



Production of Hydrolyzed Collagen from Common Carp *Cyprinus carpio* L. Scales by Synergistic Enzyme Systems

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Received 25th July 2019; Accepted 11th October 2021; Available online 4th March 2022

Abstract: Fish skin, bones and scales are a good source of collagen, for the sake of exploitation these fish wastes of high commercial worth and convert them into food, therapeutic and pharmaceutical products. Therefore, the study included extracting collagen with acetic acid at a concentration 0.5 M and pepsin enzyme 0.1% from fish *Cyprinus carpio* scales and then producing hydrolyzed collagen protein. Chemical content of the scales was estimated from the percentage of moisture, protein, fat and ash, as it was found (55.36, 35.85, 2.47 and 5.99) %, respectively. The metallic elements represented by (Ca, P, Fe and Zn), they reached (153430, 1887.5, 71.455 and 48.045) $\mu\text{g.g}^{-1}$. The collagen yield by the two methods mentioned above was 16.40% and 5.30%, respectively. Diagnosis of collagen extracted with acid using FT-IR technique and determination of the active groups in it, the presence of amides A, B, I, II and III was observed at frequencies 3413.39, 2924.52, 1653.66, 1548.56 and 1240 cm^{-1} , respectively. Hydrolysed collagen was prepared using Collagenase enzyme followed by Pepsin enzyme, the highest degree of hydrolysis reached 43.56% after four hours. The electrophoresis of collagen extracted with acid and enzyme was carried out, as three bundles of collagen α_1 , α_2 and β were obtained with weights (126, 100 and 180) KD, respectively.

Keywords: Extracted Collagen, Fish scales, Diagnosed bioactive compounds, Protein hydrolysates.

Introduction

Fish wastes have received considerable attention because they contain many components, including amino acids, fatty acids, vitamins and mineral elements. Fish skin, bones, and scales are good sources of collagen, and utilizing these fish by-products minimizes pollution while also converting these wastes into high-value commodities. (Arvanitoyannis & Kassaveti, 2008; Olatunji & Denloye, 2017).

Collagen is the basic protein of connective tissue, as it consists of three polypeptide chains wrapped in a helix, in which proline or hydroxyproline is the basic structure to form the helix. Collagen and its peptides have been used in many foods, medical and pharmaceutical applications due to their biocompatibility and good biodegradability (Shoulders & Raines, 2009; Sorushanova *et al.*, 2018).

Proteolytic enzymes are the products of the hydrolysis of proteins using chemical and enzymatic methods aim to obtain peptides, or amino acids of technological importance, as these products used in the field of food, pharmaceutical and medical industries (Udenigwe & Aluko, 2011). Many studies have indicated that hydrolysed collagen is easy to digest and absorb, as well as having biological efficacy, as it can be taken orally in the form of amino acids or peptides, as it reaches through the small intestine and the bloodstream to the rest of the body parts that work on the formation of collagen fibers. Peptides also act the short chain linkage of fibroblast receptors and stimulates the production of new collagen, hyaluronic acid and elastin.

The hydrolysate is characterised by low viscosity, colourless and odorless with its ability to emulsify foam and dissolve (Denis *et al.*, 2008). Given the importance of the nutritional, medicinal and commercial importance of collagen and its use as an alternative source for pig and cow collagen because it is disease-free and far from religious beliefs. Therefore, the study aimed to extract collagen from carp fish scales. Study its characteristics, and preparing hydrolysed collagen with collagenase enzyme and pepsin.

Material and Methods

Common carp *Cyprinus carpio* L. was collected from fish breeders in Basrah province, Iraq, within average weight and length around 1900-2000 g and (45-50) cm, respectively. After transporting them to the laboratory, the scales were removed with a knife, then washed with cold water and the scales were cut into small pieces, which were kept in polyethylene bags at freezing temperature until use.

Acid Solubilized Collagen (ASC)

Collagen was extracted according to the method of Zhang *et al.* (2010) with some modification. The scales were immersed in 0.5 M NaOH solution at a mixing ratio of 1:10 w/v for 48 hours with stirring at a temperature of 4°C and replacing the solution every eight hours to remove non-collagenous proteins. The scales were washed with cold water until the pH reached neutrality, and then scales were immersed in 0.5 M EDTA-2Na solution at pH 7.4 for 48 hours at ratio 1:10 w/v with stirring and replacing the solution, every 48 hours, The scales were rinsed several times in cold water before being immersed in 0.5 M acetic acid at a 1:15 w/v mixing ratio for 48 hours with agitation. Filtered the solution via cheesecloth, then added 2.5M NaCl Tris-solution to the filtrate. At pH7, the HCL concentration was 0.05 M, and the sample was centrifuged 10,000 times for 30 minutes at 4°C. The precipitate was placed in 8-14 KD dialysis bags for 12 hours in 0.1 M acetic acid solution, then distilled water was replenished and immersed for 48 hours, with replacement every six hours, the samples were lyophilised using the Freeze Dryer (Christ, Germany).

Pepsin Solubilized Collagen (PSC)

Collagen was extracted using the Nagai *et al.* (2001) with some modification. The ASC residues were collected and suspended in 0.5 M acetic acid, followed by the addition of 0.1 % pepsin enzyme and stirring for two days. The samples were then centrifuged at 4°C for one hour at 10,000 cycles. The supernatants were taken and dialysed against 0.02M Na₂HPO₄ (PH 7.2) for three days to inhibition pepsin. The precipitate was dissolved in 0.5 M acetic acid and placed in 8-14 KD dialysis bags in 0.1 M acetic acid solution for 12 hours, the acid was replaced with distilled water and immersed for 48 hours, with replacement every six

hours, and then the samples were lyophilised using the Freeze Dryer (Christ, Germany).

Chemical content

Chemical content of scales and collagen included moisture, protein, fat and ash was

Collagen Yield

Collagen yield was estimated according to the Fehing (2016).

$$\% \text{ collagen yield} = \frac{\text{Wt of Lyphilized collagen (gm)}}{\text{Wt of Scales (gm)}} \times 100$$

Diagnosis of collagen by FT-IR

Mixing 2 mg of extracted collagen and standard collagen were mixed separately with KBr, then placed in an (FT-IR) device cell, recorded the rays spectra at (400 - 4000) cm⁻¹. This examination Conducted at the Polymer Research Center, University of Basrah, Iraq, according to the method mentioned in Yan *et al.* (2008).

Preparation of hydrolysed collagen

Hydrolysed collagen was prepared according to Giménez *et al.* (2009) with some modifications. Dissolve 2% of lyophilised collagen in 0.1M acetic acid with stirring until the mixture is well homogeneous. Adjust the pH and the optimum temperature for each enzyme to work (collagenase with specific activity 125 units.mg⁻¹ and pepsin with specific activity 250 units.mg⁻¹. The enzyme was added to the collagen solution at a concentration of 0.1 percent (w/v) and left to hydrolyze for four hours with stirring. The enzymes were inhibited for 10 minutes at 95°C, followed by 15 minutes of cooling centrifugation at 8000-10000 rpm. Every 30 minutes, the degree of hydrolysis is estimated.

Degree of hydrolysis

Identification of protein bands

estimated according to the method mentioned in AOAC (2016). The concentration of metallic elements was calculated using the atomic absorption spectrophotometer (shimadzu, Japan) according to the method (APHA, 1998).

The degree of decomposition was measured with an OPA method (Sigma, USA) by measuring the free amino nitrogen (Church *et al.*, 1983), which is dependent the interaction of the liberated amino groups with the OPA reagent. A 400 µl of the sample was added to 3 ml of reagent and left two minutes. Then absorbance was measured at 340 nm. The standard curve of glutathione refers to the released peptide bonds. The degree of degradation is defined as the percentage of peptide bonds released:

$$DH = \frac{h}{htot} \times 100$$

As (h) Represent for the number of peptide bonds released.

Htot Represent for the total number of peptide bonds released.

Calculated of Hydrolysis degree according to equation (Nielsen *et al.*, 2001), with some modification:

$$DH = \frac{(X - \beta) / \alpha \text{ megv/g protien}}{htot} \times 100\%$$

X = values of released peptide bonds extracted from the standard curve equation for glutathione, β, α and h_{tot} stand for constant values for protein raw materials, α=1 β=0.40 h_{tot} =8.6

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was

used to determine the protein bands of both extracted and standardised collagen samples with some modification (Chang *et al.*, 1991; Hames, 1998). 10% separating gel and 5% stacking gel were used, the samples were mixed in 5% SDS solution, they were added to buffer solution (HCL 0.0625 M at pH 6.8, 2% SDS (w/v), 5% Mercaptoethanol, 10% (v/v) glycerol and 0.002% bromophenol blue). The samples were heated in a water bath at boiling point for 3 minutes, then cooled and centrifuged for 5 minutes at 10,000 rpm. After the migration is complete, inject 10l samples onto an acrylamide gel. Coomassie blue dye was applied to the samples until the bands appeared. Treated samples with Coomassie blue dye until the bands appear. The gel was kept in 5% acetic acid solution for the appearance of the bands.

Statistical analysis

Statistical analysis of the results was carried out using a complete randomised design

(CRD) within the Ready Statistical Program (SPSS) and the test of the least significant

difference at the level of 0.05 for samples with three replicates (Al-Rawi & Khalafallah, 2000).

Results & Discussion

Chemical content

Table (1) shows the chemical content of carp fish scales from protein, moisture, ash and fat, as the percentage of moisture and protein was (55.36 and 35.85) %, respectively, while the percentage of fat and ash reached (2.47 and 5.99%), respectively. These results are similar to those of Mahboob *et al.* (2014) who recorded that the chemical composition of common carp scales amounted to 29.91 and 55.13% for protein and moisture, respectively. Minh-thuy *et al.* (2014) found that the percentage of protein in the scales of Japanese jack mackerel *Trachurus japonicus* was 35.50%. Mahboob (2014) noted that the content of *Catla catla* scales of moisture, protein, fat and ash was 67.36%, 22.26% and 2.88 % and 5.11%, respectively.

Table (1): Chemical content of carp fish scales.

Chemical Content of Scales(%)		Minerals elements $\mu\text{g.g}^{-1}$	
moisture	55.36±0.05	Ca	153430±3
protein	35.85±0.04	P	1887.5±0.1
Fat	2.47±0.03	Fe	71.455±0.002
Ash	5.99±0.01	Zn	48.045±0.004

*The results are an average of three replicates

Table (1) shows the Ca, P, Fe and Zn concentration in common carp scales 153430, 1887.5, 71.455 and 48.045 $\mu\text{g.g}^{-1}$, respectively. The difference in the concentrations of minerals elements may be attributed to too many biological factors such as environment, water, location of the food chain, and physiological characteristics of

fish. Toppe *et al.* (2007) that the concentration of the minerals calcium, phosphorous, iron and zinc in the bones of mackerel and trout (233 g.kg^{-1} , 111 g.kg^{-1} , 56 mg.kg^{-1} , 70 mg.kg^{-1}) and (147 g.kg^{-1} , 87 g.kg^{-1} , 32 mg.kg^{-1} and 126 mg.kg^{-1}), respectively.

Yield and protein content of the collagen

Table (2) demonstrates the collagen output yield of carp fish scales extracted with 0.05 M acetic acid, 0.1% pepsin enzyme, and the protein content of collagen. The results of the statistical analysis showed that the percentage of the yield differed significantly depending on the method of extraction at (p<0.05), as the percentage of the yield was 16.40 and 5.30%, respectively. The difference in yield percentage between the enzymatic and acid extraction methods could be attributable to the effectiveness or quantity of pepsin utilized, which varies depending on the fish species and collagen composition. Or it may be attributed to the fact that the collagen extracted by the acidic method did not completely dissolve when treated with 0.5M acetic acid compared to Using the enzyme that caused its complete dissolution. According to a study, collagen cross-links through covalent connections as well as molecular bonding in the telopeptide region, resulting in a decrease in collagen solubility with acid compared to enzymatic treatment (Singh *et al.*, 2011). These results were higher than what was found by Bhagwat & Dandge (2016) when extracting collagen by acidic method from the scales of common carp,

which amounted to 9.79%. Wu *et al.* (2019) found that the collagen yield of *Hypophthalmichthys molitrix* fish scales was 5.09. Matmaroh *et al.* (2011) reported that the yield of collagen extracted by the enzymatic method from the scales of the golden-spotted goatfish *Parupeneus heptacanthus* was 1.20%. Chuaychan *et al.* (2015) indicated that the collagen extracted from the scales of the seabass *Lates calcarifer* was 1.06%.

The proportion of protein in collagen extracted with acid and enzyme was 92.07 percent and 92.41 percent, respectively, according to the table. With a protein concentration of 93.56 percent, the results were identical to those obtained by Wang *et al.* (2013) when extracting collagen from Croceine Croaker fish scales. Because of the high collagen production and the high proportion of protein recovered using acidic methods.

Diagnostics of amide compounds by FT-IR technology

The wavenumber and infrared spectrograph of the amide groups of the ASC and standard collagen are shown in table (3), figs. (1 and 2).

Table (2): yield and protein content of collagen extracted from common carp scales.

Yield	
(%) the protein of acid- soluble collagen (ASC)	92.07 ±0.01 ^a
(%) the protein of Pepsin soluble collagen (PSC)	92.41±0.01 ^a
acid soluble collagen (ASC)	16.40 ±0.02 ^a
Pepsin soluble collagen(PSC)	5.30 ±0.01 ^b

*a, b Means in a column followed by different letters differ significantly (P < 0.05)

The amides A, B, I, II, and III for ASC are at wavenumbers 3413.39 cm-1 and 2924.52 cm-1, 1653.66 cm-1, 1548.56 cm-1, and 1240

cm-1, respectively, according to the table. Standard collagen's amide bands A, B, I, II,

and III appeared at wavenumbers 34 and 35, respectively.

The results also revealed that the ratio between amide III and the wavenumber 1454 cm⁻¹ is 1.17 for both acid-extracted collagen and standard collagen, which is close to 1.0, indicating that collagen has a helical structure. A number of studies have found that amide III determines collagen's helix structure; as the

more the ratio approaches 1.0, it indicates the helix structure of collagen (Sionkowska *et al.*, 2015; He *et al.*, 2019). The result was in agreement with Chen *et al.* (2016) observed the ratio between the absorption of amide III and 1454 cm⁻¹ for collagen extracted with pepsin from the scales of red drum *Sciaenops ocellatus* ranged 1-1.1, which indicates the integrity of the helical structure of collagen.

Table (3): Wave numbers and effective groups of ASC and standard collagen estimated by the technology of FT-IR amide type.

Amide type	Wavenumber cm ⁻¹ for ASC collagen	Wavenumber cm ⁻¹ for standard collagen	Effective group
A	3413.39	3413.39	N-H
B	2924.52	2924.52	CH ₂
I	1653.66	1642.09	C=O
II	1548.56	1564.95	N-H,C-N
III	1240	1241.39	NH,C-N

A, B, I, II, III: amide bands infrared spectroscopy (FTIR) of scale collagen and standard collagen.

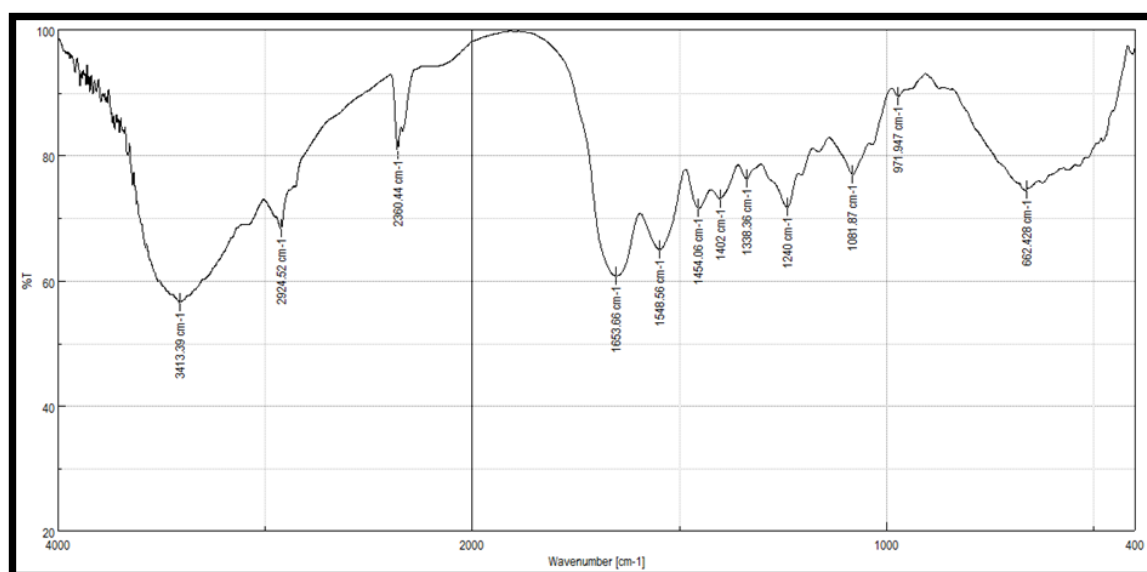


Fig. (1): FT-IR spectrum of ASC.

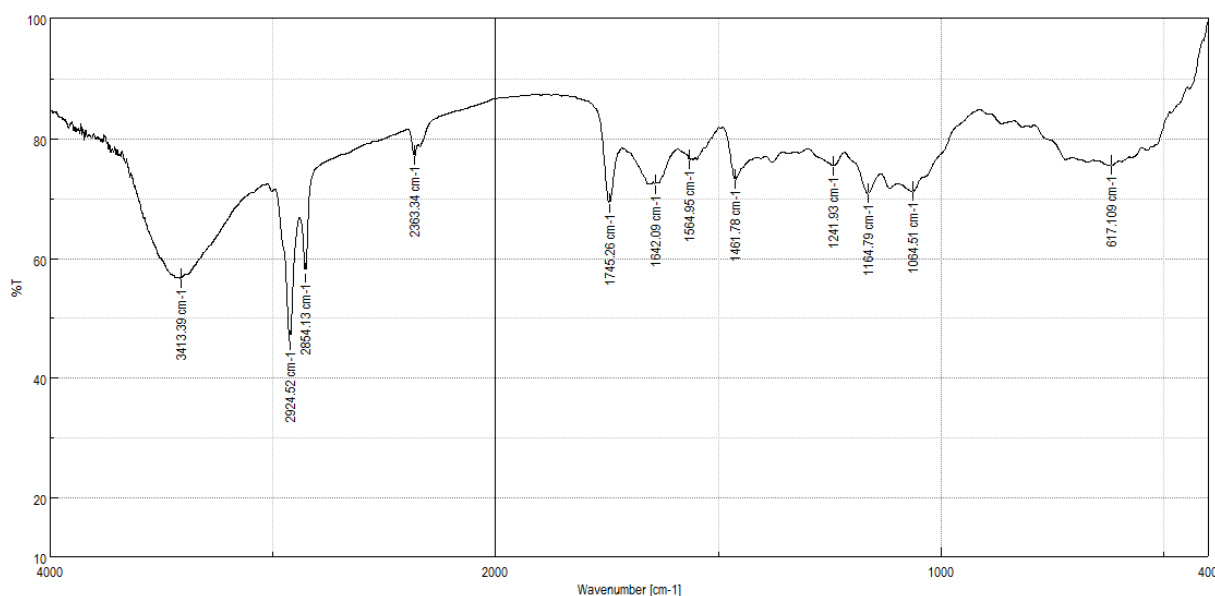


Fig. (2): FT-IR Spectrum of Standard Collagen.

The appearance of the amide band A occurred because of the elastic vibration of the NH group in the range $3400\text{--}3440\text{ cm}^{-1}$. At the same time, the amide band B formed due to the asymmetric elastic vibration of the CH₂ group. The band I was caused by the stretching vibration of the carbonyl group C=O, which indicates the secondary structure of the peptide. The presence of band II occurred as a result of the bending vibrations of the NH group and the stretching vibration of the CN group, and it should be noted that the appearance of this band in ASC at wavenumber $1500\text{--}1600\text{ cm}^{-1}$ is evidence that the hydrogen bonds in collagen are abundant and strongly interconnected. These results were confirmed by (Chen *et al.*, 2016). The presence of the amide III bands in ASC attributed to the vibration of the flexural plane of the NH group and the stretchy vibration of the CN group resulting from the amide bonds and the absorption resulting from the oscillatory vibration of the CH₂ group, these results agreement with (Sionkowska *et al.*, 2015 ; He *et al.*, 2019). Zhang *et al.* (2009) mentioned that amide III is responsible for fusing the NH and CN group jointly along

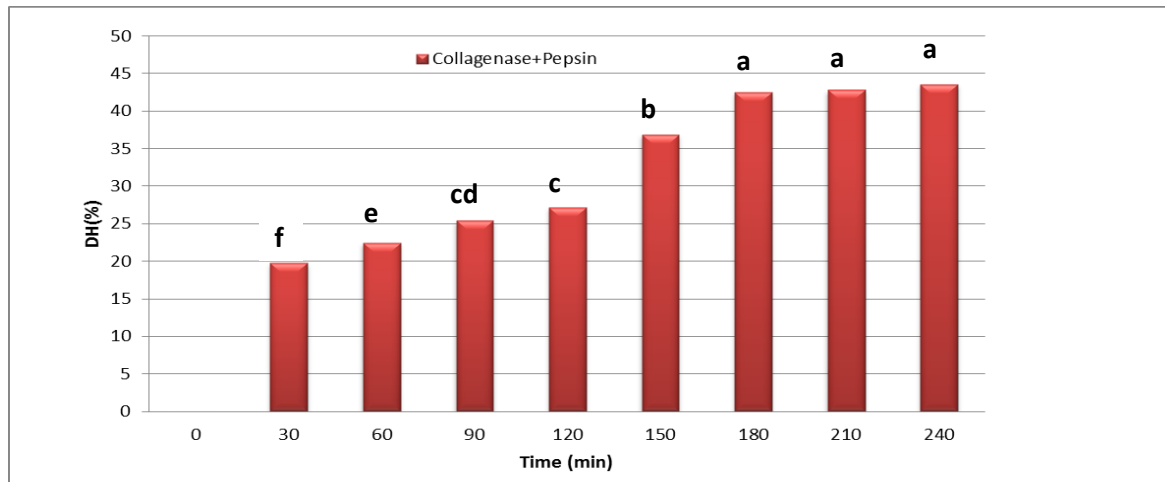
with the triple helix. According to Bet *et al.* (2001) the existence of peaks near wavenumber 1454 cm^{-1} shows the vibrations of the pyrrolidine ring of proline and hydroxyproline. These results agreed with several studies (Krimm & Bandekar, 1986; Payne & Veis, 1988; Surewicz & Mantsch, 1988).

Degree of hydrolysis

Fig. (3) shows the degree of hydrolysis of ASC after 240 minutes of treatment with Collagenase and Pepsin enzymes at the optimum temperature and pH for each enzyme. For 0 to 120 minutes, a progressive increase was seen, with the highest degree of hydrolysis 27.21 percent after two hours of treatment with collagenase, which was thereafter inhibited. The Pepsin enzyme was then introduced, and it was discovered that by following up on the degree of hydrolysis significantly increased after 30 minutes of treatment, this increase continued until it reached 43.56% for 240 minutes. The high degree of hydrolysis after adding pepsin may be attributed to the fact that collagenase is a specialised enzyme that cleaves the helical peptide chains of collagen and converts them into chains that make it easier for the

proteases to reach and attack them, splitting large peptides and converting them into

amino acids and smaller units.



**a, b, c, d, e, f Means in a column followed by different letters differ significantly (P < 0.05)

Fig. (3): Collagen hydrolysates by collagenase + pepsin enzymes.

Wang *et al.* (2013) found that the degree of hydrolysis by pepsin and trypsin enzymes with each other at concentrations 2.5% and 3%, respectively, a period of four hours for collagen extracted from the scales of large yellow crocker *Pseudosciaena crocea* (*Larimichthys crocea*) reached 25.11%.

Yi *et al.* (2016) noted that the degree of hydrolysis of collagen prepared from the skin of grass carp using Collagenase enzyme followed by Trypsin enzyme reached 13.80% for three hours. According to these studies, Collagenase's function is limited to cleaving the peptide chains of the triple helical structure of collagen, then converting the chains that become easy for proteolytic enzymes to access and attack.

Electrophoresis

The electrophoresis method will diagnose the collagen bands by polyacrylamide with existence SDS for standard collagen, ASC,

and PSC. Fig. (4) shows the occurrence of three protein bands represented by α_1 , α_2 , and β with molecular weights of 126, 100, and 180 KD for ASC and PSC, respectively. In contrast, the standard collagen showed the presence of two α_1 and α_2 bands with a molecular weight of 130 and 126 KD, respectively. Terzi *et al.* (2019) pointed that the existence of different bands of α indicates that collagen is a type I, the absence of any other types of protein refer to the purity of collagen. Many investigations had found that the collagen of bighead carp *Hypophthalmichthys nobilis* scales had two α_1 and α_2 bands with a molecular weight of 126 and 116 KD, respectively, and two different chains of β (Liu *et al.*, 2012). The electrophoresis of collagen enzymatically extracted from the scales of *Oreochromis niloticus* showed three bands α_1 , α_2 , and β with molecular weight 128, 123, and 200 KD, respectively, which indicated that type of I collagen (El-Rashidy *et al.*, 2015).

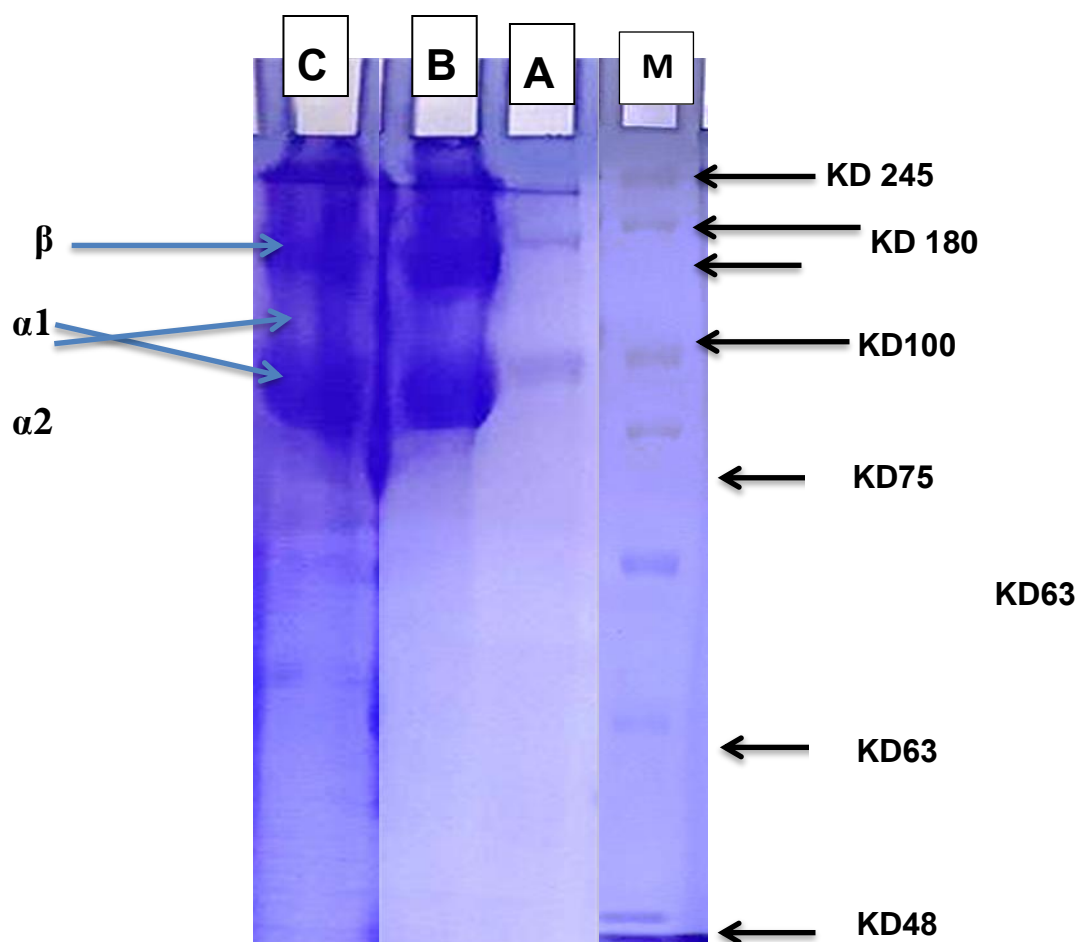


Fig. (4): Electrophoresis - (A) Standard Collagen, (B) ASC, (C) PSC. (M) Standard Proteins

Conclusion

The results were concluded from the current study that it is possible to exploit fish waste, especially fish scales, to produce compounds of high commercial value represented by collagen. As well could be produce hydrolyzed collagen that is safe for consumption and bioactive that utilized as a nutritional or pharmaceutical supplement.

Acknowledgements

We offer sincere thanks and appreciation to the staff of the Department of Food Sciences. We would like to thanks the Polymer

Research Centre, University of Basrah, for assistance in the analysing of samples.

Conflicts of Interest: The authors declare no conflicts of interest.

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انتاج متحلل كولاجيني من قشور سمك الكارب *Cyprinus carpio* بالنظم الانزيمية المتازرة

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المستخلص: تعد جلود الأسماك وعظامها وقشورها مصدراً جيداً للكولاجين ، من أجل استغلال المخلفات السمكية وتحويلها إلى منتجات غذائية وعلاجية وصيدلانية ذات قيمة تجارية عالية . لذا شملت الدراسة استخلاص الكولاجين بحامض الخليك بتركيز 0.5 مولار وإنزيم البيبسين 0.1% من قشور سمك *Cyprinus carpio* ثم درست قابلية إنتاج المتحلل الكولاجيني. قدر المحتوى الكيميائي للقشور من نسبة الرطوبة والبروتين والدهون والرماد حيث وجد 55.36 و 35.85 و 2.47 و 5.99 % على التوالي. أما العناصر المعدنية الممثلة Ca و P و Fe و Zn فقد بلغت 153430 و 1887.5 و 71.455 و 48.045 ميكروغرام. غم⁻¹. بلغ الحاصل الكولاجيني بالطريقتين المذكورتين 16.40% و 5.30% على التوالي. تم تشخيص الكولاجين المستخلص بالحامض باستخدام تقنية FT-IR وتحديد المجاميع النشطة فيه، اذ لوحظ وجود الأميدات A و B و I و II و III عند الترددات 3413.39 و 2924.52 و 1653.66 و 1548.56 و 1240 سم⁻¹ على التوالي. حضر المتحلل الكولاجيني باستعمال إنزيم الكولاجيناز يليه إنزيم البيبسين حيث بلغت أعلى درجة تحلل 43.56% بعد 4 ساعات. اجري الفصل الكهربائي للكولاجين المستخلص بالحامض والإنزيم، حيث لوحظ وجود ثلاث حزم من الكولاجين تمثلت $\alpha 1$ و $\alpha 2$ و β بأوزان (126 و 100 و 180) كيلودالتن على التوالي.

الكلمات المفتاحية : المستخلص الكولاجيني، قشور الأسماك، تشخيص المركبات الفعالة، التحلل البروتيني.