Isolation of lipoxygenase enzyme from patients with allergic rhinitis and study of the effect of natural products isolated from buckthorn plants (rhamnaus L) on its effectiveness

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Summary

This thesis included the isolation and purification of the enzyme lipoxygenase (LOX) from the blood serum of a person with allergic rhinitis disease, the study of several factors affecting its effectiveness, in addition to determining its molecular weight, and then the effect of natural products isolated from buckthorn (rhamnaus L) on the effectiveness of the enzyme partially purified from the blood serum. In which the DEAD-Cellulose ion exchanger was used for the protein leachate produced by deposition with ammonium sulfate (65-0%) after membrane sorting (dialysis). The package showed high efficacy of the enzyme, as the specific effectiveness was (0.105U/mg) and the number of purification times was (2.77) and the package was relied on in determining the molecular weight of the enzyme by electrical migration technology using SDS-PAGE, which amounted to (66,000) kiloDalton approximately.Then the optimum condition of the effectiveness of the partially purified enzyme were studied and the results showed that the highest effectiveness was at the sixth minute, pH=8, temperature (40°C), (0.6 mmol/L) concentration of substrate (linoleic acid), that the maximum velocity (*V*max) was (184 units / liter), and the value of (*K*m) by applying Michales – Menton equation and Lineweaver-Burke plot was (0.18) and (0.19) mM respectively. As for the concentration of the enzyme, the results showed that the higher enzyme concentration will increased the activity of enzyme The study also involved isolating natural products from buckthorn (sidr) (flavonoids and oils) by a soxhlet device using various solvents, then the oils were diagnosed with GC gas chromatography and flavonoids were diagnosed with high-performance liquid chromatography technology (HPLC) The results showed that there were several bands in GC chromatography, two of them belong to Eicosenoic acid (Cis11-Eicosenoic acid), while the HPLC was characterized by the presence of five bands belonging to (P-Coumaric acid , Qurectine , Apigenin, Rutin and Chlorogenic acid).

(1). Introduction

Allergic rhinitis (AR) is a chronic inflammatory immunoglobulin (Ig) E-mediated illness of the nasal mucosa that can be induced by seasonal or perennial allergens inhalation(Drazdauskaitė et al., 2021). Sneezing, rhinorrhea, nasal irritation, nasal congestion, and allergic conjunctivitis symptoms are common. AR affects up to 40% of the global population, and its incidence has been growing over the last 20 years. AR symptoms include rhinorrhea, sneezing, nasal irritation, and congestion. Seasonal AR (SAR) is caused by outdoor allergens, specifically grass, tree, or weed pollen. Perennial AR (PAR) symptoms remain throughout the year as a result of consistently prevalent indoor allergens such as house dust mites (HDM), animal dander, insects, and mold(Blaiss et al., 2018) The disease's pathogenesis is defined by an early phase of sensitivity to a single allergen. Individuals who are sensitive to the allergen have distinct immunological responses after exposure. The activation of T helper 2 (Th2) cells contributes to disease establishment and maintenance. The major effector cells of AR are eosinophils, mast cells, and basophils, which are innate immune response cells. Inflammatory mediators such as histamine, prostaglandins, cytokines, tryptase, leukotrienes, and eosinophilic cationic protein are released by these cells. Most of the degenerative processes that occur in the nasal mucosa are caused by these mediators. that occur in the nasal mucosa.(Costa *et al.*, 2021)

Lipoxygenases (EC: 1.13.11.12), LOXs are a vast protein family present in mammals, plants, and fungus. These enzymes also are present in cyanobacteria(Kuhn *et al.*, 2015), but only rarely in other prokaryotes. LOXs have attracted the interest of many researchers due to their functions in a variety of disorders such as asthma symptoms, allergic rhinitis, inflammatory bowel disease and rheumatoid, osteoporosis, stroke heart disease, Alzheimer's disease and cancers.(Khan *et al.*, 2021) Also, LOX products, leukotrienes, & lipoxins play important roles in metabolic activities such as organelle breakdown. transcription controls As a result, LOX inhibition is regarded as a significant target for the therapy of LOX-related disorders.(Cengiz Şahin & ÇAVAŞ, 2020).

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(2).An aim of this study

Isolate the enzyme lipoxygenase (LOX) from a serum with allergic rhinitis, using ion exchange technique, estimating the molecular weight of the enzyme lipoxygenase (LOX) using electrical migration technology. Stabilize optimal conditions for the enzyme lipoxygenase (LOX).

(3). Substances and methods

(1.3).Isolation and purification of the enzyme lipoxygenase (LOX)

(.1.3.3). Estimation of the effectiveness of the enzyme lipoxygenase

The effectiveness of the enzyme lipoxygenase (LOX) was estimated based on the method followed by the researchers (Shastry & Rao., 1975)

(2.3.3). Serum preparation

A blood sample of (25 ml) was drawn from a 37-year-old donor with allergic rhinitis disease who was selected to be the study model according to the instructions of the field supervisor and specialist doctor for allergies and asthma in the consultant of Al-Salam Teaching Hospital, after the moral license of the patient and conformity with the controls of the Nineveh Health Department. After separation and obtaining the serum, the protein concentration in it was estimated by the modified lauri method as well as the effectiveness of the enzyme lipoxygenase (LOX), and then the serum was kept in the freezer to later undergo a series of purification processes necessary to separate and study the properties of the enzyme.

(3.3.3). Isolation and deposition of the enzyme lipoxygenase using ammonium sulfate:

Ammonium sulfate was added in its solid state and gradually (Robyt F.J. & White J. B. (1987). To serum and saturation, (65%) subject to stirring with magnetic motor and low temperature (4°C) for 60 minutes. Then put the solution in the refrigerator for 24 hours to settle, and then the precipitate was separated from the leachate with the cooled centrifuge for (min 10) and at a speed of xg 4000, after which the precipitate was dissolved with the lowest possible amount of regulated phosphate solution 0.02M), (pH = 6.8,where the amount of protein and the effectiveness of the enzyme in the resulting protein precipitate solution was estimated and then the precipitate solution was kept at -20 ° C until used again. (Muhaisen et al. (2008)

(4.3.3)Membrane sorting

The solution from the previous stage was membrane sorted in order to remove ammonium sulfate using the(Robyt F.J. & White J. B. (1987). Where (15 ml) the solution is placed within the bag and thereafter closed from the upper edge and unfortunately tightly with immersion there in phosphate regulated solution at a concentration of (mm 20) and an acid function (ph=6.8) with stirring with permanent magnet motor and for a time frame of (24) hours as well as a temp of 4 ° C (taking into consideration the replacement of the regulated solution each (4) hours and then upon conclusion of the final quantity of the resulting solution where the ability.

(5.3.3).An exchange of ions chromotograph

An exchange of ions chromotograph: weight (30 g) of negative ion exchange (DEAE-Cellulose then activated with acid, then with base, and rinsed with water several times until pH = 7, then added to it the solution of controlled phosphate pH = 6.8 (0.02M) If the resin was extracted and put into in the separation column, which is 2.5 cm in diameter & 40 cm long and slanted to avoid the creation of air bubbles, then the controlled solution had passed for at least one hour until passing the models..(Plummer,T.D. (1978).

(4).Isolation of natural products from buckthorn

(1.4). Preparation of the plant

This herb was obtained from one of the popular markets in Baghdad, weighed (370 grams) of it and then ground with a blender to obtain a lower volume and inserted into a bag of gauze to be soaked and extracted with three different solvents (petroleum ether, ethanol, water).

(1.1.4). Isolation of natural products from buckthorn

(1.1.1.4.). Isolation of Oils

Fatty acids and oils were extracted from the fruits of the dry buckthorn plant weighing (370 g) from it and placed in the thimble of the extraction device thimble (Soxhlet), about 48 hours after soaking the plant with solvent (petroleum ether 60 – 80 °C) (3) days ,then the process of evaporation of the solvent was performed using the rotary evaporator and the calculation of the percentage of oil extracted from the plant. (Al- Samarrai, 2018).

(2.1.1.4). Isolation of Flavonoids

The flavonoids were isolated by taking the resulting residual (pepper) after isolating the oil in the extraction device for about 48 hours and for 3 days after it dried from the effect of petroleum ether and soaked with ethanol for a whole day in the dark. The ethanol was then evaporated using a rotary evaporator and then the resulting material was placed in an airtight tube for the purpose of HPLC diagnosis. (Al-Chalabi *et al.*,2020)

(3.1.1.4). Isolation of Alcaloides

Isolate alkaloides from the plant by taking (pepper produced after isolating flavonoids) and placing it in the Soxhelt extraction device using distilled water for 48 hours after it has dried from the trace of ethanol and soaking in distilled water for a whole day and

then put the resulting solution in the eyelid device to get rid of the water and get the precipitate in powder form and then stored frozen in a tightly lied tube for the purpose of knowing its effect on the effectiveness of the enzyme partially purified from the blood serum.(Sezgin and Artk, 2010)..

(5). Statistical analysis

The results were statistically analyzed using the statistical program 18SPSS (Burton, 1994) and the data were statistically analyzed using the analysis of the ANOVA test and the Duncan test to compare more than two variables and find the difference at the probability level p ≤0.05.

(6). Study of isolation and purification of the enzyme lipoxygenase (LOX) from the blood serum

6.1.. Partial purification of the enzyme lipoxygenase (LOX) by deposition with ammonium sulfate (saline displacement).

Isolation of the enzyme lipoxygenase-5 (LOX-5) from the serum of a patient with allergic rhinitis and using different life techniques, as the first technique used to purify the enzyme is sedimentation using ammonium sulfate salt (neutral salt), which is preferred to be used in the first step of the process of purifying enzymes, because it is a salt consisting of di-charged ions ($So4^{-2}$) and ($NH4^{-2}$) so its ionic strength is high and its efficiency in the deposition of proteins is more compared to The rest of the neutral single-charge salts such as sodium chloride because the ionic force is directly proportional to the charge square of salt ions (Al-Fayyad & Al-lehebe, 2021)The results shown in Table (1-1) indicate that the specific effectiveness of the enzyme lipoxygenase increased after sedimentation to (0.061 U/mg) compared to the specific effectiveness in the raw serum before sedimentation, which was (0.0379 enzymatic units / mg protein), i.e. the number of purification times increased after sedimentation by (1.64) times compared to what was in the raw serum before purification, while the amount of recovery of the total effectiveness of the enzyme amounted to (44.75%) compared to the total effectiveness of the raw enzyme

Table 6.1: Steps to purify the enzyme lipoxygenase (LOX) from serum of patients with allergic rhinitis (AR).

Purificationsteps	The size (m1)	protein mg/ml)(	Totalprotein(mg)	Effectiveness (U/ml)	Effectivenesstotal) U/mg)	The specific effectiveness	The number ofpurification	Recovery%
Raw serum	15	4.6	69	0.243	3.645	0.0528	___	100
Precipitation by Ammonium Sulphate (before dialysis)	6.5	4.1	26.65	0.251	1.6315	0.061	1.15	44.75
Sorting of membrane (after dialysis)	9.5	3.7	35.15	0.263	2.4985	0.070	1.32	68.54
Ion exchange package	24	0.9	21.6	0.095	2.28	0.105	1.98	62.55

*Enzymatic unit (U): It travels to the amount of enzyme that releases one micromol from the base substance (linoleic acid) per minute.
** Specific efficacy (U/mg): The number of enzyme units found in one mg of protein.

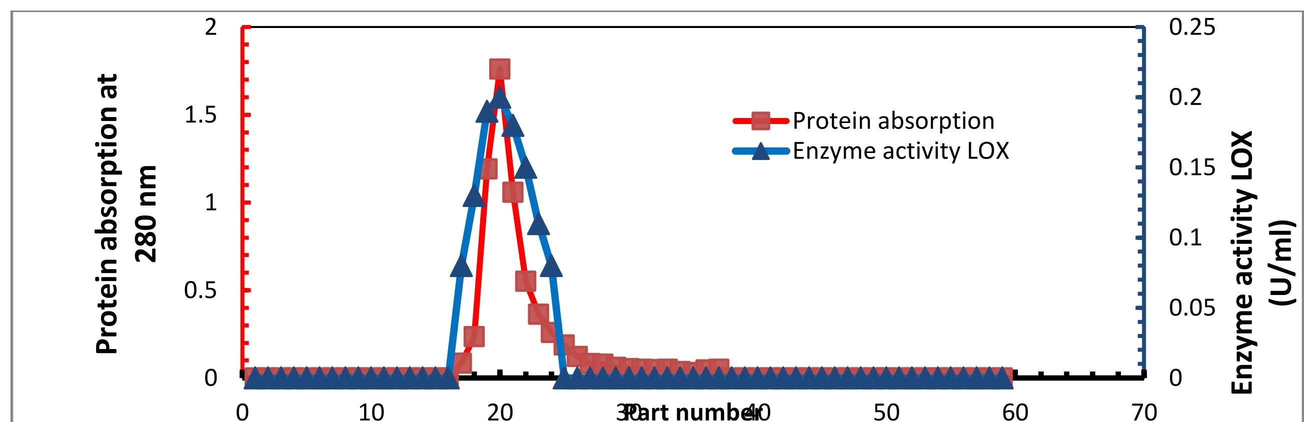


Figure 1.1: Profile of Rogan of the enzyme Lipoxygenase partially purified from serum by ion exchange chromatography technique

(7.)Study of a number of factors affecting the effectiveness of the enzyme lipoxygenase partially purified from the blood serum

Effect of enzyme concentration: -) ..(7.1

The effectiveness of the enzyme (LOX) was measured using different concentrations of the molecule-purified enzyme from allergic rhinitis patients ranging from (0-100 μL) of the solution of protein beams It was found that

the reaction speed of the enzyme is directly proportional to the concentration of the enzyme when the base material is abundant in the reaction perimeter after the conditions of temperature and the base material pH (Ahmed and crescent (and crescent, 2010: the Fleih, 2000)

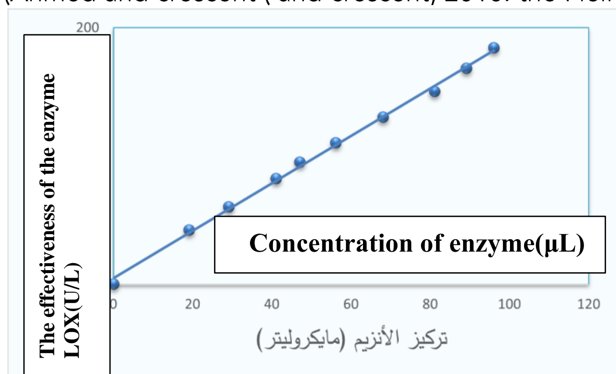


Figure 7.1: Effect of enzyme concentration on the effectiveness of LOX partially purified from serum

).(2.7).Effect of pH:-

Each enzyme has a pH or acid function at which the enzyme appears at the highest efficacy called the optimum pH function and that the use of high or very low pH will lead to a loss of the effectiveness of the enzyme as a result of the occurrence of a mutant or abnormalities in the nature of the enzyme, where the effectiveness of the partially purified enzyme (LOX) was measured from patients . solution KH2PO4, K2 (HPO4), as shown in Figure 7-2, if the effectiveness of the enzyme LOX was at hydrogen (8).As compared to previous studies (Kamal & Hasan, 2019), the optimal pH for the enzyme LOX isolated from the blood serum is pH(8).

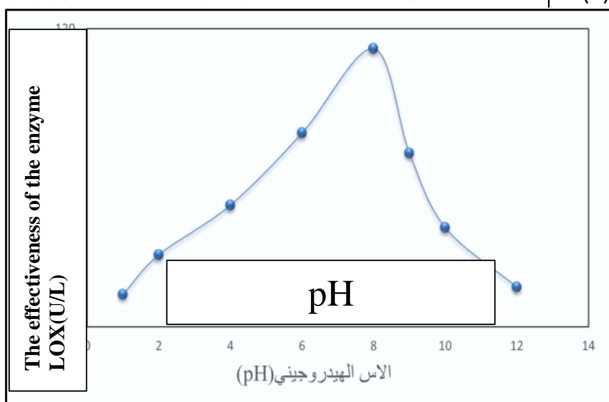


Figure 7-2: Effect of pH on the effectiveness of the enzyme lipoxygenase (LOX) partially purified from the blood serum.

) (3.7)Effect of temperature:-

The effectiveness of the enzyme lipoxygenase (LOX) was measured at different times and different temperatures to choose the optimal duration of time and the results appeared as shown in Figure (7-3) that the best optimum temperature for the enzyme LOX is (40◦C) (Kamal & Hasan, 2019) and then the effectiveness of the enzyme decreased significantly due to the occurrence of the denaturation mutant in the nature of the enzyme due to the breakdown of hydrogen bonds and other forces that are responsible for maintaining the triple structure of the protein and thus losing its effectiveness the enzyme (Lu et al., 2020) This finding is consistent with the findings of both researchers (Aanangi et al., 2016)

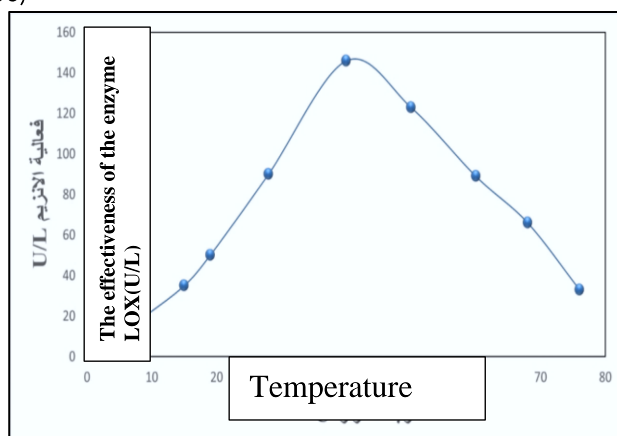


Figure 7-3: Effect of temperature on the effectiveness of the enzyme Lipoxygenase (LOX) partially purified from serum

(4.7.)Effect of reaction time on LOX activity

The effectiveness of the enzyme (LOX) was measured at different times from the start of the reaction to choose the optimal duration of time, where the results shown in Figure (7-4), indicate that the best effectiveness of the enzyme (LOX) was at the sixth (6) minute of the enzyme incubation period at different times of time, and then the effectiveness begins to decrease due to the decrease in the concentration of the base substance due to the saturation of the site with the base material. (Ahmed and Hilali, 2010) This is consistent with previous studies. (Ma et al., 2010)

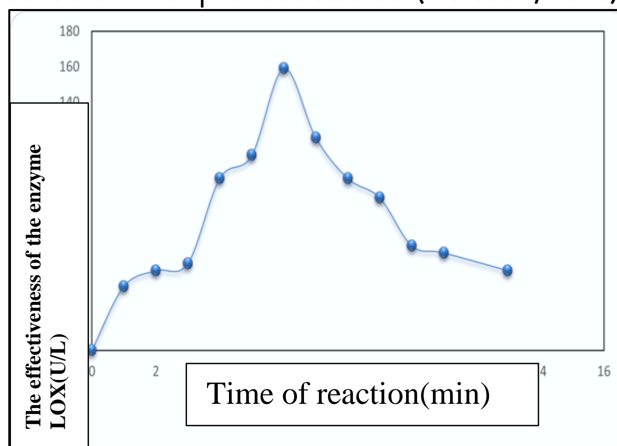


Figure 4-7: Effect of reaction time on the effectiveness of the partially purified LOX enzyme from the blood serum

:)(5.7).Effect of substrate concentration(

The effectiveness of the enzyme lipoxygenase (LOX) was measured using different concentrations of the base material linoleic acid ranging in concentration between (0-1.2 mmolar / L) and the results in Figure (7-5) showed that increasing the concentration of the base material leads to an increase in the speed of the enzymatic reaction, until it reaches a constant value after which there is no increase in the rate of the speed of the enzymatic reaction by increasing the concentration of the base material (linoleic acid) and the speed at the highest concentration of the base

material is called the maximum speed (Vmax) of the enzyme. (Al Faleih, 2000) (Elemo *et al.*, 2021) Using the Lineweifer-Burke diagram, the maximum speed value (Vmax), Km appeared at (184 units/l) either the value (Km and Vmax) for the Michels-Mentin equation and the Lineweiver–Burke equation were (0.18 mmolar) (184 units/l), meaning that the enzyme has a high affinity to bind to the base material (linoleic acid), since the value of (Km for enzymes is usually between (100 – 0.1) millimular identical to a previous study (Al Fleih, 2000) The value of (Km for most enzymes is between (10^{-1}-10^{-6}) Mular

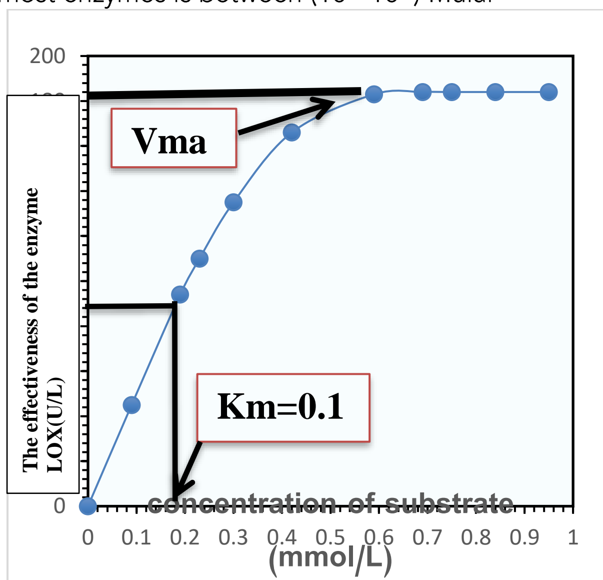


Figure 7-5: Effect of the concentration of the base material on the effectiveness of the enzyme lipoxygenase (LOX) partially purified from the blood serum.

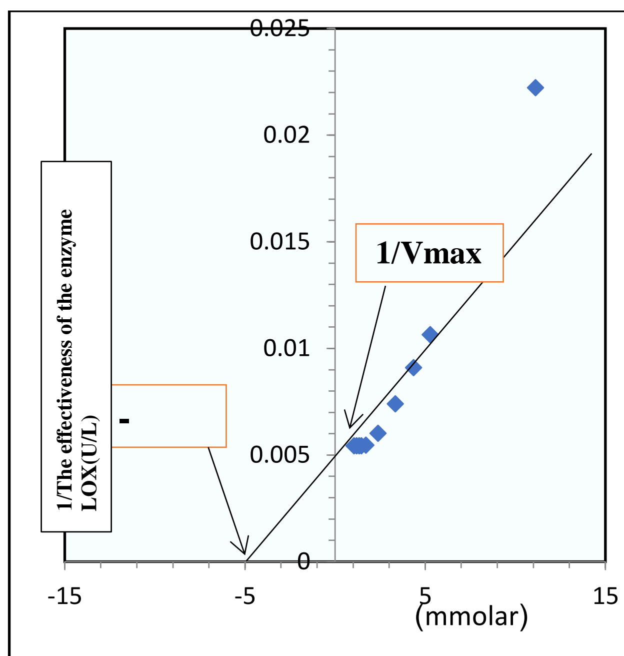


Figure 7-6: Lineweifer-Burke diagram to illustrate the value of the Micheles-mentin constant and the maximum velocity of the enzyme lipooxygenase (LOX) partially purified from the blood serum.

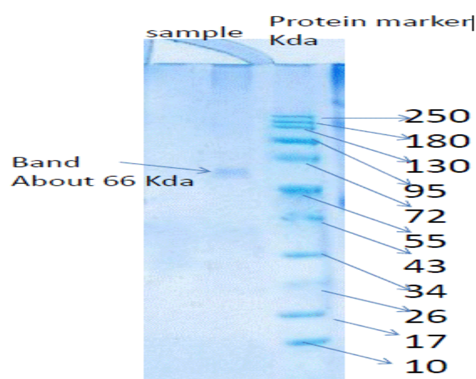
LOX Optimum condition for assay of Lipoxygenases8.

The summary of the optimal conditions for measuring the effectiveness of the enzyme LOX partially purified from a male blood serum with asthma is shown in Table(8.1). Table (8.1): Optimal conditions for the partially purified serum enzyme LOX

concentration of)mmolar(substrate	Temperature) ◦C(	pH	time of reaction(minute)
0.6	40	8	6

9. Finding the molecular weight of the enzyme lipooxygenase (LOX) in the serum with the technique of electrical migration (Electrophoreses)

The approximate molecular weight of the enzyme epoxygenase separated from the serum of patients with allergic rhinitis was estimated by the electrical migration technique containing PAGE-SDS, where the model was injected into the protein solution of the isolated package from the separation process using a technique applying ion exchange chromatography, and through this process the protein beam was found at a distance of (4.0) cm from the point of origin as shown in Figure (9-1), This package was adopted in estimating the approximate molecular weight of the enzyme equal to (66) k.This result is an approach to previous studies (Dinh et al., 2020; Gilbert et al., 2020)



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