Cyclopenta(cd)pyrene and benzo(b)naphtho(2,1-d)thiophene are considered indicators of contamination from traffic emissions. these compounds were found in varying concentrations in most of the samples studied.

Apparently the main source of PAH contamination of fats and oils is man-induced combustion. The atmospheric fallout of adsorbed PAH compounds is deposited on the plants and other raw materials used in fat production and the contaminants are partly transferred to the food products. As mentioned above, drying processes in which large volumes of hot ambient air are blown through the seeds may essentially raise the contamination level.

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Isolation of Lipase from Germinating Oilseeds for Biotechnological Processes

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Germinating oilseeds have been explored as a possible source of lipases (glycerol ester hydrolase, EC.3.1.1.3) for the biotechnological processing of oils and fats. Seedlings of rape (*Brassica napus*) and mustard (*Sinapis alba*) at day 4 of germination and cotyledons of lupine (*Lupinus albus*) seedlings at day 3 of germination yield active crude lipase preparations upon homogenization with Tricine buffer (pH 7.5) followed by centrifugation at 23,000 g. The major portion of the lipase activity, determined with an emulsion of sunflower oil as substrate, is recovered in the supernatant fraction. These crude lipase preparations exhibit highest activity between pH 8 and 9, but they are inactive in acidic pH or at pH > 10. Each of the crude lipase preparations is highly specific for the *sn*-1,3 positions of triacylglycerols. The crude lipase preparations exhibit excellent stability on storage at −10 C, but about 50-60% of their activity is lost upon freeze-drying. Dialysis of the crude lipase prior to freeze-drying does not prevent the loss of activity. However, acetone powder obtained from the seedlings exhibits a lipase activity as high as the undialyzed crude lipase preparation.

Seeds generally contain proteins and, depending on the plant species, mainly starch or triacylglycerols as food reserve for germination. In the mobilization of these three major reserves during germination, they are hydrolyzed initially by specific proteases, amylases and lipases, respectively.

Most investigations on plant lipases have been carried out on oleaginous seeds in which lipase activity is generally found to become prominent upon germination. During the germination of oilseeds the utilization of the storage fats is initiated by stepwise hydrolysis of the triacylglycerols to free fatty acids and glycerol. These primary reactions commonly are assumed to be catalyzed by the enzyme lipase (glycerol ester hydrolase, EC.3.1.1.3) which has been demonstrated to be active in seedling tissues of many different plant species (1).

Particularly in the endosperm of germinating castor bean seeds, lipolysis has been investigated in great detail (2-4). The castor bean lipase is localized in the membrane of the lipid bodies and is already active in ungerminated seed (5). This enzyme is rather unique among oilseeds, because most of the other oilseeds examined possess no detectable activity of lipase in extracts of ungerminated seeds (6).

Theimer and Rosnitschek (7) examined the development of lipase activity in the cotyledons of rape seedlings. They found that lipase activity reaches its maximum at day 4 and has its pH optimum at 9.0. Lin and Huang (8) studied the lipase in lipid bodies from the seeds of rape and mustard. They found that lipase activity is absent in the ungerminated seeds and increases during seedling growth. Lipase activity

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increased to a peak at day 4 of seedling growth, and the maximum activity as measured with N-methylindoxylmyristate was found at pH 8.0 for rapeseed lipase and 9.0 for mustard seed lipase.

In the present work we isolated the lipase preparations from germinating seeds of rape, mustard and lupine and studied the properties of these preparations during seedling growth with the aim of exploring their possible applications in biotechnological processes.

EXPERIMENTAL

Material. Rapeseed (*Brassica napus*, cv. Quinta) and mustard seed (*Sinapis alba*, cv. Albatros) were obtained from Saaten-Union GmbH, Hannover, Federal Republic of Germany, and lupine seed (*Lupinus albus*) was obtained from the local market in Zagazig, Egypt. All reagents and chemicals used were of analytical grade and were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany.

Seed germination. Rapeseed and mustard seeds were soaked in water for 24 hr at 26 C and allowed to germinate on moist papers at 26 C in darkness for five days. Lupine seed was allowed to germinate under identical conditions without prior soaking in water.

Enzyme preparations. All operations for the isolation of the enzymes were carried out at 4 C. The entire germinating seed tissue of rape and mustard was used in the enzyme preparations, but in the case of lupine seed only the cotyledons were used.

The seed tissues were washed three times with distilled water and homogenized for 10 min in grinding medium (4 ml/g fresh weight) using an Ultra-Turrax apparatus (Janke & Kunkel GmbH, Hohenstaufen, Federal Republic of Germany). The grinding medium contained 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol and 0.15 M Tricine (N-[2-hydroxyl 1,1-bis (hydroxymethyl) ethyl] glycine) buffer adjusted to pH 7.5 with KOH (8). The homogenate was filtered through one layer of cheesecloth and centrifuged for 30 min at 23,000 g, yielding a fat layer (spherosomal fraction), a supernatant liquid and a pellet (crude particulate fraction) (7). The fat layer was carefully removed with a spatula and the supernatant liquid separated from the pellet. Aliquots of the enzyme preparation were used immediately after their isolation for the assay of lipase activity. The bulk of the preparations was either stored at −10 C or freeze-dried and the resulting powder stored at 4 C.

In one experiment, the crude lipase preparation (80 ml) obtained from 27 g seedlings of mustard at day 4 of germination was dialyzed against distilled water for 24 hr, which resulted in the appearance of a white precipitate. This precipitate was separated from the clear liquid by centrifugation. Both the precipitate and the supernatant liquid were freeze-dried and the resulting powders stored at 4 C.

Acetone powder was prepared from mustard seedlings at day 4 of germination. The seedlings (27 g) were washed three times with distilled water and homogenized using an Ultra-Turrax with 40 ml acetone at 4 C. The acetone extract was separated from the residue by centrifugation and the residue reextracted four times with 20 l each of acetone at 4 C. The residue was dried at room temperature to yield the acetone powder.

Assay of enzyme activity. Lipase activity (glycerol ester hydrolase, EC 3.1.1.3.) was determined by the titrimetric method according to Theimer and Rosnitschek (7).

For preparation of the substrate, 5 g sunflower oil (Deutsche Thomy GmbH, Karlsruhe, Federal Republic of Germany), 5 g gum arabic and 95 ml 0.89% (w/v) NaCl solution were emulsified with an Ultra-Turrax homogenizer at room temperature for 5 min. Emulsions were always prepared immediately before use. The assay mixture for lipase activity contained 5 ml substrate emulsion, 0.5 ml 10 mM sodium desoxycholate, 0.2-1.0 ml enzyme preparation and 0.89% NaCl solution, made up to a final volume of 10 ml. The mixture was incubated in screw-capped tubes at 37 C with continuous stirring, usually for a period of 10 min. The incubations were terminated by immersing the tubes in a boiling water bath for 10 min. The fatty acids liberated were measured by titration with 0.01 M KOH. Corrections were made for endogenous fatty acid production (assay mixture without substrate emulsion) and nonenzymatic fatty acid production (assay mixture without enzyme preparation).

The lipase activity was expressed as μmol triacylglycerols hydrolyzed per mg protein in 10 min.

The titrimetric method was also used for the determination of the pH optimum of lipase activity. The test mixture contained in a final volume of 10 ml:5 ml substrate emulsion, 0.5 ml 10 mM sodium desoxycholate, 0.8 ml enzyme preparation and 3.7 ml Tris buffer, adjusted to different pH values.

Protein determination. Protein content of lipase preparations was determined according to the method of Markwell et al. (9).

Lipid analysis. Aliquots from the products of lipolysis were extracted according to Folch et al. (10) in order to isolate the lipids. The lipolysis products were fractionated by thin layer chromatography (TLC) on silica gel H containing boric acid (11) in order to detect unesterified fatty acids, 2-acylglycerols, 1,2(2,3)-diacylglycerols, 1,3-diacylglycerols, and unhydrolyzed triacylglycerols.

RESULTS AND DISCUSSION

Lipase activity in various fractions. In the seedlings of rape and mustard and in the cotyledons of lupine seedlings, a major part of the lipase activity observed in the homogenate, using sunflower oil as a substrate, is recovered in the supernatant fraction (Table 1). Both oil bodies and the particulate fraction exhibit considerably lower lipase activity (less than half at the peak of lipase activity, i.e. at day 3-4) compared to the supernatant fraction. Therefore, the supernatant fraction, designated as "crude lipase," was used in most of the experiments.

Lipase activity during germination. In the seedlings of rape, mustard and lupine, the lipase activity during seedling growth was determined in both homogenate and crude lipase using sunflower oil as substrate. The data given in Table 1 show that the maximum lipase activity in both homogenate and crude lipase is reached at day 4 of seedling growth for rape and mustard, and at day 3 for lupine. These data also show that the lipase

activity is highest for mustard, followed by rape and lupine. The development of lipase activity during seedling growth, as observed for rape and mustard (Table 1), is in good agreement with earlier findings (7,8,12).

pH Optimum. Figure 1 gives the effect of different pH values on the activity of the crude lipase preparations, using sunflower oil as substrate. The preparations from 4-day-old rape and mustard seedlings display maximum lipase activity at pH 9.0, whereas the preparation from lupine at day 3 of seedling growth exhibits highest lipase activity at pH 8.5. With all the three lipase preparations no hydrolysis of triacylglycerols is observed below pH 6, and the lipase activity decreases sharply at pH 10 (Fig. 1). These findings are in agreement with earlier observations on lipase from rape and mustard seedlings (7,8,12).

Effect of enzyme concentration. The effect of concentration of the crude lipase preparations from seedlings of rape, mustard and lupine on the initial rate of hydrolysis of sunflower oil emulsion is shown in Figure 2. For each lipase preparation the rate of hydrolysis plotted against varying protein concentration at a constant substrate concentration yields a straight line that passes through the origin. These data are in close agreement with those found by Wetter (12) and Lin and Huang (8) for lipase from rape and mustard seedlings.

Effect of time. The effect of time of reaction on the rate of hydrolysis of sunflower oil by the crude lipase preparations from rape, mustard and lupine seedlings is shown in Figure 3. Apparently, the hydrolysis proceeds at a linear rate initially up to about 10 min.

Enzyme stability. In order to study the stability of the lipase preparations, the crude lipases from the seedlings of rape, mustard and lupine were held at -10 C and asayed over a 5-week period. Figure 4 shows that crude lipases from mustard and rape lose about 10-12% of activity and that crude lipase from lupine loses about 15% of activity over a period of five weeks. It also shows that each of the crude lipase preparations loses as much as 50-60% of its activity by freeze-drying.

Ory et al. (3) studied the stability of purified castor bean acid lipase. They found that the purified lipase barely lost any activity when stored for 60 days in the cold as a suspension. Sanders and Pattee (13) found that loss of activity in a crude alkaline lipase extract from peanuts was noticeable within 6-8 hr, but stability was extended to several days when the enzyme was purified by passage through Sephadex.

In one experiment, we subjected the crude lipase isolated from 4-day-old mustard seedlings to dialysis, which yielded a precipitate and a supernatant solution. Each of these fractions was freeze-dried and their lipase activities were determined. It was found that in comparison to the crude lipase with an activity of 15.8 μmol triacylglycerols hydrolyzed/mg protein · 10 min,

TABLE 1

Lipase Activity,[a] (μmol Triacylglycerols Hydrolyzed/mg Protein · 10 min) During Seedling Growth

Day	Fraction	Rape	Mustard	Lupine
1	Homogenate	0.5	0.7	0.3
	Supernatant	1.5	2.5	2.0
2	Homogenate	1.1	1.3	1.0
	Supernatant	6.0	7.2	5.1
3	Homogenate	3.0	3.2	1.8
	Supernatant	10.0	10.2	8.9
4	Homogenate	4.0	4.5	1.7
	Supernatant	13.5	15.8	8.1
5	Homogenate	3.0	3.8	1.5
	Supernatant	12.0	14.0	7.5

[a]Determined at pH 7.5.

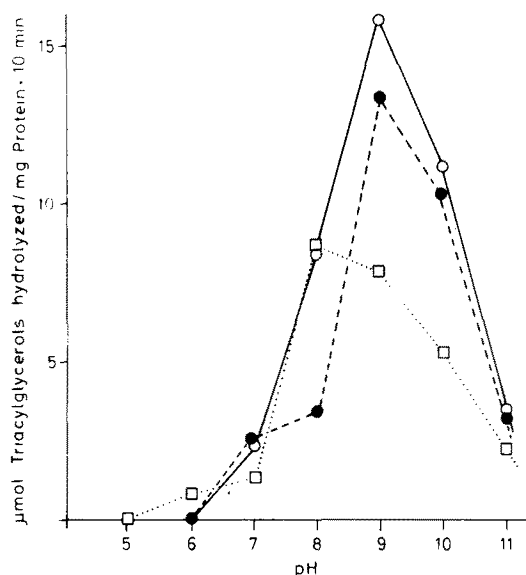

FIG. 1. Effect of pH on the activity of crude lipase isolated from seedlings of rape (● - - - ●) and mustard (○ ——— ○) at day 4 of germination, and from cotyledons of lupine seedlings (□ · · · □) at day 3 of germination.

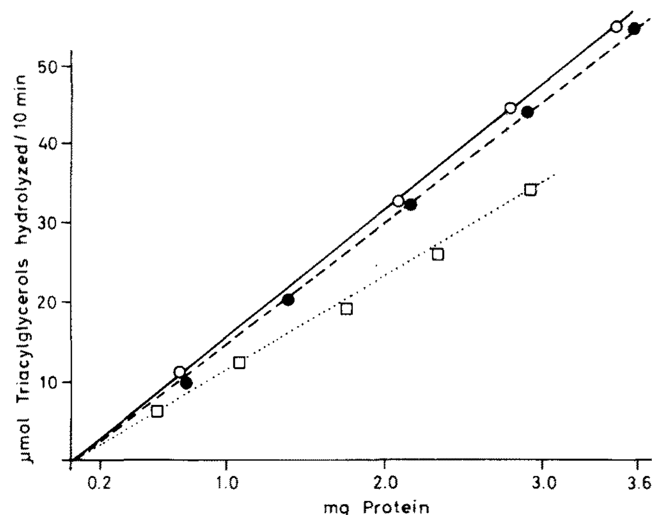

FIG. 2. Effect of protein concentration on the rate of hydrolysis of triacylglycerols catalyzed by crude lipase isolated from seedlings of rape (● - - - ●) and mustard (○ ——— ○) at day 4 of germination, and from cotyledons of lupine seedlings (□ · · · □) at day 3 of germination.

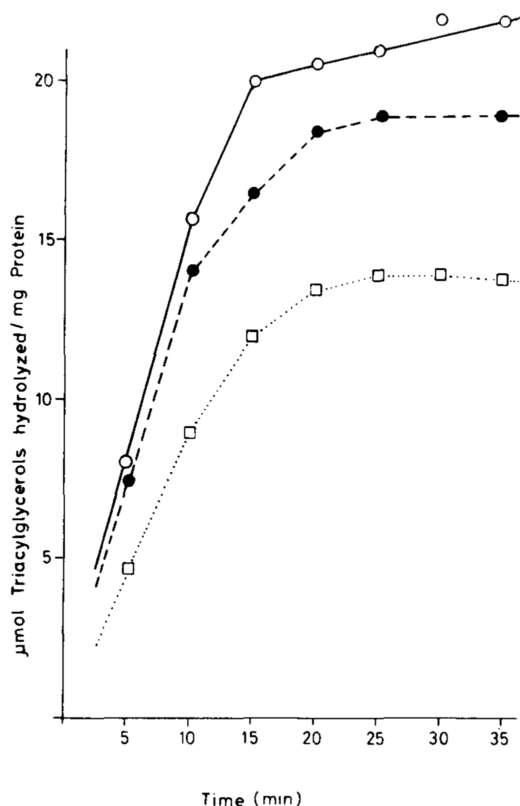

FIG. 3. Effect of reaction time on the extent of hydrolysis of triacylglycerols catalyzed by crude lipase isolated from seedlings of rape (● - - - ●) and mustard (○ ——— ○) at day 4 of germination, and from cotyledons of lupine seedlings (□ · · · □) at day 3 of germination.

the precipitate and the supernatant obtained after dialysis have lipase activities of 8.4 and 11.2 μmol triacylglycerols hydrolyzed/mg protein · 10 min, respectively. Thus, dialysis does not seem to improve the activity of the lipase preparation.

In comparison, the acetone powder obtained from 4-day-old mustard seedlings and assayed after one week of storage at 4 C exhibits a lipase activity as high as 17.4 μmol triacylglycerols hydrolyzed/mg protein · 10 min. Obviously, the simple technique of preparation of acetone powder could be quite suitable for technical use. This aspect should be further explored.

Positional specificity. In order to assess the positional specificity of lipases, the freeze-dried crude lipase preparations isolated from the seedlings of rape, mustard and lupine were used for the hydrolysis of sunflower oil for 5 and 15 min and the products of lipolysis examined by TLC (Fig. 5). It is quite evident from the chromatogram that the lipolysis products obtained with each lipase preparation are almost exclusively composed of 2-acylglycerols, 1,2 (2,3)-diacylglycerols and fatty acids. Obviously, each of the preparations is highly specific for the *sn*-1,3 positions of triacylglycerols. Minor proportions of 1,3-diacylglycerols detected in the products of lipolysis are attributed to acyl migration during incubation and lipid extraction.

Current interest in biotechnology has been extended recently to the exploration of lipase-catalyzed reactions for the commercial processing of oils and fats (14). So far, only microorganisms have been considered as sources of lipase. The findings reported here show that germinating seedlings of oil-bearing seeds have a good potential as a source of lipase for biotechnological processes. Seedlings are easy to grow and harvest. Crude lipase preparations can be obtained from the seedlings by simple processes, such as extraction with

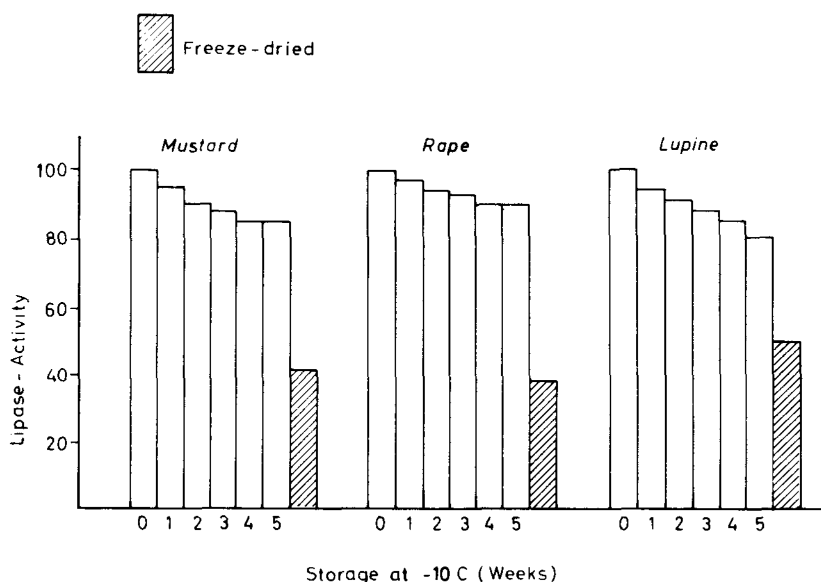

FIG. 4. Effect of storage and freeze-drying on the activity of crude lipase isolated from seedlings of rape and mustard at day 4 of germination, and from cotyledons of lupine seedlings at day 3 of germination.

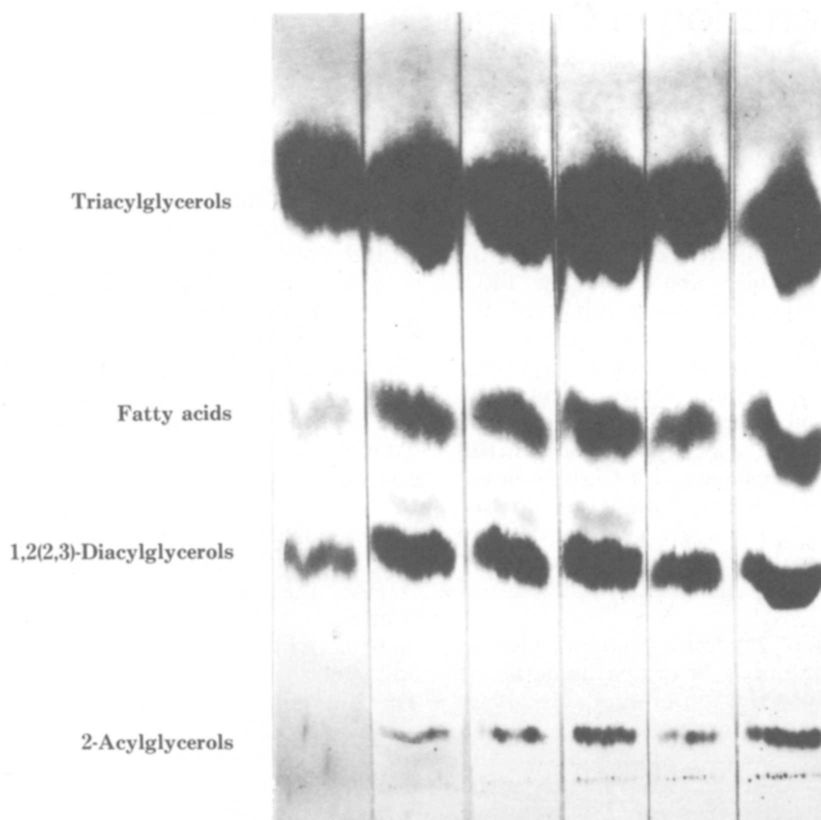

FIG. 5. Thin layer chromatogram of lipolysis products of sunflower oil catalyzed for 5 and 15 min, respectively, by freeze-dried crude lipase isolated from seedlings of rape (lanes 5 and 6) and mustard (lanes 1 and 2) at day 4 of germination, and from cotyledons of lupine seedlings (lanes 3 and 4) at day 3 of germination.

buffer solutions or acetone. Activity and stability of these crude lipase preparations are more than satisfactory to warrant further studies on their use in hydrolysis, esterification and interesterification of fats and oils.

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