

**EXTRACTION, PURIFICATION AND CHARACTERIZATION OF LIPASE
FROM THE DIGESTIVE DUCT OF COMMON CARP
CYPRINUS CARPIO L.**

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ABSTRACT

This study was aimed to use digestive duct of common carp *Cyprinus Carpio* L. as economic source for fish lipase extraction . The enzyme was extracted using potassium phosphate buffer (pH 7 , 0.2 M). The crude extract then precipitated at the saturation range between 40 – 80 % and dialyzed . Further purified carried out through ion exchange chromatography using DEAE-Cellulose column (2.5 × 30 cm) and gel filtration chromatography by Sephadex G-100 column (1.5 x 60 cm). The purified lipase had a molecular weight of 37.150KDa ,and optimum pH and temperature for enzyme activity about 45°C and 6.5 using olive oil as substrate .The specific activity, number of purification folds and yield of purified Lipase were 146.48U/mg, 9.76 and 40.50% respectively.

Keywords: lipase activity, Ion exchange, gel filtration, molecular weight.

ألبياتي وآخرون

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استخلاص وتنقية وتوصيف إنزيم اللابيز من القناة الهضمية لسماك الكارب العادي

CYPRINUS CARPIO L.

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المستخلص

تهدف الدراسة إلى استعمال القناة الهضمية لأسماك الكارب العادي *Cyprinus Carpio* L. كمصدر اقتصادي لاستخلاص إنزيم اللابيز. تم استخلاص الإنزيم باستخدام محلول فوسفات البوتاسيوم (0.2 M ، pH 7) ثم ترسيب المستخلص الخام عند نسبة تشبع بين 40-80% كبريتات الامونيوم . تم إجراء الديلزة ثم تنقية إضافية من خلال كروماتوغرافيا التبادل الأيوني باستعمال عمود DEAE-Cellulose بأبعاد (2.5 × 30) سم و كروماتوغرافيا ترشيح الهلام بواسطة عمود Sephadex G-100 بأبعاد (1.5 × 60 سم). كان لإنزيم اللابيز المنقى وزن جزيئي قدره 37.150 كيلو دالتون ودرجة حرارة مثالية لنشاط الإنزيم حوالي 45 درجة مئوية و ورقم هيدروجيني أمثل 6.5 باستعمال زيت الزيتون كركيزة. الفعالية النوعية وعدد مرات التنقية والحصيلة الإنزيمية لإنزيم اللابيز المنقى كانت 146.48 وحدة / مجم ، 9.76 وحدة. و 40.50% بالتتابع.

الكلمات المفتاحية: فعالية اللابيز، التبادل الأيوني، الترشيح الهلامي، الوزن الجزيئي.

INTRODUCTION

Enzymes are vital catalysts that accelerate the reactions in which they participate with high degrees of specialization when the appropriate conditions for their work are available. Additionally, the enzymes work to increase the quality of products, reduce production costs, and less impact on the environment, and consequently, the use of enzymes increased in the applied fields. Lipases were first discovered in 1956 by Claude Bernard when he studied the role of pancreas in fat digestion (16). Lipases works on the hydrolysis of fats and oils to their components of glycerol and fatty acids. Lipase(triacyl glycerol acyl hydrolase, EC 3.1.1.3) catalyzes the hydrolysis of the carboxyl ester bonds in triacylglycerols to produce diacylglycerols, monoacylglycerols fatty acids and glycerol under aqueous conditions and the synthesis of esters in organic solvents (10). It is characterized by its ability to stimulate the hydrolysis of the aqueous bonds at the interface of the insoluble matter and the water phase in which the enzyme is dissolved, it also have ability to reverse reactions with the presence of organic solvents, so that the esters is being or exchange of acetyl groups between various acetyl glycerol, alcohols, esters and amines(26). Lipases has attracted much attention in both food industrial and biological catalysts. In food industry, lipases were played a major role as catalysts for biological processes and are safer and more environmentally friendly. It has been used in food manufacturing increasingly in the past few decades as a result of its useful functions in processing and improving the quality and improving the quality of products (8). Studies conducted on fish lipase indicate that they may have different reactions towards fatty acids than those found in mammals, plants, and microbes, as fish lipase works very actively on the analysis of long-chain poly-unsaturated fatty acids as well as its advantage because it works with a wide range of pH numbers. It does not need auxiliaries and is stable at high temperatures. Fish tissues by-products such as the head, frame viscera, the skin and especially the digestive glands are rich source of enzymes such as lipase. however, the

studies focusing on the purification of fish tissue lipases appear to be much more limited (18). Due to the economic importance of the Lipase this study aimed to identify the optimum conditions to extract and purify the enzyme from the digestive duct of common carp *Cyprinus carpio* L. and some of its characteristic.

MATERIALS AND METHODS

Extraction of the enzyme

The extraction process carried out according to Naser (19) method mentioned by Abadi (1). The digestive duct of common carp *Cyprinus carpio* L. were collected from a local market at Baghdad city and washed with a cooled distilled water to remove fat and any other unwanted materials. Fresh digestive duct were blended with 0.2 M Potassium phosphate buffer (pH 7) in a mixing ratio (1: 4), homogenized for 2 minutes, large parts filtered by Watt man filter No.1 and centrifuged at 5000 xg for 30 minutes to remove the coarse parts. The supernatant was designated as crude extract, then its volume, enzymatic activity, and protein concentration were determined and the specific activity was calculated

Enzyme activity assay

The enzymatic activity was estimated according to the (5) method mentioned by (3) using olive oil as a substrate.

Protein concentration

Bradford method was used to determine the protein concentration using Bovine Serum Albumin (BSA) as standard protein (Bradford,1976).

Enzyme purification

Ammonium sulfate saturation (20, 40, 60, 80 and 90) % were set up to precipitate the crude enzyme. The precipitation allowed to continue for about 4 hrs. Subsequently, the mix was centrifuged at 10,000 xg for 30 min, carefully the supernatant poured off and the pellets dissolved in appropriate volume of 0.2 M Potassium phosphate buffer at pH 7. Whole process achieved in a cold room. The ammonium sulfate precipitate enzyme was dialyzed against Potassium phosphate buffer (pH7, 0.2 M) for 24 hours with three changes of dialysis buffer. DEAE – Cellulose-A50, column 2.5 × 30, was prepared according to GE Healthcare company instructions, where 4 gm of dry powder was suspended in Potassium

phosphate buffer (pH7, 0.2 M). The slurry was prepared with binding buffer in a ratio of 75% settled medium to 25% buffer. After column equilibration, concentrated enzyme was applied gently onto the surface of the Cellulose beads. The column was washed by Potassium phosphate buffer to make unbinding proteins pass through. Fraction collector was set up to collect 3ml for each fraction at a flow rate of 40 ml / hr. Elution is achieved using a continuous increasing salt gradient to release the other proteins bounded to the column, NaCl (0.1-1M) dissolved in 0.2 M Potassium phosphate buffer solution (pH 7.0) was used for this purpose. The absorbance and the enzymatic activity of each fraction were measured by using spectrophotometer at 280 nm and Lipase activity assay respectively. Fractions with Lipase activity were pooled and stored at 4°C for the next step of purification. Sephadex G-100 was used to prepare the gel filtration column (1.5 x 60 cm dimensions), it was prepared according to GE Healthcare instructions. The medium suspension diluted with 0.2 M Potassium phosphate buffer at pH 7, and stirred with a glass rod to make a homogeneous suspension free from aggregates. The column was equilibrated using the same buffer at a flow rate of 40 ml / h for 5 hours. After column preparation, 3 ml of the enzyme solution was added gently to column surface and eluted using 0.2 M Potassium phosphate buffer pH 7 with a flow rate of 40 ml / hr (3ml for each fraction). Optical density (at 280nm), and enzyme activity were determined for each fraction. Fractions represent activity were pooled and kept at 4°C.

Enzyme characterization

Determination of the molecular weight of the lipase by gel filtration method

Use a Sephadex G-100 gel column (1.5 x 60 cm). Estimate the void volume (V_0) of the column by passing the blue dextran solution (at a concentration of 5 mg / ml) and calculate the sum of the volumes of the separate fractions from the beginning of the passage of the dextran solution to its absorption peak at a wavelength of 600 nm and the recovery volumes (V_e) for the standard proteins (Bovine Serum Albumin, 67000), (Pepsin 35000), (Trypsin, 23000) and (Lysozymes

14400) was estimated to collect the parts in the same way and read the optical absorption at a wavelength of 280 nm, then the linear relationship between the ratio of the recovery volume of each standard protein to the void volume (V_e / V_0) versus the logarithm of its Molecular weight.

Determination of the optimum pH for the lipase activity: The optimum pH for the activity of the enzyme was determined using sodium acetate buffer solution of (0.2 M) with pH values ranging from 3.5-5.5, sodium phosphate buffer solution with pH value ranging between 6.0-7.5, and Tris-HCL buffer solution with a pH value (8.0) to prepare the substrate solution. The reaction carried out by incubating the substrate with enzyme for each pH (3.5-8.0) in test tubes for 15 minutes in a water bath at 37 ° C, then it was cooled directly in an ice bath to stop the enzymatic reaction and the enzymatic activity determined as explained previously and then the relationship between the enzyme activity and the pH values is plotted .

Determination of the optimal pH for enzyme stability

A particular volume of the pure enzyme was incubated with an equal volume of buffer solutions with different pH values (3.5-8.0.) in test tubes for 15 minutes in water bath at 37 ° C, then cooled directly in an ice bath and the activity was measured. The remaining enzyme activity as a percentage of the activity out of the highest activity .The relationship between the remaining enzyme activity versus the pH values was plotted to determine the optimal pH for the enzyme stability.

Determination of the optimum temperature for enzyme activity

The activity of the enzyme was estimated at different temperatures ranging from 20-90 ° C at the optimum pH for the reaction.

Determination of the thermal stability of the enzyme: The enzyme was incubated in a water bath at different temperatures ranging from 20-90 ° C for 15 minutes, then the tubes were cooled directly in an ice bath and the reaction solution was added to them (with the optimum pH).The residual enzymatic activity is estimated and the relationship between the remaining activity

and different temperatures is drawn to determine the optimum temperature for the enzyme stability.

RESULTS AND DISCUSSION

Extraction, concentration and purification of the enzyme

Results of the fish lipase extraction from the digestive duct of the local common carp by potassium phosphate buffer 0.2 M at pH 7 at 4 °C, showed a higher enzymatic activity of 33.33 units/ml, protein concentration 2.2227 mg/ml, specific activity 14.99663 units/mg, total activity 8333.25 units and yield 100% (Table 1). Figure 1 shows the enzyme activity through ammonium sulfate precipitation of crude enzyme extract. It has been noticed that the

enzyme precipitated out between 20 - 80% of ammonium sulfate saturation and the highest activity for the precipitated fraction were achieved at 80% saturation. It was clear that as the enzyme activity increased in precipitated fraction, it is decreased in supernatant fractions. The enzyme units and the specific activity in this step was 317 U/ml and 104 U/mg respectively. After dialysis step the enzymatic activity was 245.7 U/ml. Iijima *et al.* (1998) reported that the Lipase can be isolated in the 40-60% saturation using fractional precipitation with ammonium sulfate. Another study demonstrated that Lipase precipitated out between 30- 90% of ammonium sulfate saturation.

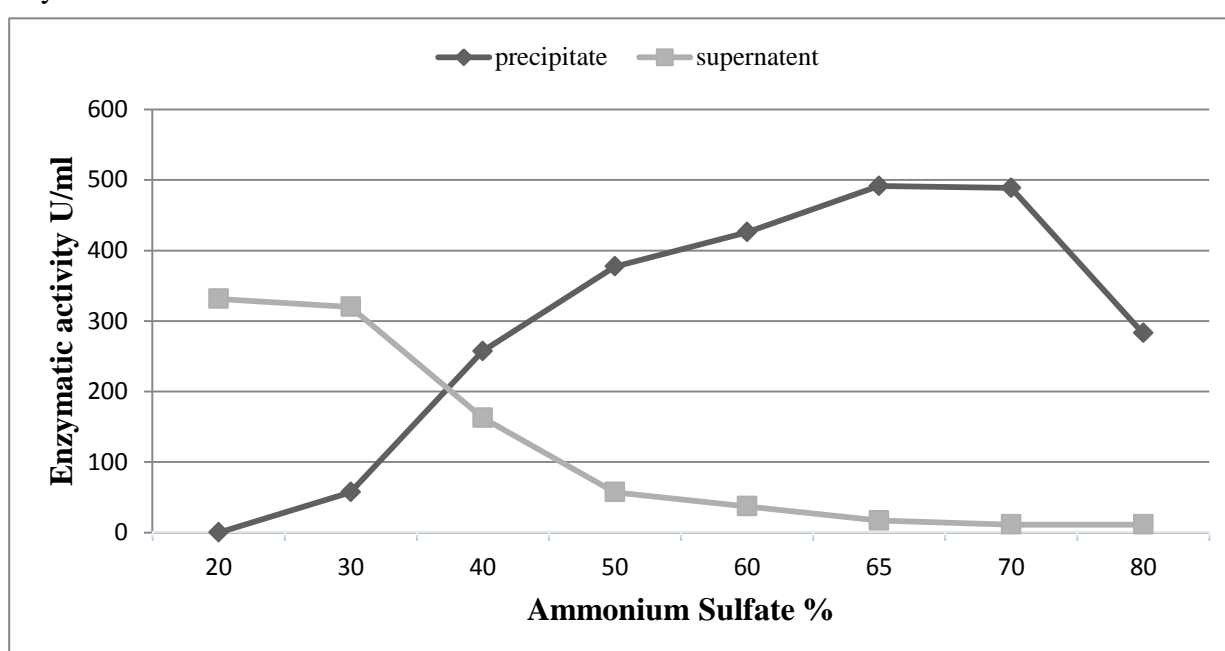


Figure 1. Ammonium sulfate salt Precipitation of Lipase.

The results in Figure 2 show a single peak in a wash step (major peak), and another peak at 0.2 mM gradient. Both peaks had lipase activity, the major peak fractions were pooled and dialyzed against phosphate buffer and concentrated using polyethylene glycol. The specific activity, enzyme activity and the yield after DEAE chromatography, were 122.69 U/mg, 100 U/ml and 64.8% respectively (Table 1). Figure (3) illustrates the gel-filtration fractionation for the experimental lipase, two protein peaks appeared, only one peak possesses enzyme activity (located at tubes 23-38) with 146.48 U/mg specific activity and 40.50% yield (Table 1), those fractions were pooled and

concentrated to be used for enzyme characterization. Al-Mousawi (3) results, found three peaks with lipase activity, produced by polymorph nuclear from mastitis milk, one peak in the washing step, and the other eluted with the increasing molarities of sodium chloride over DEAE Sephadex A-50 column fractionation, which was previously equilibrated with 0.2 M phosphate buffer pH 7.

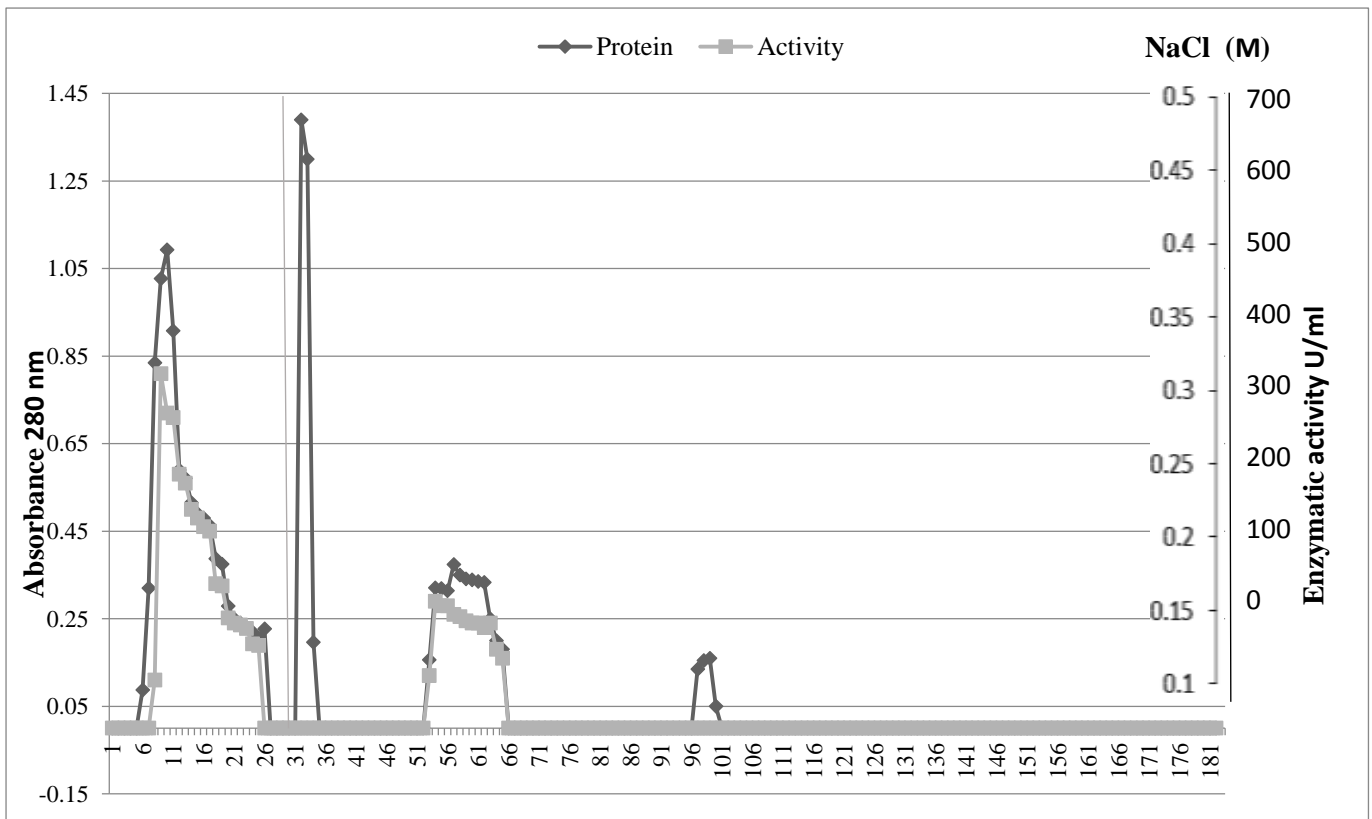


Figure 2. Ion- exchange Chromatography using DEAE Sephadex A-50. The column (2.5 × 30) was equilibrated with 0.2 M phosphate buffer (pH 7). The gradient elution was done as indicated and fractions of 3 ml were collected at flow rate 40 ml/hr.

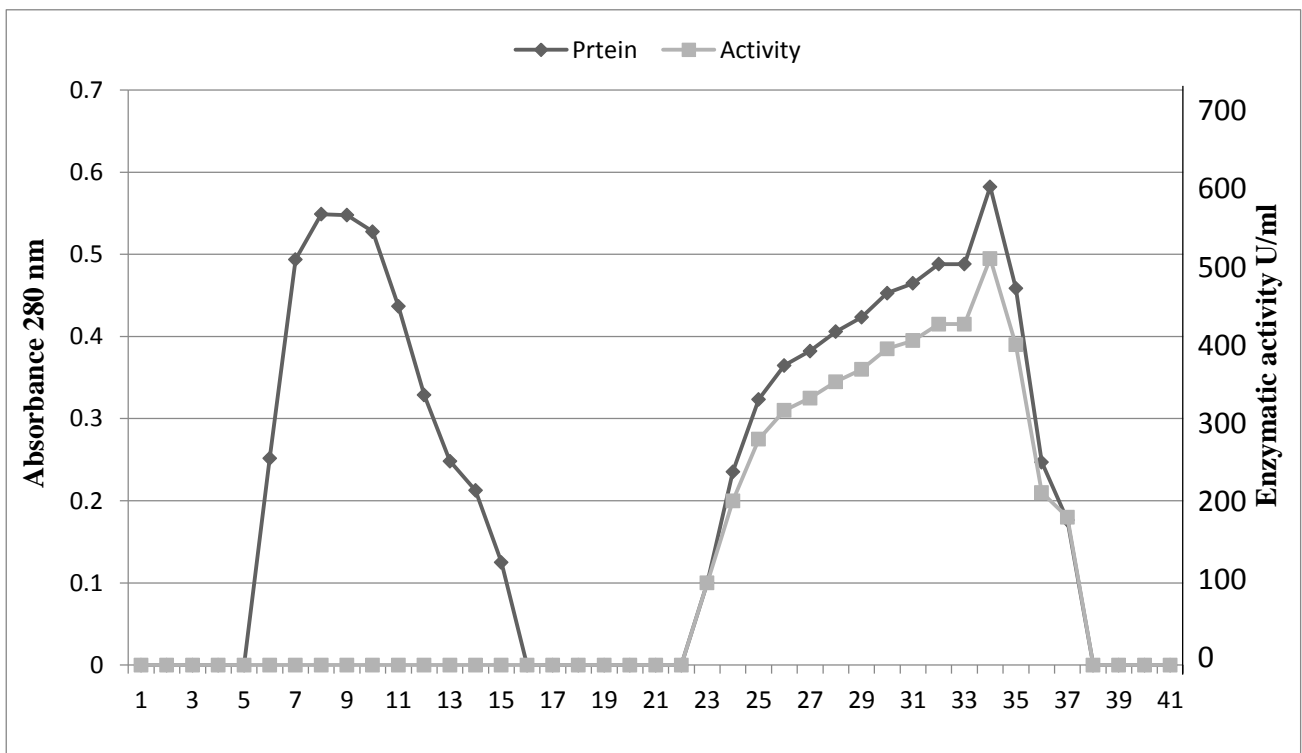


Figure 3. Gel filtration chromatography using Sephadex G-100 (1.8 x 68 cm) equilibrated with phosphate buffer at pH 7, fraction volume was 3ml at a flow rate of 30 ml/hr.

(6) stated, an extracellular lipase was isolated and purified from the culture broth of *Pseudomonas aeruginosa* SRT 9 to apparent homogeneity using ammonium sulfate precipitation (30-90%) followed by

chromatographic techniques on phenyl Sepharose CL- 4B and Mono Q HR 5/5 column, resulting in a purification factor of 98 fold with a specific activity of 12307.8 U/mg

Table 1. Lipase purification summary

Purification step	Enzymatic activity (U/ml)	Volume (ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	No. of folds	Yield %
Crude extract	33	250	2.22	14.99	8333.25	1	100
Ammonium Sulfate 40-80%	317.	22	3.02	104.68	6944	6.98	83.68
After dialysis	241.66	28	2.22	108.85	6766.64	7.25	81.20
Ion exchange DEAE-Sephadex	100	54	0.815	122.69	5400	8.18	64.80
Gel filtration Sephadex G-S100	75	45	0.512	146.48	3375	9.76	40.50

Characterization of the enzyme

Molecular weight

Fig (4) indicates that the molecular weight of the purified lipase was 37153 Dalton. This finding was close to that found by (27), the molecular weight of the purified lipase from *Pseudoalteromans sp.* was 37000 Dalton using PAGE -SDS. Tako, *et al.* (25) found the molecular weight of the purified lipase

from *Rhizopus oryzae* was 35000 Dalton. These findings were different from the result recorded by (7, 24) that the molecular weight of the purified lipase from Antarctic Krill was 71270 Dalton and *Aspergillus terreus* reached 46300 Dalton. These variation in molecular weight of lipase could be due the source of enzyme and the methods of determination (2).

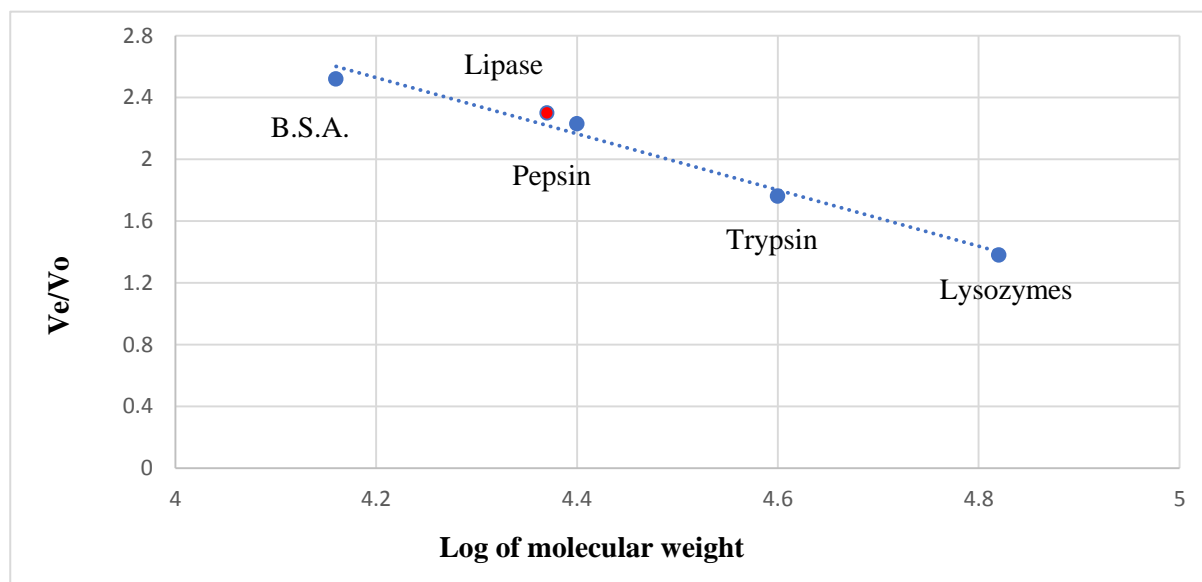


Figure 4. Standard curve for estimating the molecular weight of the lipase enzyme by gel filtration method through Sephadex G-100 column with dimensions (1.5 x 60) cm.

The optimum pH for lipase activity

Figure (5) shows the pH profile of the lipase activity (pH range 3.5 – 9). It has been noticed that the optimum pH for the activity of the enzyme was (6.5). The enzymatic activity decrease remarkably at extreme values of pH (below 4 and above 8). This may

be due to the effect of the pH changes on the ionic properties of the enzyme molecule or the matrix and other components of the reaction medium. As a result, there is a change in the composition of the three-fold stacked shape to a more random composition, meaning a

change in the natural state of the enzyme occurs. This may weaken the activity of the enzyme to the extent that the enzyme completely loses its effectiveness. The effectiveness of the enzyme may also decrease due to a change in the shape, or arrangement of the molecules of the base material. As a result, the tendency of the enzyme to interact with the base material will decrease, which

leads to a decrease in the enzymatic activity (23, ; 20).The results of the current study were close to the results of other related studies about lipase from different sources. It was mentioned (13) that the optimum pH for the activity of lipase extracted from *Bacillus subtilis* was 7. Whereas, (17) mentioned that the optimal pH for the Chinook salmon lipase was 8.5 .

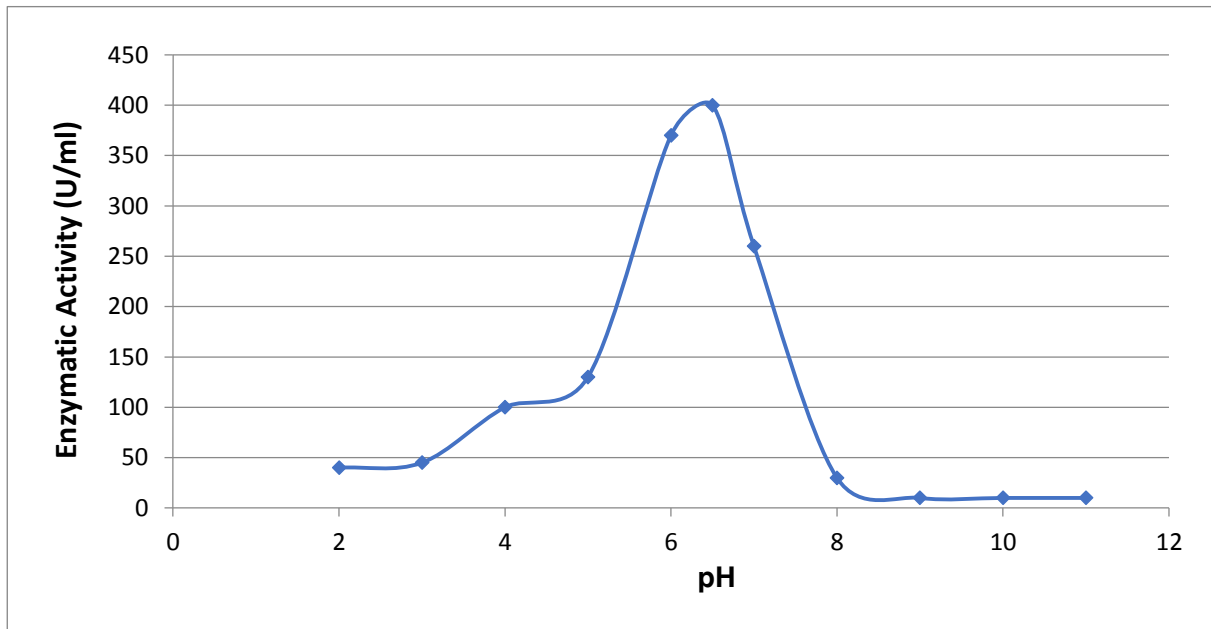


Figure 5. The optimal pH for the common carp digestive duct lipase activity

Optimum pH for lipase stability

Figure. 6 represents the stability of the experimental lipase at different pH values. The enzyme stability was noticed at pH ranges from 5 – 7.5, as the enzyme retained 100% of its original activity when incubated at above pH values for 15 minutes at 37°C.

The enzymatic activity remarkably decreased at pH value above 8 and below 5. These results were in agreement with (16) findings that Chinook salmon (*Oncorhynchus tshawytscha*) lipase was at pH values ranged between (5 - 8) .

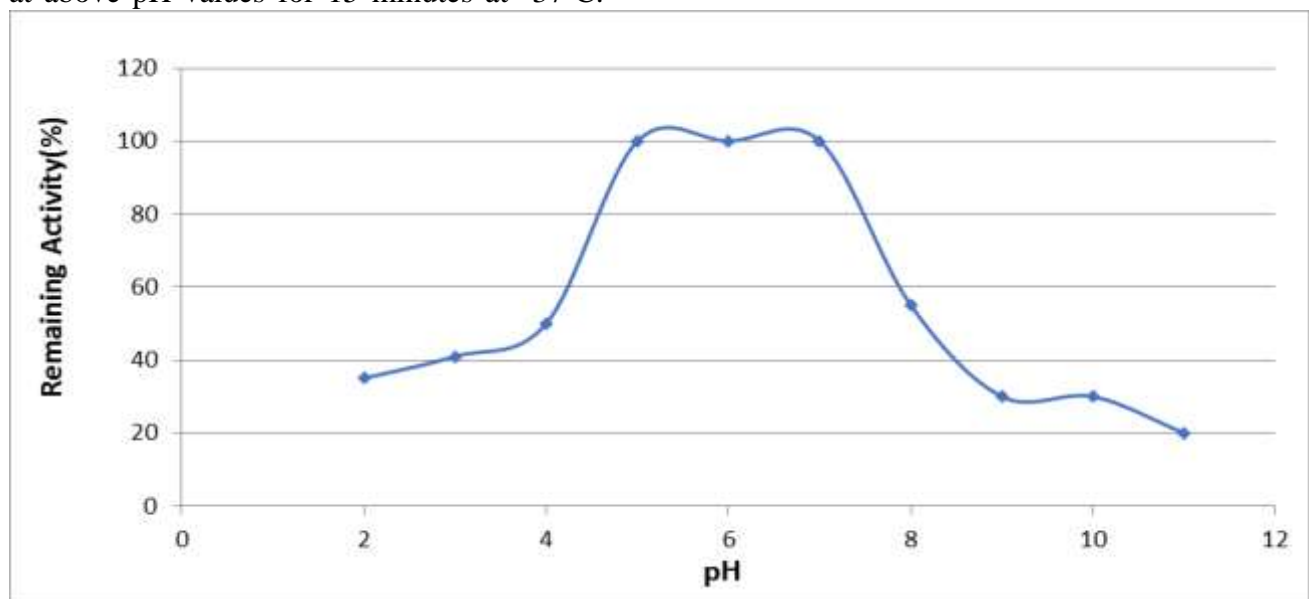


Figure 6. The optimal pH for the common carp digestive duct lipase stability

Optimum temperature for lipase activity

Figure (7) shows the effect of temperature on the activity of the lipase. The enzymatic reaction carried out at different temperature ranging between (20 - 90 ° C) with a difference of 5 degrees between one treatment and another. The results showed that the enzyme activity increased as the reaction temperature increased up to 45 ° C when the activity reached the highest value. Then it gradually decreased when the temperature increased to above 45°C . This could be attributed to the increase in contact between the enzyme molecules and the substrate due to the increase in the kinetic energy of the

molecules (20). The enzymatic activity decreases due to a change in the nature of the enzyme or the substrate or both, which in turn leads to a decrease in enzyme activity and reaction rate . reaction decreases, and the heat may affect the base material itself and become not subject to the enzyme (23). The results of this study were in accordance with the results of Sae and Benjakul (22) ,who found the optimum temperature for activity of the lipase extracted from the *Latescalcarifer*, was at 50 ° C . In contrast to (9) results ,they found the optimum temperature of lipase purified from *Klebsiella pneumoniae* was 30 ° C.

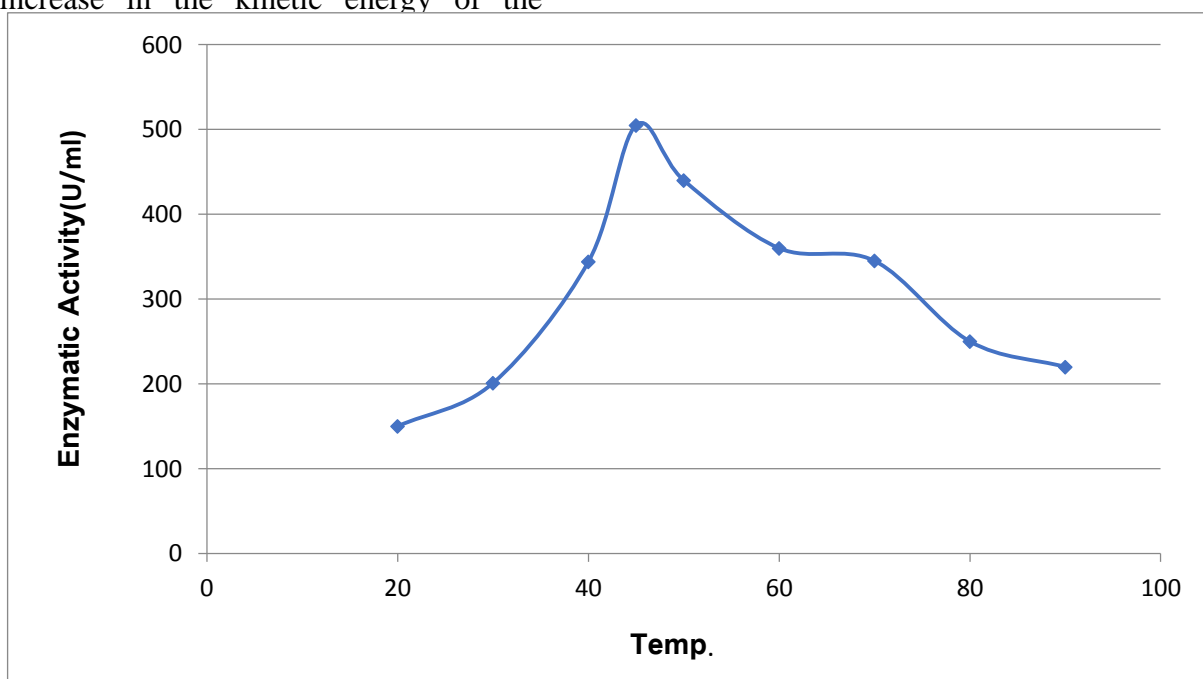


Figure 7. Optimal temperature for Lipase activity extracted from common carp.

Thermal stability of the lipase from common carp

Figure (8) shows the thermal stability of the purified lipase when incubated for 15 minutes at temperatures ranging between (20 - 80) C, with a difference of 5 degrees between each treatment. The enzyme retained its entire activity when incubated at temperatures ranging between (20 - 50 C) for 15 minutes, then the activity started to decrease as the temperature increased above 50C.. The enzyme retained approximately 25% of its activity at 70 C. This could be due to the

effect of heat on the triple structure of protein, which may lead to deformation of the protein and loss of its activity. The results of this study were similar to (16) ,they found the purified lipase from Chinook salmon (*Oncorhynchus tshawytscha*) was stable at temperatures ranging between 20 - 50 ° C. Whereas (15) found the lipase enzyme extracted from Indian Major Carp *Catla catla* (Catla) was stable at temperatures ranging from 30 - 60 ° C.

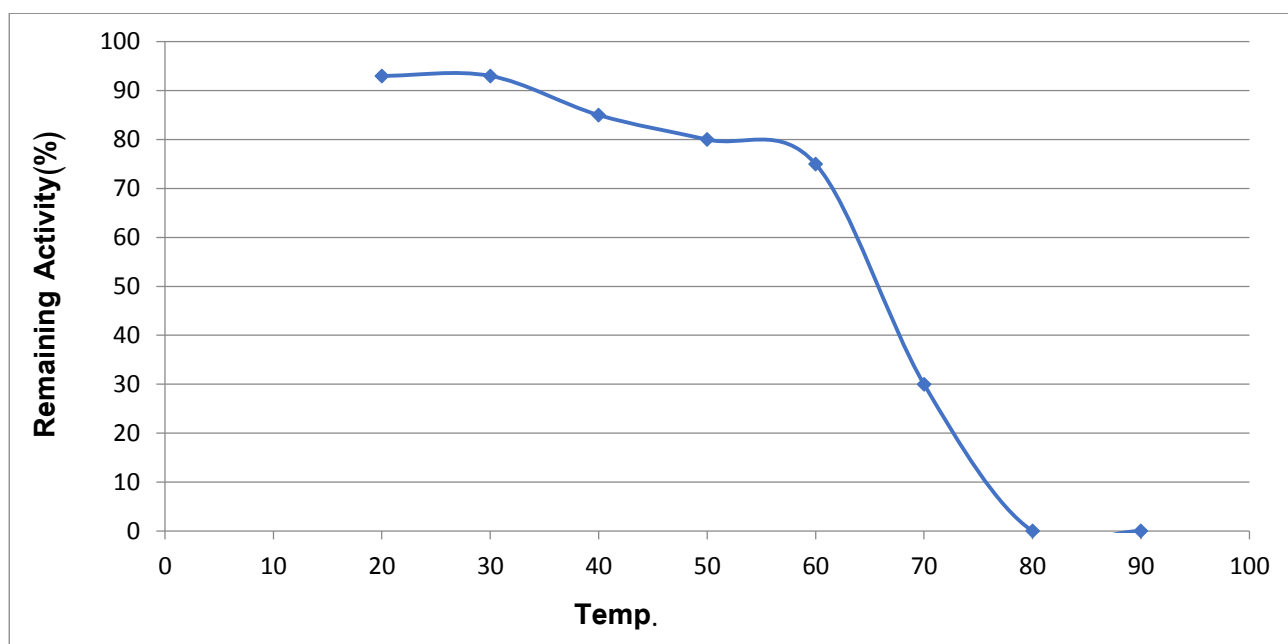


Figure8. Thermal stability curve of Lipase extracted from common carp.

Conclusion

The digestive duct of common carp *Cyprinus carpio* L. Is a promising source for lipase. Phosphate buffer was appropriate solution for extracting the Lipase- from common carp digestive duct tissue. The ammonium sulfate at 40% - 80% of saturation was best choice to concentrate the crude extract . Using ion-exchange and gel filtration chromatography were efficient for the lipase purification.

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