adjusted to 9 using NaOH solution by a pH meter and sterilized for 30 minutes in an auto clave (Tuttnauer steam 121^0C sterilizer).The media was then allowed to cool under room temperature after which inoculation was done with a 5% overnight grown seed bacterial culture. Temperature was set 36^0C, and stirrer was set at 120 rpm. After four days of continuous fermentation the enzyme was harvested, centrifuged in Sorvall ST16R centrifuge (1500rpm, 4^0C for 4 minutes) and placed in the cold room (4^0C) ready for use in the hydrolysis process.

2.3 Gelatin extraction

200g of scales from the three marine fish species were weighed and packed separately in 1L conical flasks and 500mLs of enzyme solution was added to each. The pH was adjusted to 12 after which the conical flasks were plugged using cotton wool and covered with aluminum foil. The hydrolysis process was done at a temperature of 50^0C (Jiang, 2012). After every 24 hours, the pH for was re-adjusted to 12 and stirring done, to ensure uniform and complete hydrolysis in the conical flasks. The hydrolysis process took 20 days, 22days and 23 days for black spot emperor, red snapper and blue barred parrot fish respectively. Gelatin solution was filtered and freeze dried.

2.4 The yield

The yield of gelatin for the scales of each species was calculated using the formulae;
%Yield= (Mass of the dried gelatin / Mass of the dry scales hydrolyzed) × 100 (Zakaria and Bakar, 2015).

2.5 Fourier Transform Infrared Spectrometry

This analysis was done for the pure gelatin from the three marine fish species using a Fourier transform infrared spectrometer IR Affinity-1S Shimadzu model. The process was carried as described by Ahmad and Benjakul (2011), but the spectra obtained in the 500 to 4500 cm^{-1}range.

2.6 Amino acid composition analysis

Amino acid composition analysis of gelatin from the three marine species understudy was performed using a narrow bore, (2.1 x 200 mm, Hypersil AA-ODS, 5 µm reverse phase column) purchased from Thermo Electron (part # 30105-202130). Samples were weighed and placed in a 13 x 100 mm Pyrex tube along with 1mL N HCl and 11 µmoles of Internal Standards (Norvaline and Sarcosine). After adding Internal Standards, the samples along with controls and blanks were exposed to liquid-phase 6N HCl for 22 hrs hours at 100^0C. Amino acids were separated on an Agilent 1260 with column heater, automatic injection programming UV and Fluorescence detection. 5µL of the hydrolysate was dried down and resuspended in 250 µL of 0.4 M Borate buffer. 1µL was injected. The G1367E auto sampler was used to perform pre-column derivatization and multiple sample handling. The derivatized amino acids were then eluted from the reverse phase column. Primary amino acids (tagged with OPA, Agilent #5061-3335) were detected at 338/390 nm by the Variable Wavelength (UV) detector (G1365D) and secondary amino acids (tagged with FMOC, Agilent 5061-3337) at 266/324 nm. The fluorometric detector (G1321B) was used to monitor the primary ones at excitation/emission

340/450nm and the secondary ones at 266/305nm. The assay was calibrated by a standard (Agilent 5061-3331) which was subjected to the same treatment as the samples and control, including hydrolysis. The assay was controlled by a known protein, Human Serum Albumin. An aliquot from the same batch of HSA was run with every assay.

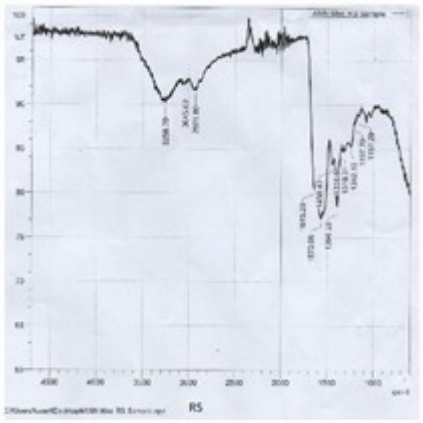
III. RESULTS AND DISCUSSION

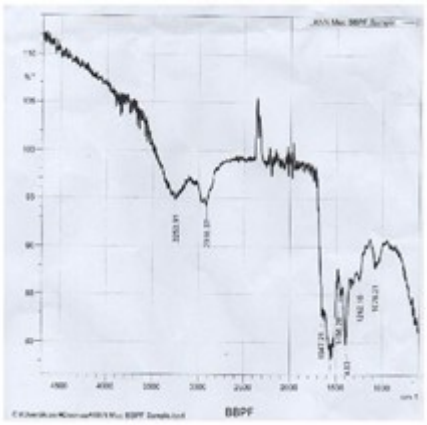
3.1 Yield

Gelatin obtained was slightly yellow and very hygroscopic. The yield was 28.2%, 38.7% and 41.4% for the scales of red snapper (RS), black spot emperor (BSE) and blue barred parrot (BBPF) fish respectively. In general, higher yields were obtained, for long extraction periods used for the enzyme hydrolysis reaction (up to 23 days). However, even though extended extraction duration leads to increased yield, the expected downside is that the gelatin obtained could have short chains (Ahmad and Benjakul, 2011). Peptide bonds are broken alongside hydrogen bonds connecting the chains in the triple helix structure. Consequently, high reduction in the size of the peptide chains leads to the lowering of the gelling strength of gelatin (Liu *et al.,* 2008). The large size of the scales of blue barred parrot fish also contributed to the higher yield when compared to the other two species. The low yield in red snapper scales is attributable to the compactness of the scales compared to the other fish species (black spot emperor and blue barred parrot fish) investigated.

3.2 Fourier Transform Infrared Spectrometry

The Fourier transform infrared spectrometry spectra for the three species showed five Amide bands namely Amide A, B, I, II, III and two other bands as shown in Figure 1;





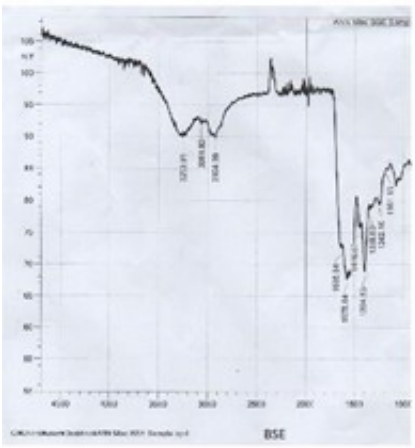


Figure 1: Fourier Transform Infrared spectra for the Red snapper, Blue barred parrot fish and Black spot emperor respectively.

Amide I bands for BSE, RS and BBPF were 1635.64, 1645.28 and 1647.21 cm-1 respectively corresponding to C=O stretching vibrations. The low amplitude were due to the interactions between C=O and adjacent chains via hydrogen bonding (Ahmad and Benjakul, 2011). This hydrogen bonding is also responsible for reduction in the frequency from the expected 1715 cm-1. Amide II bands for BSE, RS and BBPF were at 1575.84, 1570.06 and 1572.95 cm-1 respectively corresponding to C-N stretching and N-H in-plane bending. Amide III bands were similar for the three species occurring at 1242.16 cm-1 representing C-N and N-H in-plane bending as well. Amide A bands for BSE, RS and BBPF were 3253.91, 3253.91 and 3259.70 cm-1 respectively, representing N-H stretching vibration. BSE and RS exhibited Amide B bands at 3066.82 and 3045.60 respectively, corresponding to N-H stretching vibrations, with BBPF spectra missing this band. These N-H stretching vibrations bands were of low amplitudes and lower frequency than the expected 3400-3550 cm-1 due to hydrogen bonding. The other bands exhibited by BSE, RS and BBPF were 2924.09, 2931.80 and 2916.37 cm-1 which are attributable to the C-H stretching vibrations,

for the methylene groups of Glycine and Alanine indicating their relative abundance relative to other present amino acids present. The same methylene group exhibited bending vibrations at 1394.53 cm-1 for the spectra of all three species. These FTIR spectra were similar to the spectra for gelatin extracted from chicken feet by acid method which exhibited the Amide I, II, III, A and B bands at 1658.74cm-1, 155.44cm-1, 1236.36cm-1, 3322.12cm-1 and 2924.36cm-1 respectively (Widyasari and Rawdkuen, 2014).

3.3 Amino acid composition

The HPLC chromatograms showing the retention time for the different amino acids are as shown in figures 2 to 4 with the different peak heights showing the relative abundance of a given amino acid.

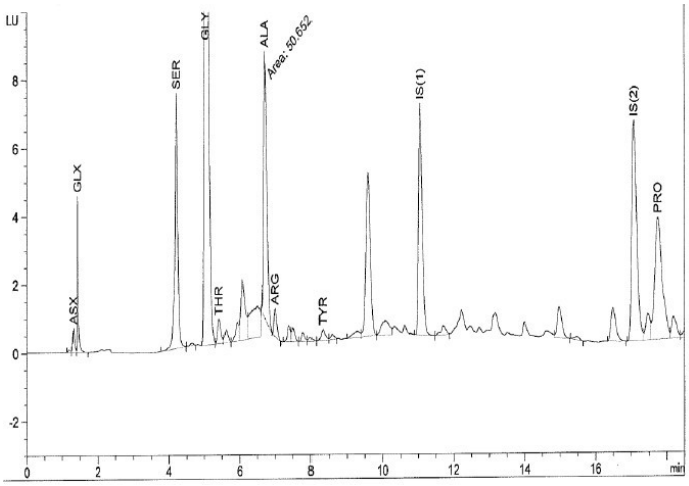


Figure 2: HPLC chromatogram of gelatin obtained from scales of Red snapper.

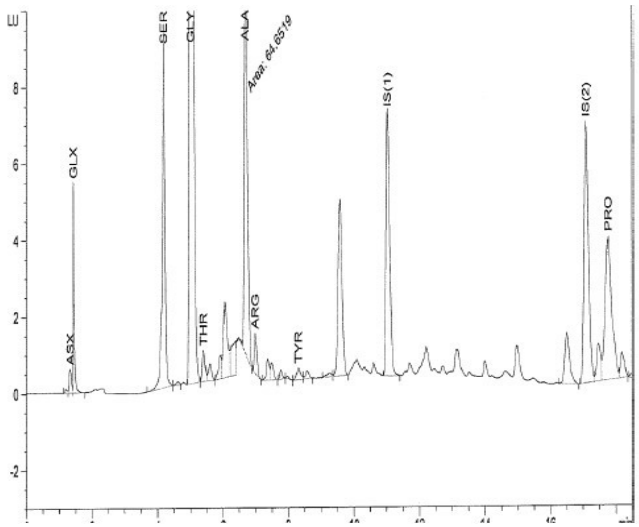


Figure 3: HPLC Chromatogram for gelatin obtained from the scales of Blue barred parrot fish

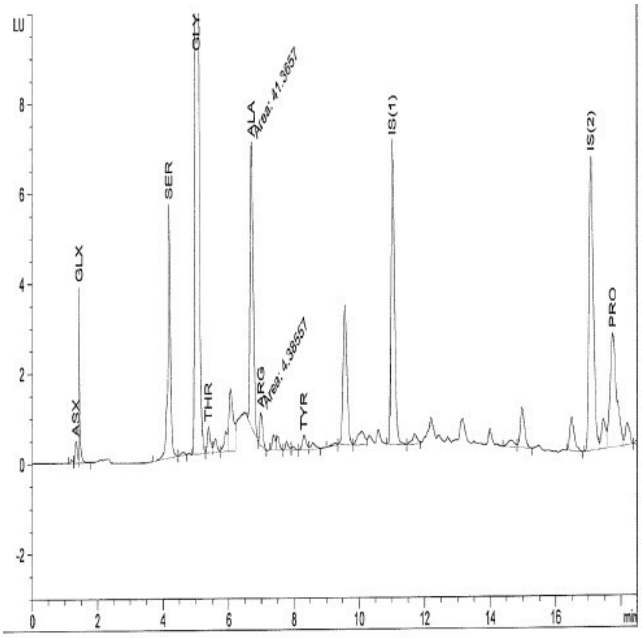


Figure 4: HPLC Chromatogram for gelatin obtained from the scales of Black spot emperor

The amino acid composition (weight %) for the three fish species is shown in table 1. The samples were analyzed in duplicates and the standard deviation (SD) is also indicated.

Table 1: Amino acid composition (%) of gelatin from the scales of Red snapper, Black spot emperor and Blue barred parrot fish.

Amino acid	% Composition ± SD in RS scales	% Composition ± SD in BBPF scales	% Composition ± SD in BSE scales
Glycine	34.84± 0.0288	35.79± 0.045	35.85± 0.0968
Alanine	14.81± 0.0005	15.42± 0.0072	15.15± 0.0085
Proline	14.16± 0.0002	11.05± 0.2048	11.63± 0.0481
Glutamic acid	8.67± 0.0013	9.05± 0.0025	8.93± 0.0001
Aspartic acid	5.15± 0.0013	5.62± 0.0013	5.58± 0.0072
Arginine	3.51± 0.0001	3.15± 0.0005	3.58± 0.0018
Leucine	2.93± 0.0002	3.26± 0.0032	2.89± 0.0005
Lysine	2.73± 0	2.84± 0.0025	2.87± 0.0001
Valine	2.55± 0	2.81± 0.0001	2.64± 0.0041
Serine	2.45± 0.0001	2.76± 0.0002	2.50± 0.0041

Methionine	2.09± 0.0098	1.62± 0.0001	2.09± 0.0265
Phenylalanine	1.92± 0.0002	2.06± 0.0032	2.04± 0.0001
Isoleucine	1.38± 0	1.64± 0	1.29± 0.0001
Threonine	1.21± 0.0018	1.09± 0.0001	1.27± 0.0013
Tyrosine	0.87± 0.0005	0.98± 0.0001	0.94± 0.0013
Histidine	0.76± 0.0008	0.94± 0.0001	0.86± 0.0008
Cysteine	Trace	Trace	Trace
Total	100	100	100

The results show that Glycine is the most abundant amino acid. This high amount of Glycine is attributed to the repeating sequence of (Glycine-X-Y) amino acid triplets in the constituent chains of gelatin, which is specific to gelatin, where X and Y are mainly Proline and Hydroxyproline respectively. The key difference of the gelatin extracted from the scales of the marine fish species under study using the operating conditions used here, when compared to the gelatin extracted from other sources like porcine skin and Grass carp fish scales (Zhang et al., 2011) and Pangasius catfish skin (Mahmoodani et al., 2014), is the absence of Hydroxyproline. The absence of Hydroxyproline as well as Hydroxylysine has also been reported by Jamilah and Harvinder (2002) in gelatin obtained from black and red Tilapia skin. The presence of Proline and Hydroxyproline in gelatin is important as this enhances gelatin's thermal stability. Zang et al., (2011) reported that the amount of Hydroxyproline is low in fish gelatin when compared to mammalian gelatin due to the low hydroxylation of Proline in turn leading to the low thermal stability. Of particular importance to note, is that Proline was higher in gelatin from the scales of red snapper at 14.16% compared to gelatin from scales of black spot emperor and blue barred parrot fish at 11.63% and 11.05% respectively. Cysteine does not take part in the structure of type I collagen; its presence in the gelatin from the scales of the three species could indicate the presence of another type of protein such as elastin or keratin (Gimenez et al., 2009). Methionine content was greater than 1.6%, higher than that found in porcine gelatin said to be 0.5%, supporting the conclusion that fish gelatin has higher amounts of Methionine compared to mammalian gelatin (Zhang et al., 2011). Gelatin from the three species under study was also low in amino acids Tyrosine and Histidine which is an inherent property of all gelatins (Mahmoodani et al., 2014).

IV. CONCLUSION

The yield of gelatin obtained (28% to 41%) shows that fish scales are a good source of gelatin. The use of alkaline protease enzyme at pH 12 and at 500C was effective in extraction of gelatin from fish scales. The amino acid composition of the different species varied slightly especially in the amount of Proline. The key difference between the gelatin extracted in this study and the mammalian sourced gelatin is the absence of Hydroxyproline. However, the two gelatins share key attributes meaning that the scales of these three marine fish species abundant in Kenya can be used as sources of gelatin on a large scale.

V. REFERENCES

[1]. Ahmad, M. & Benjakul, S. (2011). Characteristics of gelatin from the skin of unicorn leatherjacket (Aluterus monoceros) as influenced by acid pretreatment and extraction time. Food Hydrocolloids, 25(3), 381-388.

[2]. Gimenez, B., Gomez-Estaca, J., Alemán, A., Gomez-Guillen, M. & Montero, M. (2009) Physico-chemical and film forming properties of giant squid (Dosidicusgigas) gelatin Food Hydrocolloids, 23(3), 585-592.doi:10.1016/j.foodhyd.2008.07.003

[3]. Gomez-Guillen, M., Turnay, J., Fernández-Dıaz, M., Ulmo, N., Lizarbe, M. & Montero, P. (2002) Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocolloids, 16(1), 25-34. doi:10.1016/s0268-005x(01)000352

[4]. Jamilah, B. & Harvinder, K. (2002) Properties of gelatins from skins of fish—black tilapia (Oreochromis mossambicus) and red tilapia

[5]. (Oreochromis nilotica). Food Chemistry, 77(1), 81-84. doi:10.1016/s0308-8146(01)00328-4

[6]. Jiang, L. (2012). Preparation of Fish-scale Gelatins by Mild Hydrolysis and Their Characterization. Journal of Polymers and the Environment, 21(2), 564-567. doi:10.1007/s10924-012-0546-y

[7]. Kim, S.-K., Perera, U., Rajapakse, N. & Kim, S. (2014). Seafood Processing By-Products: Springer.146-156.

[8]. Lakshmi, B. K., Ratna Sri P. V., Devi, K. A. & Hemalatha K. P. J. (2014). Media optimization of protease production by Bacillus licheniformis and characterization of alkaline protease. International journal of current microbiology and applied science ISSN: 2319-7706 Volume 3 Number 5 (2014) pp. 650-659

[9]. Liu, H. Y., Li, D. & Guo, S. D. (2008) Extraction and properties of gelatin from channel cat fish (Letalurus punetaus) skin. LWT-Food Science and Technology, 41(3), 414—419. Doi:10.1016/j.lwt.2007.03.027

[10]. Mahmoodani, F., Ardekani, V. S., Fern, S. S., Yusop, S. M. & Babji, A. S. (2014). Optimization of extraction and physicochemical properties of gelatin from pangasius catfish (Pangasius sutchi) skin. Sains Malaysiana, 43(7), 995-1002.

[11]. Sevinc, N. & Demirkan, E. (2011). Production of protease by Bacillus sp. N-40 isolated from soil and its enzymatic properties. Journal of Biological and Environmental Sciences, 5(14).

[12]. Shoulders, M. D. & Raines, R. T. (2009). Collagen structure and stability. Annual review of biochemistry, 78, 929-958.

[13]. Wanyonyi, W. C., Onyari, J. M., Shiundu, P. M. & Mulaa, F. J. (2014) Enzymatic Decolorization of Malachite Green Dye by a Newly Isolated Bacillus Cereus Strain wwcp. IOSR Journal of Environmental Science, Toxicology and Food Technology, 8(12), 58-64. doi:10.9790/2402-081235864

[14]. Widyasari,. R & Rawdkuen, S. (2014). Extraction and characterization of gelatin from feet by acid and ultra sound assisted extraction. Food and bio science journal 2(1): 85-97.

[15]. Zakaria, S., andBakar, N. H. A. (2015). Extraction and Characterization of Gelatin from Black Tilapia (Oreochromis niloticus) Scales and Bones. International Conference on Advances in Science, Engineering, Technology & Natural Resources (ICASETNR-15) Aug. 27-28, 2015 Kota Kinabalu (Malaysia).

[16]. Zhang, F., Xu, S., andWang, Z. (2011). Pre-treatment optimization and properties of gelatin from freshwater fish scales. Food and Bioproducts Processing, 89(3), 185-193.