

## HAZELNUT SEED LIPASE: EXTRACTION, PURIFICATION, AND CHARACTERIZATION

İsmail Kılıç<sup>1</sup> and Ayten Sağıroğlu<sup>2</sup>

**Abstract:** Interest in lipases has markedly increased to their potential industrial applications. The most of lipases produced commercially are obtained from animal and microbial sources. Nowadays, also obtained from plant seeds such as sunflower, soybean, peanut, castor bean and hazelnut. Hazelnut is one of the most important foods in majority of the world and Turkey is largest hazelnut producer. In this study, It was aimed that Lipase from hazelnut seed identified as yomra species isolated, purified and characterized. Lipase from hazelnut seed was purified 1255 fold to homogeneous state by ammonium sulfate precipitation, dialysis and Sephadex G-100 gel filtration chromatography after by defatting from hazelnut proteins. The purified enzyme showed single band when it was subjected to SDS-PAGE. The molecular weight of the determined by SDS-PAGE was 20 kDa. Purified lipase from hazelnut seed exhibited the maximum activity at 9.0 and 50°C and stable under alkaline conditions (pH 7.0-10.0) and at temperatures between 20-55°C. Lipase from hazelnut seed more specified versus triolein and tributyrin and olive oil among the nature oils as substrate. The enzyme activity was measured by using 0.1 ml of enzyme solution for 5 min. To determine the storage stability of lipase from hazelnut seed, the activity assays carried out for a period of one year. it was observed that about 83% of its activity was retained of 9 months at -20°C. Purified lipase from hazelnut seed versus triolein as substrate calculated  $K_m$  and  $V_{max}$  values, 4.545mM and 80 U/dk.mg.Enzyme, respectively.

**Key Words:** Hazelnut (*Corylus avellana* L.) seed, lipase, purification, characterization, substrate specification.

### INTRODUCTION

Hazelnut seeds (*Corylus avellana* L. ) are used to derive edible oil and meal (oil cake) that are

<sup>1</sup> Address correspondence to ismail Kılıç, Department of Science Education, Faculty of Education , Trakya University, 22030, Edirne, Turkey; E-mail: iso\_kilic@hotmail.com

<sup>2</sup> Department of Chemistry, Faculty of Science, Trakya University, Edirne, Turkey

rich in proteins, vitamins, and carbohydrates. Seeds having shells contain 55–60% oils, 16% proteins, 16% carbohydrates, 17% ash, and 4.45% moisture. The presence of lipase in the seed proteins results in enzymatic hydrolysis at ambient temperature during harvesting, handling, and processing of oil (Mukherjee and Hill, 1994; Maeshima and Beevers, 1985).

Many investigators have studied the enzymatic hydrolysis of fats and oils as an attractive alternative to the currently used high pressure and high temperature processes for the industrial production of fatty acids (Moullin et al., 1994; Teissere et al., 1995; Jonhson and Fritz, 1989). Lipases (triacylglycerol hydrolases (EC 3.1.1.3) are part of a large group of enzymes which catalyze the hydrolysis of a wide range of carboxylic esters (Swern, 1982; Ferrato et al., 1997). Lipases can be obtained from animal, microbial, and plant sources. Manufacture of lipases by microbial fermentations constitutes a process that is currently popular (Sugiura et al., 1977; Chen et al., 1992).

Plant lipases, which exhibit high activity above room temperature, are inexpensive and easy to obtain, are particularly attractive for some industrial applications. Some researchers have studied rice bran (*Oryza sativa*) lipase (Mirosław and Shrang, 1997; Mayordomo et al., 2000), black cumin (*Nigella sativa L.*) seed lipase (Anita et al., 1999; Aizono et al., 1976), castor bean seed (*Ricinus communis L.*) lipase (Munshi et al., 1993; Dandik and Aksoy, 1992; Mert et al., 1995), oat seed (*Avena sativa L.*) lipase (Tüter, 1998) and rapeseed (*Brassica napus*) lipase (Rao and Paulose, 1992) and Sunflower seed lipase (*Heliantus annuus L.*) (Sagiroglu and Arabaci, 2005). However lipases have, so far, found many industrial uses in detergents, surfactants, oils, fats, and in the dairy, pharmaceutical, and textile industries; they can be produced on a large scale by using different sources. In addition, immobilized lipases have been widely used in large scale industrial applications (Sagiroglu and Telefoncu, 1993). There is still a great interest in new lipases with commercially useful properties.

The purpose of the present paper is to study the extraction, purification, and characterization of hazelnut seed lipase for enzymatic hydrolysis of natural vegetable oils and simple triglycerides.

## **EXPERIMENTAL**

**Materials:** Hazelnut seeds were obtained from the West Blacksea region in Turkey (Yomra). All of the chemicals that have been used for the experiments were analytical grade (Merck, Darmstadt, Germany). The electrophoresis chemicals, Sephadex G-75 gel, cellulose dialysis tubes, molecular weight Dalton's markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Chem. Co. (St. Louis, MO).

**Apparatus:** Enzymatic hydrolysis reactions were carried out in a three necked flask (100 mL), equipped with sampling pipette, temperature and pH controller, and automatic burette. The reaction mixture was stirred with a magnetic needle to produce a stable emulsion. The data presented are the averages of duplicate determinations, which varied by no more than 0.1%. Electrophoresis was performed in an SE 600 vertical slab gel electrophoresis unit, scanning of gel with GS-300 dual speed scanning densitometry with GS 365W electrophoresis data systems (Hoefer Sci. Inst., California, USA).

**Defatting of Hazelnut Seed:** For defatting, hazelnut seed was dehulled and its 50g was grounded. It was stirred for 30min each, in batches, with 150 mL of cold solvent. At the end of each stirring period, the solvent phase with oil was decanted. This defatting was followed in each experiment unless otherwise mentioned.

**Extraction and Purification of Hazelnut Seed Lipase:** All of the extractions were carried out at 4°C. The defatted seed powder was allowed to air dry for about one hour so that all solvent was practically removed. Fifty grams of the residue was suspended overnight in 350 mL of phosphate buffer (100 mM and pH 7) containing 1.0 mM of CaCl<sub>2</sub> (as a lipase activator). Concentration of the buffer, as well as calcium chloride, was varied in the preliminary experiments. The phosphate buffer concentration of 100 mM with 1.0 mM calcium chloride was found to give maximum activity in the extract. The buffer suspension was gently centrifuged in order to remove the residues of seed cells. The total supernatant was subjected to fractional salt precipitation with the solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Three precipitates were obtained by fractional salting precipitation; they were 0-30%, 30-60%, 60-90%, 0-90% precipitates. All of them were suspended in the above phosphate buffer and lipase activity was measured. The highest lipase activity was found in the 0-90% salt saturation fraction. The active precipitate was dialyzed at 4°C in 50mM phosphate buffer (pH 7.0) for 24h with three changes of buffer during dialysis through a cellulose membrane. This dialyzed material was put onto a Sephadex G-100 column, equilibrated with 50mM phosphate buffer (pH 7), after washing with the same buffer. The lipase enzyme was eluted with 100 mM phosphate buffer (pH 8) containing 1.0 mM CaCl<sub>2</sub>, with a flow rate of 0.5 mL/min, and 3 mL were collected.

**Protein Measurement:** The protein content was determined by the method of Lowry, using bovine serum albumin as a standard (Lowry et al., 1988). The protein contents of elution samples from the column were measured at 280nm (Caspar, 1988).

**Lipase Assay:** Lipase lipolytic activity was measured using low acidic olive oil as substrate (or other substrates) in various emulsifying agents such as gum arabic and poly-(vinyl alcohol). Various compositions of assay mixtures were investigated. The following method yielded the highest and most reproducible activity for the lipase. Standard reaction mixture contained 10mL

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 bidistilled water), 2 mL of deoxycolate solution (1.6% w/v sodium deoxycolate), and 4mL Tris HCl (5mM Tris buffer /3.2 $\mu$ M NaCl solution) buffer, pH 7. This mixture was placed on a water bath at stable temperature, was stirred with a magnetic needle and the pH was adjusted to 7 with 0.01 M NaOH solution. Then, 100  $\mu$ L of enzyme solution was added. Next, 0.5 mL aliquots of the reaction mixture were removed at specified intervals, and the reaction was quenched using 10mL methanol. This was then titrated with NaOH with phenolphthalein as an indicator. Blank assays were performed in the above reaction mixture, without enzyme solution but, instead, the same volume of the above 50 mM buffer solution was added. The reported values of activity were within +3% of the average value. One unit of the hydrolytic activity was defined as the amount of enzyme which is necessary to liberate one micromole of free fatty acid by the hydrolysis of oil per minute, under the assay conditions. The amount of acid released can be calculated from the difference of titration values for a sodium hydroxide solution of known molarity. Specific activity was calculated as the units per mg protein.

**Incubation Time:** The reaction mixture was prepared as defined above in the enzyme assay section. In order to determine the effect of incubation time for enzyme activity, these mixtures were titrated with 0.1 M NaOH solution for different time periods (1–20 min), and the enzyme activities were calculated.

**Electrophoretic Analyses:** SDS-PAGE was carried out in polyacrylamide gel (20% T, 0.5% C) in the presence of 0.1% SDS, as described by Laemmli (Laemmli, 1970). and stained with Coomassie Brilliant Blue R-250. Sample protein bands and markers on the gel were determined by GS-300 scanning densitometry and a related computer program. Purified enzyme sample was applied in the SE-600 vertical slab gel electrophoresis unit (Giulian and Graham, 1985).

**Substrate Specificity:** To investigate the substrate specificity of the enzyme, six substrates were selected. Four of them were natural vegetable oils, such as hazelnut seed oil, corn kernel oil, olive oil, sunflower oil and another two were simple triglycerides such as tributyrin and triolein. Each of the substrates was used as previously described in the enzyme assay section.

**Effects of Temperature and pH on Enzyme Activity and Stability:** The relative activity of hazelnut lipase at several temperatures (20–70°C) was determined by using the standard procedure described above. Thermal stability experiments were carried out in the temperature range of 20-80°C. The enzyme samples were incubated in 100 mM phosphate buffer (pH 7) at each chosen temperature for 30min and cooled on ice; the residual activity was measured as before.

To determine the optimum pH, 50 mM of solutions consisting of sodium acetate (pH 3-4), phosphate (pH 5-7.5), Tris-HCl (pH 8-9) and glycine-NaOH (pH 9.5-13) buffer were used in the standard assay. To test pH stability, 10  $\mu$ L of the enzyme was incubated at 37°C for 1/2h with 60  $\mu$ L of following buffers, phosphate (pH 5-7.5), and Tris-HCl (pH 8-9.5). Samples were then diluted (final dilution: 1/10) in 100 mM phosphate buffer (pH 7), and the remaining activity was assayed as before.

## RESULTS AND DISCUSSION

The direct aqueous extraction of proteins in the oil seeds is rather difficult because of the presence of the fatty material. For this reason, the defatting of hazelnut seeds constituted the first step of the extraction process of hazelnut seed lipase.

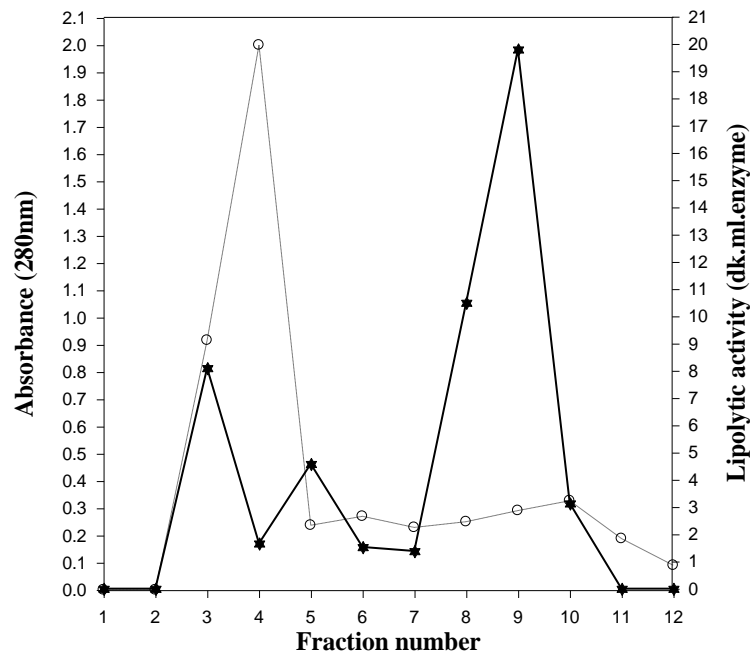
Acetone was selected as a solvent that could be easily vaporized from the residue and, also, it effectively aids the recovery of oil. The crude enzyme extract was obtained by the extraction of the acetone extracted powder with 20 mL of 100 mM phosphate buffer (pH 7), three times, for 1 h each. Then, the extract was tested for hydrolytic activity. It was found that the amount of lipase in this extract was negligible when compared to the overall yield.

The highest lipase activity was found in 0-90% salt precipitates. The others have much less hydrolytic activity. The activity of the active fraction increased after dialysis. Consequently, it was seen that lipolytic activity of active eluate recovered from the Sephadex G-100 column (fig. 1) is highest when compared with the activities of salt precipitate and dialysate (Table 1).

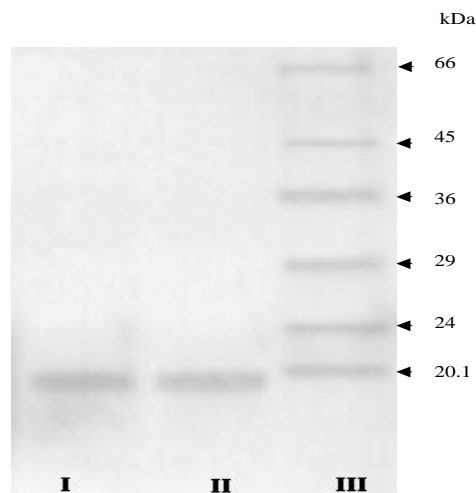
In Fig. 2, the discontinuous buffer system SDS-PAGE profile is shown at different stages of purification of hazelnut seed lipase with molecular weight markers. The lipase fraction which was purified from the column was homogeneous and contained a single Coomassie-stained protein band. Lipase molecular size was determined to be 20kDa from a standard curve generated by plotting the  $R_f$  of each versus its  $\log_{10}$  molecular weight with gel densitometry.

**Table 1.** Lipolytic activities of purification fractions

<b>Purification steps</b>	<b>Lipolytic activity (U/dak.ml.enzyme)</b>	<b>Purification (-fold)</b>
Crude enzyme extract	0.0131	1
0-90% salt fraction	0.8245	62.9
Dialysate	1.1	83.9
Sephadex G-100 column	16.44	1254.9



**Figure 1.** Gel Chromatography of the fractions of hazelnut seed lipase on a Sepha-rose G-75 Column, (O) absorbance at 280 nm; (●) lipolytic activity, column equilibrated with 10mM phosphate buffer, pH 7. After washing with the same buffer, the lipase enzyme was eluted with 0.1 M phosphate buffer, pH 8.0, containing 1.0 mM CaCl<sub>2</sub>, with a flow rate of 0.5 mL/min; 3 mL fractions were collected.

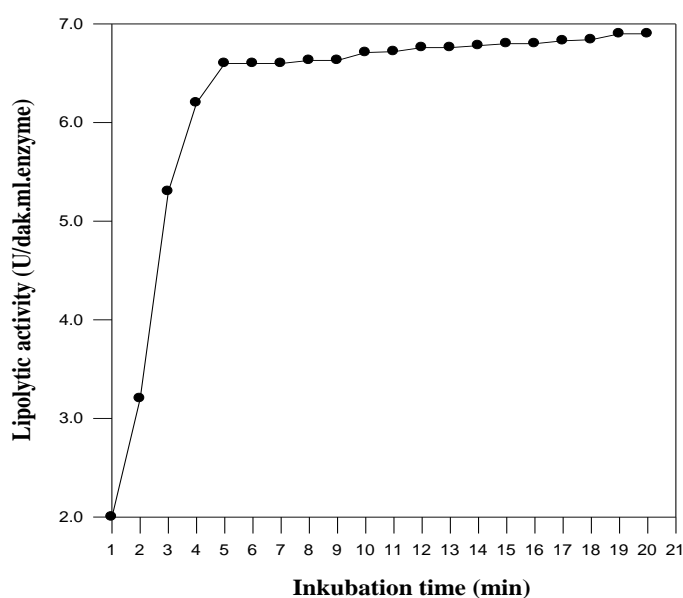


**Figure 2.** The SDS-PAGE of purification steps of hazelnut seed lipase III: Dalton's protein markers; from top to bottom, bovine serum albumine (66 kDa), ovalbumin (45 kDa), Glyceraldehyde-3-phosphate dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa), Trypsinogen (24 kDa), Trypsin inhibitor (20100 Da) I and II: 40 mL Chromatography on Sephadex G-100 column by gradient elution with 100mM phosphate buffer, pH 8.0, containing 0.5mM CaCl<sub>2</sub>.

### Enzymatic Characterization

To determine the effect of incubation time, hydrolysis was performed with seven different reaction mixtures, for the different time periods (1-20min), in a continuous system. The lipolytic activities, as a function of incubation time, are shown in Fig. 3. The hydrolysis triglycerides to produce fatty acids reached the highest value when incubation had been carried out for 5min.

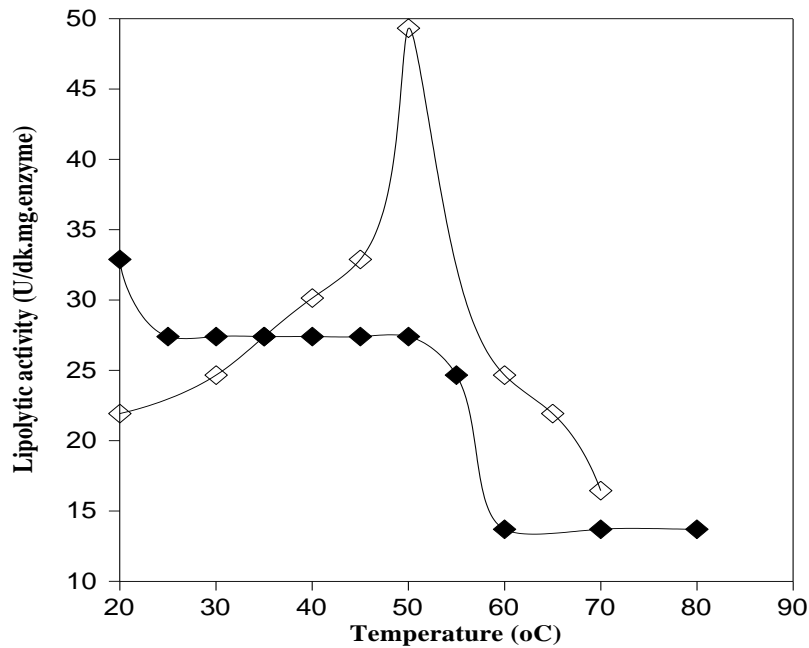
The substrate specificity of hazelnut seed lipase on various substrates was investigated. Simple triglycerides, such as tributyrin, triolein, and various natural oils such as hazelnut oil, sunflower oil, corn kernel oil, and olive oil were used as substrates. The results are summarized in Table 2. It is clear that the rate of hydrolysis is the highest in the case of olive oil. Among the substrates, it is seen that triolein and tributyrin are the same lipolytic activity with hazelnut seed lipase. Other similar studies on seed lipases have yielded similar results (Sagiroglu and Arabaci, 2005).



**Figure 3.** Effect of incubation time on activity of hazelnut seed lipase

**Table 2.**Effect of substrates on lipolytic activity of hazelnut seed lipase.

Substrates	Lipolytic activity (U/dk.ml.enzyme)
Hazelnut oil	10.96
Sunflower oil	19.12
Corn kernel oil	21.92
Olive oil	24.66
Tributyryn	30.14
Triolein	30.14

**Figure 4.** (□) Effect of temperature on activity of hazelnut seed lipase; (■) temperature stability of lipolytic activity.

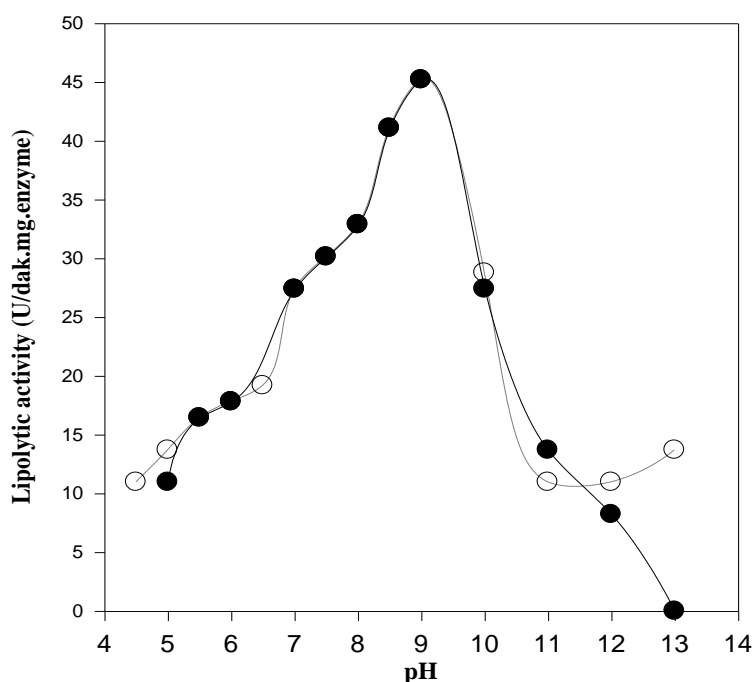
The apparent optimal activity of enzyme was observed at 50°C, when assayed at pH 7.5, although the enzyme showed the ability to maintain high reactivity at lower temperatures (Fig. 4).

Stability towards high temperature is perhaps one of the most important aspects considered before application of an enzyme; also, a thermally stable enzyme is likely to be stable, in general, towards other conditions. A thermal stability test of hazelnut seed lipase was carried



out using olive oil as the substrate, as defined above. It was observed that the loss in activity is practically negligible up to 50°C. These results are similar to the thermal properties of some plant lipases reported by various researchers (Chen et al., 1992; Dandik and Aksoy, 1992)

The activity of the hazelnut seed lipase showed a high dependence on the pH. The optimum pH of the enzyme was found to be pH 9.0. The pH stability profile revealed that the activity was especially conserved at alkaline pH values over a 10 min period (Fig. 5).



**Figure 5.** (○) Effect of pH on activity of hazelnut seed lipase; (●) pH stability of lipolytic activity.

In addition, effects of storage time on the activities of purified enzyme were investigated. The activity of purified enzyme was very stable around neutral pH, at 25°C, in a deep freeze for more than four months.

Hazelnut lipase has been extracted from defatted seed and our extraction method provides the use of cheap, readily available materials. In this work, purification of lipase was achieved in three steps by simple, classical methods. It is found that lipolytic activity of purified lipase is rather high at optimized operating conditions, and enzyme is rather stable at the higher temperatures, alkaline pH's, and for four months of storage time. All of these properties make this enzyme very interesting for a number of biotechnological applications.

## ACKNOWLEDGMENT

We thank Trakya University Research Foundation for its financial support of this research.

## REFERENCES

- Aizono, Y.; Funatsu, M.; Fujiki, Y.; Watanabe, M. Purification and characterization of rice bran lipase II. *Agric. Biol. Chem.* 1976, 40, 317–324.
- Anita, V.P.; Sheehas, P.T.; Neena, N.G.; Sudhir, B.S.; Jyeshtharaj, B.J. Rice brain lipase: extraction, activity, and stability. *J. Am. Chem. Soc.* 1999, 14 (6), 1083–1089.
- Caspar, L. *Methods* ; Kerese, I., Ed.; Ellis Horwood: Chicheser, 1988; 30-45.
- Chen, J.; Ishii, T.; Shimura, S.; Kirimura, K.; Usami, S. Lipase production by tri-chosporon fermentens WU-C12 newly isolated yeast. *J. Ferment. Bioeng.* 1992, 73, 412–414.
- Dandik, L.A.H.; Aksoy, H.A. The kinetics of hydrolysis of *Nigella sativa* (Black cumin) seed oil catalyzed by native lipase in ground seed. *JAOCS.* 1992, 69 (12), 250–253.
- Ferrato, F.; Carriere, F.; Sarah, L.; Verger, R. A critical reevaluation of the phenomenon interfacial activation. *In Methods in Enzymology, Part B*; Rubin, B., Dennis, E.A., Eds.; Academic Press: New York, 1997; Vol. 286.
- Giulian, G.G.; Graham, J. Resolution of low molecular weight polipeptides in a nonurea SDS slab polyacrylamide gel system. *J. Fed. Proc.* 1985, 44, 686-692.
- Johnson, R.W., Fritz, E., Eds. *Fatty Acids in Industry: Processes, Properties, Derivatives, and Applications*; Marcel Decker Inc.: New York, 1989; 51–59.
- Laemli, U.K. Cleavage of structural proteins during the assembly of head of bacteriophage. *Nature* 1970, 227, 680-685.
- Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the phenol reagent. *J. Biol. Chem.* 1951, 193, 265–275.
- Maeshima, M.; Beevers, H. Purification and properties of glyoxysomal lipase from castorbean. *Plant Physiol.* 1985, 79, 489-493.
- Mayordomo, I.F.; Rande-Gil, J.A.; Prieto, J. Isolation, purification, and characterization of a cold-active lipase from *Aspergillus nidulans*. *J. Agric. Food Chem.* 2000, 48, 105–109.
- Mert, S.; Dandik, L.; Aksoy, H.A. Production of glycerides from glycerol and fatty acids by native lipase of *Nigella sativa* seed. *Appl. Biochem. Biotechnol.* 1995, 50, 333–342.
- Mirosław, C.; Shrag, J.D. Structure as basis for understanding interfacial properties of lipases. *In*

- Methods of Enzymology*; Rubin, B., Dennis, E.A., Eds.; Academic Press: New York, 1997; Vol. 284, Lipases, Part A, Biotechnology.
- Moulin, A.; Teissere, M.; Bernard, C; Pieroni, G. Lipases of the Euphorbiaceae family: purification of a lipase from euphorbia characias latex and structure-function relationships with the B chain of Ricin. *Proc. Natl. Acad. Sci. U.S.* 1994, A91, 11328–11332.
- Mukherjee, K.D.; Hill, M.J. Lipases from plants. In *Lipases their Structure, Biochemistry and Application*; Woolley, P., Peterson, S.B., Eds.; Cambridge University Press: Cambridge, 1994; 49-75.
- Munshi, S.K.; Bhatia, N.; Sekhon, B.S.; Sulhia, P.S. Inactivation of rice brain lipase with metal ions. *J. Chem. Technol. Biotechnol.* 1993, 57, 169–174.
- Rao, K.S.V.A.; Paulose, M.M. A process for splitting of castor oil at ambient temperature using homogenised castor seed as lipase source. *Res. Ind.* 1992, 37, 36–37.
- Sagiroglu, A., and Arabaci, N. Sunflower Seed Lipase: Extraction, Purification, and Characterization. *Preparative Biochemistry and Biotechnology.* 2005, 35: 37-51.
- Sagiroglu, A.; Telefoncu, A. Synthesis of stereospecific esters and resolution of racemic alcohols with immobilized lipases. *Ind. J. Chem.* 1993, 32B, 85–87.
- Sugiura, M.; Oikawa, T.; Hirano, K.; Innukai, T. Purification, crystallization and preparation of a triacylglycerol lipase from *P. fluorescens*. *Biochim. Biophys. Acta.* 1977, 352–358.
- Swern, D. Ed. Bailey's Industrial Oil and Fat Products, 2, 4th Ed.; John Wiley & Sons: New York, 1982; 110–112.
- Teissere, M.; Borel, M.; Calliol, B.; Nari, J.; Gardies, A.M.; Noat, G. Purification and characterization of a fatty acyl-ester hydrolase from post-germinated sunflower seeds. *Biochim. Biophys.* 1995, 35, 121–125.
- Tüter, M. Castor bean lipase as a biocatalyst in the esterification of fatty acids to glycerol. *JAACS.* 1998, 75 (3), 417–420.