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## Extraction and Purification of Lipases Enzyme from Germinating Seeds of Four Crops

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### Abstract

Lipase enzyme has attracted a lot of attention in recent years because of its diverse biotechnological applications. The present study was conducted to screen germinated seeds of four crops, namely sunflower (*Helianthus annuus*), flax (*Linum usitatissimum*), peanut (*Arachis hypogaea*) and castor bean (*Ricinus communis*), for the activity of their lipases. The study also included the extraction and purification of lipase from the seeds of the most promising crop using different solvents.

The results indicated that the maximum enzymatic activity (0.669 U/ml) was obtained when 0.1 M Tris-HCl buffer extract was used after 3 days of seed germination of all the tested species, as compared to the other test solvents (acetone and water). Sunflower germinated seeds showed the highest lipase activity, which was higher by 159.67, 185.32, and 285.90 % over the activities of castor bean, flax, and peanut seeds, respectively. Among the used ranges of saturation of ammonium sulfate, the ratio of 70% was the best in precipitating the crude enzyme, showing a highest specific activity of 2.576 U/ mg protein.

The first stage of gel filtration chromatography column by Sephadex G-200 indicated the presence of two non-identical peaks, one for protein and another for lipase activity, between the fractions of 18 to 23. The active fractions were pooled and loaded again in the Sephadex G-200 column and the eluted fractions showed two identical peaks, one for protein and another for lipase activity, between the fractions of 19 to 23. The final purification step by gel filtration showed a specific activity of 6.482 U/mg proteins with a yield of 38.75 % and 11.33 folds of time of purification. The study revealed that sunflower seeds are a better source of lipase as compared to the other used plant seeds, which can be used in different industries.

**Keywords:** Lipase, Extraction, Purification, Different types of plant seeds.

### استخلاص وتنقية انزيمات اللايباز من البذور النامية لأربعة محاصيل زراعية

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### الخلاصة

لأنزيم اللايباز أهمية كبيرة وخاصة خلال السنوات الأخيرة بسبب تنوع التطبيقات الحيوية التي يستخدم بها، لقد تركزت الدراسة الحالية على دراسة فعالية هذا الإنزيم في البذور النامية لأربعة أنواع نباتية هي زهرة

الشمس, الكتان, فستق الحقل والخروج ثم استخلاص باستخدام مذيبات مختلفة وتنقية الانزيم من بذور النبات الذي اعطى اعلى فعالية.

بينت النتائج ان فعالية الانزيم تزداد في البذور بعد بدء عملية الانبات وتصل الى اعلى فعالية بعد 72 ساعة من الانبات وفي جميع الانواع النباتية والمذيبات المستخدمة. واعطى المحلول الدائري Tris-HCl اعلى فعالية للانزيم مقارنة بالمذيبات الاخرى المستخدمة (الاسيتون والماء المقطر). واعطت البذور النابتة لزهرة الشمس اعلى فعالية لانزيم اللايباز والتي تفوقت على بذور الكتان والخروج وفستق الحقل بنسبة 185.32, 159.67, 285.90 % على التوالي. وكان اعلى نشاط للانزيم قد سجل في بذور زهرة الشمس النابتة (0.669 يونت /مل) والمستخلصة بالمحلول الدائري Tris-HCl.

وسجلت نسبة التشبع ( 70 %) بكبريتات الامونيوم اعلى فعالية نوعية ( 2.567 يونت/ملغم بروتين) عند ترسيب انزيم اللايباز المستخلص مقارنة بنسب التشبع المستخدمة. وتبين نتائج المرحلة الاولى للفحص الكروماتوغرافي بالترشيح الهلامي باستخدام الـ Sephadex G-200 وجود قمتين غير متطابقة واحدة للبروتين والاخرى للفعالية الانزيمية بين الاجزاء 18-23 وتم جمع الاجزاء الفعالة ووضعها مرة اخرى في عمود التنقية بوجود الـ Sephadex G-200. تم الحصول على قمتين متطابقة واحدة للبروتين واخرى للفعالية الانزيمية عند الاجزاء 19-23. وتبين نتائج المرحلة الاخيرة للتنقية بواسطة الترشيح الهلامي ان الفعالية النوعية كانت 6.482 يونت/ملغم بروتين ويحصل 38.75% وان عدد مرات التنقية كان 11.33.

اوضحت الدراسة ان بذور نبات زهرة الشمس تعد مصدرا جيدا لانزيم اللايباز لاستخدامه في الصناعات المختلفة اكثر من غيره من بذور الباتات المستخدمة في الدراسة.

## Introduction

Lipases (triacylglycerol ester hydrolase, EC3.1.1.3) are enzymes that catalyze the total or partial hydrolysis of fats and oils, releasing free fatty acids, diacylglycerol, monoglycerol and glycerol [1]. The purified enzyme is used in a wide range of food industries [2], detergent industries [3], personal care products and cosmetics industries [4]. Also, It is used in biodiesel production [5], pharmaceutical industry [6], polyesters industry [7] and fatty acids production [8].

Lipases can be extracted from microorganisms, including bacteria [9] and fungi [10], and from animals [11] and plants [12, 13]. Plant lipases are extracted from different parts of the plant, such as fruits, latex and seeds. However, seeds, especially oil-seeds, are the main parts which contain higher concentrations of lipases. The lipases in seeds play an important role in the growth of the embryo, providing it with energy for growth and development. Therefore, lipases are present in higher levels during the period of seeds germination [14].

Many researches reported that lipases activities in plants are varied according to the species under study and the condition of extraction; therefore, the screening process and the determination of the optimum conditions of extraction are essential steps for the selection of plant species with the higher activity of lipases [15,16].

The present study was conducted to screen seeds of different crops, namely sunflower (*Helianthus annuus*), flax (*Linum usitatissimum*), peanut (*Arachis hypogaea*) and castor bean (*Ricinus communis*) for their lipase activities. We also determined the best solvent for extraction and selected the most promising species to purify its lipase and study its characteristics.

## Materials and Methods

### Seeds collection and germination

Seeds of four plant species were obtained from the Directorate of Seeds Certificate, Abu-Ghariab, Baghdad. Uniform and healthy looking seeds were taken from each plant species, soaked separately in distilled water for six hours, placed on moist filter paper inside Petri dishes and allowed to germinate for 24, 48, 72, 96 and 120 h under dark condition at room temperature (28±2 °C). The emergence of radicles was considered as an indication of germination.

### Lipase extraction

Seed coats were removed from the germinated seeds and 20 g of fresh weight of cotyledons at each germination time were separately homogenized for 5 minutes with 80 ml of Tris- HCl buffer (0.1M, pH = 7.5), distilled water (DW), and cold acetone solvents. The extracts of Tris- HCl buffer

and DW were filtered through four layers of cheese cloth and centrifuged at 10000 rpm using cooling centrifuge (4°C) for 30 minutes. The upper fat layer was removed by a spatula and the supernatant was taken as a source of crude lipases [12].

For lipase extraction by acetone, the homogenized extract was centrifuged at 3000 rpm using cooling centrifuge at 4°C for 30 min. The pellet obtained was dissolved in 100 ml of DW then centrifuged at 7500 rpm. The supernatant was obtained as a source of crude lipase [16].

#### **Assay of lipases activity at different germination times**

Lipase activity in the extract of each plant species was determined according to Rahman *et al.* [17]. The reaction mixture consisted of 1ml of crude enzyme and 2.5 ml of olive oil emulsion (10 g of each of olive oil and Arabic gum was mixed with 200 ml of 0.1M Tris-HCl buffer, pH = 7.5 and homogenized by vortex for 10 min.). A volume of 0.02 ml of 20 mmol CaCl<sub>2</sub> .2H<sub>2</sub>O was added to the mixture and shaken at 150 rpm by a shaking water bath for 20 min at 30 °C. The reaction was stopped by adding 5 ml of isoctane and 1 ml of 6 N HCl. The upper layer (about 4 ml) was taken in another test tube and 1 ml of the reagent (5% cupric acetate/ pyridine) was added. The free fatty acids were determined by using a spectrophotometer at 715 nm. A standard curve of oleic acid was made to determine the lipase activity according to the amount of free fatty acids. The amount of lipase enzyme that liberated 1 μ mol free fatty acids in 1 min at 30°C was defined as one unit of lipase enzyme activity [17].

Based on the results of the previous experiments, the highest lipases activity appeared in seeds of all test plants at 72 h after sowing and started to slightly decrease thereafter; also, the seeds of sunflower showed the highest lipase activity as compared to the other plant species. Therefore, all the subsequent experiments of purification, characterization, and application were carried out on sunflower lipases.

#### **Protein estimation**

Lowry's method [18] was used to determine protein concentration of the enzyme supernatants using a standard curve of Bovine Serum Albumin (BSA) at 1mg/ml. Different concentrations (0- 100 μg/ml) of BSA were prepared and the absorbance was read at 600 nm to obtain the standard curve. The concentration of protein in the extract of sunflower seeds was estimated from the standard curve.

#### **Purification of Sunflower lipases**

##### **Ammonium sulfate precipitation and dialysis**

For the purification step, ammonium sulfate, which is required to precipitate the lipase enzyme, was optimized by adding it at varying concentrations of saturation (40%, 50%, 60%, 70% and 80%) to the crude extract of lipases [19].

Ammonium sulfate was slowly added, initially at 40% saturation, to precipitate the protein while being kept in ice with gentle stirring. The precipitate protein was obtained by centrifuging the 40% saturation solution at 10000 rpm for 15 min at 4°C. The precipitated protein was re-suspended in 0.1 M Tris-HCl buffer (pH= 7.5) and dialyzed overnight against the same buffer [16]. The process was repeated similarly with the other test concentrations of ammonium sulfate to find out the most efficient salt concentration required for protein precipitation.

##### **Gel filtration chromatography**

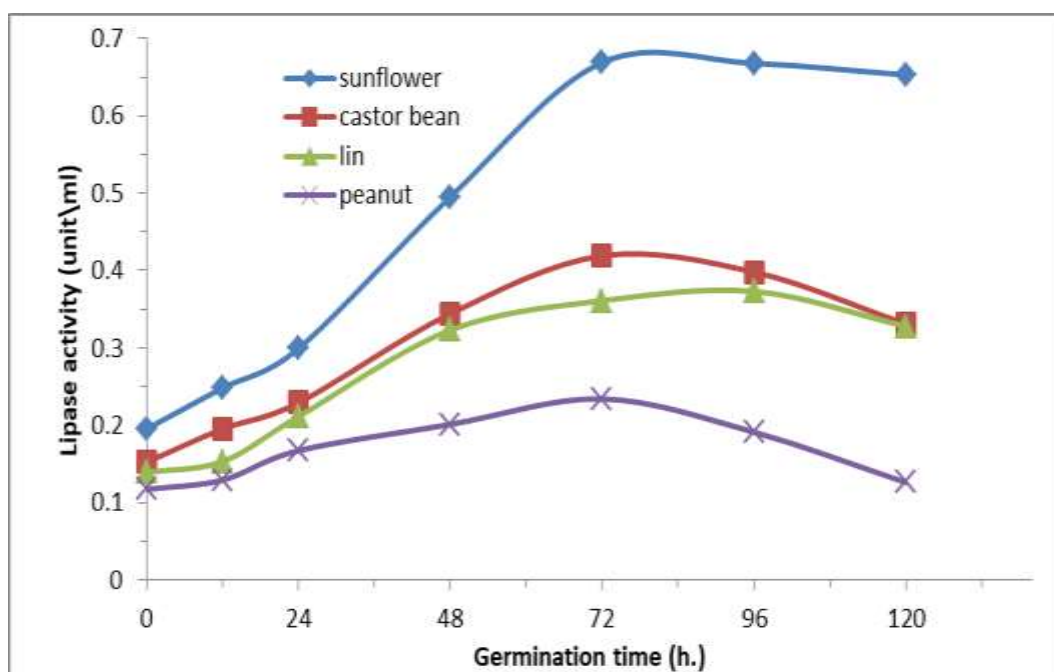
The partially purified enzyme from the ammonium precipitation step was further purified by gel filtration chromatography using Sephadex G-200 column (2.5x 30 cm) and Tris-HCl buffer (0.1 M, pH= 7.5) for elution. The fractions (each of about 3 ml) were collected to determine protein concentration using a spectrophotometer at 280 nm and the enzyme activity of each fraction was determined by the above mentioned method [20].

The fractions that demonstrated the highest enzyme activity were pooled together and used for another purification step by gel filtration against Sephadex G-200 column. The fractions of the second purification step were used to measure the enzyme activity, as in the above mentioned method, and protein concentration at 280 nm using a spectrophotometer [20].

#### **Results and discussion**

##### **Extraction of lipase**

The results showed that all the germinated seeds demonstrated the highest enzyme activity after 3 days (72 h.) of germination time. The maximum lipase activity was recorded by sunflower (0.669 U/ml), which was higher than those of castor bean, flax and peanut seeds by 159.67, 185.32 and 285.90 %, respectively, as shown in Figure-1.



**Figure 1**-Lipases activity at different germination times of test crop seeds. The seeds at zero time were soaked for 6 h before extraction.

The results in Table-1 revealed that the 0.1M Tris-HCl buffer, pH= 7.5, exhibited the best ability to extract lipase compared with the other solvents. Moreover, sunflower seeds also produced the maximum levels of lipase enzyme (0.669, 0.520 and 0.427 U/ml) when extracted by Tris-HCl buffer, acetone and DW, respectively, compared with the other used seeds.

**Table 1**- Lipases activity in germinated the seeds of different crops as extracted by different solvents.

Seeds of Plant species	Lipases activity (U/ml)*			
	Solvent of extraction			
	Tris- HCl buffer	Distilled water	Acetone	Average
sunflower	0.669	0.427	0.520	0.535
flax	0.361	0.281	0.160	0.276
peanut	0.234	0.161	0.089	0.161
castor bean	0.419	0.094	0.384	0.352
Average	0.417	0.240	0.163	
LSD $P \leq 0.05$	$P = 0.05$	$S = 0.05$	$P \times S = 0.09$	

\* Enzyme activity was determined at 72 h. after seed germination.

Various previous studies described the extraction of lipase from different plant seeds at different germination times with different solvents. Sammour [21] extracted lipase from germinated linseeds every 12 h for 5 continuous days with 165 mM tricine-NaOH buffer (pH 7.5) and cold grinding medium consisting of 0.4 M sucrose, 10 mM KCl, 2 mM EDTA, 2 mM dithiothreitol, and 1 mM MgCl<sub>2</sub>). His results showed that the highest lipase activity was obtained after 72h. of seeds germination and remained constant at 84 and 96 h..

Another study by Ezema [20] extracted lipase from four days-germinated seeds of *Cucumeropsis mannii* with two separated buffers (150 mM Tris- HCl buffer, pH= 7.5, and 150 mM phosphate buffer, pH= 5.9) and a cold grinding medium containing 0.4M sucrose, 0.5mM EDTA, 2.0mM  $\beta$ -mercaptoethanol, and 1.95% (w/v) Tween 80. Okunwaye *et al.* [12] used Tris-HCl buffer as a solvent during lipase extraction from the fruit mesocarp of *Raphia palm*, which demonstrated the highest value of lipase activity (23.05 U/ ml),.

#### Purification of lipase

The crude lipase was extracted by Tris-HCl buffer from sunflower germinated seeds which were

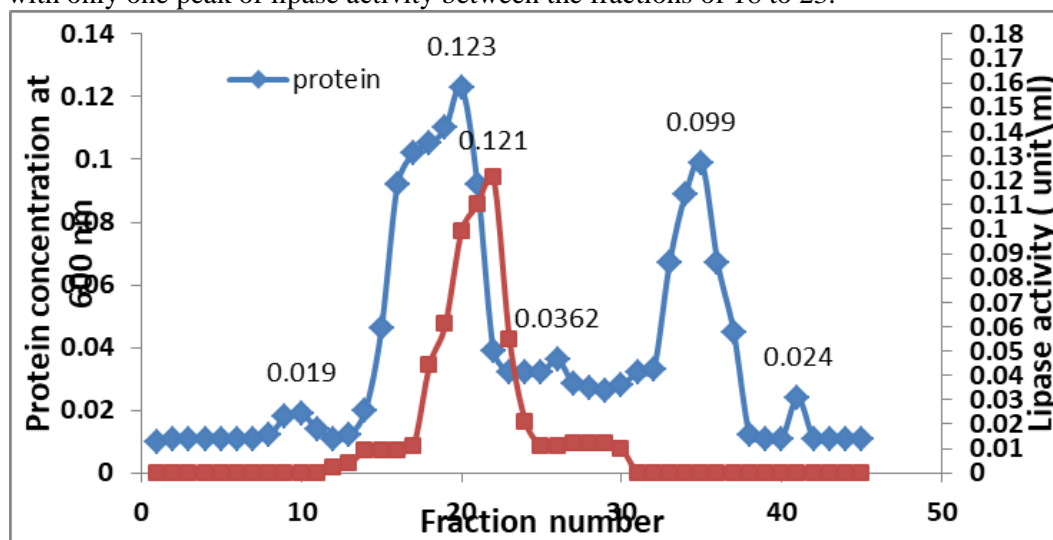
subjected to a purification protocol. The purification involved ammonium sulfate precipitation followed by two steps of gel filtration by Sephadex G-200 column, as in Table-3. Table-2 shows the results of crude enzyme precipitation in different saturation ranges of ammonium sulfate. The highest enzymatic activity was observed in the ratio of 60-80% ammonium sulfate compared with the other used ratios. Also, the results revealed that 70% ammonium sulfate demonstrated the best specific activity (2.576 U/mg protein) with a yield of 69.21%.

**Table 2-Lipases precipitation by different concentrations of ammonium sulfate**

Purification Steps	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude enzyme	31	0.885	27.435	1.601	49.631	0.552	100.00
40%	8	0.362	2.896	0.509	4.072	0.711	10.55
50%	8	0.637	5.384	0.691	5.528	0.973	19.62
60%	8	1.215	9.720	0.811	6.488	1.498	35.42
70%	8	2.373	18.99	0.921	7.368	2.576	69.21
80%	8	2.400	19.21	1.330	10.46	1.805	70.02

Other studies found that using 80% ammonium sulfate is the best to precipitate the crude lipases [12]. Madhikar *et al.* [16] used 80% of ammonium sulfate saturation for protein precipitating from sunflower seeds. Ammonium sulfate was reported to be an appropriate salt because it is highly soluble in water and a cheap chemical material compared with other salts [22]. Nevertheless, other researchers used other compounds in the precipitation process, such as Eze and Ezema [23] who used ammonium sulfate and cold acetone for protein precipitation. One half of the crude extract of white melon seeds was precipitated with ammonium sulfate at 70% saturation, whereas the second half of the crude was saturated with cold acetone at 50% (v/v).

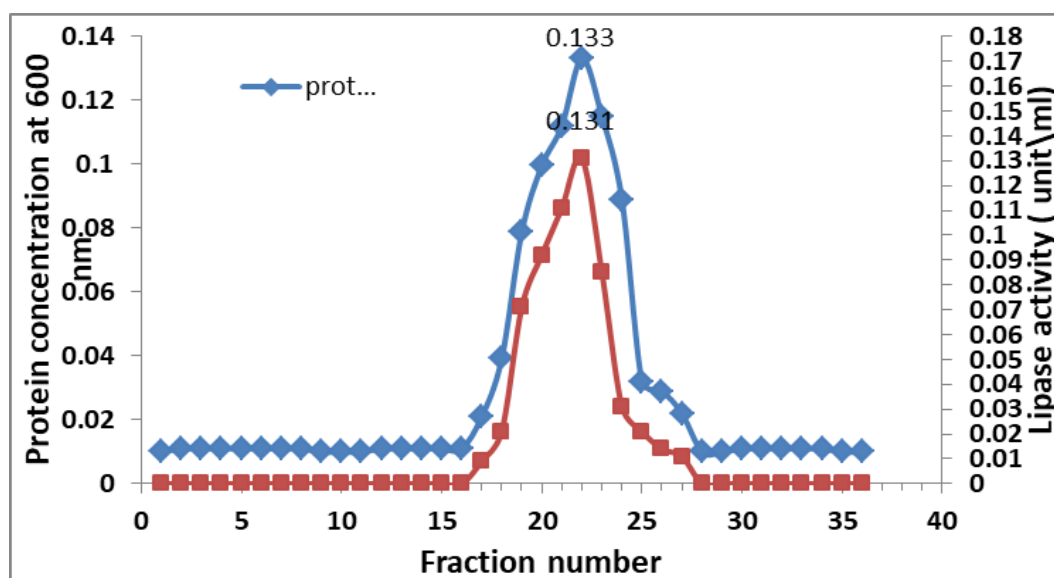
The enzyme obtained from 70% ammonium sulfate precipitation was again purified by gel filtration chromatography column, as in Figure-2. The results indicated the presence of two protein peaks with only one peak of lipase activity between the fractions of 18 to 23.



**Figure 2-**The first step of lipase purification using Sephadex G-200 gel filtration column chromatography.

The active fractions from the first step of gel filtration were again pooled and loaded into the Sephadex G-200 column.

The results in Figure-3 revealed the presence of two identical peaks for protein and enzyme activities, which is a good indicator on the purification steps. Also, the active fractions for the enzymatic activity were found between the fractions of 19 to 23.



**Figure 3**-The second step of lipase purification using Sephadex G-200 gel filtration column chromatography.

Further results can be concluded from Table-3, which shows the results of the final purification. The first and second stages of the gel filtration column demonstrated the maximum specific activity (4.644 and 6.482 U/ mg protein) with purification fold that reached to 8.12 and 11.33 times, respectively.

**Table 3**-Purification steps of lipases enzyme extracted from sunflower seeds.

Purification Steps	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein concentration (mg/ml)	Total protein (mg)	Specific Activity (U/ mg)	Purification Fold	Yield (%)
Crude enzyme	50	0.562	28.100	0.982	49.100	0.572	1.00	100.00
70% of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	11	1.825	20.075	0.724	7.964	2.520	4.40	71.44
Chromatography on Sephadex G-200 (first step)	18	0.692	12.456	0.149	2.682	4.644	8.12	44.32
Chromatography on Sephadex G-200 (second step)	15	0.726	10.890	0.112	1.680	6.482	11.33	38.75

These results showed that the optimum activity was obtained from the lipase enzyme extracted from sunflower seeds using Tris-HCl buffer and then purified in two steps by gel filtration. The extracted enzyme can be employed in different industrial applications.

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