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Isolation, Purification, and Characterization of Pecan Nut Lipase and Studying its Affinity towards Pomegranate Extracts and 1, 4-Diacetoxybenzene

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Abstract

This study included partial purification of the lipase enzyme from the karnel of the pecan nut. After applying ion exchange chromatography technique using CM-Cellulose, two lipase isoenzymes were observed with specific activity values of 2.66 and 1.7 units/mg protein. The highest activity of both isoenzymes appeared at the optimum pH values of 8 and 6 and at temperatures of 40 and 50 °C, respectively. A pure single band was obtained by using electrophoresis technique and it was found that the approximate molecular weight was 42 kDa for the two isoenzymes. The study dealt with the preparation of the diester compound 1,4-diacetoxybenzene, which was diagnosed by GC-MS and ^{1}H NMR spectrometry. When studying the inhibition mode, the above compound showed a competitive inhibition of the lipase, as the value of Km increased from 0.013 to 2.857 mM, and Vmax was 0.25 enzymatic units / ml / min. Inhibition constant Ki was calculated and its value was 0.0319 mM. Pomegranate peel extracts with chloroform and ethyl acetate revealed an activating effect on lipase. This research aims to detect the presence of lipase in the pecan nut karnel and purify it by biochemical methods to know its properties and molecular weight. Then we attempted to inhibit it with a laboratory-prepared compound that has a structure similar to the basic substance on which the enzyme works.

Keywords: Pecan, Lipase, 1,4-diacetoxybenzene, Pomegranate.

فصل، تنقية انزيم ليبيز جوز البقان ودراسة الفته تجاه مستخلصات الرمان و 4،1-ثنائي اسيتوكسي بنزين

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الخلاصة:

تضمنت الدراسة الحالية التنقية الجزئية لأنزيم الليباز من جوز البقان. بعد تطبيق تقنية كروماتوغرافيا التبادل الأيوني باستخدام CM–Cellulose ، لوحظ وجود اثنين من متماثلات الليباز وبفعالية نوعية 2.66 و 1.7 وحدة / ملغم بروتين. ظهر أعلى نشاط لكلا المتماثلين عند دالة حامضية مثلى 8 و 6 وعند درجة حرارة

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40 و 50 درجة مئوية على التوالي. تم الحصول على حزمة منفردة نقية باستخدام تقنية الهجرة الكهربائية ووجد أن الوزن الجزيئي التقريبي هو 42 كيلو دالتون لكلا المتماثلين.
تناولت الدراسة تحضير المركب ثنائي الاستر ، 1.4–دياسيتوكسي بنزين ، والذي تم تشخيصه بواسطة GC– MS و ^{1}H NMR الطيفي. عند دراسة نوع التثبيط ، أظهر المركب أعلاه تثبيطا تنافسيا للانزيم ، حيث زادت قيمة Km من 0.013 إلى 2.857 ملي مولار ، وكانت قيمة Vmax تساوي 0.25 وحدة إنزيمية / مل / دقيقة. تم حساب ثابت التثبيط K وقيمته كان 0.0319 ملم. اظهرت مستخلصات قشور الرمان بالكلوروفورم وخلات الإيثيل تأثيرا تنشيطيا لفعالية الليباز. يهدف هذا البحث الى الكشف عن وجود انزيم اللايبيز في لب جوز البقان وتنقيته بالطرق الكيموحيوية لمعرفة خصائصه ووزنه الجزيئي ومن ثم محاولة تثبيطه بمركب محضر مختبريا له تركيب مشابه للمادة الاساس التي يعمل عليها الانزيم.

1.0 Introduction

The pecan [*Carya illinoinensis* (Wangenh.) K. Koch] is a vegetable that belongs to Juglandaceae family. The pecan tree is a big tree that grows to be about 20 to 30 meters tall, with a crown diameter of 12 to 20 meters and a trunk circumference of 2 meters in ideal conditions. Pecan is a deciduous tree with alternate, pinnate leaves that grow up to 45 centimeters in length and are made up of nine to fifteen leaflets [1-2]. Pecans are notable among foods for their medical benefits, as they contain phytosterols, mono- and polyunsaturated fatty acids, phenolic compounds, and essential nutrients that decrease the severity of diseases [3]. According to Eagappan & Sasikumar [4], daily intake of nuts has been linked to several health benefits, including protection from oxidative damage, reduction of LDL-C, and prevention of chronic diseases, in addition to their antidiabetic and anticancer properties. They can help to reduce inflammation by decreasing the amount of inflammatory mediator molecules produced. Also, they can counterbalance the pro-inflammatory effects of a diet high in saturated fatty acids, which are commonly overconsumed in the Western diet [5].

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are monomeric proteins with molecular weights ranging from 19 to 60 kDa that are ubiquitous and important in biology and industry. Natural lipases hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Esterases, as well as lipases, can hydrolyze carboxylic esters bonds [6-7]. Lipase enzymes have the unique ability to hydrolyze the ester bonds of poorly soluble substrates at the interaction between substrate and water. It has attracted a lot of consideration in latest years because of its assorted biotechnological applications [8-9]. Lipase is stable and can be obtained from animals, plants, and various microorganisms. Nature's catalysts, not only microbial lipases, are widely used commercially [10].

The lipase activity relies on many factors like surface area, extreme mild conditions, and pH. Purification entails not only isolating lipases from contaminants and sources, but also improving their stability, activity, and shelf life. Only when lipases have been purified to homogeneity can various studies on their structural orientation be conducted [11]. Because of their vast properties and ease of mass production, lipases among the most important enzymes. Lipases are useful for synthesis and have a wide range of applications in food preparation, fats and oil processing, paper manufacturing, and pharmaceuticals and cosmetics production [12]. Pomegranate peel is food waste obtained during the production of pomegranate juice and is unfit to eat. The peels make up about 40% of the total fruit weight. As a result, processing waste such as peel has emerged as an ideal substrate for extracting valuable bioactive compounds [13]. Phytochemicals have been found in various parts of the pomegranate fruit, including the juice, seeds, and peels, according to previous research [14]. Many polyphenolic compounds, such as gallic acid, caffeic acid, and coumarin, were separated from pomegranate peels by HPLC [15]. Solvent extraction is a method for isolating plant antioxidants [16]. Due

to its various chemical and polar properties, the phenolic content of various plant materials is dependent on the nature of the solvent used [17]. Many studies have discovered that the phenolic content of plants varies depending on the polarity of the solvent [18]. Pomegranate peel extracts have recently drawn attention due to their potential as natural food preservatives [19].

2.0 Materials and methods

2.1 Plant material and Chemical

In this paper, pecan nut was used, which was seasonally obtained from the local markets in the city of Ankara in November, 2020, and was classified in the Faculty of Education, Department of Life Sciences (*C. illinoinensis*) (Picture 1). The process of isolating the shell from the pulp was conducted directly for subsequent experiments on the pulp. All chemicals used were of analytical grade and obtained from Sigma and Molecule Chemical Company.

Picture 1- Photographs of the Pecan Tree and Fruits

2.2 Protein Determination

Lowery method was used to estimate the concentration of protein by using bovine serum albumin as a standard [20].

2.3 Preparation of crude Lipase extraction

Fifty gram of pecan nut karnel was mixed with cold distilled water in a ratio of 1:4 weight: volume and crushed for ten minutes using the Blender machine. Then, it was frozen by adding liquefied nitrogen and allowed to melt at room temperature and the process was repeated three times. The mixture was filtered through several layers of gauze and the extract was separated by a refrigerated centrifuge for 15 minutes at a speed of 10,000 xg. The volume of the filtrate was measured and was found to be 70 ml.

2.4 Lipase assay

The activity of lipase enzyme was estimated using the method of Winkler and Stuckman [21]. The method includes the decomposition of the substrate p-nitrophenyl acetate, a reaction that is stimulated by the lipase enzyme. The method is based on measuring the absorbance of the para-nitrophenol at the wavelength of 410 nm.

2.5 Purification of Lipase

The procedures for lipase purification from pecan involved the following steps:

2.5.1 Step 1: Ammonium sulfate precipitation

Solid ammonium sulfate was gradually added to the crude extract, with a degree of saturation ranging 0-70%, taking into account the continuous stirring for one hour by the magnetic stirrer at 4 °C. The solution was left in the refrigerator for 24 hours to settle. The precipitate was separated with a refrigerated centrifuge for 20 min at 10,000 xg. The filtrate was discarded and then dissolved with the lowest possible amount of buffer phosphate buffer pH 7.1 at a concentration of 10 mM [22].

2.5.2 Dialysis

Dialysis was achieved by putting enzyme solution in a dialysis tube against the buffer on a stirrer at 4˚C with six-time buffer changes. This step was performed to remove the excess of ammonium sulfate ions.

2.5.3 Step 2: CM-cellulose column chromatography

The enzyme solution was placed onto CM-cellulose column (2×25 cm) before being equilibrated with buffer. The purification was accomplished by stepwise elution with increasing concentration of buffer at a flow rate of 1 ml/ min. Fractions showing tyrosinase activity were collected after monitoring at intervals of an individual fraction (3ml) and then lyophilized [23].

2.6 Characterization of Lipase

By using 1.5 mM of P- nitrophenyl acetate as substrate, the determination of lipase activity was carried out at temperatures between 20 and 50 °C. The optimum pH was measured by using 10 mM potassium phosphate buffer (pH of 6-8).

2.7 Determination of Molecular weight

The molecular mass of the lipase purified after ion-exchange chromatography was estimated by using polyacrylamide of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by following the Laemmli method [24]. In bathwater, the protein solution was heated for 5 minutes at 100 °C. The stacking gel was run at a voltage supply of 70 V for about 45 minutes, and the separating gel was run at a voltage supply of 100 V for about 90 minutes. The Coomassie Blue staining technique was used to visualize the protein bands, and the molecular weight was calculated using eight proteins of known molecular weights that ranged 14-116 kDa.

2.8 Preparation of 1,4-diacetoxybenzene

In a round bottomed flask, concentrated sulphuric acid (1 drop) was added to mixture of hydroquinone (2.2 g, 20 mmol) and acetic anhydride (4 ml, 40.4 mmol). Then, at room temperature, the reaction mixture was stirred until the hydroquinone was dissolved (exothermic reaction). The reaction was cooled and poured unto ice-water (30 ml), to bring about the product to precipitate [25-26]. The precipitated product was collected by filtration, washed with cold H_2O, and dried in a desiccator to give, following recrystallization from ethanol, the pure diacetate product as a white solid (3.7 g, 95%). (m.p= 121 – 123 ^oC, lit.,121 and 123-124 ^oC); δ_H (500 MHz, $CDCl_3$) 7.09 (4H, s, Ar-H), 2.29 (6H, s, CH_3). m/z (GC-MS, EI^+) 194 ($[M]^+$, 10%), 152 ($[MH\text{-}CH_3CO]^+$, 25%), 110 (100%) found, 43 (30%) found.

2.9 Pomegranate peel extraction

The pomegranate was purchased from local supermarket in Mosul. The pomegranate peel was isolated from seeds manually then washed with water and dried under dark. A weight of 50 g of dried peel was smashed using a mortar, then extracted by cold percolation with 350 ml of methanol for 12 h to offer, after reducing the solvent under vacuum, the desired methanol extract of 19 g. Water (150 ml) was then added and extraction was performed with 50 ml chloroform three times to offer the $CHCl_3$ extract of 0.303 g. The residue of water from the first extraction above was extracted three times by adding ethyl acetate (50 ml) to yield an ethyl acetate extract of 0.73 g.

2.10 Measurement of lipase activity against the extracts or 1,4-diacetoxybenzene

A volume of 0.2 mL of partially purified lipase was incubated with 0.1 mL of 1,4-diacetoxybenzene (1-10 Mm) and pomegranate extracts (0.5-4 mg/ml) for 30 minutes at 37 °C separately. After that, the activity of lipase enzyme was estimated in the presence of the substrate at a concentration of 1.5 mM.

2.11 Inhibition type test

The enzymatic reaction was carried out in order to verify the type of inhibition of the partially purified lipase by incubating 0.1 ml of 1,4-diacetoxybenzene (6 mM) with 0.2 mL of the

enzyme for 30 minutes [27]. The enzyme activity was estimated using different concentrations of the substrate that ranged 0.25-3 mM.

2.12 HPLC analysis

The sample was analyzed by high performance liquid chromatography, model (SYKAM) Germany. Pump model: S 2100 Quaternary Gradient Pump, Auto sampler model: S 5200, Detector: UV (S 2340), and Column Oven model: S 4115. ODS C-18 column (25 × 4.6 mm). An aliquot (10 µl) was injected and eluted at the temperature of 40°C according to Muchuweti *et al. (2007)* [28], using a solvent of Methanol: D.W: formic acid (70:25:5 V/V) as the mobile phase. At the wavelength of 280 nm, the analysis was accomplished at a flow rate of 1 mL/min.

3.0 Results and Discussion

3.1 Purification of lipase from pecan karnel

The results shown in Table 1 indicate that the specific activity of lipase after the sedimentation process was 1,047 units/mg protein, meaning that it was multiplied by 7.2 times. The amount of recovery of the total activity of the enzyme was 79.2% compared to the crude extract. After the precipitation process, the dialysis technique was applied as a second step in purifying the enzyme by removing ammonium sulfate used in the previous step, as well as peptides, amino acids, ions, and some small molecular weight compounds. The results shown indicate that lipase specific activity after this process is 1.66 units/mg protein, that is, it was multiplied by 10.84 times compared to that of the crude extract. After passing the protein solution, which was produced from the dialysis, through the column containing cation exchanger CM-cellulose, we obtained tow lipase isoenzyme (I, II) (Figure 1) with specific activity values of 2.66 and 1.7 U/mg protein, respectively (Table 1).

Table 1- Lipase purification steps from pacan karnel

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Specific activity (U/mg protein)	Yield %	Purification Fold
Crude extract	70	3.08	0.476	0.153	%100	**1**
Ammonium sulfate	13	0.351	0.377	1.047	79.2	**7.2**
Dialysis	13	0.078	0.13	1.66	27.3	**10.84**
CM-Cellulose column						
Peak I	50	0.60	1.6	2.66	336.1	**17.38**
Peak II	**50**	**1.65**	**2.8**	**1.7**	**588.2**	**11.11**

Sunflower seeds contain a high percentage of oils, which suggests the possibility of the presence of the enzyme lipase significantly. The lipase was purified from sunflower seeds by Al-Haidari and his group [29], where two peaks were shown using gel filtration chromatography. They had 4.644 and 6.482 units/mg protein. The lipase was identified from pistachio khunjuke karnel, and the purification fold was 8.216 times compared to that of the crude enzyme [30]. Priyanka *et al.* [31] isolated the lipase enzyme from *Pseudomonas reinekei* bacteria, and it was observed that one peak appeared, with a specific activity of 4.23 units/mg protein, while the activity recovery was 13.72%.

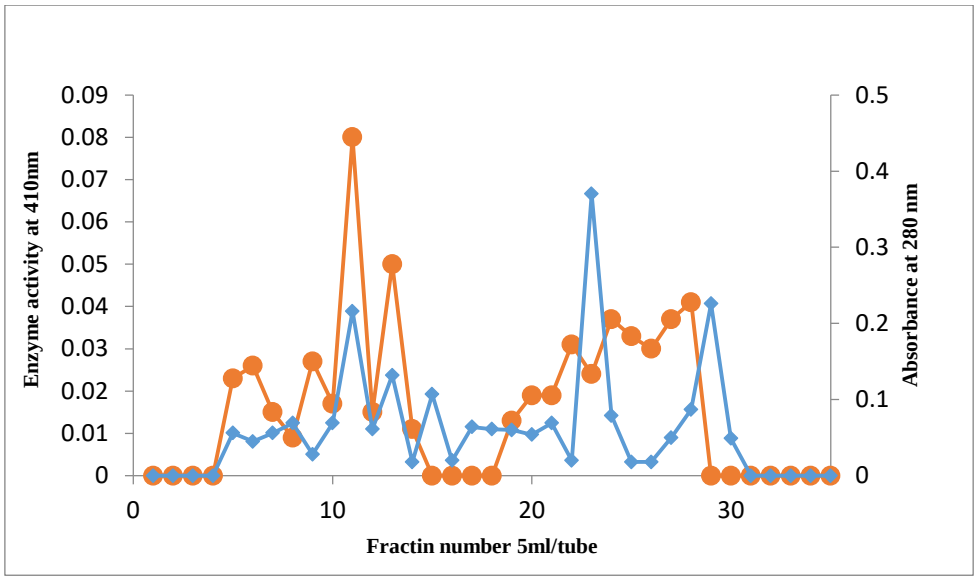


Figure 1- Purification of lipase by CM- Cellulose chromatography

The results obtained indicate that the highest activity of both purified isoenzymes was achieved at pH = 8 and 6 by using the buffer solution KH_2PO_4-Na_2HPO_4, as shown in Figures 2 and 3, respectively.

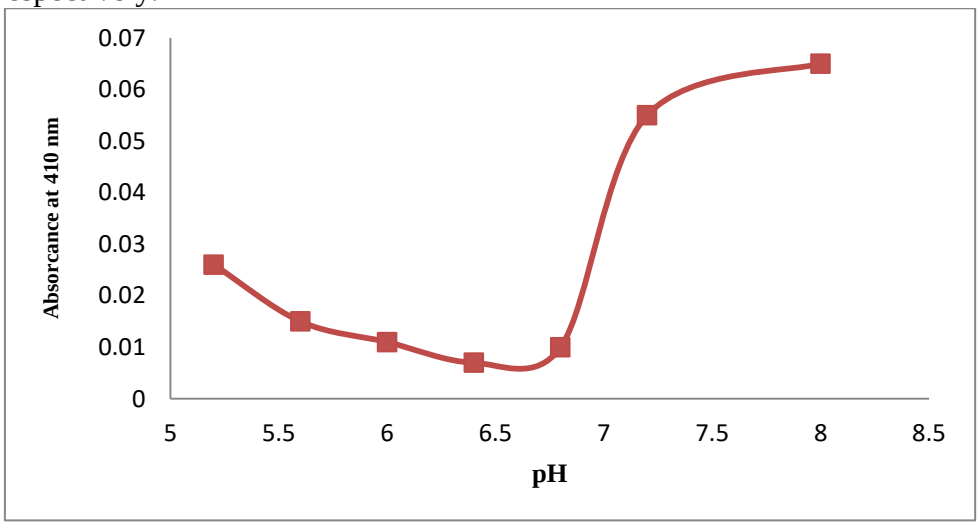


Figure 2- Effect of pH on the purified lipase activity (peak I).

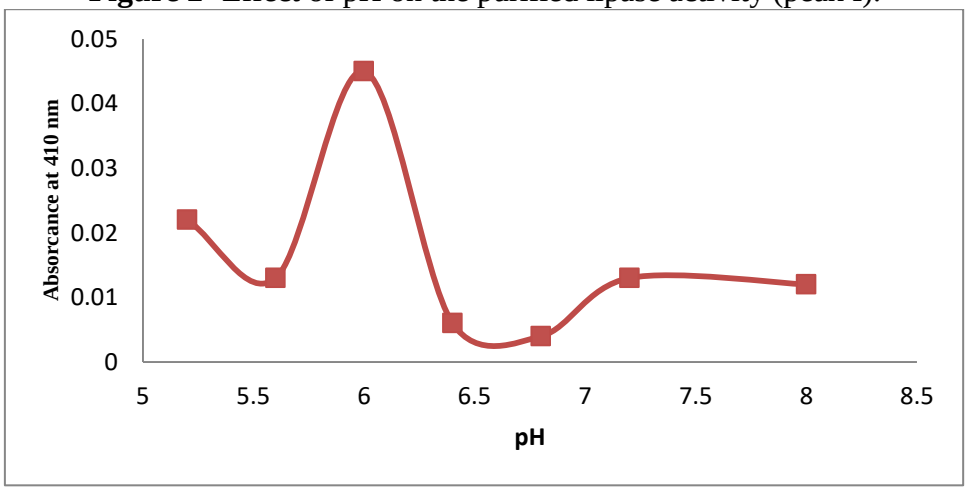


Figure 3- Effect of pH on the purified lipase activity (peak II).

These results are close to those which found that the optimum pH of the enzyme extracted from the palm tree mesocarb is 7 [32]. It was also noted that lipase purified from pistachio khinjuke karnel had a maximum activity at pH 7.5 [30]. While the maximum activity of lipase from *Aspergillus fumigatus* was at pH 9 [33].

Regarding the effect of pH, as it is known, each enzyme has an optimum pH at which it shows the highest activity. At higher or lower values than the optimum pH, the secondary and tertiary structures of the enzyme molecule as a whole or of the active site of this enzyme change, thus affecting its activity. Generally, enzymes are affected by increasing or decreasing the temperature because the enzymes, due to the nature of proteins, are sensitive to thermal changes [34].

In this study, lipase activity was estimated at different temperatures, ranging 20-50 °C, and incubation time of 15 minutes. An initial increase in temperature was obtained, which led to an increase in the enzyme activity for each of the two peaks of the pecan karnel, reaching a maximum of 40 and 50 m^{o}, as shown in Figures 4 and 5, respectively**.**

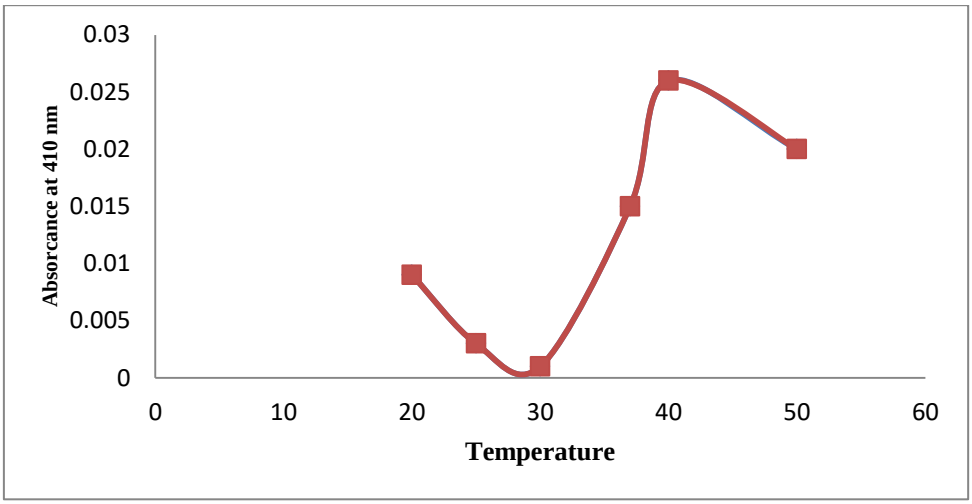


Figure 4- Effect of temperature on the purified lipase activity (peak I).

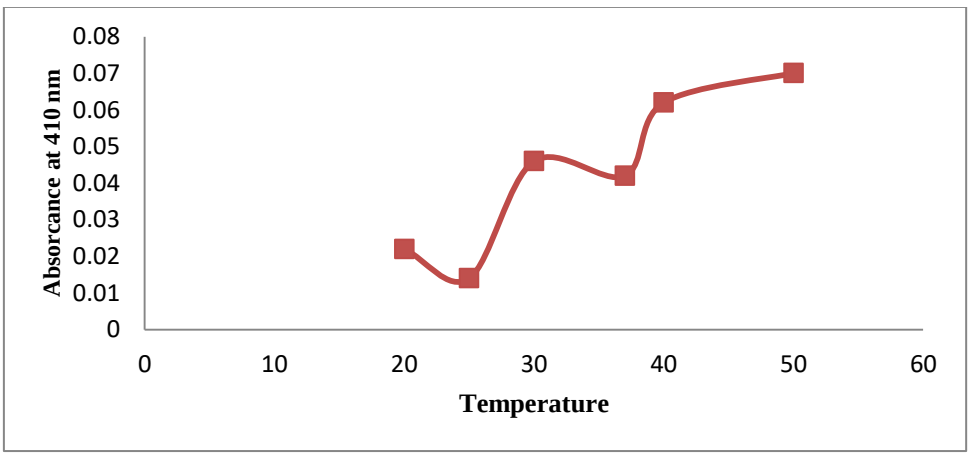


Figure 5- Effect of temperature on the purified lipase activity (peak II).

The results for peak I are consistent with what was found in two previous studies of purified lipase from pistachios khinjuke karnel and *Aspergillus fumigatus*, respectively, where the optimum temperature was at 40°C [30,33]. Okunwaye and his group [32] found that the optimum temperature of the enzyme molecule purified from a fine palm tree was 40 °C. Also, lipases were purified from yeast, where the optimal activity was achieved at 35 °C [35]. Prajapati *et al.* [36] reported that the extraction of lipase from the bacteria *Cellulomonas*

flavigena UNP3 yielded an optimally activity at 30 °C. However, the optimal activity of lipase extracted from *Acinetobacter sp*. was found to be reached at 50°C [37].

3.2 Molecular weight

The results indicated that the peaks of the activity of lipase extracted from the pecan karnel, which were generated by ion exchange chromatography, appeared in the form of a single clear band for each isoenzyme when applying SDS-PAGE electrophoresis technique (Figure 1). The molecular weight of the partially purified lipase was approximately calculated to be 42 kDa for both bands (Figure 6). This molecular weight is close to that of lipase purified from the pistachio khinjuke karnel, as its molecular weight was approximately 43.5 kDa [30]. However, it is higher than that of lipase purified from palm, which had a molecular weight of 35 kDa [32]. While it was lower than that of the enzyme purified from *Pseudomonas reinekei* using ion exchange chromatography, which was found to be 50 kDa [31].

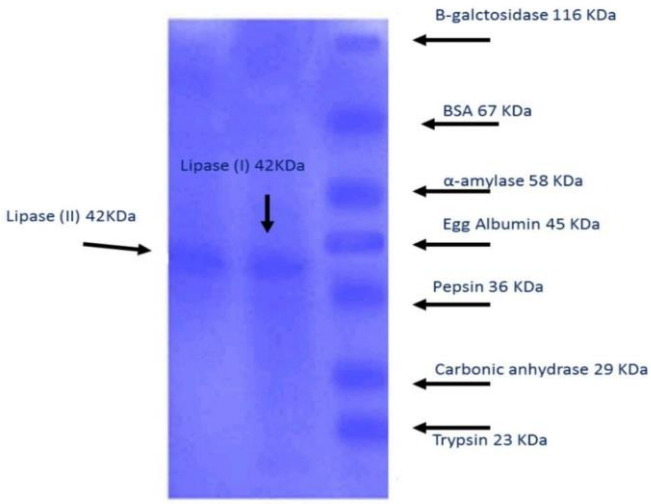
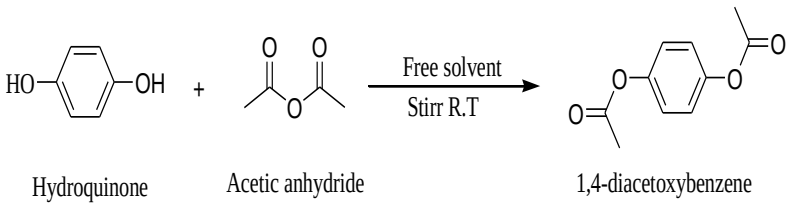


Figure 6-SDS-PAGE - electrophoresis of lipase isoenzymes purified from pecan karnel with standard proteins.

3.3 Preparation of 1,4-diacetoxybenzene

A literature search disclosed several methods described by Prichard, in which 1,4-diacetoxybenzene could be prepared by adding acetic anhydride to hydroquinone in the presence of sulfuric acid, to give, following recrystallization from ethanol, the desired product in 95% (Scheme 1) [25].



Scheme 1-Preparation steps of 1,4-diacetoxybenzene.

Confirmation of diacetate product was obtained by GC-MS, which displayed a 100% conversion of SM and the M^+ peak was observed at Rt = 13.149 with m/z = 194 ($[M]^+$) and 152 ($[M\text{-}CH_3CO]^+$). Furthermore, the ^1H NMR spectrum displayed a characteristic CH_3 signal

of acetyl group at δ= 2.29 and lacked the characteristic OH signal at δ= 8.6 [38] for hydroquinone (Figures 7 and 8).

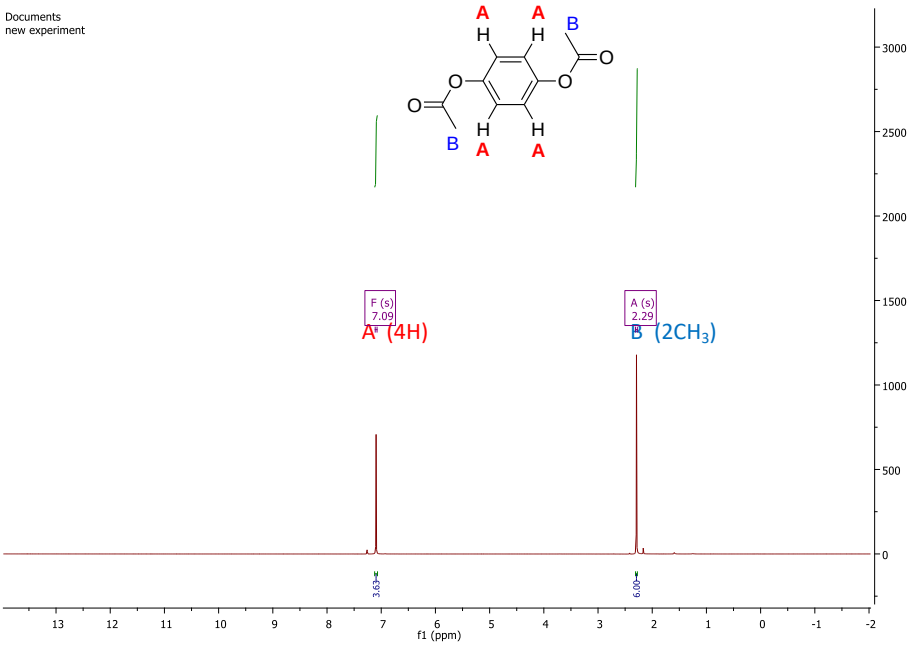
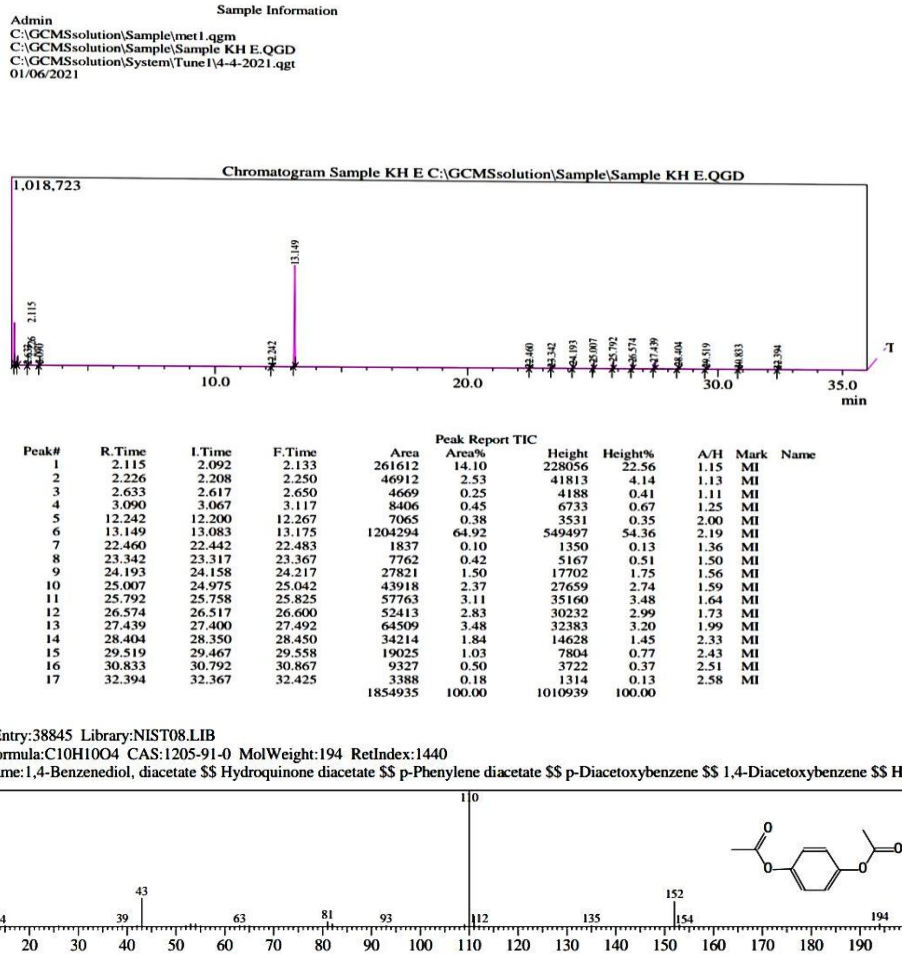


Figure 7- ^{1}H NMR (500 MHz, $CDCl_3$) for 1,4-diacetoxybenzene.



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.115	2.092	2.133	261612	14.10	228056	22.56	1.15	MI	
2	2.226	2.208	2.250	46912	2.53	41813	4.14	1.13	MI	
3	2.633	2.617	2.650	4669	0.25	4188	0.41	1.11	MI	
4	3.090	3.067	3.117	8406	0.45	6733	0.67	1.25	MI	
5	12.242	12.200	12.267	7065	0.38	3531	0.35	2.00	MI	
6	13.149	13.083	13.175	1204294	64.92	549497	54.36	2.19	MI	
7	22.460	22.442	22.483	1837	0.10	1350	0.13	1.36	MI	
8	23.342	23.317	23.367	7762	0.42	5167	0.51	1.50	MI	
9	24.193	24.158	24.217	27821	1.50	17702	1.75	1.56	MI	
10	25.007	24.975	25.042	43918	2.37	27659	2.74	1.59	MI	
11	25.792	25.758	25.825	57763	3.11	35160	3.48	1.64	MI	
12	26.574	26.517	26.600	52413	2.83	30232	2.99	1.73	MI	
13	27.439	27.400	27.492	64509	3.48	32383	3.20	1.99	MI	
14	28.404	28.350	28.450	34214	1.84	14628	1.45	2.33	MI	
15	29.519	29.467	29.558	19025	1.03	7804	0.77	2.43	MI	
16	30.833	30.792	30.867	9327	0.50	3722	0.37	2.51	MI	
17	32.394	32.367	32.425	3388	0.18	1314	0.13	2.58	MI	
				1854935	100.00	1010939	100.00			

Figure 8- GC-MS, EI^{+} for 1,4-diacetoxybenzene.

3.4 Lipase inhibition

The inhibitory effect of 1,4-diacetoxybenzene at different concentrations (1-10 mM) on the activity of the purified lipase was investigated. Table 2 shows that these concentrations led to a decrease in the activity of the enzyme compared to its activity in the absence of the inhibitor. The highest percentage of enzyme inhibition was 38.3% at 6 mM concentration. Accordingly, this concentration was adopted in the study of the type of inhibition.

Table 2-Effect of 1,4-Diacetoxybenzene on the inhibition of lipase enzyme purified from pecan.

Inhibitor conc.	Activity at 410 nm	Inhibitory effect %
Control	0.738	%100
1	0.574	22.3
2	0.607	17.7
3	0.490	33.6
4	0.494	33.1
5	0.500	32.2
6	0.455	38.3
7	0.614	16.8
8	0.614	16.8
9	0.665	9.9
10	0.513	3.5

3.4.1 Inhibition mode

The inhibition of the purified lipase activity was studied by the presence of the inhibitor 1,4-diacetoxybenzene at a concentration of 6 mM. The inhibition mode was verified by drawing Lenweaver-Burk plot (Figure 9) using different concentrations of the substrate (para-nitro phenyl acetate) ranging 0.25-3 mM. The results indicate that the mode of inhibition was competitive, as the value of Vmax was constant and (0.25 enzymatic units /ml/ min), while the value of Km increased from 0.013 mM to 2.857 Mm. The value of the inhibition constant Ki was calculated to be 0.0319 mM.

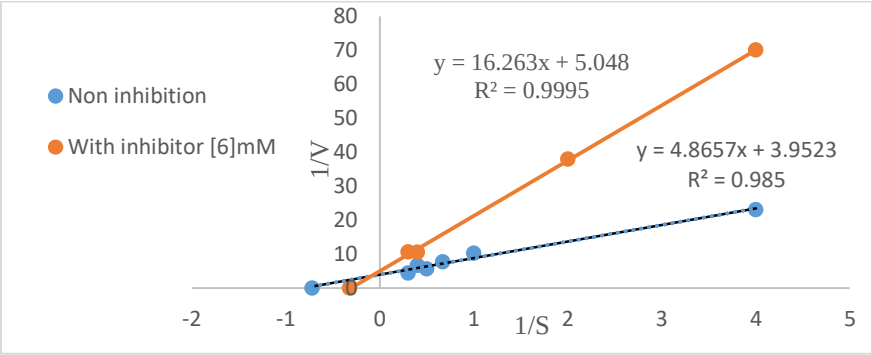


Figure 9- The inhibition mode of the purified lipase by 1,4-diacetoxybenzene.

The compound 1,4-diacetoxybenzene prepared in the laboratory is a diester compound, as it contains two ester groups in the Para site. This basis can explain that the type of inhibition of this compound was competitive inhibition. In a previous study, the inhibition type was found to be non-competitive for the lipase extracted from the pistachio khinjuke karnel, by using the two compounds of Quercetin rutinoside and Melatonin, where the values of Ki were 1.025 and 1.006 mM, respectively [30]. It was also revealed that the extract of *Levisticum officinale* showed mixed inhibition of pancreatic lipase enzyme, while the inhibition was non-competitive when using extracts of *Quercus infectoria*, *Eucalyptus galbie*, and *Rosa damascene* [39]. Also, the inhibition of lipase enzyme isolated from the fungus *Candida*

rugosa and wheat seeds were followed up by polysaccharide chitosan, and it was found that the inhibition constant values were 1.4 and 0.9 mM, respectively [40].

Effects of pomegranate peel extracts on the activity of lipase enzyme

Table 3 shows the effects of chloroform extract on enzyme activity, as the highest percentage of the extract effect was at a concentration of 0.2 mg/ml and the lowest at a concentration of 0.4 mg/ml.

Table 3- Effects of chloroform extract on the activity of lipase.

Extract conc.(mg/ml)	Activity at 410 nm	Extract effect %
Control	0.973	100%
0.1	0.992	101.95
0.2	1.042	107.09
0.4	0.979	100.61
0.6	1.008	103.59
0.8	1.002	102.98

Table 4 also shows the effects of ethyl acetate extract on enzyme activity, where the highest percentage of the extract effect was at a concentration of 0.6 mg/ml and the lowest percentage was at 0.8 mg/ml

Table 4- Effects of ethyl acetate extract on the activity of lipase.

Extract conc.(mg/ml)	Activity at 410 nm	Extract effect %
Control	0.601	100%
0.1	0.624	103.82
0.2	0.655	108.98
0.4	0.652	108.15
0.6	0.739	122.96
0.8	0.611	101.66

The results of HPLC analysis (Figures 10 and 11) indicate the appearance of several bands belonging to the chromatograms of standard phenolic compounds (gallic acid, chlorogenic acid, tannic acid, para-coumaric acid, and quercetin) with concentrations and retention times specific to each compound, as shown in Tables 5 and 6.

Table 5-Concentrations and retention times for ethyl acetate extract by using HPLC.

Standard compounds	Rt (min)	Retention time of pomegranate peel extract (min)	Peak area	Phenolic compounds conc. in extract mg/gm
Gallic acid	1.800	1.990	2227.891	5.510
Chlorogenic acid	3.000	3.033	5982.901	24.12
Tannic acid	4.300	4.300	7816.958	27.92
Para-coumaric acid	4.900	4.983	9882.073	89.84
Quercetin	6.000	6.060	8405.671	11.39

A

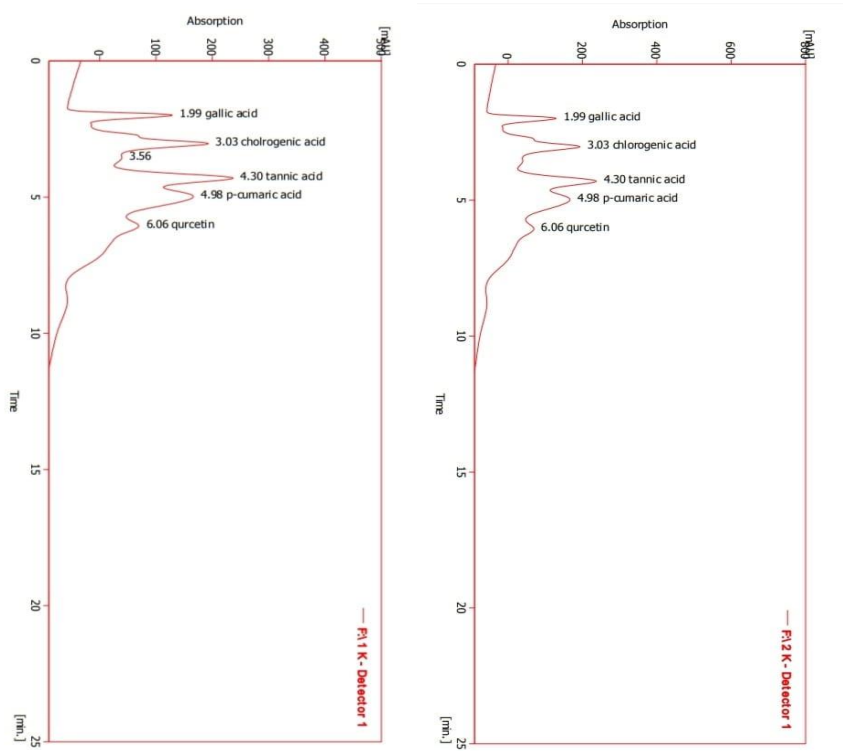


Figure 10 & 11- Phenolic compounds chromatography of pomegranate peel extracted by ethyl acetate (**A**) and chloroform (**B**)

Table 6-Concentrations and Retention times for chloroform extract from HPLC

Standard compounds	Rt (min)	Retention time of pomegranate peel extract (min)	Peak area	Phenolic compounds conc. in extract mg/gm
Gallic acid	1.800	1.990	846.5080	2.095
Chlorogenic acid	3.000	3.033	1375.782	5.550
Tannic acid	4.300	4.300	917.9320	3.280
Para-coumaric acid	4.900	4.983	1384.202	12.58
Quercetin	6.000	6.060	130.9350	0.177

It is evident from the two tables that the concentration of para-coumaric acid was the highest in the two extracts, reaching 89.84 and 12.38 mg/g, respectively, while quercetin was the lowest, reaching 11.39 and 0.177 mg/g, respectively.

Pomegranate peel is a rich source of tannins, flavonoids, and other phenolic compounds [41-42]. HPLC analysis was used in a previous study to identify phenolic compounds in pomegranate peel extracts using ethanol and isopropanol. Polyphenolic compounds, such as p-coumaric acid, caffeic acid, ellagic acid, cinnamic acid, quinic acid, and iso-ferulic acid, were discovered [13]. The extraction of various phenolic compounds from pomegranate peels was demonstrated [43]. Mansour *et al.* [44] exhibited the occurrence of caffeic, ellagic, vanillic, gallic, and p-coumaric acids as well as quercetin in pomegranate peel extract. HPLC was used to find chlorogenic acid, ferulic, coumaric, and cinnamic acids in pomegranate peel methanolic extract [18].

Conclusions

In the current study, two peaks of lipase were purified and characterized from pecan karnel by using paranitro phenyl acetate as substrate. The molecular mass of both isoenzymes was found to be 42 kDa by using electrophoresis. The enzyme was inhibited competitively by

using 1,4-diacetoxybenzene compound, but activated with pomegranate peel extracts. Also, some phenolic compounds were identified in pomegranate peel extracts by HPLC technique. Several peaks appeared that belong to the chromatograms of gallic acid, chlorogenic acid, tannic acid, para-coumaric acid and quercetin, with concentrations and retention times specific to each compound.

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