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OPTIMUM METHOD FOR COMMON CARP ELASTIN EXTRACTION AND THE ANTI-OXIDANT ACTIVITY OF ELASTIN ENZYMATIC HYDROLYSATE

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ABSTRACT

This study aimed to compare five protocols to optimize elastin purification, where purification protocols for elastin generally result in greatly damaged elastin fibres and this likely influences the biological response. The optimal protocol for elastin purification from common Carp bulbus comprised 4 M of urea solution, pH 7.2 containing 1 % β-mercaptoethanol, washing with distilled water then autoclaving at 121 C° for 15 minutes depending on amino acid analysis, SDS-PAGE electrophoresis, scanning electron microscopy and sulfhydryl content. Then elastin hydrolysate was prepared using partially purified Catfish elastase and the degree of hydrolysis (DH %) and antioxidant activities were recorded, the degree of hydrolysis rapidly increased in the first three hours (reaching 50.43 %) then gradually increasing was shows to reach 65.53 % after 10 hours. Antioxidative properties of elastin hydrolysate were based on three different assays: radical scavenging activity of DPPH, reducing power activity and total antioxidant capacity. The antioxidant activity of elastin hydrolysate increased with increasing the degree of hydrolysis, Where the IC_{50} of radical scavenging activity of elastin and EC_{50} of reducing power and total antioxidant capacity reached 2.309, 2.734 and 0.158 mg respectively after 10 hours of hydrolysis.

Keyword: DPPH, enzymic hydrolysate, functional properties, tropoelastin
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INTRODUCTION

Besides collagen and polysaccharides, Elastin is a major extracellular protein component of animal tissue like humans, livestock, fish and birds that provides elasticity to tissues that experience repeated stretching and recoiling, like blood vessels, skin, lungs, cartilage and arteries (Jenkins et al, 2021) which is represented 90 % of the Elastic fibers and the remaining components are primarily comprised of fibrillin glycoproteins (Ozsvar et al, 2021). It is believed play important role in regulating the cell properties like cell adhesion, cell proliferation and cell signalling (Abdel-Aty *et al*, 2024, Kamaruzaman &Yusop, 2021). It's prepared commercially from bovine ligaments; however, bovine spongiform encephalopathy (BSE) has led to concern regarding the use of elastin of bovine origin (Nakaba *et al*, 2006). So, many of researcher studying more social acceptability and free from BSE from natural sources like fishes (Nakaba *et al*, 2006; Shigemura *et al*, 2012; Shiratsuchi *et al*, 2013;h Shiratsuchi *et al*, 2016) and poultry (Nadalian et al, 2013; Nadalian *et al,* 2019; Yusop et al, 2019).The produced wastes represent 30-40 % of industrial fish processes in different forms (bones, scales and meat) which cause pollutants and pollute the environment and coastal areas .These waste concept a good source of valuable compounds like proteins, fat and bioactive compound extraction (gelatine, elastin and chitosan), In addition it contains enzymes, polyunsaturated fatty acids, antioxidant compounds and minerals (Blanco *et al*, 2019, Hernández-Ruiz *et al,* 2022). The Carp concept is the major species is currently cultured in mid and southern Iraq production ranged from 18819 to 21737 tons in the last

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four years, presenting 0.49 % of the total world production in 2022 (FAO, 2013). Carp is important nutrients such as proteins, fats, vitamins and minerals (Ahmed et al, 2020). Carp production ranged from the bulbus arteriosus of fish is a thick-walled tissue that connects the single cardiac ventricle to the ventral aorta, because of its central location in the single circulation of the fish, the bulbus can regulate the impedance faced by the ejecting ventricle and the pressure and flow occurring in the systemic arteries and in the gill capillaries (Trębacz & Barzycka, 2023). Also reported that the bulbus arteriosus contains significant amounts of Desmosine and Isodesmosine, so it's used to isolate elastin from the fish, where harsh experimental approaches designed to remove non-elastin contaminates are employed to generate an insoluble product (Pure elastin) that has the amino acid composition expected of elastin (Mecham, 2008).Elastin may contain bioactive peptides with a sequence of amino acids encoded in the parent protein molecules and remain inactive until released by enzymatic hydrolysis (like elastase) during food processing and/or gastrointestinal digestion (Nadalian *et al*, 2019). Besides the nutritional value of its amino acids, many researchers tried to use elastin and solubilized elastin in medicine, biomedical field and few in the food system, like drug delivery (Nadalian et al, 2019; Cobb *et al*, 2021), improve the blood vessels and skin conditions (Shiratsuchi et al, 2013; Shiratsuchi *et al*, 2016), genetically-engineered protein (Kowalczyk et al, 2014), ingredients in lotions, creams, skincare products, jells regenerative medicine (37) and improve cosmetic formulation stability (Kamaruzaman & Yusop, 2021). In addition, bioactive peptides prepared from poultry skin and skipjack tuna elastin were shown antioxidant properties and beneficial effects on human health (Shiratsuchi et al, 2013; Nadalian et al, 2019). Also, improving taste, flavour and texture, reducing the percentage of fat and salt in products, packaging techniques and improving the pathogen detection system (Sallam et al, 2004; AL-Ghanimi & AL-Rubeii, 2020).Using synthetic antioxidant agents led to concern of consumers about the safety due to their instability and have a negative effect on health, so this concern has led to arouse a great interest in avoiding purchasing products containing synthetic additives and preferring foods with natural additives (Sallam *et al*, 2004; Al-Zubaidi *et al*, 2021,). The present study aimed to identify the optimum condition for elastin extraction from bulbus arteriosus of common Carp using different methods to prepare the enzymic elastin hydrolysate then evaluate the antioxidant activities of enzymic elastin hydrolysate.

MATERIALS AND METHODS

Fresh bulbus arteriosus was collected from the fish market in Baghdad/ Iraq and stored in a polyethylene bag at -18 C° for further analysis.

Preparation high elastin powder (HEP)

High elastin Powder (HEP) was prepared by removing the soluble protein as mentioned in the (Kamaruzaman & Yusop, 2021) method with some modifications. Bulbus arteriosus from common Carp was thawed and
washed with distilled water then suspended two times with 1 M of NaCl at a mixing ratio 1: 10 (w: v). then homogenate using mixture at high speed then homogenized using a homogenizer at 15000 ×g for 5 minutes. Then left for two hours at 4 C° and the precipitate was collected at 11000 ×g for 20 minutes. HEP was defatted by (Li et al, 2023) method. HEP was macerated in ethanol for one hour and in acetone for a second hour at a mixing ratio of 1: 20 (w: v) with a stirrer at room temperature. Then the supernatant was removed by centrifugation at 11000 ×g for 20 min. then left to dry in a vacuum oven at 40 C° for 6 hours and stored at -18 C° for further analysis.

Optimum conditions for elastin extraction

Five methods were used to extract elastin from the bulbus arteriosus of common Carp. These methods depend on hot alkali treatment, autoclaving, chaotropic agent, ionic strength buffers and protease to remove non-elastin protein as described below:

Procedure A

Method described in (Halabi & Mecham, 2018; Kamaruzaman & Yusop, 2021) with modifications using hot alkali treatment. Elastin was purified by mixing HEP with 0.1

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N of NaOH solution at a 1: 20 (w: v) mixing ratio, placed in a shaking water bath at 90 C° for 45 minutes, lifting to cool at room temperature. Then the elastin was precipitated at 11000 ×g for 20 minutes and resuspended in distilled water four times then centrifugating at 11000 ×g for 20 minutes after each washing step and lyophilized and stored at -18 C° for further analysis.

Procedure B

Daamen *et al*, (2007) and Mecham (Nadalian et al, 2019) methods were used to extract elastin with some modification using autoclave treatment. A HEP was mixed with distilled water at a mixing ratio of 1: 20 (w: v) and autoclaved at 121 C° for 15 minutes three times and precipitated by centrifugation at 11000 ×g for 20 minutes after each time. Then washed with distilled water four times by centrifuging at 11000 ×g for 20 minutes then lyophilized and stored at -18 C° for further analysis.

Procedure C

Elastin was extracted by (Daamen *et al*, 2007) method with some modifications using reducing, chaotropic agents and autoclave treatment. A solution of 4 M of urea, pH 7.2 containing 1 % β-mercaptoethanol was mixed with HEP at a 1:10 (w: v) mixing ratio. Left for 24 h at 4 C°. Then diluted up to four-fold with distilled water and the precipitate was collected by centrifugation at 11000 ×g for 20 minutes. Followed by an autoclave at 121 C° for 15 minutes and washing with distilled water four times by centrifugating each washing step then lyophilized and stored at -18 C° for further analysis.

Procedure D

Elastin was extracted using a chaotropic agent and enzyme digestion as described in (Daamen, *et al*, 2001) with some modification. A solution of 4 M of urea containing 0.02 % sodium azide was mixed with HEP at a 1: 10 (w: v) and kept for 4 h at 4 C°. Then precipitate was collected by centrifugation at 11000 ×g for 20 minutes and washed with distilled water two times then mixed with 0.1 M Tris-HCl buffer, pH 8.2 containing 0.02 M $CaCl_2$ at mixing ratio 1: 10 (w: v) and incubated with 1000 u/ gm HEP of trypsin at 37 C° for 4 h with constant shaking. Then the trypsin was inactivated by heating at 95 C° for 2 minutes and the precipitate was collected by centrifugation at 11000 ×g for 20 minutes. Then washed with distilled water four times by centrifugation each washing step at the same condition above then the elastin was lyophilized and stored at -18 C° for further analysis.

Procedure E

This method depends on the use of ionic strength buffer, autoclave treatment and enzyme digestion as described in the (Halabi & Mecham. 2018) HEP was mixed at a mixing ratio 1: 10 (w: v) with 0.05 M phosphate buffer, pH 7.6 containing 1 % NaCl and 0.1 % EDTA for 12 h at 4 C° three times and the precipitate was collected by centrifugation at 11000 ×g for 20 minutes. Then washed with water two times by centrifugation and autoclaved at 121 C° for 15 minutes using distilled water at a mixing ratio 1: 20 (w: v) then the precipitate was collected by centrifugation at 11000 ×g for 20 minutes and mixed with 0.1 M Tris-HCl buffer containing 0.02 M $CaCl_2$ at pH 8.2 and mixing ratio 1: 10 (w: v) and incubated with 1000 u/ gm HEP of trypsin at 37 C° for 8 h with shaking. Then the trypsin was inactivated by heating at 95 C° for 2 minutes and the precipitate was collected by centrifugation at 11000 ×g for 20 minutes followed by washing with distilled water four times with centrifuging each washing at the same condition above then the elastin was lyophilized and stored at -18 C° for further analysis.

Assessment of the elastin purity

Four methods were used to compare the various methods (Scanning Electron Microscopy examination, Assessment of disulphide group, Amino Acid composition and SDS-Page Electrophoresis) to assess the purity of elastin as described below:

Assess of sulfhydryl content

Elman reagent was used to determination of the total protein sulfhydryl content in purified elastin as mentioned in (Hashmi et al, 2023). First, the protein was denatured by dissolving 10 mg of purified elastin with 3 ml of 0.08 M sodium phosphate buffer, pH 8 containing 2 % SDS and 0.5 mg/ ml EDTA. Then 0.1 ml of DTNB (40 mg of DTNB in 10 ml 0.1 M

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sodium phosphate buffer, pH 8) and left at room temperature for 15 minutes. The absorbance was read at 410 nm. The sulfhydryl content of the samples was calculated by comparison of a standard curve with 3.125–50 Mg L-cysteine.

SDS-PAGE Electrophoresis

Electrophoresis technique was used to assess the purity of elastin extracted by the five methods described above due to using SDS-PAGE electrophoresis as mentioned in (Westermeier, 2016). The contaminant and elastin degradation products will penetrate the gel and are visualized by Colloidal Coomassie blue stain. Samples were dissolved 10 mg in 1 ml of loading buffer with /without a reducing agent. Denaturation was performed for 10 minutes at 95 °C. The concentration of Running gel and stacking gel was 12 % and 4.5 % respectively. The starting voltage was 50 V; after the passage of samples to the running gel, the potential was increased to 100 V. The protein was fixed with 12 % trichloroacetic acid for one hour then washed with water for 5 minutes. Staining for protein was performed overnight using Colloidal Coomassie blue solution (0.1 % Coomassie blue G-250. 10 % ammonium sulphate, 2 % phosphoric acid and 20 % ethanol). Destaining in distilled water with several changes.

Scanning electron microscopy examination

Scanning electron microscopy (SEM) was conducted as described in (47). The purified elastin was coated with gold to enhance electron interaction with the elastin. Then TESCAN, VEGA III microscope with accelerating voltage 10 kV electron beams was projected on the elastin. The resolution range carefully chosen to scan the sample was between 2-500 mm. Different elastin SEM images were taken to study topographical allied with the surface features of elastin.

Amino acid composition

Elastin samples were hydrolysed in 6 M HCl at 110 C° for 24 h followed by derivatising the amino acids using phenyl isothiocyanate as described in (35) using High Performance Liquid Chromatography (HPLC), SHIMADZU (LC 2010 A model). The optimization of separation was performed on HPLC column (analytical), NUCLEODUR 100-5 C18 ec, 5 µm, 250 x 4.6 mm with binary mobile phases gradient (0 min-0 % B, 0.1 min-15 % B, 15 min-50 % B, 30 min-100 % B and 37 min-0 % B) whereas 0.115 M ammonium acetate, pH 6 was solvent A and 0.23 M ammonium acetate, pH 6 in acetonitrile: methanol: water 44:10:46 (v/v) was solvent B. The flow rate was 1 mL/ min, temperature 50 C°, injection volume 20 µL and Spectrophotometric detection was 254 nm.

Preparation of elastin hydrolysate

Partially purified elastase was prepared previously as described by (Kadhim & Shakir, 2024). Elastase was added to elastase solution (2 % of elastin in 0.1 M Tris-HCl buffer, pH 8) at a ratio 1: 50 (Unit: v) and incubated at 40 C° for 1-10 hours, then samples were taken each hour. The enzyme was inactivated by heating at 100 C° for 2 min. The resulting hydrolysate was then rapidly cooled and centrifuged at 10000 ×g for 20 minutes at 4 C°. Then the supernatant was collected and stored at -18 C° for further analysis.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH %) of elastin hydrolysate was determined as described in with some modifications where all hydrolysate diluted to a concentration of 2.5×10^{-3} amino equivalent/L with distilled water. A 2 ml of 0.2125 M phosphate buffer pH 8.2 was added to 0.250 ml of diluted hydrolysate in a test tube then 2 mL of 0.1 % TNBS was added and incubated in a shaking water bath at 50 C° for 60 minutes in a dark place. The reaction was terminated by adding 4 mL of 0.1 M HCl and kept at room temperature for 15 min before reading the absorbance against water at 340 nm. A 5-55 mM of L-leucine solution was used for standard curve preparation. The following question is used to calculate the degree of hydrolysis:

$$\mathbf{DH}\ (\%) = \frac{\boldsymbol{Lt - L0}}{\boldsymbol{Lmax - L0}} \times \mathbf{100}$$

Where:

L_t: the specific amino acid at the time.

L_0: is the amount of the specific amino acid at time zero.

L_{max}: is the maximum amount of the specific amino acid in the substrate obtained after hydrolysis using 6 N HCl at 120 C° for 24 hours.

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Assessment the antioxidant activities

Free radicals scavenging activity (RSA)

The DPPH radical scavenging of elastin hydrolysate was carried out according to the (Laohakunjit *et al*, 2017) method. one millilitre of DPPH (0.1 mM in 95 % ethanol) was added to (25, 50 and 100 µL) of sample solution and 900 µL of distilled water in test tubes; the mixture was mixed vigorously, placed in the dark place for 30 min at room temperature and centrifuged at 10000 ×g for 5 min. The resultant color was measured at 517 nm using a spectrophotometer. Butylated hydroxytoluene (BHT) (0.1-0.3 mg/ ml) and Ascorbic acid (10-30 µg/ ml) were used for comparison. The scavenging activity was calculated using the following equation as described in (20):

$$\boldsymbol{RSA}\ (\%) = \frac{\boldsymbol{C - A}}{\boldsymbol{C}} \times \mathbf{100}$$

Where:

A: (sample) is the absorbance value of 0.1 ml of sample solution + 1 mL of 0.1 mM DPPH.

B: (blank) is the absorbance value of 0.1 ml of sample solution + 0.9 ml of distilled water + 1 mL of 95 % ethanol.

C: (control) is the absorbance value of 1 mL of distilled water + 1 mL of 0.1 mM DPPH.

Reducing power activity: The reducing power test was carried out according to the method described by (Zhang *et al*, 2018) with some modifications. An Aliquot of 250, 500 and 750 µL of sample solutions was mixed with 2.5 mL of 0.2 M phosphate buffer, pH 6.6 and 2.5 mL of 1 % potassium ferricyanide. The mixtures were mixed vigorously and incubated in a water bath at 50 C° for 20 minutes. Subsequently, 2.5 mL of 10 % trichloroacetic acid (TCA) was added to the mixture and centrifuged at 10000 ×g for 10 minutes. Then 2.5 mL of supernatant was transferred to an empty tube and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % (w: v) ferric chloride. After standing at room temperature for 10 min, the absorbance was measured at 700 nm. An increase in absorbance of the mixture indicates an increase in reducing power as measured by the reduction of ferric ions. Butylated hydroxytoluene (BHT) and Ascorbic acid (0.1-0.3 mg/ ml) were used for comparison.

Total antioxidant capacity: Total antioxidant capacity (TAC) was determined by (26) method with modifications. TAC is based on the reduction of Mo IV in acidic pH to form a green complex of phosphate Mo V. A 1 ml of phosphomolybdic solution (28 mM sodium phosphate, 0.6 M sulfuric acid and 4 mM ammonium molybdate) was added to the test tube containing (62.5, 125 and 187.5 µL) of sample solution or (0.1, 0.2 and 0.3 mg/ ml) of BHT or ascorbic acid for comparison. Then incubated at 95 C° for 90 minutes and cooled at room temperature, the absorbance was measured at 695 nm. A 20-300 µg/ ml of ascorbic acid was used as standard.

Statistical analysis: The collected data were statistically analyses as mentioned in (Al-Salmany et al, 2020) by Minitab software 17th version using analysis of variance (ANOVA). Differences among treatment means were compared using the Least Significant Difference (LSD) P≤ 0.05 probability level. The IC_{50} was calculated by linear regression between concentrations and inhibition, whereas inhibition = 50. The EC_{50} was calculated by the following equations:

Halfway Value = Y max – (Y max – Y min)/2

EC_{50} = (X range, Y range, Halfway Value)

Where:

X: Logarithm 10 (Log 10) of Concentration

Y: Reducing power values or total anti-oxidant values Y: Reducing power values or total anti-oxidant values

RESULTS AND DISCUSSION

Elastin extractability from common Carp

The elastin recovery at various extraction methods was illustrated in Table (1), which is based on dissolving most non-elastin proteins (using hot alkali, autoclave, reducing agent, chaotropic agent and enzyme digestion) and reducing the damage to elastin molecules at the same time. The elastin yield ranged from 71.35, 38.02, 36.11, 33.08 and 20.16 % (dry weight basis per high elastin powder weight) from procedures C, D, B, E and A, respectively. The obtained results were higher than the results reported by (Daamen *et al*,2007) who reported that elastin yield varied from 20-23 % (dry weight basis per high elastin powder weight) for five different

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procedures, also (Nakaba *et al*,2006) reported that elastin presented more than 34.8 % of the total protein extracted from Yellowtail bulbus arteriosus.

Table 1. elastin extraction percent of the common Carp

Procedure	Principles	Elastin yield (%)
A	hot alkali treatment	20.16
B	autoclave treatment	36.11
C	reducing, chaotropic agents and autoclave treatment	71.35
D	chaotropic agent and enzyme digestion	38.02
E	ionic strength buffer, autoclave treatment and enzyme digestion	33.08

Assessment the sulfhydryl content

Table (2) shows the sulfhydryl content in elastin at different extraction methods. Elastin prepared by procedures E and D shows the lowest sulfhydryl content 0.36 and 0.53 mg/ gm respectively, followed by A and B procedures which show similar results 0.64 and 0.63 mg/ gm respectively. The highest amount of sulfhydryl content was recorded by using procedure C, where reached 1.55 mg/ gm. This result could be attributed to residual 2-mercaptoethanol in the extracted elastin that reacts with DTNB and may add to the sulfhydryl content (Daamen *et al*, 2007).

Table 2. Sulfhydryl content of common Carp elastin

Procedure	mg L-cysteine/ gm elastin
A	0.64
B	0.63
C	1.55
D	0.53
E	0.36

SDS-PAGE Electrophoresis: Figure (1) shows the electrophoresis of prepared elastin using five extraction methods. Elastin couldn't dissolve by extraction buffer so it couldn't penetrate the gel and still on the holes of stacking gel. While other protein molecules (contaminant) dissolve by extraction buffer and penetrate the gel as shown after staining the gel. So, electrophoresis could be used to assess the purity of elastin. From the figure noticed that there were no major contaminations with soluble proteins in all procedures used in this study.

Scanning electron microscopy examination

SEM was used to identify impurities like collagen and microfibrils in the purified elastin. Figure (2) shows the scanning electron microscopy of purified elastin using various extraction methods. As noticed in Figure 2 (A), elastin prepared by procedure A was impure, which could be attributed to representing (part of) the microfibrillar components of the elastic fibre (Daamen *et al*, 2007), similar results were noticed by (Nadalian et al, 2013) who reported that that to extract elastin by Lansing methods still contains non-elastinous material of microfibrillar nature. Figure 2 (B, D, E) shows a rough surface without fibrous content but it contains holes. These results agreed with (Daamen *et al*, 2007) who reported that trypsin is capable to digest elastin leading to degradation.

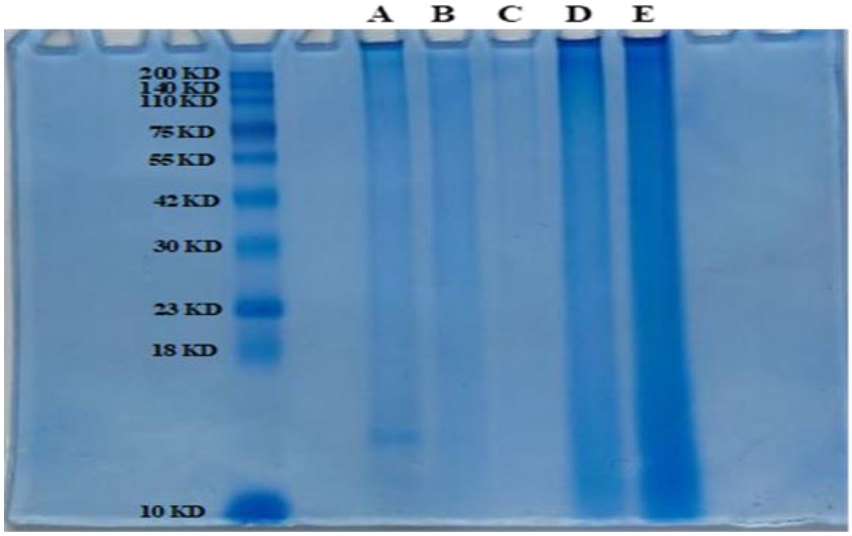

Figure 1. SDS-PAGE electrophoresis of extracted elastin

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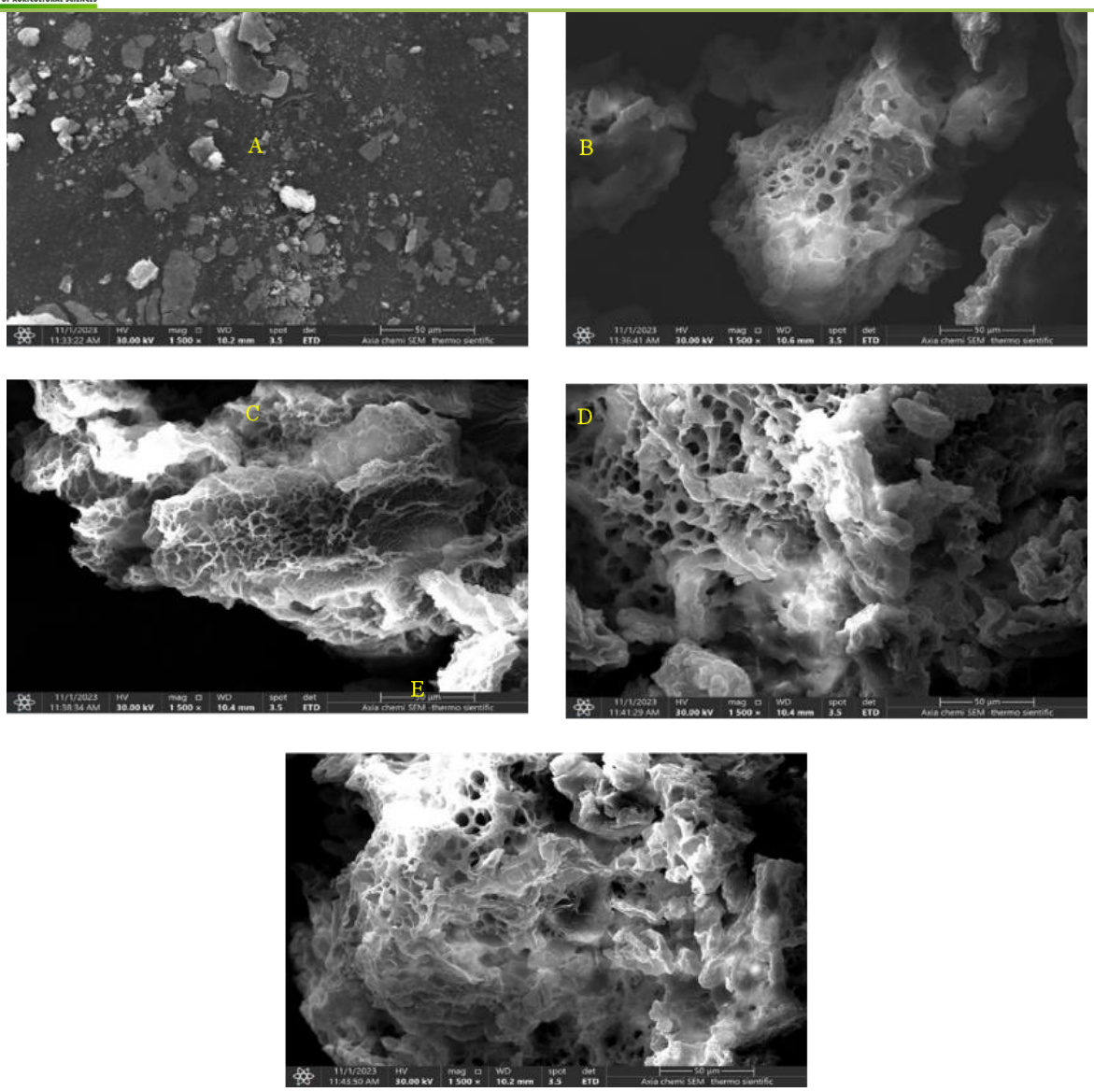

Figure 2. Scanning electron microscopy of purified elastin

Amino acid composition

The variation in amino acid content of elastin using various extraction methods and commercial elastin (from bovine ligament) was illustrated in Table (3). The amino acid analysis showed that the bulbus arteriosus Carp elastin rich in glycine and proline content which precent 13.66, 19.39, 23.71, 18.51, 21.77 % and 9.34, 12.81, 6.34, 10.73, 13.33 % of the total amino acid in extracted elastin using procedure A, B, C, D, E and bovine nick ligament elastin respectively. These results agreed with (31, 49) who reported that glycine was the most dominant amino acid in broiler skin, spent hen's and Yellowtail elastin (which is represented one third of the total amino acid). The Met and His content were very low, were ranged from 2.32-4.94 % and 0-2.98 % of the total amino acid, these results agreed with (Kamaruzaman &Yusop,2021) results who reported that the methionine and histidine contents (related to the collagen fibrils and microfibril residues) in purified elastin were 0.3 and 1.2 % respectively.

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Table 3. Amino acids percent (%) of common Carp elastin

Amino acid	A	B	C	D	E	Bovine nick ligament elastin
Ala	4.18	4.73	3.52	4.83	5.53	0.76
Arg	7.82	7.18	5.24	6.57	8.29	12.21
Asp	4.11	3.72	6.16	3.27	1.13	4.19
Cys	1.10	1.33	1.50	0.43	0.78	0.41
Glu	2.32	2.73	4.70	2.78	2.80	3.19
Gly	13.66	19.39	23.71	18.51	21.77	24.51
His	0.00	0.00	0.91	2.45	2.98	1.60
Hyp	4.69	1.23	1.66	6.41	7.11	1.89
Ile	14.90	8.90	1.50	12.30	9.61	10.79
Leu	3.51		10.66	4.31		3.99
Lys	7.50	6.42	4.26	5.35	6.65	0.30
Met	2.32	2.94	4.94	2.62	2.71	4.97
Phe	4.99	5.19	5.91	3.14	1.52	3.43
Pro	9.34	12.81	6.34	10.73	13.33	8.09
Ser	3.20	2.80	3.76	2.59	2.46	3.01
Thr	5.05	5.47	2.93	3.12	3.21	4.69
Trp	1.77	2.47	0.00	0.70	0.28	1.58
Tyr	7.04	6.43	5.36	6.11	6.71	4.61
Val	2.48	5.80	6.96	3.78	3.14	5.76

The optimum procedure for elastin extraction: The purity of prepared elastin was assessed using sulfhydryl content, SDS-PAGE, amino acid analysis and scanning electron microscopy as illustrated in Table (4). The Sulfhydryl Content was used to select the optimum procedure for purification (Daamen *et al*, 2007). From the figure notice that only procedures D and E gave a highly purified elastin preparation. The SDS-PAGE can't be used to indicate to optimum procedure after elastin purification, where all procedures have shown a positive result for purification. The SEM results showed that highest purity of elastin was obtained using procedure C. The amino acid analysis showed that procedure C resulted in elastin with a high content of glycine 24.07 % while the histidine less than 1 % of the total amino acids. Which is similar to bovine nick ligament elastin.

Table 4. Overview of purity of elastin using different preparation methods

Procedure	Sulfhydryl Content	SDS-PAGE	Amino acid analysis	Scanning electron microscopy
A	-	+	±	-
B	-	+	±	-
C	-	+	+	+
D	±	+	±	-
E	±	+	±	-

Sulfhydryl content is - if > 0.5 mg /g elastin; ± if 0.1 < value < 0.5; + if < 0.1. SDS PAGE is + if no bands are seen on the gel. Amino acid analysis is + if His was less than 1, Gly was more than 20 %. SEM is + if no microfibrillar structures or holes are present.

Preparation of elastin hydrolysate

Elastin contains a highly cross-linked polymer to form sheets or fibers in the extracellular matrix, leading to insoluble matter in water, saline and all non-hydrolytic solvents (Halabi & Mecham, 2018; Trębacz & Barzycka, 2023). So, it's important to hydrolyse elastin using chemical or enzymic methods to improve its functionality. In this study, partially purified Catfish elastase was used to obtain soluble elastin at 37 C°, pH 8 for 1-10 hours. Figure (3) illustrates the degree of hydrolysis of elastin using elastase at 37 C°, pH 8 for 10 hours. From the figure noticed that the degree of hydrolysis increased with time, increasing from 30.77 % after one hour to 65.53 % after 10 hours of hydrolysis. On the other side, the degree of hydrolysis was rapidly increased through three hours of hydrolysis (reaching 50.43 %) then increased slowly to reach 65.53 % after 10 hours. The increasing DH % with time could be explained to elastase (serine protease) acting on cleaving peptide bonds on the carboxyl-terminal side of amino acids with a small alkyl side chain and then protein solubilization. Yusop *et al*, (2016)

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reported that the DH % of spent hen and Broiler water-soluble elastin decreased in the early stage of hydrolysis then a rapid increase was observed until 12 h in both enzymes, after that slightly decreased and remained constant until the end of hydrolysis using Elastase and Alcalase. The bitterness of elastin hydrolysate was tested each hour of hydrolysis, a small bitterness taste was developed after nine hours of hydrolysis and increased with time until 18 hours of hydrolysis where the deuterate symbol was noticed.

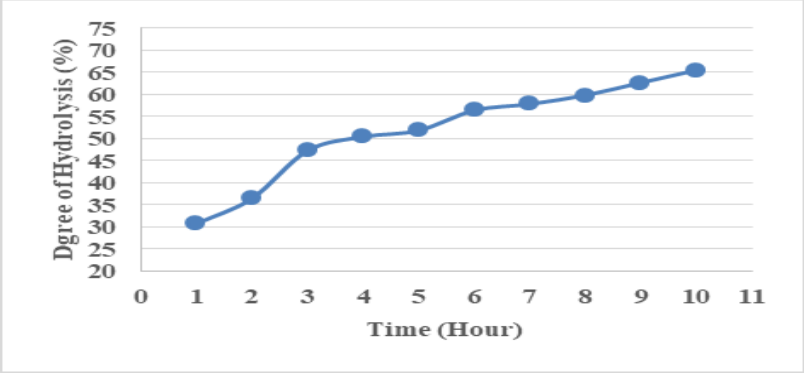

Figure 3. Degree of hydrolysis of elastin at variant times

Antioxidant activities of elastin hydrolysate

DPPH concept is a stable radical in ethanol that has been widely used to assess the ability of some compounds as proteins to act as free radical scavengers or hydrogen donors (Tang et al, 2009). The antioxidant activity was expressed as IC_{50} for radical scavenging activity as shows in Figure (4). Where IC_{50} defines the concentration of protein amount (mg) at which 50 % inhibition of free radical activity is observed (16). The lower IC_{50} value indicates the greater overall effectiveness of the antioxidant. From the figure noticed that IC_{50} of the radical scavenging activity decreased with time, where reached 2.309 mg after 10 hours of hydrolysis this could be attributed to that the side chain of liberated amino acids increased with time of hydrolysis, thus providing additional amino acids that donate protons and electrons to maintain a relatively high redox potential (Yusop et al, 2016; Kadhim & Shakir, 2019). Also (Nadalian et al, 2019) mentioned that increasing the hydrolysis time surges liability of peptide bonds to the elastase and consequently releasing significant antioxidant peptides leading to IC_{50} increased. A similar result was obtained by (Hangun-Balk & McKenney, 2012) who stated the IC_{50} of hemp protein hydrolysate was 2.8 mg/ mL obtained by using Protamex. The statistical analysis showed that there was significant analysis ($P \leq 0.05$) between RSA of BHT and Ascorbic acid and the RSA of elastin hydrolysate, moreover there are a significant analysis among RSA of elastin hydrolysate after 4-10 hours of hydrolysis and other RSA of elastin hydrolysate.

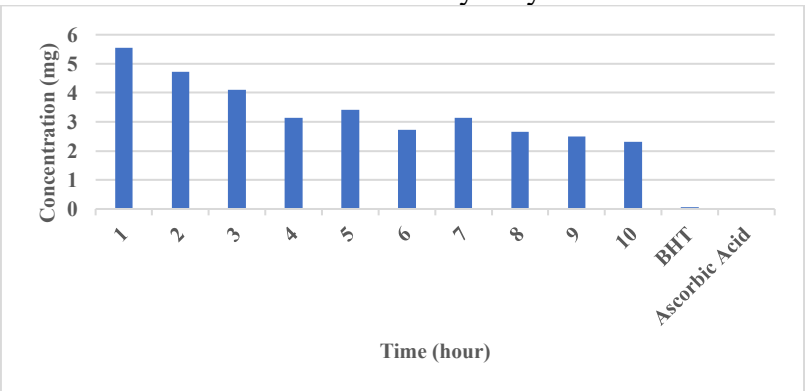

Figure 4. Radical scavenging activity of elastin hydrolysate using DPPH

The reducing power activity of elastin hydrolysate depends on the reduction of the Fe3+/ferricyanide complex to the Fe2+ form, which is monitored by measuring the formation of Perl's Prussian blue at 700 nm (3, 14). Figure (5) illustrates EC_{50} of reducing

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power of elastin hydrolysate. Where EC_{50} defines the concentration of protein (mg) on a dose-response curve where the response equals 50 % of the maximum response (Noel et al, 2018), the lower EC_{50} value indicates the greater overall effectiveness of the antioxidant. From the figure noticed that EC_{50} of reducing power decreased with time, where reduced from 1.716 mg in first hour to 2.734 mg after 10 hours of hydrolysis. The BHT and Ascorbic acid showed the highest reducing power (the EC_{50} reached 0.158 and 0.179 mg respectively) which was significantly ($P \leq 0.05$) higher than Elastin hydrolysate activities, similar findings were observed by (14) when they studied the antioxidative potential of mung bean vicilin protein. The obtained results agreed with (Sun *et al*, 2011; Kadhim & Shakir, 2019) who reported that the side chain of liberated amino acids increased with time of hydrolysis, thus providing additional amino acids that donate protons and electrons to maintain a relatively high redox potential. Statistically, the hydrolysis time didn't show a significant difference in elastin hydrolysate after 10, 9, 8, 7, 6, 5, 4, 2 hours of hydrolysis at significant level ($P \leq 0.05$).

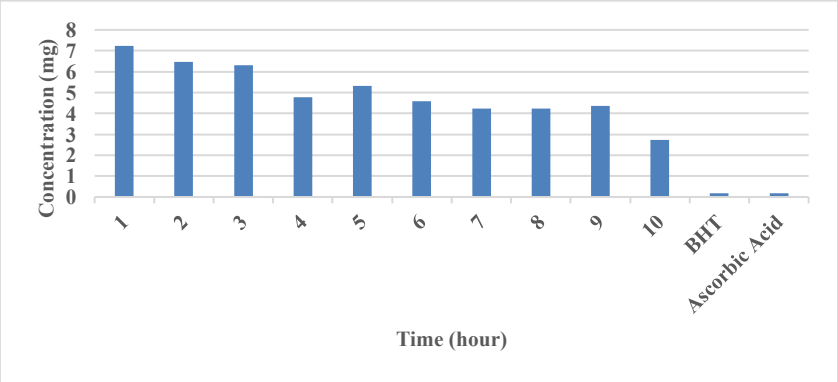

Figure 5. Reducing power of elastin hydrolysate at variant times

Total antioxidant capacity based on the reduction of MoVI to MoV by samples and formation of green-coloured phosphate/MoV complex at acidic pH measured at 695nm (Rahman *et al*, 2015). Figure (6) shows the total antioxidant capacity of elastin hydrolysate expressed as EC_{50}. The total antioxidant capacity of elastin hydrolysate showed similar results to radical scavenging activity and reducing power, where the EC_{50} decreased with hydrolysis time. Where raised from 1.716 mg after one hour to reach the optimum (0.747 mg) after 10 hours of hydrolysis. Meanwhile, EC_{50} of BHT and ascorbic acid was 0.182 and 0.191 mg respectively which was significantly better than the EC_{50} of elastin hydrolysate at all hydrolysis hours. In addition, the statical analysis at ($P \leq 0.05$) shown there are significant (differences in elastin hydrolysate after 4-10 hours and the other hours of hydrolysis. Depending on the degree of hydrolysis, bitterness and antioxidant activities: the elastin hydrolysate prepared by partially purified elastase at 2:100 (W elastin: V 0.1 N Tris-HCl buffer, pH 8) mixing ratio at 37 C° for 8 hours was chosen as the optimum reaction time for further experiments.

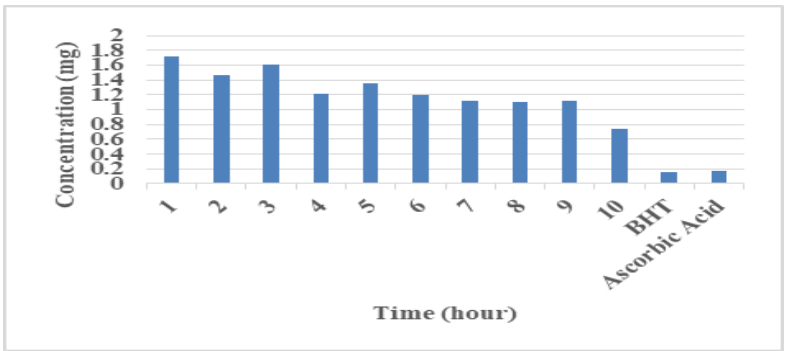

Figure 6. Total antioxidant capacity of elastin hydrolysate at variant times

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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الظروف المثلى لاستخلاص ايلاستين الكارب الشائع ودراسة الخصائص المضادة للأكسدة لمتحللات الايلاستين الانزيمية

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المستخلص

هدفت هذه الدراسة الى دراسة خمس طرق تنقية مختلفة لاستخلاص الايلاستين اذ من الممكن ان تؤدي طريقة الاستخلاص إلى احداث ضرر كبير في ألياف الايلاستين وبالتالي التأثير على الاستجابة البيولوجية لها. وجد ان أفضل الطرق لتنقية الايلاستين من bulbus اسماك الكارب الشائع باستخدام 4 مولار يوريا عند رقم هيدروجيني 7.2 الحاوية على 1 % بيتا–ميركابتوايثانول ثم الغسل بالماء المقطر ثم استخدام المؤصدة على درجة حرارة 121 م° لمدة 15 دقيقة وذلك بالاعتماد على محتوى مجاميع السلفاهيدريل والاحماض الامينية وفحص الهجرة الكهربائية SDS–PAGE والمجهر الالكتروني الماسح. تبعها تحضير متحللات الايلاستين باستخدام انزيم ايلاستيز الجري المنقى جزئيا ومتابعة التغير في درجة التحلل (DH %) والفعالية المضادة للاكسدة، اذ ازدادت درجة التحلل بشكل سريع خلال الثلاث ساعات الأولى لتصل الى 50.43 % بعدها ازدادت بشكل ابطئ لتصل الى 65.43 % بعد 10 ساعات من التحلل. تبعها دراسة الخصائص المضادة للأكسدة لمتحللات الايلاستين باعتماد ثلاث طرق: قابلية كبح الجذور الحرة والقوة الاختزالية والسعة المضادة للأكسدة الكلية. اظهرت النتائج زيادة الخصائص المضادة للأكسدة مع زيادة درجة التحلل لمتحللات الايلاستين، اذ وصلت IC_{50} لقابلية كبح الجذور الحرة وEC_{50} للقوة الاختزالية والسعة المضادة للأكسدة الى 2.309 و2.734 و0.158 ملغم على التوالي بعد 10 ساعات من التحلل.

الكلمات المفتاحية: تروبوايلاستين، المتحللات الانزيمية، الخصائص الوظيفية، DPPH.

***جزء من اطروحة دكتوراه للباحث الأول.**