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ORIGINAL PAPER

Biocatalytic Properties of Lipase from Walnut Seed (*Juglans regia* L.)

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Abstract Lipase (E.C. 3.1.1.3) from walnut seed was purified 28.6-fold with 31% yield using Sephadex G-100 gel chromatography. Olive oil served as good substrate for the enzyme. The optimum pH and temperature were 9.0 and 70 °C, respectively. The lipase was stable between 30 and 80 °C for 5 min. K_m and V_{max} values were determined as 48 mM and 23.06×10^{-3} U/min mg for triolein as substrate. Lipase activity was slightly reduced by Cu^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, and Ni^{2+} ions, while Mg^{2+} and Zn^{2+} had no effects. Anionic surfactant sodium dodecyl sulfate stimulated lipase activity while non-ionic surfactants Tween-80 and Triton X-100 had negligible effects on enzymatic activity. The enzyme activity was not affected by 50 mM urea and thioacetamide. Potassium ferricyanide, *n*-bromosuccinamide and potassium cyanide reduced the enzyme activity. The enzyme showed a good stability in organic solvents, the best result being in *n*-hexane (113% residual activity). The activity of dialysate was maintained approximately 80% for 1 year at −20 °C.

Keywords Purification · Characterization · Walnut seed · Lipase · Enzymatic activity · Stability

Introduction

Lipases are an important group of biocatalysts because of their ability to carry out the reactions both in aqueous and non-aqueous media. Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) catalyze the hydrolysis of triacylglycerol to release fatty acids and glycerol. This hydrolytic reaction is reversible, and in the presence of decreased amounts of water, often in the presence of the organic solvents, the enzymes are effective catalysts for various inter-esterification reactions. These find diverse applications in fats and oil hydrolysis, the food industry, the detergent industry, peptide synthesis, and the pharmaceutical industries [1–7].

Lipases have been purified and characterized from various plant, animal, and microbial sources. Most of the developed works about lipases have focused on microbial lipases [8–10]. There has been a growing interest in plant lipases in recent years since they are cheap, very versatile, and stable in organic media [11–13]. Lipases from plant families like Asclepiadaceae [14], Euphorbiaceae [15–17], Caricaceae [18–21], or Rosaceae [22] have been described as useful biocatalysts for several applications. Very little work on 'unusual' or tropical seeds has been reported [21].

Within the Juglandaceae family, *Juglans* are deciduous trees, and widely distributed in the New World and Europe. Walnuts contain arginine, which is an amino acid that the body uses to produce nitric oxide, necessary for keeping blood vessels flexible [23], are an excellent source of omega-3 fatty acids, and have been shown as helpful in lowering cholesterol.

Lipases are present with high activity in reserve tissue of many oilseed plants [22]. Lipase enzymes in many oilseeds are localized in the aleurone layer of the grain, whereas the fatty materials are dispersed in the sub-aleurone layer and the endosperm. Aqueous extraction of proteins is rendered rather difficult because of the presence of the fatty material. Therefore, the first step in the extraction process is defatting process [12]. This step resulted in rather low activity yields. In the present study, the extraction of fatty material from walnut seeds was carried out with acetone.

Lipase purification and characterization from walnut seeds has not yet been done, almost nothing is known about

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walnut seed lipase. In the present study, we describe the purification and some biochemical characteristics of a new lipase from the walnut seed in our laboratories to discover new sources of this enzyme for potential use in the food and pharmaceutical industries.

Materials and Methods

Walnut seeds were obtained from the local market of Edirne, Turkey. The walnuts used were of the 'şebin' variety of *Juglans regia*. The hulls of the seeds were removed by placing them in warm distilled water for 1 day then dried and stored at 25 °C until needed. Sephadex G-100, bovine serum albumin, and sodium deoxycholate were purchased from Sigma. All the other chemicals and solvents were obtained from Merck.

Defatting of Seeds

Walnut seed is one of the oil seeds which contain considerable amounts of fatty oil. Before the isolation of lipase from walnut seeds, the oil was removed by a defatting process. For defatting, 50 g of seeds was stirred with 150 mL of cold acetone for 30 min. The extraction procedure was repeated three times. This process was carried out in a water bath at 4 °C. At the end of every stirring period, the cold acetone phase was filtered, and fresh cold solvent was added to the seeds. All of the solvent phases were put together and evaporated under a vacuum at 40 °C. The acetone was removed from the powder standing at room temperature. The remaining precipitate, which was called seed powder, was used as the enzyme source, and stored at −20 °C until used.

Lipase Purification

Preparation of the Crude Extract

Fifty grams of the defatted seed powder was suspended overnight in 350 mL of sodium phosphate buffer (0.1 M and pH 7.0), after which the suspension was centrifuged for 30 min at 4,000×*g* at 4 °C.

Ammonium Sulfate Precipitation

The supernatant was precipitated by using solid ammonium sulfate. The ammonium sulfate precipitation of the crude extract was carried out with a range of 0–90% (w/v) saturation at 4 °C. Precipitation was allowed to occur for 1 h at 4 °C and followed by centrifugation at 13,000×*g* for 30 min. All of the samples were suspended in the above sodium phosphate buffer and lipase activity was measured. The highest lipase activity was found in the 60–90% salt saturation fraction. The precipitate obtained was dissolved in a small volume of sodium phosphate buffer (0.1 M, pH 7.0).

The active precipitate was dialyzed at 4 °C for 24 h with three changes of sodium phosphate buffer (0.05 M, pH 7.0) in cellulose membrane dialysis tubing (Sigma Chemical Co., St. Louis, MO, USA).

Gel Filtration Chromatography

The dialyzed lipase sample was applied to a Sephadex G-100 column (1.0 cm × 60 cm) which was pre-equilibrated with 0.1 M of sodium phosphate buffer (pH 7.0). The lipase was eluted with the same buffer at a flow rate of 20 mL h^{-1}. The fractions of 3.0 mL were collected. These were assayed for both lipase activity and protein (A_{280}).

Lipase Assay

Lipase activity was assayed by alkali titration, as described by Nahas with some modifications [24]. Olive oil was used as the substrate and 0.01 mol L^{-1} NaOH solution for titration. The standard reaction mixture contained 10 mL of substrate solution (gum arabic 10% w/v in 30 mL water, 3.5 mL of olive oil, 2.5 g of ice pieces that were prepared from distilled water), 2 mL of deoxycholate solution (1.6% w/v sodium deoxycholate, 32 μM NaCl in 100 mL of distilled water), and 4 mL of Tris–HCl buffer (50 mM). This mixture was placed in a water bath at 37 °C. The reaction was initiated by addition of 1 mL enzyme to the substrate emulsion. When the enzyme was added, pH decreased (below 7), the fatty acids were liberated, and the mixture was stirred with a magnetic needle for 5 min and the pH was adjusted to 7 with 0.01 M NaOH solution. One unit of lipase activity was defined as the number of micromoles of fatty acid hydrolyzed per minute under these conditions. Enzyme activity was expressed as enzyme unit per milligram of protein (U mg $protein^{-1}$).

Protein Estimation

The protein concentration was determined by the Lowry method using bovine serum albumin as the standard protein [25].

Statistical Analysis

All analyses were run in triplicate. The results were presented as the means ± SEM. Statistical analyses were performed according to the GraphPad Prism 4 Demo (trial) program. Analyses of variance were performed using the ANOVA procedure. Significant differences ($P < 0.05$)

between the means were determined using Dunnett's post hoc test.

Substrate Specificity

Lipase activity was determined by using refined five substrates (olive oil, sunflower oil, corn oil, soy bean oil, and hazelnut oil). The enzyme activity was measured under standard assay conditions.

pH Optimum

The pH optimum for the walnut seed lipase was determined at various pH from 3 to 11. The buffer systems used were: 0.1 M sodium acetate buffer (pH 3.0–4.0), 0.1 M sodium phosphate buffer (pH 5.0–7.0), 0.1 M Tris–HCl buffer (pH 8.0) and 0.1 M glycine-sodium hydroxide buffer (pH 8.5–11.0). Lipase activity of each sample in each buffer was determined using the lipase assay described previously. The highest activity obtained was taken to be 100% for calculating the relative activities.

Temperature Optimum

To determine the effect of temperature on lipase activity, the enzyme assay was measured at various temperatures (30–80 °C) using a circulation water bath. The lipase extract and substrate emulsion were incubated for 15 min at the different temperatures indicated above at the optimum pH (9.0). Then lipase activity was determined as previously mentioned.

Effect of Metal Ions and Additives on Lipase Activity

The effect of metal ions on lipase activity was studied at pH 7.0 by incubating the enzyme in presence of 10 mM of metal ions (Ca^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+}) and in presence of 50 mM of additives (*n*-bromosuccinamide, urea, potassium ferricyanide, thioacetamide, and potassium cyanide). Incubation of mixture was carried out at 30 °C for 30 min and assayed for lipase activity. Relative lipase activity (%) was calculated taking that without metal ions/additives as 100%.

Surfactant Stability

The surfactant stability of enzyme was assessed by incubating the enzyme with sodium dodecyl sulfate, Triton X-100, and Tween 80 at 5, 15, 25, and 35 mM concentrations. The mixture was incubated at 30 °C for 30 min. Relative lipase activity (%) was determined taking the control (enzyme incubated without the surfactants) as 100%.

Organic Solvent Stability of Lipase

Lipases are known for their ability to work in aqueous as well as in organic solvents. The stability of walnut lipase was thus investigated in various polar and non-polar organic solvents. The effect of organic solvents of different log *P* values (the logarithm of partition coefficient of that solvent between *n*-octanol and water) on the stability of lipase was investigated. One millilitre of organic solvent was added to 3 mL of lipase in a sealed glass vial. The mixture was incubated for 24 h at 30 °C with shaking at 200 rpm. The residual lipase activity was determined under standard assay conditions [4]. Residual lipase activity was calculated taking that of 0 h as 100%.

Determination of K_m and V_{max}

K_m and V_{max} of the lipase were determined by measurement of the enzyme activity with various concentrations of triolein substrate. The rate of the lipase reaction was measured at various substrate concentrations under standard conditions (pH 7.0, 37 °C). The kinetic data were plotted between activities and substrate concentrations. Kinetic constants were calculated from Lineweaver–Burk plot.

The Effect of Storage Time

The lipolytic activity of walnut lipase in crude extract and dialysate was assayed at certain intervals for 1 year of storage at −20 °C. The lipase activity was determined at certain intervals under optimum conditions for each of them during this storage period.

Results and Discussion

Purification of Walnut Lipase

Walnut seed is one of the oil seeds which contain a considerable amount of fatty oil. Before the isolation of lipase from walnut seeds, the oil was removed by a defatting process. The lipase was purified by Sephadex G-100 gel filtration chromatography. Table 1 summarizes the results of lipase purification. About 28.6-fold purification with 31% recovery was achieved. In our previous study, we reported that the almond lipase was partially purified by ammonium sulfate fractionation, followed by dialysis [22].

Characterization of Walnut Lipase

Substrate Specificity

Substrate specificity of lipases may be attributed to differences in the geometry and size of their active sites [4].

Table 1 Purification of walnut seed lipase

Purification step	Protein (mg)	Lipase activity (IU)	Specific activity (IU mg^{-1})	Fold purification
Crude extract	309.5	1949.7	6.3	1
Ammonium sulfate precipitation	39.5	730.8	18.5	2.9
Sephadex G-100	3.5	633	180.2	28.6

As shown in Table 2, the substrate with the highest activity was olive oil. The lipase exhibited broad substrate specificity towards various oils, which makes the walnut lipase on ideal biocatalyst to the food and oleochemical industry. The seed storage lipid is enriched in a particular fatty acid, and the lipase produced by that seed displays a corresponding substrate specificity. The seed lipids of the walnut are composed of almost entirely of triolein. The Euphorbiaceae family showed high lipolytic activity toward medium-long acyl chain triacylglycerols [15].

Effect of pH on Lipase Activity

The optimum pH of the walnut lipase was found to be 9.0, and the lipase retained 84% of its maximum activity at pH 9.5 (Fig. 1). The enzyme was more stable at higher pHs. In our previous study, this value was found to be 8.0 for almond lipase [22]. Ng and Tsai reported that the optimum pH of *Carica papaya* occurred at 8.5 [21]. Plant lipases have been reported as having optimal activity at neutral or basic pH values. The optimum pH depends on nature of substrate and enzyme source.

Temperature Optimum

The lipase showed rather narrow activity in temperature range of 60–75 °C. Maximal activity was observed at 70 °C and the activity dropped of rapidly at temperatures above 75 °C (Fig. 2). A similar temperature optimum was reported as 65 °C for almond lipase using soy bean oil as the substrate [22]. The optimum temperatures for different *C. Papaya* lipases have been generally reported between 40 and 50 °C by using olive oil as the substrate [21].

Table 2 Activity of walnut seed lipase towards various substrates

Substrate	Relative activity (%)[a]
Olive oil	100 ± 1.2
Sunflower oil	74 ± 1.5
Corn oil	91 ± 1.5
Soybean oil	82 ± 1.0
Hazelnut oil	82 ± 0.0

Relative activity was expressed as the percentage of the activity towards olive oil. All measurements were repeated three times

[a] Data are presented as means ± standard errors of the means (SEM)

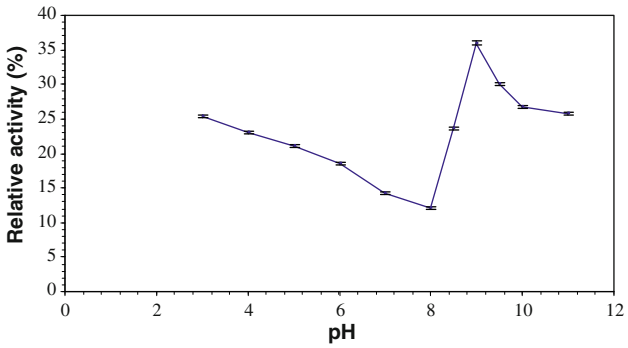

Fig. 1 Effect of pH on lipase activity. The activity was determined at 37 °C at different pH values using different buffers. The activity at pH 7.0 was set as 100%. Significant differences ($P < 0.05$) between the means were determined using Dunnett's post hoc test

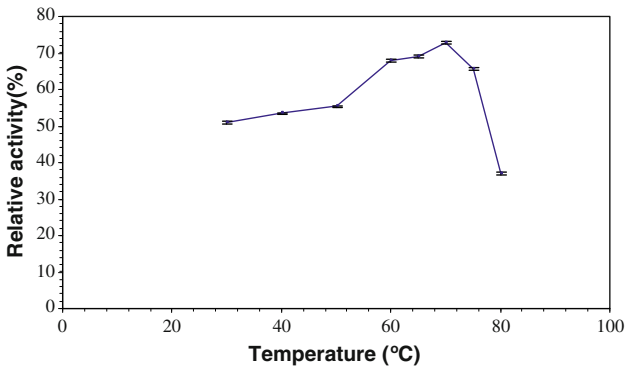

Fig. 2 Effect of temperature on lipase activity. The activity was determined at different temperatures at pH 7.0 in 50 mM Tris–HCl buffer. The activity at 70 °C was set as 100%. Significant differences ($P < 0.05$) between the means were determined using Dunnett's post hoc test

Effect of Metal Ions and Additives on Lipase Activity

Effect of metal ions and additives (oxidizing and reducing agents) was tested. Lipase activity was slightly reduced by Cu^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, and Ni^{2+} ions, while Mg^{2+} and Zn^{2+} had no effects (Fig. 3). These results are different from those obtained for almond seed lipase. Ca^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, and Ba^{2+} ions stimulated lipase activity while Mg^{2+}, Ni^{2+}, and Cu^{2+} caused inhibition for almond seed lipase [22]. These kinds of studies should be addressed very carefully, since the effect of those ions, and other compounds, on lipase activity depends on the system and substrate used.

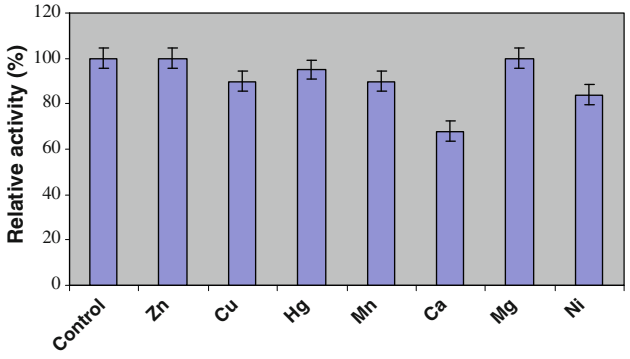

Fig. 3 Effect of metal ions on lipase activity. Activity without metal ions was set as 100%. Significant differences ($P < 0.05$) between the means were determined using Dunnett's post hoc test

Potassium ferricyanide, *n*-bromosuccinamide, and potassium cyanide caused inhibition of the enzyme activity (Fig. 4). The inhibition of activity by potassium ferricyanide and potassium cyanide confirms the presence of sulfur-containing amino acids in the active site of the lipase. Thioacetamide and urea had no effects on the enzyme activity. These results are in agreement with our previous study [22]. Sammour has reported linseed lipase activation at high concentration of salts [26].

Effect of Surfactants on Lipase Activity

It was well known that lipase activities from different sources are affected by surfactants [8]. To examine the effect of surfactants on the activity of walnut lipase, a few surfactants were chosen based on our previous report. As shown in Fig. 5, lipase activity was enhanced (142% relative activity) at high concentrations (15–35 mM) of anionic surfactant sodium dodecylsulfate, however it caused moderate inhibition of lipase activity at 5 mM. Among the non ionic surfactants tested, Triton X-100

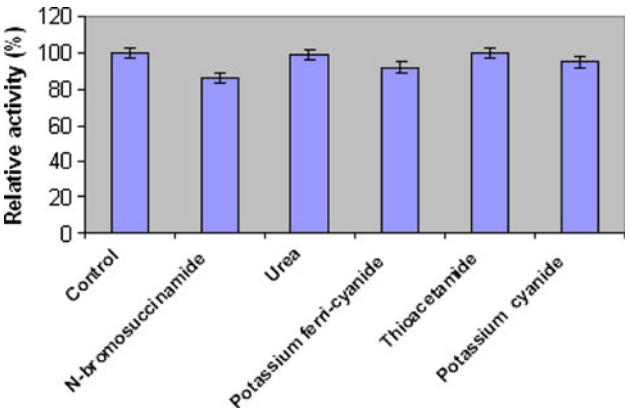

Fig. 4 Effect of additives on lipase activity. Activity without additives was set as 100%. Significant differences ($P < 0.05$) between the means were determined using Dunnett's post hoc test

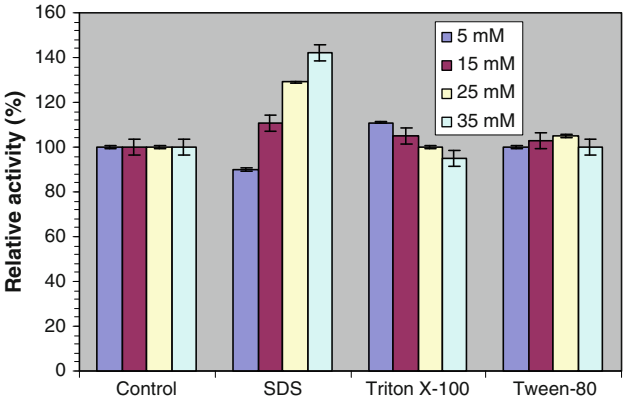

Fig. 5 Effect of surfactants on lipase activity. Activity without surfactants was set as 100%. Insignificant differences ($P > 0.05$) between the means were determined using Dunnett's post hoc test

caused only 5% reduction in activity, even at 35 mM concentration while Tween 80 caused moderate stimulation at all concentrations tested. Surfactants are known to increase the lipid–water interfacial area, which in turn, enhances the observed rate of lipase-catalyzed reactions [4]. So, it gives a simple method of improving the reaction efficiency by the addition of surfactants.

Organic Solvent Stability of the Enzyme

Employing lipases for bioconversions in organic solvents is advantageous from a biotechnological point of view, hence activity and stability in solvents are considered novel attributes in a lipase. The effect of the organic solvent depends on the nature of both enzyme and solvent. As shown in Table 3, the enzyme had a good stability of 100% for isopropanol, 96% for butanol, 88% for acetone, 83% for *n*,*n*-dimethyl formamide, and 75% for methanol. Similar result was found for the *Pseudomonas aeruginosa* lipase [4]. The lipase was also stable in hexane (113% residual activity) and this result being in agreement with Lima et al. [9]. It is reported that polar solvents strip off the essential water molecules from the active site of enzymes. For this reason, use of polar solvents is avoided and hydrophobic solvents are more often employed in non-aqueous enzyme-catalyzed reactions. The polar solvent tolerant lipases therefore appear promising for catalysis in a low water medium. This property is also observed for *B. megaterium* CCOC-P263 (97% after 1 h in neat isopropanol), and *P. mendocina* (83% after 2.5 h in neat ethanol) [4].

This conclusively demonstrates the solvent stability of walnut lipase. The good stability of the enzyme with organic solvents justifies the search for potential applications of the enzyme in biocatalysis in many synthetic reactions.

Table 3 Stability of the walnut seed lipase in organic solvents

Organic solvent	Log *P*	Residual activity (%)
Phosphate buffer pH 7.0		100 ± 0.0
Methanol	−0.76	75 ± 1.1
Iso propanol	−0.28	100 ± 0.5
Acetone	−0.23	88 ± 1.4
N,*N*-dimethyl formamide	−0.87	83 ± 1.2
Butanol	0.8	96 ± 1.1
Hexane	3.5	113 ± 0.6

Log *P* values were parameters of Lima et al. [9]

[a] Data are presented as means ± standard errors of the means (SEM)

Kinetic Parameters

The kinetic parameters for walnut lipase were determined by a Lineweaver–Burk plot (Fig. 6). It showed K_m of 48 mM and V_{max} of 23.06×10^{-3} U/dk mg towards triolein as substrate. Based on these, the corresponding catalytic efficiency (V_{max}/K_m) was determined as 4.80×10^{-5} U dk^{-1} mg. When the substrate was triolein, the K_m values for almond lipase [22] and rice bran lipase [27] were 25 and 6.71 mM, respectively.

The Effect of Storage Stability

Storage stability is a very important parameter to consider if an enzyme is to be commercialized or used in industry at both high or low temperatures. The storage stability of walnut lipase for crude extract and dialysate was investigated at −20 °C. Lipase activity remained relatively stable for 1 year. The crude extract had lost about 10% of its initial activity. The activity of dialysate had decreased by approximately 21% at the end of that period (Fig. 7). The activity of almond lipase had decreased by about 50% after 12 months [22]. Similar results were found by Lopes et al. [28] with a *Lactobacillus plantarum* extracellular lipase, which was stable at −80 °C for 4 weeks.

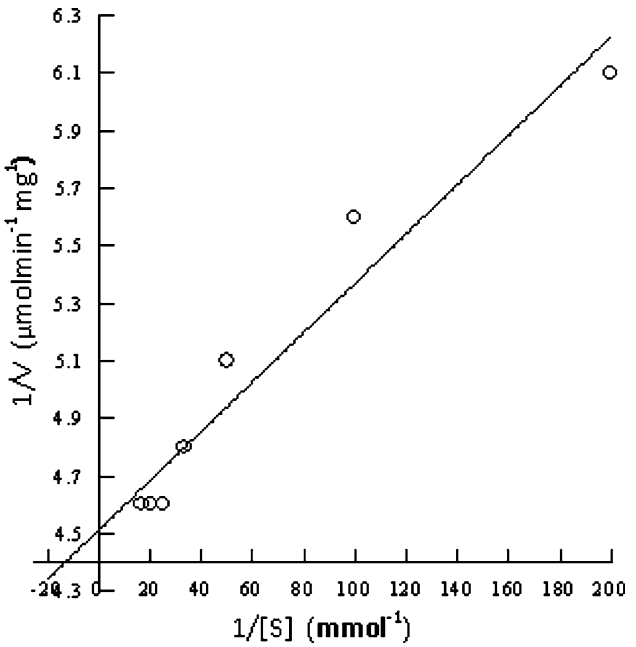

Fig. 6 Lineweaver–Burk plot for walnut lipase. Walnut lipase was assayed under standard conditions using varying concentrations of triolein. Lineweaver–Burk plot was used to calculate the kinetic parameters, K_m and V_{max}

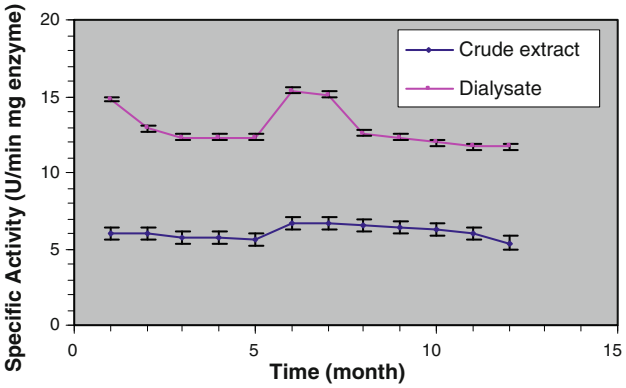

Fig. 7 Effect of storage time on the activity of lipase. The activities of enzyme solutions (crude extract and dialysate) stored at −20 °C were measured at certain intervals under optimum assay conditions. Significant differences ($P < 0.05$) between the means were determined using Dunnett's post hoc test

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