Germinating Rapeseed as Biocatalyst: Hydrolysis of Exogenous and Endogenous Triacylglycerols†

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Germinating oilseeds are well-known sources of lipases (triacylglycerol acylhydrolases, EC 3.1.1.3). Seedlings of low-erucic rape (*Brassica napus* cv. Ceres) at day 3–5 of germination have been homogenized in Tris-HCl buffer and the homogenate used as biocatalyst for the hydrolysis of low-erucic rapeseed oil. The rape seedlings were found to be highly active in the hydrolysis of exogenous low-erucic rapeseed oil. The optimum rates of hydrolysis were found at day 4 of germination, pH 8.0, and a temperature of 30 °C. The oil (storage triacylglycerols) contained in the seeds of low-erucic rape has also been efficiently hydrolyzed by homogenization of the germinating seedlings in Tris-HCl buffer (pH 8) followed by incubation at ambient temperature. The fatty acids formed undergo very little catabolism via β-oxidation, and they can be recovered by extraction with hexane or by centrifugation. These findings open up an alternative mild biotechnological approach for the production of fatty acids from plant oils via lipolysis of the seed storage triacylglycerols *in situ* as opposed to the conventional fat splitting at high temperatures and pressures.

Keywords: *Germinating rapeseed; lipase from rapeseed; biocatalyst; Brassica napus*

INTRODUCTION

The storage triacylglycerols of oil-rich seeds are hydrolyzed during germination of such seeds by the action of the endogenous lipase, and the lipolysis products, i.e. fatty acids and glycerol, are metabolized further in glyoxysomes and other organelles. It is well-known that germinating seedlings of many oilseeds are a rich source of lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), which have been isolated, purified, and characterized from such tissues, and some of these purified enzyme preparations have been used as catalysts in the biotransformation of lipids (Mukherjee, 1994; Mukherjee and Hills, 1994). Especially, lipase preparations from germinating seedlings of the very common oilseed rape (*Brassica napus*) have been isolated (Theimer and Rosnitscheck, 1978; Hassanien and Mukherjee, 1986), partially purified (Hills and O'Sullivan 1989; Weselake et al., 1989), and used as a biocatalyst after partial purification and immobilization (Hills et al., 1989, 1990a,b, 1991; Hills and Mukherjee, 1990; Ncube et al., 1993).

Here we report the use of germinating rapeseed homogenates—without any isolation or partial purification of the lipase—as a biocatalyst for the hydrolysis of added triacylglycerols as a possible biotechnological alternative to conventional fat splitting at high temperatures and pressures for the production of fatty acids. Moreover, we show the possibility of using the rape seedlings as biocatalyst for hydrolysis of endogenous triacylglycerols *in situ*.

MATERIALS AND METHODS

Materials. Seeds of low-erucic rape (*B. napus*) cultivar Ceres were provided by Norddeutsche Pflanzenzucht, Hohenlieth, Germany. Low-erucic rapeseed oil (0.1% free fatty acids) was from Noblee & Thörl, Hamburg, Germany. All chemicals of analytical grade and adsorbents were purchased from E. Merck, Darmstadt, Germany. Distilled solvents were used throughout.

Preparation of Biocatalyst. Seeds of low-erucic rape were germinated on moistened filter paper in shallow plastic trays, covered with aluminum foil provided with perforations, for various periods at temperatures between 20 and 25 °C. The whole seedlings were harvested, and routinely the seedlings (1 g) were homogenized in an ice–water bath with 1 mL of Tris-HCl buffer (50 mM) at different pH values using an Ultra-Turrax homogenizer (Janke & Kunkel, Hohenstaufen, Germany). The homogenate was used in hydrolysis reactions as such.

Hydrolysis Reactions. Routinely, 1 g of the rape seedlings of different ages, homogenized with Tris-HCl buffer, was added to 1 g of low-erucic rapeseed oil and incubated for various periods at room temperature or in a thermostatically controlled glycerin bath by magnetic stirring. Alternatively, the homogenate containing only the endogenous oil, without any addition of exogenous oil, was incubated for different periods.

In several experiments 10 g of the ground dormant low-erucic rapeseed was incubated as described above with 10 g of low-erucic rapeseed oil in the presence of 10 mL of Tris-HCl buffer (50 mM, pH 7.0). Moreover, some incubations were carried out with mixtures of 10 g of the ground dormant low-erucic rapeseed together with various proportions of germinating rape seedlings in the presence of 10 mL of Tris-HCl buffer (50 mM, pH 8.0).

Lipid Extraction and Analysis. Lipids were extracted from the rape seedlings at different stages of germination as well as from the products of hydrolysis by extraction according

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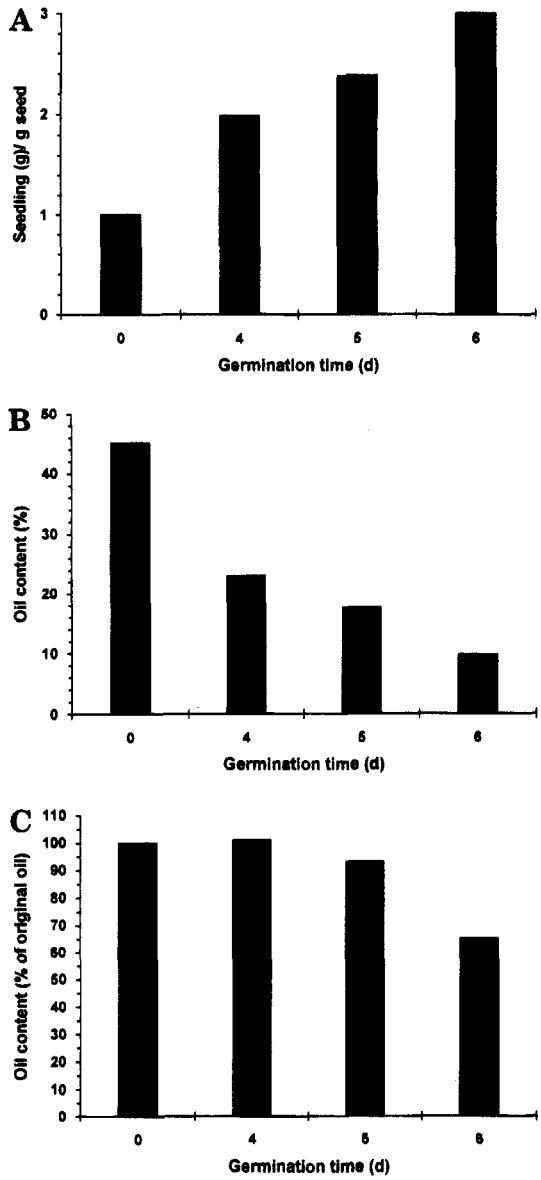

Figure 1. Seedling weight (A), oil content (B), and percentage of original oil remaining (C) during germination of rape.

to the method of Bligh and Dyer (1959). Subsequently, the residual lipids were recovered from the residue by repeated extractions with a mixture of hexane/diethyl ether (1:1 v/v). The lipid extracts were combined.

The proportion of fatty acids formed by hydrolysis of triacylglycerols was determined titrimetrically (IUPAC, 1987). Values of fatty acids formed are reported as a percentage of that theoretically possible. The lipids from the germinating seedlings were converted to methyl esters (Schulte, 1993), which were analyzed by gas chromatography in a Perkin-Elmer F-22 instrument equipped with flame ionization detectors. The separations were carried out on a 30 m × 0.25 mm i.d. DB 23 fused silica capillary column of 0.25 μm film thickness (methyl/50% cyanopropyl silicone, J&W, Fisons Instruments, Mainz, Germany) using a temperature program from 150 to 230 °C at a rate of 4 °C/min and nitrogen (10 mL/min) as carrier gas.

RESULTS

Changes in the weight and oil content of rape seedlings during germination show a steep increase in weight during days 4 and 6 of germination (Figure 1A), whereas the oil content with respect to the fresh weight of the seedlings continuously decreases (Figure 1B). However, the data presented in Figure 1C show that

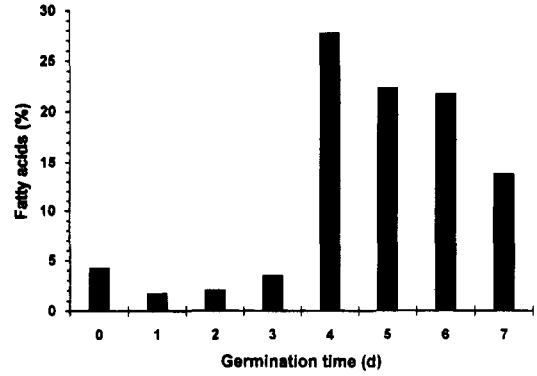

Figure 2. Lipolytic activity of homogenized rape seedlings of different ages, measured as percent fatty acids formed upon 24 h of incubation of 1 g of seedlings with 1 g of low-erucic rapeseed oil in 1 mL of Tris-HCl buffer (50 mM, pH 7.0) at room temperature.

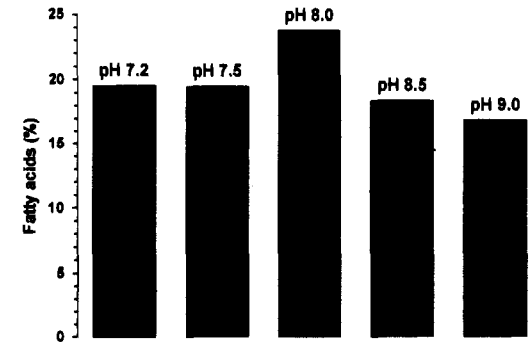

Figure 3. Effect of pH on lipolytic activity of homogenized rape seedlings at day 4 of germination, measured as percent fatty acids formed upon 24 h of incubation of 1 g of seedlings with 1 g of low-erucic rapeseed oil in 1 mL of Tris-HCl buffer (50 mM) at 30 °C.

the total amount of oil, present in the original seeds, remains essentially unchanged till day 4 of germination. Obviously, utilization of the storage triacylglycerols does not begin until this time. From day 4 of germination onward, the storage triacylglycerols are catabolized, as evident from a steep decrease in the amount of total oil with respect to the oil originally present in the seeds (Figure 1C). Obviously, the decrease in oil content of the seedlings during germination is predominantly due to accumulation of moisture (Figure 1B). Some variations in the initial oil content among different batches of the seedlings were observed that are attributed to variations in the conditions of germination, such as temperature and humidity.

Lipolytic activity of the homogenates of rape seedlings at various stages of germination was examined using a large excess of added low-erucic rapeseed oil as substrate. The results given in Figure 2 show that the peak of lipolytic activity is attained at day 4 to day 5 of germination, after which time the extent of formation of fatty acids per gram of seedlings decreases. These results agree with the steep increase in the catabolism of storage triacylglycerols after day 4 of germination (Figure 1C).

The results given in Figure 3 show that the pH of the buffer has a strong effect on the extent of lipolysis catalyzed by homogenates of 4-day-old rape seedlings; the highest lipolytic activity is observed at pH 8.0. These data agree well with the known pH optimum of partially purified rapeseed lipase (Theimer and Rosnitscheck, 1978; Hassanien and Mukherjee, 1986; Weselake et al., 1989).

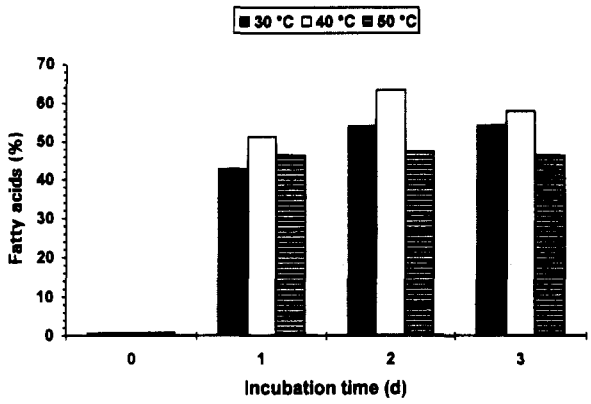

Figure 4. Effect of temperature on lipolytic activity of homogenized rape seedlings at day 5 of germination, measured as percent fatty acids formed upon incubation of 4 g of seedlings with 1.3 g of low-erucic rapeseed oil in 4 mL of Tris-HCl buffer (50 mM, pH 8.0) at different temperatures.

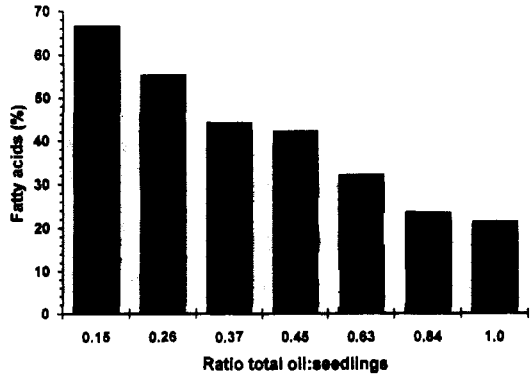

Figure 5. Percent fatty acids formed during lipolysis of triacylglycerols upon incubation (24 h) of homogenized 4-day-old rape seedlings (1 g) with different proportions of low-erucic rapeseed oil in 1 mL of Tris-HCl buffer (50 mM, pH 8.0) at 22 °C.

The data on the effect of temperature on the lipolysis of triacylglycerols, catalyzed by homogenates of rape seedlings, show some increase in the extent of hydrolysis when the temperature is raised from 30 to 40 °C; further increase of temperature to 50 °C led to a decrease in lipolysis (Figure 4).

In one set of experiments, the extent of lipolysis catalyzed by homogenates of rape seedlings was measured by adding various amounts of low-erucic rapeseed oil at different ratios of total oil, i.e. endogenous plus exogenous oil, to seedlings. The data presented in Figure 5 show that the percent fatty acids formed increases when the total oil to seedling ratio is lowered. The absolute free fatty acid content increases from 96 to 232 mg/g of seedlings when the ratio of grams of oil to grams of seedlings is increased from 0.15 to 1.0.

To examine the extent of hydrolysis of exogenous oil by the rape seedlings, homogenates of the seedlings at day 4 of germination were incubated with added low-erucic rapeseed oil at a ratio of about 2 to 1 of exogenous to endogenous oil for various periods. The results given in Figure 6 show that both exogenous and endogenous triacylglycerols were extensively hydrolyzed to fatty acids after 3–4 days of incubation.

Several experiments were carried out to determine whether lipolysis of triacylglycerols also occurs when ground dry rapeseeds are incubated together with added low-erucic rapeseed oil in the presence of Tris-HCl buffer. The results of these experiments, presented in Figure 7, show that the extent of hydrolysis is quite low

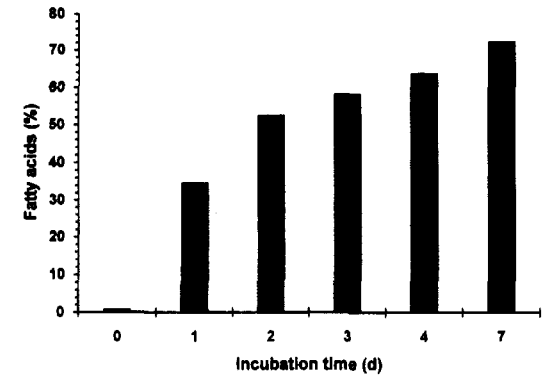

Figure 6. Percent fatty acids formed during lipolysis of triacylglycerols upon incubation of homogenized 4-day-old rape seedlings (5 g) with 1.35 g of low-erucic rapeseed oil in 5 mL of Tris-HCl buffer (50 mM, pH 8.0) for different periods at 22 °C.

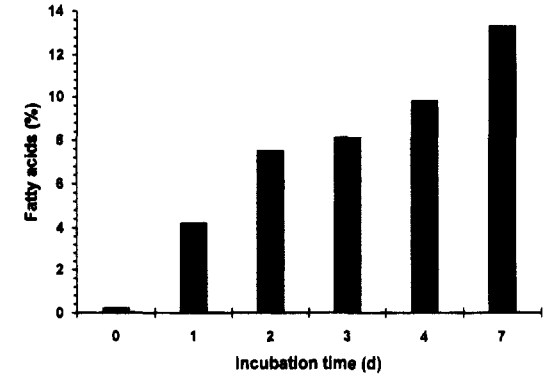

Figure 7. Percent fatty acids formed during lipolysis of triacylglycerols upon incubation of ground dormant rapeseeds (10 g) with 10 g of low-erucic rapeseed oil in 10 mL of Tris-HCl buffer (50 mM, pH 7.0) for different periods at 22 °C.

when the dormant seed, rather than germinating seedlings, is used as a biocatalyst. These findings agree with the data given in Figure 2 on lipolytic activity at various stages of germination.

Figure 8 shows the lipolytic activity of the homogenates of rape seedlings without any addition of exogenous oil at three different stages of germination. If the lipolytic activity is expressed as percent fatty acids formed, the highest activity is observed for the 6-day-old seedlings, followed by 5- and 4-day-old seedlings (Figure 8A). However, if the lipolytic activity is expressed as yield of fatty acid per gram of seedling, the highest activity is observed in incubations up to 3 days for the 5-day-old seedlings, followed by the 6- and 4-day-old seedlings. The apparent discrepancy between the results given in parts B and A of Figure 8 is attributed to the fact that the amount of oil per gram of seedlings is lower for the 6-day-old seedlings than for the 5-day-old seedlings (Figure 1B), with the consequence that less triacyglycerol substrate per weight of seedling was available, which led to the observed higher lipolytic activities (Figure 8A). If the lipolytic activity is expressed as yield of fatty acids per gram of original seeds, essentially no difference is observed between the seedlings at days 5 and 6 of germination, whereas the yield of fatty acids is distinctly lower at day 4 of germination (Figure 8C).

Finally, one set of experiments was designed to evaluate whether the germinating rape seedlings can also be used as a biocatalyst for hydrolysis *in situ* of the oil present in dry dormant seed, rather than hydrolysis of the oil after it has been recovered from

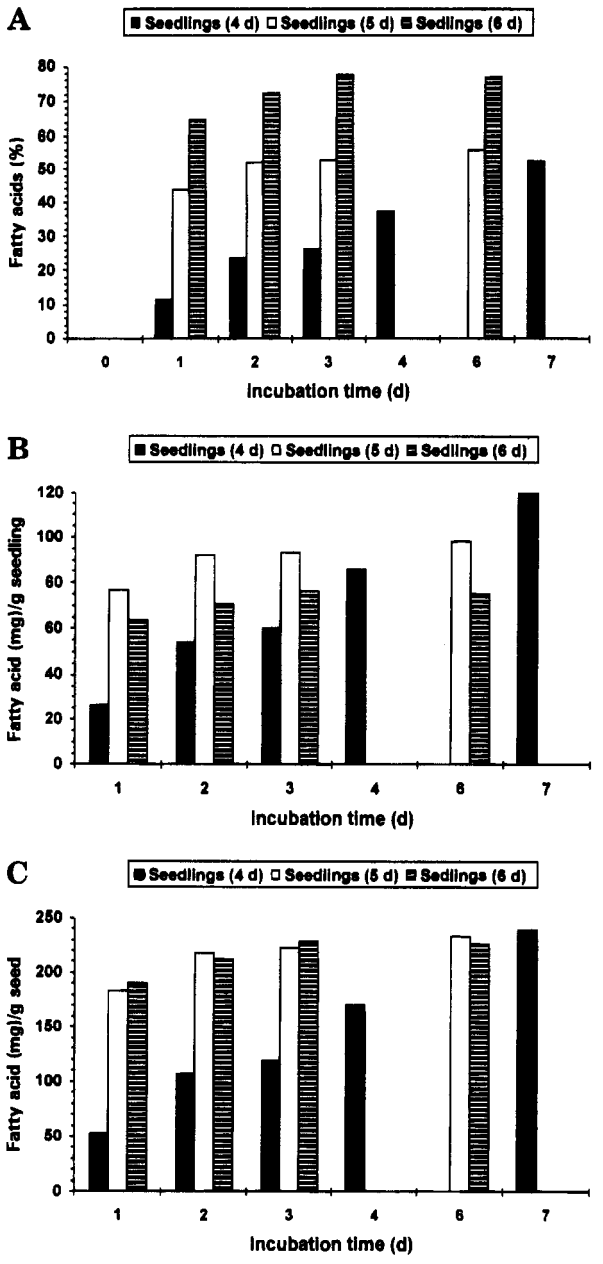

Figure 8. Percent fatty acids formed (A), yield of fatty acids per gram of seedlings (B), and yield of fatty acids per gram of original seed (C) during lipolysis of endogenous triacylglycerols upon incubation of homogenized rape seedlings of different ages. Incubations were carried out at 22 °C in Tris-HCl buffer (50 mM, pH 8.0) using 19.9 g of seedlings plus 25 mL of buffer (seedlings at day 4), 23.8 g of seedlings plus 30 mL of buffer (seedlings at day 5), or 60.0 g of seedlings plus 60 mL of buffer (seedlings at day 6).

the seed by solvent extraction or other processes. For this purpose homogenates of germinating rape seedlings were incubated with ground dry rapeseed in the presence of Tris-HCl buffer. The results presented in Figure 9 show that substantial hydrolysis of the triacylglycerols occurs after prolonged incubation. As expected, the extent of hydrolysis increases with decreasing ratio of dry seed to seedlings.

DISCUSSION

The results of our studies show that during germination of rapeseed very little catabolism of the endogenous storage triacyglycerols occurs until 4–5 days of germination (Figure 1C).

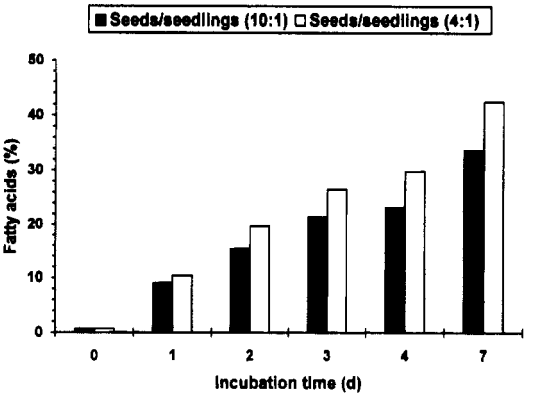

Figure 9. Percent fatty acids formed during lipolysis of triacylglycerols upon incubation of ground dormant rapeseeds (10 g) with different proportions of 4-day-old rape seedlings in 10 mL of Tris-HCl buffer (50 mM, pH 8.0) for different periods at 22 °C.

Optimum parameters for the hydrolysis of exogenous oil are 4-day-old seedling age (Figure 2), incubation time of 4–5 days (Figure 6), pH of 8.0 (Figure 3), temperature of 40 °C (Figure 4), and a ratio of total oil to seedlings of 0.3–0.5 (Figure 5).

The results presented in Figures 2–7 show that homogenates of rape seedlings can be used efficiently as a biocatalyst for hydrolysis of exogenous oils. In contrast to homogenates of germinating rape seedlings, those of dormant seeds have very little lipolytic activity, as evident from the data presented in Figure 7. The data presented in Figure 9 show, however, that homogenates of germinating seedlings can also be used as a biocatalyst for the hydrolysis of the storage oil contained in the dormant seed by incubation of the ground seed with the seedling homogenate.

Homogenization of the rape seedlings at 5–6 days after germination followed by incubation for 2–3 days leads to extensive (50–80%) hydrolysis of the endogenous oil (Figure 8A). Obviously, homogenization enables the triacylglycerol lipase of the seedlings to hydrolyze the storage triacylglycerols, which leads to rapid accumulation of the fatty acids. It appears that the fatty acids formed are not subjected to catabolic processes that are known to occur in cell organelles, such as glyoxysomes. It seems likely that the catabolic activities of various cell organelles are lost due to disruption by homogenization, although the lipolytic activity is unaffected. These findings indicate that germination of rapeseed, followed by homogenization and incubation, may offer an alternative biotechnological approach for the production of fatty acids from the storage oil utilizing the endogenous lipase of the seedlings as biocatalyst.

An accompanying paper describes the substrate selectivity of the homogenates from germinating rapeseed as a biocatalyst for the hydrolysis of oils containing common and unusual fatty acids as constituents of the storage triacylglycerols.

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