EXTRACTION, PURIFICATION AND CHARACTERIZATION OF ELASTASE FROM THE DIGESTIVE DUCT OF CATFISH (*SILURUS TRIOSTEGUS)*

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ABSTRACT

This study was aimed to extract, purify and characterize the pancreatic elastase from the Catfish (*silurus trioseegus)* digestive duct. Different extraction solutions were used to optimize the extraction condition being (Distilled water, 200 mM NaCl, Phosphate buffer (100 mM, pH 8), the latter buffer Tis-HCl (100 mM, pH 8) both containing 0-100 mM NaCl or $CaCl_2$ separately). Tris-HCl buffer containing 100 mM $CaCl_2$ was the best among the rest extraction solutions. The crude extract was precipitated using a 30 % - 55 % of ammonium sulfate saturation ratio. The crude precipitate was dissolved in the same buffer and dialyzed. Then the dialyzed precipitate was concentrated and applied to CM-52 column (3.5 ×10 cm), elastase activity was appeared in two protein peaks. Each peak fraction were pooled individually and loaded on a Sephadex G-75 column (1.6×62 cm). Elastase activity was recovered only from one peak with 29.54 % yield, 530.70 U total activity and 17.89 U/mg specific activity. The optimum temperature for elastase stability and activity was 0-30 C° and 55 C° respectively. while the optimum pH for the enzyme activity and stability were 9 and 4-9 respectively. The molecular weight was 21898 Da as determined by Gel filtration.

Keyword: fish elastase . serine proteinase. size exclusion.
***Part of Ph.D. Dissertation for the 1st author.**

استخلاص وتنقية وتوصيف انزيم ايلاستيز من القناة الهضمية للجري (*Silurus triostegus*)

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المستخلص

هدفت هذه الدراسة الى استخلاص وتنقية وتوصيف انزيم الايلاستيز البنكرياسي من القناة الهضمية لأسماك الجري. استعملت محاليل استخلاص مختلفة تمثلت بـ (ماء مقطر، 200 ملي مولار محلول كلوريد الصوديوم، محلول دارئ الفوسفات (100 ملي مولار، pH 8) والدارىء الاخير و دارئ Tris–HCl (100 ملي مولار، pH 8) الحاويات على 0–100 ملي مولار كلوريد الصوديوم او كلوريد الكالسيوم كل على انفراد) لغرض تحديد افضل الظروف لاستخلاص الايلاستيز وكان محلول دارئTris–HCl (100 ملي مولار، pH 8) الحاوي على 100 ملي مولار كلوريد الكالسيوم هو الافضل. تم ترسيب الانزيم الخام باستعمال كبريتات الامونيوم بنسب اشباع متدرجة 30 % – 55 % بعدها أذيب الراسب النهائي في دارئ الاستخلاص أعلاه تلتها عملية الديلزة وتركيز المستخلص المديلز وامراره في عمود كاربوكسي مثيل سليلوز (CM–52) (3.5×10 سم)، اذ ظهرت فعالية الايلاستيز في قمتين. بعدها جمعت اجزاء القمتين على انفراد وتم امرارهما كل على انفراد على عمود الترشيح الهلامي (السيفادكس G–75) (1.6×62 سم). لوحظ وجود فعالية الانزيم فقط في واحدة من القمم المستحصلة من الخطوة السابقة وكانت بحصيلة انزيمية 29.54 % وفعالية كلية 530.70 وحدة و فعالية نوعية 17.89 وحدة/ملغم . كانت الحرارة المثلى لثباتية ولفعالية الانزيم كانت عند 0–30 م° و55 م° , وكان الرقم الهيدروجيني لفعالية وثباتية الانزيم 9 و4–9 على التوالي. بلغ الوزن الجزيئي للأنزيم 21898 دالتون عند تقديره بالترشيح الهلامي.

الكلمات المفتاحية: ايلاستيز السمك، بروتيزات السيرين، حدود الاقصاء.
***جزء من اطروحة دكتوراه للباحث الأول.**

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INTRODUCTION

Catfish is one of the most popular seafood especially in Africa and Asia (1). In Iraq, the Catfish (*Silurus triostegus)* concept is one of the widespread fish in the Tigris, Euphrates rivers and Shatt al-Arab (3, 22). Which its generally considered a good food for people as it's a rich source of important nutrients like proteins (than any other animal source), fats, vitamins and minerals (19, 22). Pancreatic elastase is one of the serine-protease family with enzyme code EC 3.4.21.39 (7). It works to cleave the carboxylic end of the neutral aliphatic amino acids of a fibrous protein in connective tissue called elastin (18). It's synthesized and stored in the pancreas as a zymogen and it's activated by trypsin (28). The elastase reported in the literature have molecular weights in the range of 24-28 kilodalton (kDa), consisting of a single polypeptide chain and most of them have a basic potential isoelectric point (pI) near pH 9 (5, 10, 18). In 1878, Walchli was the first one to report the elastase activity, where he found that Ox elastase could digest the nuchal ligament elastin, followed him Kuhne who found that crude pancreatic trypsin could dissolve elastin (24). some authors reported many benefits of elastase in food systems such as meat tenderization (2, 12) or medical applications like curing hyperlipidemia and arteriosclerosis (23), preparation of elastin hydrolysate which has beneficial effects on the skin and blood vessels (26) and induced of the abdominal aortic aneurysm (13). Besides trypsin and pepsin, elastase could be used in the preparation of elastin hydrolysate leading to improve their functionality and preparation of natural antioxidative bioactive peptide (6, 17, 20). fish Elastases species have been studied from Carp (10), Catfish (29), Rainbow Trout (7), Atlantic Cod (14) and North Atlantic Salmon (8). Many of solvents were used for the extraction of elastase from fish like Distilled water (28), 0.05 M ammonia buffer (pH 4.5) (21), 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl (7), 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM $CaCl_2$ (29, 30). The optimum temperature and pH for elastase activities and stabilities were varied depending on the fish species. Yoshinaka *et al*. reported that the optimum pH and temperature of the elastase were found at pH 8.0 and 50-60 C° respectively, and pH 5-9 and below 60 C° for Catfish elastase stabilities. Otherwise, the activity and stability of Cod elastase reached a maximum at a temperature of 40 C° and below 60 C° respectively (5). Also it was noticed that the optimum pH for stability was at pH 5 and above. (7) found that the optimum pH of elastase Rainbow Trout (*Oncorhynchus Mykiss*) elastase was 8.0 and 45 C° optimum temperature, and the enzyme was stable up to this temperature. The aim of this study was to extract, purify and characterize the elastase from the digestive duct of Catfish and utilize it in preparation of elastin hydrolysate with functional properties for food applications.

MATERIALS AND METHODS

Catfish waste was collected from fish sellers at the local market (Baghdad/Iraq) in November/2022. The Catfish pancreas was separated by removing the stomach, intestine and liver, and stored at -18 C° for further analysis. The following chemicals: N-Succinyl-Ala-Ala-Ala-p-nitroanilide (STANA) from Sigma-Aldrich (Germany); Tris-base, Ammonium Sulphate, Sodium Chloride and Calcium Chloride anhydrous from BDH (England); Sephadex G-75 from Pharmacia (Sweden); Carboxy Methyl Cellulose (CM-52) from Whatman (England); Bovine Serum Albumin, Sodium carbonate from MERCK (Germany).

Extraction of elastase

Catfish pancreas was mixed with Distilled water, 200 mM NaCl, 100 mM Phosphate buffer and 100 mM Tris-HCl buffer containing 0-100 mM of NaCl or $CaCl_2$ separately at a mixing ratio 1:6 (W: V) sample: buffer. Then the mixture was homogenized at 15000 ×g for 2 minutes in an ice water bath and held for 3 h at 8 C°. Then the supernatant was collected by centrifugation at 10000 ×g for 20 minutes at 4 C°, elastase activity was measured as described below.

Elastase activity assay

The N-Succinyl-Ala-Ala-Ala-p-nitroanilide (STANA) was used for elastase activity as described in (9, 11) methods with some modifications. A 50 µl of 2 mg/ml (STANA) was added to a glass Cuvette containing 0.675 ml of 100 mM Tris-HCl buffer pH 8 at 25 C° and the absorbance was measured at 410 nm

(presenting the blank). Then 25 µl of elastase extract was added to the mixture and reading the change in absorbance every 1 minute for 4 minutes. One unit of the activity was defined as one unit hydrolyzed one micromole of STANA per minute under specified conditions. The following equation was used to calculate the elastase activity:

$$\left(\frac{U}{ml}\right) = [(\Delta A410/\text{min Sample} - \Delta A410/\text{min blank}) \times 0.75 \times df] \div [8.8 \times 0.025]$$

where:

0.75 = Total volume (mL) of assay

df = Dilution factor

8.8 = Millimolar extinction coefficient of p-nitroaniline at 410 nm at pH 8.0

0.025 = Volume (mL) of Enzyme Solution used

Determination of protein content

Protein content was determined by the Bradford method mentioned in (25). Aliquot of 100 µl of the elastase extract was mixed with 5 ml of Coomassie brilliant blue G-250 solution in a glass test tube and then kept at room temperature for 5 min.. Then the absorbance was read at 595 nm. A series of diluted Bovine serum albumin was prepared from the stock solution of 1 mg/ml for the standard curve.

Precipitation of elastase

Various saturation ratios of ammonium sulfate were used to precipitate elastase as described in (15) method with some modification. A certain weight of ammonium sulfate was gradually added to 10 ml of elastase extract to reach 20-80 % saturation percent. Left overnight at 4 C°, then centrifugated at 10000 ×g for 20 minutes at 4 C°, elastase activity was measured as described above in the supernatant and the precipitate to access the optimum saturation percent for elastase precipitation. Then the precipitate was collected and dissolved in 100 mM Tris-HCl buffer, dialyzed against distilled water using a dialysis tube cut off 1000 KDa and concentrated.

Purification of elastase

Preswollen Carboxy Methyl Cellulose (CM-52) from whatman was used to purify the elastase as described by (16). The CM-52 was first equilibrated with 200 mM acetate buffer pH 5.6. then packed to form a 3.5×10 cm column. Then 2 ml of dialyzed and concentrated elastase extract was loaded into the column and 3 ml fractions were collected at a flow rate of 30 ml/h NaCl gradient 0-0.8 M solutions in the same buffer were used to elute the protein peaks. The absorbance was measured at 280 nm and the protein peaks were tested for elastase activity as described above. Gel filtration chromatography was the second step to purify the elastase as described in (18) The concentrated elastase fractions from the previous step were applied on a 1.6×62 cm column of Sephadex G-75 and eluted with 100 mM Tris-HCl buffer (pH 7.2) containing 50 mM NaCl at a flow rate of 30 ml/h and the eluted fraction collected 3 ml/tube, then the absorbance was read at 280 nm. Fractions with elastase activity was pooled, dialyzed and concentrated then stored at -18 C° for further analysis.

Determination of molecular weight by gel filtration: The molecular weight of elastase purified from Catfish was determined by gel filtration on Sephadex G-75 column (1.5×60 cm) equilibrated with 100 mM Tris-HCl buffer (pH 7.2) containing 50 mM NaCl. The column was calibrated with protein standards (BSA 66500 Da, Ovalbumin 43000 Da, Trypsin 24000 Da and Lysozyme 14000 Da), then the calibration curve was plotted according to the relationship between the ratios of (Ve/Vo) and the logarithm of the molecular weight of each standard protein.

Characterization of elastase

Optimum temperature for elastase activity and stability: The effect of the temperature on elastase activity and stability was tested according to the method described in (16) at different incubation temperatures ranged from 20-80 C°. For optimum temperature of activity, the substrate and the enzyme were incubated at 20-80 C° for 30 min.. while for enzyme stability, an aliquot of 0.5 ml of purified elastase was held at 20-80 C° for 30 min. then the activity was measured as described in (4, 21). A 100 µl of the purified elastase was added into a test tube containing 10 mg Congo-elastin and 200 mM Tris-HCl buffer pH 8. Mixing the mixture and incubated at 37 C° for one hour then centrifugated at 10000 ×g for 5 min then the absorbance was read at 495 nm. The enzyme unit was equivalent to the quantity of enzyme causing a

rise in absorbance at 495 by 0.01 after 1 hour of incubation under assay conditions.

Effect of pH on elastase activity and stability: The effect of the pH values on elastase activity and stability was tested following the method described in (16). For the activity test, at a pH range of 2.6-10.6. 2 ml of 100 mM of acetate buffer, Tris-HCl buffer and Carbonate buffer at pH values (2.6-10.6) was added to 10 mg congo-elastin, then 100 µl of the purified elastase was added and incubated at 50 C° for one hour, then centrifugated at 10000 ×g for 5 min and the absorbance was read at 495 nm. For the effect of the pH on elastase stability test, 0.1 ml of the purified elastase mixed with 0.6 ml of 100 mM acetate buffer, Tris-HCl buffer and Carbonate buffer at pH range 2.6-10.6 and incubated at 40 C° for 30 minutes. Then the elastase activity was measured as described above.

Statistical analysis

The obtained data were statistically analyzed using analysis of variance (ANOVA). Differences between treatment means were compared using the Least Significant Difference (LSD) ≤ 0.05 probability level using Minitab® 17.3.1 software program.

RESULTS AND DISCUSSION

Table 1 illustrates the optimum solution for elastase extraction from the digestive duct of Catfish. The highest activity was achieved using Tris-HCl buffer (100 mM, pH 8) containing 100 mM $CaCl_2$, this could be attributed to the presence of trypsin in the crude extract which resulted in elastase activation in the presence of $CaCl_2$ (27, 29). The specific activity and total activity reached 4.90 U/mg and 17.93 U respectively. Yoshinaka *et al.* (29) reported that the elastase was autoactivated by bovine trypsin at 4 C° over 4 days in 50 mM Tris-HCl buffer containing 50 mM $CaCl_2$ at pH 8. The statistical analysis revealed that there was a significant difference ($p \leq 0.05$) for specific and total activities between 100 mM Tris-HCl buffer containing 100 mM $CaCl_2$ and the other extraction solutions.

Table 1. Total and specific activities of elastase using different extraction solutions

Extraction solvents	Activity (U/ml)	Protein (mg/ml)	Volume (ml)	Sp. Activity (U/mg)	Total Activity (U)
Distilled water	0.16	0.24	8.80	0.66^{b}	1.41^{b}
200 mM NaCl	0.55	0.25	6.60	2.22^{ab}	3.63^{b}
Phosphate buffer	0.62	0.34	6.60	1.82^{ab}	4.11^{b}
Phosphate, 100 mM NaCl	0.39	0.24	8.40	1.67^{ab}	3.31^{b}
Phosphate, 50 mM NaCl	0.54	0.41	8.10	1.33^{ab}	4.38^{b}
Phosphate, 100 mM $CaCl_2$	0.88	0.34	9.00	2.61^{ab}	7.88^{ab}
Phosphate, 50 mM $CaCl_2$	0.33	0.16	9.00	1.98^{ab}	2.93^{b}
Tris-HCl buffer	0.72	0.33	9.70	2.20^{ab}	6.97^{b}
Tris-HCl, 100 mM NaCl	0.33	0.45	1.10	0.73^{b}	0.36^{b}
Tris-HCl, 50 mM NaCl	0.89	0.34	9.20	2.61^{ab}	8.14^{ab}
Tris-HCl, 100 mM $CaCl_2$	1.83	0.37	9.50	4.90^{a}	17.39^{a}
Tris-HCl, 50 mM $CaCl_2$	0.65	0.38	7.20	1.73^{ab}	4.69^{b}

***Values are from duplicate**

***Different letters mean there are significant differences at (≤ 0.05)**

Figure 1 shows the total activity of elastase at different saturation of ammonium sulfate. The elastase activity increased in the precipitate and decreased in the supernatant with increasing ammonium sulfate saturation. The heights elastase activity recovered in the precipitant from 50 % and above saturation. Therefore, ammonium sulfate of 30 % and 55 % saturation were chosen for gradient precipitation of elastase.

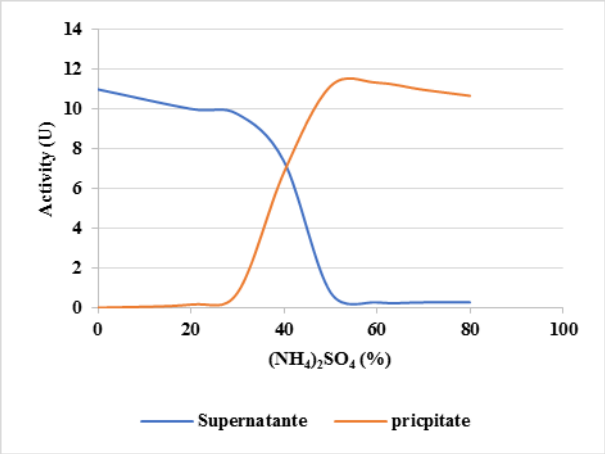

Figure 1. Precipitation of elastase at various salt saturation of ammonium. Sulphate

Figure 2 represent the elution curves of elastase extract on CM-52 column. Elastase activity appeared in three peaks, one minor peak represented by tubes (43-52) and two major peaks represented by tubes (52-60) and (61-72) respectively, which eluted with acetate buffer (200 mM, pH 5.6) containing 0.4 M, 0.5 M and 0.6 M NaCl respectively. The two major peaks are named elastase Ⅰ and elastase Ⅱ. These results agreed with Yoshinaka *et al.* (29) who identified 4 isoenzymes of Catfish elastase using a 1.2×20 cm CM Cellulose column. Similarly, Bassompierre *et al.* (7) found three isoenzymes of elastase from Bluefin Tuna and Yellowtail extract and two isoenzymes were found in elastase extracted from Carp and Eel using CM Cellulose Column.

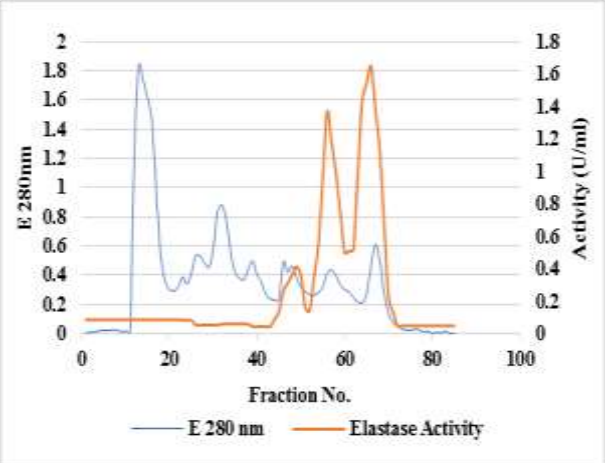

Figure 2. Purification of elastase by ion exchange chromatography, using CM-52 column (3.5×10 cm) which eluted with 200 mM acetate buffer, pH 5.6 and then with a linear gradient from 0 to 0.8 M NaCl in the same buffer. The flow rate was 30 ml/h. and fractions of 3 ml/ tube were collected

Figure 3. shows the elution curves of elastase Ⅰ and elastase Ⅱ on the Sephadex G-75 Column. Elastase Ⅱ represented in Figure 3.B, more than one protein peak appeared, only one peak fraction had elastase activity (represented by tubes 26-33). These results agreed with Lei *et al.* (16) Findings who reported that elastase produced by *Chryseobacterium indologene* appeared in one peak eluted at the same tubes above. Figure 3.A represents the fractionation of elastase Ⅰ through the Sephadex G-75 column, there was no elastase activity in eluted protein peaks. A similar result was noticed by Yoshinaka *et al.* (29) who reported that repeating the CM-52 cellulose fractionation for elastase A was unsuccessful. Further work is needed to extract, purify and characterize elastase I.

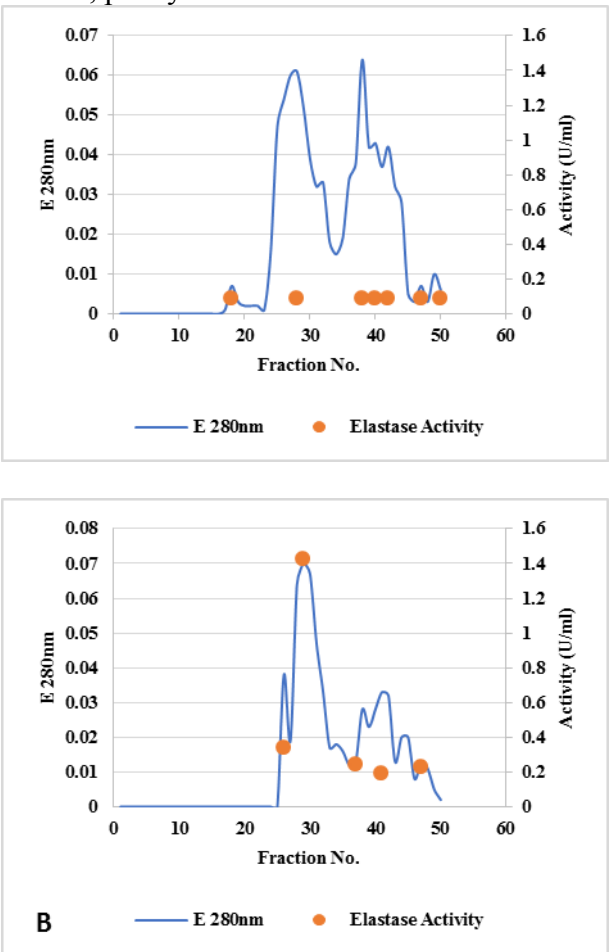

Figure 3. Fractionation of elastase Ⅰ (A) and elastase Ⅱ (B) by gel filtration chromatography using Sephadex G-75 column (1.6×62 cm), eluted with 100 mM Tris-HCl buffer, pH 7.2 containing 50 mM NaCl, flow rate was 30 ml/h. and 3 ml/ tube were collected

The obtained Catfish elastase was purified by ion exchange and gel filtration chromatography as summarized in Table 2. The purification fold of elastase was increased in each step and reached 5.12 fold after the gel filtration step and the final recovery and total activity were 29.54 % and 530.70 U respectively. The total activity of elastase was decreased with the purification steps progress and this could be attributed to the presence of more than one elastase (isoenzyme) (5, 28, 30) which might be lost through purification steps.

Table 2. Purification of elastase Ⅱ for Catfish pancreas

Steps	Activity (U/ml)	Protein (mg/ml)	Volume (ml)	Sp. Activity (U/mg)	Total Activity (U)	Yield (%)	Fold
Crude Extract	2.53	0.72	710	3.49	1796.38	100.00	1.00
Ammonium Sulphate Precipitation	32.11	6.71	50	4.79	1605.68	89.38	1.37
Ion Exchanger of Elastase Ⅰ	10.31	0.19	48	54.26	494.88	27.54	15.5
Ion Exchanger of Elastase Ⅱ	15.19	3.52	40	4.32	607.50	33.82	1.23
Gel Filtration of Elastase Ⅱ	23.07	1.29	23	17.89	530.70	29.54	5.12

Figure 4 shows that the molecular weight of elastase II was 21898 Da as determined by Gel filtration chromatography. Many authors reported various values for elastase molecular weight, the molecular weight of Atlantic Cod elastase was found to be 25 and 24.8 KDa (5, 6), 25 KDa from Carp (10), 26 KDa from Catfish (29) and 27 KD from Trout (7) using SDS-PAGE electrophoresis.

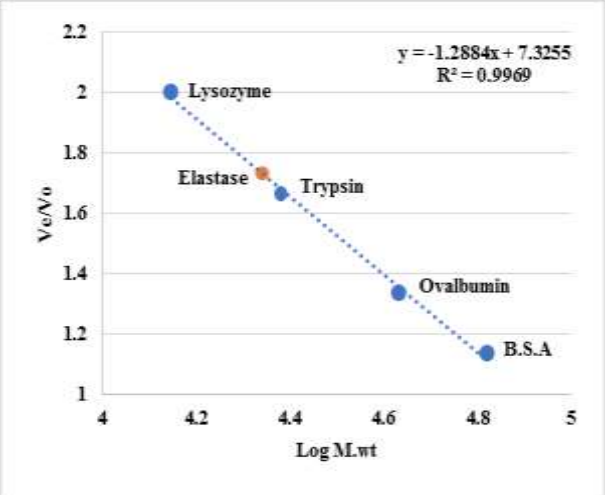

Figure 4. Molecular weight of elastase as determined by gel filtration

Effect of temperature on elastase activity and stability: The effect of temperature on elastase Ⅱ activity was studied at temperatures ranging from 20-80 C° as shown in Figure 5. The elastase Ⅱ activity increased as the reaction temperature increased from 20 C° (1.44 U/ml) to 55 C° (20.65 U/ml). Then elastase Ⅱ lost most (2/3) of its total activity over 30 min. incubation at 60 C° (7.43 U/ml) and 65 C° (6.74 U/ml), then elastase Ⅱ lost its entire activity at temperature above 65 C°. The obtained results were similar to Yoshinaka *et al.* results (29) who found that the optimum temperature for elastase B from Catfish (*Paracilurus asotus*) was between 50 to 60 C° at pH 8 and disagreed with Asgeirsson and Bjarnason (5) and Bassompierre *et al*. (7) findings who reported that the optimum temperature was 45 °C for Rainbow Trout elastase and 40 °C for Atlantic Cod elastase. Figure 6 illustrates the elastase Ⅱ stability at a temperature ranged from 20-80 C°. The maximum stability for catfish elastase was up to 30 C° when the enzyme retained its entire activity, then elastase stability decreased as the temperature increased and it reached to 52.44, 29.73, 28.96, 25.61, 13.87, 12.20, 9.76 and 9.30 % at 45, 50, 55, 60, 65, 70, 75 and 80 C° respectively. These results were similar to Sovik and Rustad (28) results who stated that Cod elastase was stable at temperatures up to 40 C° then rapidly dropped at 50 C° and above. Also, the result of this study agreed with Asgeirsson and Bjarnason (5) and Yoshinaka *et al*. (29) findings who reported that elastase from Catfish (*Paracilurus asotus*) and Atlantic Cod was stable at a temperature below 50 C° and 40 C° respectively.

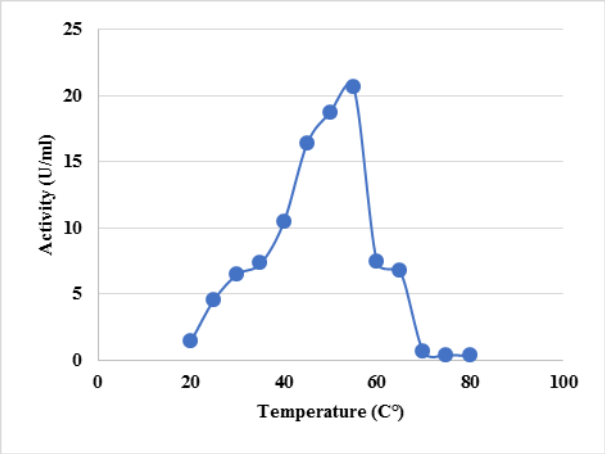

Figure 5. Optimum temperature for Catfish pancreatic elastase activity using Congo-elastin

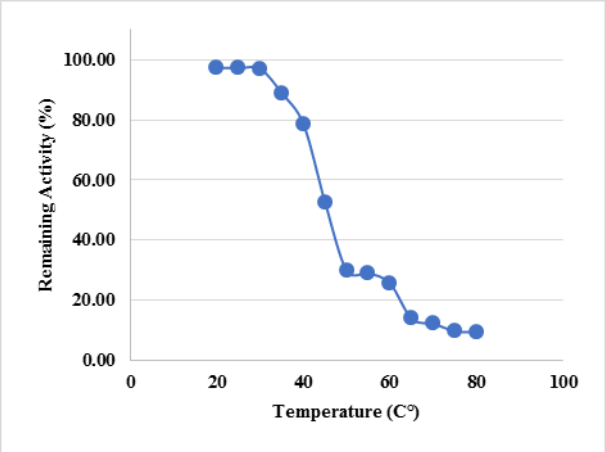

Figure 6. The effect of temperature (20-80 C°) on Catfish pancreas elastase stability using Congo-elastin

Effect of pH on elastase activity and stability: Figure 7 shows the elastase Ⅱ activity at the pH ranged from (2.6-10.6). The optimum activity was at pH 9. Meanwhile, at pH 7 and 8 the enzyme also showed high activity. Whereas the enzyme activity was very low at pH 2.6-6 and pH 10. The obtained result was similar to the optimum pH for Atlantic Cod elastase and porcine elastase (5). Bassompierre *et al* (7) and Yoshinaka *et al* (29) reported that pH 8 was the optimum pH for elastase activity from Rainbow Trout and Catfish (*Paracilurus asotus*). The stability of elastase Ⅱ at different pH values was shown in Figure 8. The highest stability seen at pH 7 (100 %). At pH 6 and 8 the enzyme retained more than 75 % of its entire activity. In contrast, more than 50 % of its activity has been lost at pH 4 and below, this could be attributed to an irreversible structural change of elastase (5). Yoshinaka *et al.* (29) reported that Catfish elastase was stable between pH values 5 and 9 at 25 C° for 6 h.

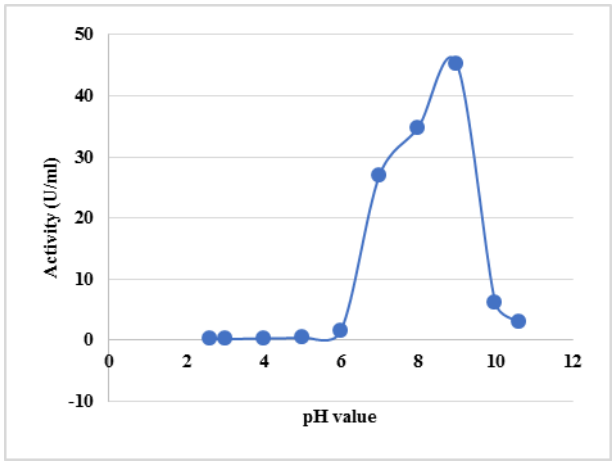

Figure 7. Optimum pH for Catfish pancreatic elastase activity using Congo-elastin

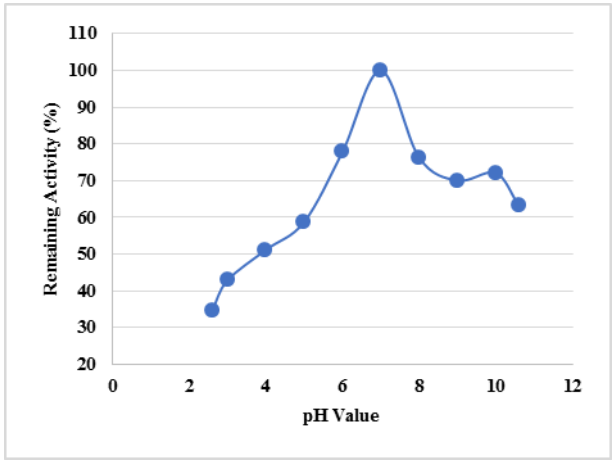

Figure 8. The effect of pH values (2.6-10.6) on Catfish elastase stability using Congo-elastin

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