Esterification and Interesterification Reactions Catalyzed by Acetone Powder from Germinating Rapeseed[1]

I. Jachmanián[2] and K.D. Mukherjee*

Institute for Biochemistry and Technology of Lipids, Federal Centre for Cereal, Potato and Lipid Research, D-48147 Münster, Germany

ABSTRACT: Acetone powder from germinating rape (*Brassica napus* L.) seedlings exhibits essentially similar activity in lipolysis of triacylglycerols as the corresponding seedling homogenates. Acetone powder from rape seedlings catalyzes the esterification of a fatty acid, such as oleic acid, with *n*-butanol or a long-chain alcohol, such as oleyl alcohol. Furthermore, the acetone powder catalyzes alcoholysis of a methyl ester, such as methyl oleate with *n*-butanol or oleyl alcohol, and acidolysis of methyl oleate with a fatty acid, such as erucic acid. However, triacylglycerols are not accepted as substrates for interesterification reactions. In esterification of fatty acids with *n*-butanol, catalyzed by the acetone powder from rape seedlings, fatty acids having an olefinic bond next to the carboxyl group as a *cis*-6 double bond, e.g., γ-linolenic, gorlic and petroselinic acids, or those having a *cis*-4 double bond, e.g., docosahexaenoic acid, are strongly discriminated against as substrates. Such substrate selectivities can be utilized for the enrichment of definite fatty acids from mixtures, derived from naturally occurring oils, *via* kinetic resolution.

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KEY WORDS: Acetone powder, esterification, interesterification, lipase, rape seedlings.

Germinating oilseeds are cheap and alternative sources of triacylglycerol lipases (EC 3.1.1.3) (1,2). Lipase from germinating seedlings of oilseed rape (*Brassica napus* L.) after partial purification and immobilization has been used as biocatalyst for esterification and interesterification of lipids (3–7). We have recently described the use of germinating rapeseed homogenates, without any isolation or partial purification of the lipase, as biocatalysts for the hydrolysis of endogenous and exogenous triacylglycerols (8,9). Competitive factors for the esterification of different fatty acids with *n*-butanol using an acetone powder of germinating rapeseed were also reported (10). Here we report the ability of the acetone powder of germinating rapeseed in catalyzing esterification and interesterification reactions.

[1]Part of the doctoral thesis of Iván Jachmanián to be submitted to Facultad de Química, Universidad de la República, Montevideo, Uruguay.

[2]Present address: Cátedra de Fisicoquímica, Facultad de Química, Universidad de la República, Montevideo, Uruguay.

*To whom correspondence should be addressed at Institute for Biochemistry and Technology of Lipids, Federal Centre for Cereal, Potato and Lipid Research, Piusallee 68, D-48147 Münster, Germany.

EXPERIMENTAL PROCEDURES

Materials. Seeds of low-erucic rape (*B. napus*) cultivar Ceres were provided by Norddeutsche Pflanzenzucht, Hohenlieth, Germany. Coriander (*Coriandrum sativum*) seed oil and borage (*Borago officinalis*) seed oil were kind gifts from Henkel, (Düsseldorf, Germany) and International Food Science Centre, (Lystrup, Denmark), respectively. *Hydnocarpus wightiana* seed oil and hake (*Merluccius hubbsi*) liver oil were obtained by extraction. The fatty acids were prepared from the above oils by hydrolysis at room temperature (11). 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 to 25°C. The whole seedlings were harvested, and the homogenate was prepared from 5 g seedlings at day 4 of germination with 5 mL Tris-HCl buffer (50 mM, pH 8.0) using an Ultra-Turrax homogenizer (Janke & Kunkel, Hohenstaufen, Germany) in an ice-water bath.

For the preparation of acetone powder, 10 g rape seedlings at day 5 of germination were first rinsed successively with 10 mL each of distilled water and acetone. Subsequently, 20 mL ice-chilled acetone were added and the mixture homogenized using an Ultra-Turrax homogenizer in an ice-water bath. The homogenate was centrifuged, the supernatant removed, and the pellet extracted four more times with acetone in a similar manner. Finally, the pellet was suspended in a small volume of acetone, transferred to a round-bottom flask, and dried using a rotary evaporator without heating. The acetone powder (about 800 mg) was stored in a refrigerator under nitrogen.

Determination of lipolytic activity. The homogenates of rape seedlings were incubated with 1.35 g low-erucic rapeseed oil for various periods at room temperature by magnetic stirring. Under the same conditions, the acetone powder (170 mg) was incubated with 1 g low-erucic rapeseed oil and 3 mL Tris-HCl buffer (50 mM, pH 8.0). Lipids were extracted from the reaction mixtures, and the amount of fatty acids formed was determined as described below.

Esterification. Reactions were carried out in glass tubes provided with Teflon-lined screw-caps using 50 mg of oleic acid with two times the stoichiometric amount of *n*-butanol, 2-butanol, or oleyl alcohol. Alternatively, 100 mg of fatty acid mixtures obtained from naturally occurring oils *via* saponification as described in reference (11) were incubated with two times the stoichiometric amount of *n*-butanol. All reactions were carried out in the presence of 50 mg of acetone powder and 1 mL of hexane by magnetic stirring at room temperature for various periods.

Interesterification. All reactions, unless stated otherwise, were carried out as described above in the presence of 50 mg of acetone powder and 1 mL of hexane except for the experiments with tripalmitin in which 3 mL of hexane were used.

Alcoholysis reactions were carried out using 50 mg of methyl oleate and double or half the stoichiometric amount of *n*-butanol or oleyl alcohol, respectively. Alternatively, 50 mg of triolein were reacted with double the stoichiometric amount of *n*-butanol or linoleyl alcohol. In a further experiment 50 mg of tripalmitin were reacted with double the stoichiometric amount of oleyl alcohol.

Under similar conditions, acidolysis reactions were carried out using 50 mg of methyl oleate and double the stoichiometric amount of erucic acid or using 50 mg each of triolein, tripalmitin, or tricaprin with double the stoichiometric amounts of erucic acid, oleic acid, and lauric acid, respectively. In one experiment 80 mg of monoolein were reacted in the presence of 25 mg acetone powder under the conditions described above.

Lipid extraction and analysis. Lipids were extracted from the products of hydrolysis according to Bligh and Dyer (12). Subsequently, the remaining lipids were recovered from the residue by repeated extractions with a mixture of hexane/diethyl ether (1:1, vol/vol). The lipid extracts were combined. In the case of reaction products formed by esterification and interesterification, the lipids were extracted with hexane. In order to determine the lipolytic activity of homogenates and acetone powders, the proportion of fatty acids formed by hydrolysis of triacylglycerols was determined titrimetrically (13).

All preparative separations of lipid classes, unless stated otherwise, were carried out by thin-layer chromatography on Silica Gel H using hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as developing solvent. The lipid fractions were made visible by exposing the edges of the chromatoplates to iodine vapor.

From the reaction products formed by esterification, the fractions containing fatty acids and butyl esters were scraped, a known amount of methyl heptadecanoate was added as internal standard, and the lipids were eluted with water-saturated diethyl ether. The fatty acids were converted to methyl esters using diazomethane, and both methyl esters and butyl esters were analyzed by gas chromatography (8,9).

The products formed by alcoholysis of methyl oleate with *n*-butanol were analyzed directly by gas chromatography to determine the proportions of methyl oleate and butyl oleate (8). From the products of alcoholysis of methyl oleate with oleyl alcohol, the wax esters formed were separated from the reaction partners by thin-layer chromatography on Silica Gel H using hexane/diethyl ether (90:10, vol/vol) as developing solvent. The unreacted oleyl alcohol and methyl oleate were eluted from adsorbent, combined, and their relative proportion determined by gas chromatography (8). The products formed by alcoholysis of triacylglycerols with *n*-butanol or long-chain alcohols were qualitatively analyzed by thin-layer chromatography, as described above, for the detection of formation of butyl esters or wax esters, respectively. From the reaction products formed by acidolysis, triacylglycerols or methyl ester were isolated by thin-layer chromatography, as described above, and analyzed by gas chromatography after triacylglycerols were converted to methyl esters.

The products formed by interesterification of monoacylglycerols were treated with diazomethane to convert the fatty acids to methyl esters and subsequently acetylated (14) to convert the mono- and diacylglycerols to the corresponding di- and monoacetates, respectively. Finally, the mixture of methyl oleate, monooleylglycerol diacetate, dioleylglycerol acetate, and triolein was analyzed by gas chromatography in a Perkin Elmer F-22 instrument (Palo Alto, CA) equipped with flame-ionization detector. The separations were carried out on a 50 cm × 4 mm internal diameter glass column, packed with 3% OV-1 on 100/120 mesh Gas Chrom Q (Applied Science Laboratories Inc., State College, PA). Immediately after injection the temperature was programmed from 120 to 360°C, 12.5°C/min, holding at 360°C for 20 min. Peaks were quantitated using correction factors that were obtained from synthetic mixtures containing known amounts of methyl oleate, monooleylglycerol diacetate, dioleylglycerol acetate, and triolein.

RESULTS AND DISCUSSION

Lipolytic activity of acetone powder. Table 1 summarizes the results of various reactions carried out with different substrates using acetone powder from germinating rapeseed as biocatalyst. Figure 1 shows the lipolytic activity, measured as the amount of fatty acid formed per gram rape seedling used as starting material, upon incubation of the acetone powder or homogenate with exogenous triacylglycerols (rapeseed oil) for various periods. Although the seedlings at day 4 of germination had the highest lipolytic activity (8), the seedlings at day 5 of germination were chosen for the preparation of acetone powder, because they were more firm, therefore easier to harvest. The data given in Figure 1 show that despite difference in the age of seedlings used the lipolytic activity is essentially similar for both acetone powder and the homogenate. It is conceivable that some lipase inhibitors were eliminated by solvent extraction during preparation of the acetone powder.

Esterification catalyzed by acetone powder. The extent of esterification of oleic acid with oleyl alcohol or *n*-butanol, catalyzed by the acetone powder from rape seedlings is

TABLE 1
Summary of Reactions Catalyzed by Acetone Powder from Rape Seedlings and the Results Obtained

Reaction	Substrate A	Substrate B	B/A[a]	Result[b]
Hydrolysis	Rapeseed oil	Water	[c]	++
Esterification	Oleic acid	*n*-Butanol	2	++
	Oleic acid	2-Butanol	2	--
	Oleic acid	Oleyl alcohol	2	++
	Mixture of fatty acids	*n*-Butanol	2	++
Alcoholysis	Methyl oleate	*n*-Butanol	2	+-
	Methyl oleate	Oleyl alcohol	0.5	++
	Triolein	*n*-Butanol	6	--
	Triolein	Linoleyl alcohol	6	--
	Tripalmitin	Oleyl alcohol	6	--
Acidolysis	Methyl oleate	Erucic acid	2	+-
	Triolein	Erucic acid	6	--
	Tripalmitin	Oleic acid	6	--
	Tricaprin	Lauric acid	6	+-

[a]Molar ratio between compound B and A.
[b]Conversion after 24 h incubation: (++) >40%, (+-) <10%, (--) ≅0%.
[c]Water in a large excess

shown in Figure 2. Although somewhat higher rate of esterification is observed initially with oleyl alcohol as compared to *n*-butanol, the esterification is essentially complete with both alcohols after 2 d of incubation (Fig. 2).

Interesterification catalyzed by acetone powder. Despite similar rates of esterification observed for both oleyl alcohol and *n*-butanol (Fig. 2), the rate of alcoholysis of methyl oleate, catalyzed by the acetone powder, is 20- to 30-fold higher for oleyl alcohol as compared to *n*-butanol (Fig. 3). As described before (15), no esterification was observed when 2-butanol was used instead of *n*-butanol.

The rates observed in the acidolysis of methyl oleate with erucic acid, catalyzed by the acetone powder from rape seedlings, were rather low (Fig. 4). The acetone powder from rape seedlings was unable to catalyze the alcoholysis reactions between triolein or tripalmitin with *n*-butanol or oleyl

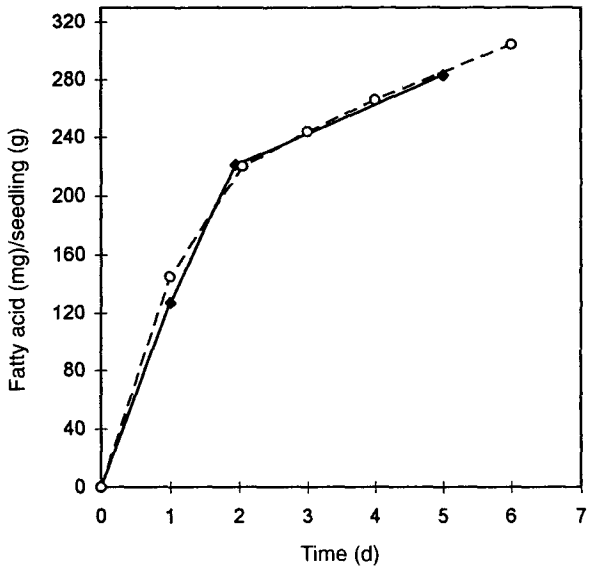

FIG. 1. Lipolytic activity of acetone powder from rape seedlings on day 5 of germination (◆) as compared to that of the corresponding homogenized rape seedlings on day 4 of germination (○). The activity was measured as the amount of fatty acids formed, per gram seedling from which the acetone powder or homogenate was prepared, upon incubation with added low-erucic rapeseed oil for different periods.

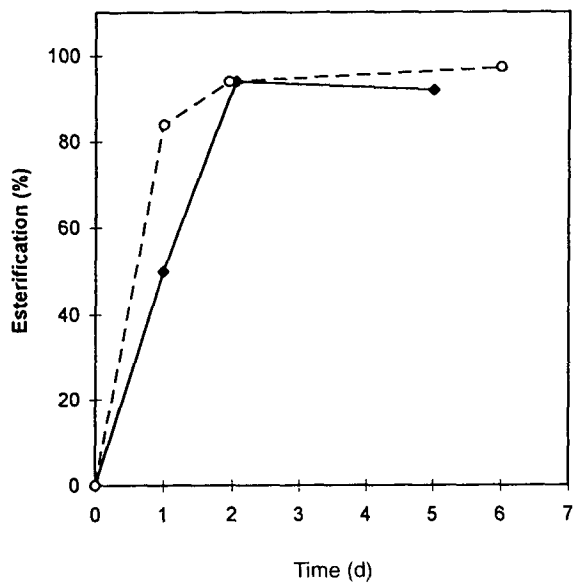

FIG. 2. Esterification of oleic acid with *n*-butanol (◆) or oleyl alcohol (○) catalyzed by acetone powder from rape seedlings.

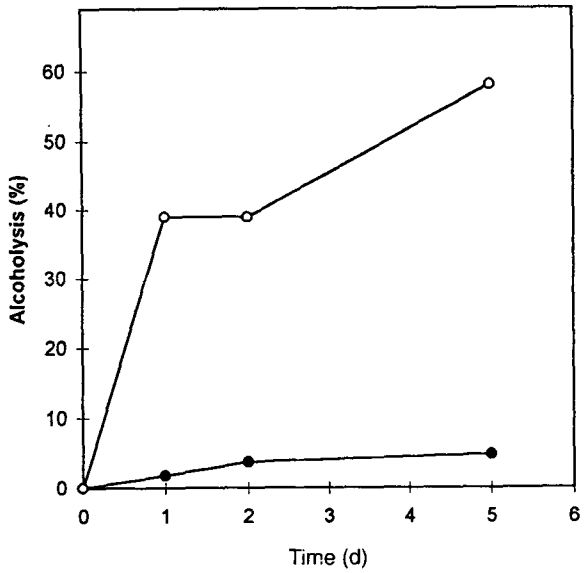

FIG. 3. Alcoholysis of methyl oleate with *n*-butanol (●) or oleyl alcohol (○) catalyzed by acetone powder from rape seedlings.

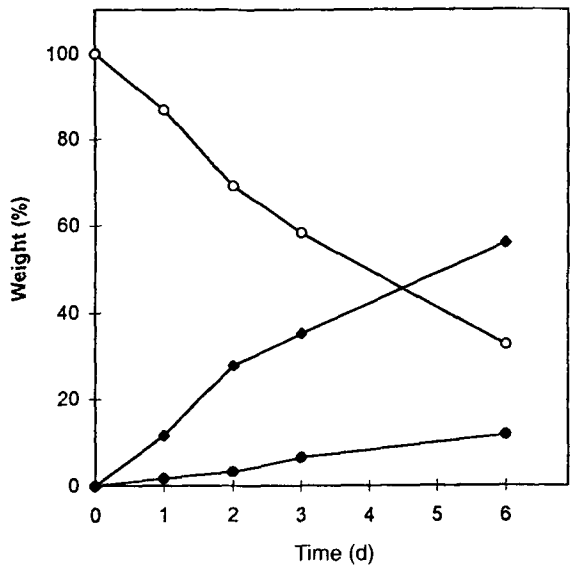

FIG. 5. Weight percentage of monoolein (○), diolein (◆), and oleic acid (●) during the course of transesterification of monoolein catalyzed by acetone powder from rape seedlings. No triolein was formed.

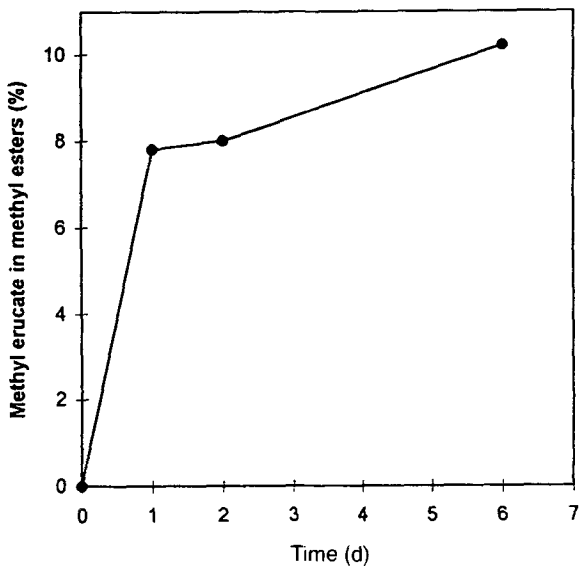

FIG. 4. Acidolysis of methyl oleate with erucic acid catalyzed by acetone powder from rape seedlings.

alcohol and between triolein and linoleyl alcohol (data not shown). Similarly, with the acetone powder as biocatalyst, no appreciable acidolysis occurred between triolein, tripalmitin, or tricaprin with erucic, oleic, and lauric acids, respectively, although a purified rapeseed lipase has been recently reported to catalyze acidolysis between tricaprin and lauric acid (7).

Incubation of monoolein with acetone powder from rape seedlings led to a progressive decrease with time in the relative proportion of monoolein with a concomitant increase in the level of diolein and minor increase in the proportion of oleic acid (Fig. 5). These data are similar to those observed with purified rapeseed lipase preparation (6) and they show that interesterification of monoolein yields diolein, but no triolein is formed.

Selectivity in esterification catalyzed by acetone powder. In esterification and interesterification reactions, catalyzed by purified lipase preparation from rape seedlings, fatty acids/acyl moieties having a *cis*-4 or *cis*-6 double bond as the first olefinic bond next to the carboxyl group have been found to be discriminated against as substrates (15). Competitive factors for esterification of fatty acids with *n*-butanol were also reported (10), showing that such substrate specificities are also exhibited by the acetone powder from rape seedlings. In this study, the substrate specificity was determined with fatty acid mixtures derived from natural oils rather than pure fatty acids as substrates, using acetone powder from rape seedlings as esterification catalyst. The reactions were carried out between *n*-butanol and fatty acid mixtures from oils that contain either a *cis*-6 fatty acid, e.g., γ-linolenic acid (borage oil), gorlic acid (*H. wightiana* oil), and petroselinic acid (coriander oil), or a *cis*-4 fatty acid, e.g., docosahexaenoic acid (hake liver oil). The data presented in Figures 6–9 show during the course of esterification an enrichment of γ-linolenic, gorlic, petroselinic, and docosahexaenoic acid, respectively, in the unesterified fatty acid fraction, whereas the relative proportion of each of these fatty acids is concomitantly reduced in the butyl ester fractions. Thus the levels of γ-linolenic, gorlic, petroselinic, and docosahexaenoic acids are elevated in the fatty acid fraction to 71, 52, 99, and 35% from initial concentrations of 20, 10, 81, and 11%, respectively (Figs. 6–9). Obviously, γ-linolenic, gorlic, petroselinic, and docosahexaenoic acids are discriminated against as substrates during esterification catalyzed by the acetone powder from germinating rape seedlings. These substrate selectivities agree

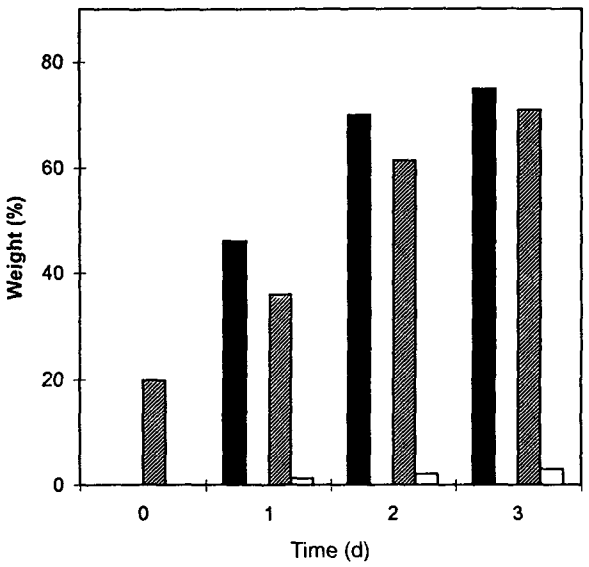

FIG. 6. Relative proportions of γ-linolenic acid in fatty acids (hatched bar) and butyl esters (open bar) in products formed by esterification of fatty acids from borage oil with *n*-butanol catalyzed by acetone powder from rape seedlings at different percentages of esterification (solid bar).

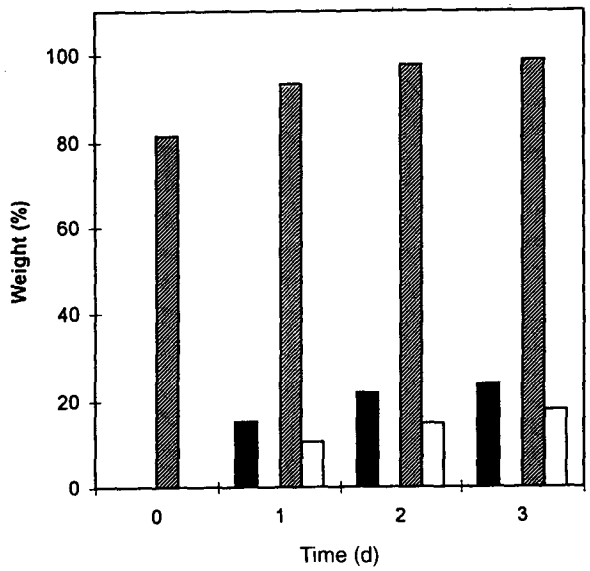

FIG. 8. Relative proportions of petroselinic acid in fatty acids (hatched bar) and butyl esters (open bar) in products formed by esterification of fatty acids from coriander oil with *n*-butanol catalyzed by acetone powder from rape seedlings at different percentages of esterification (solid bar).

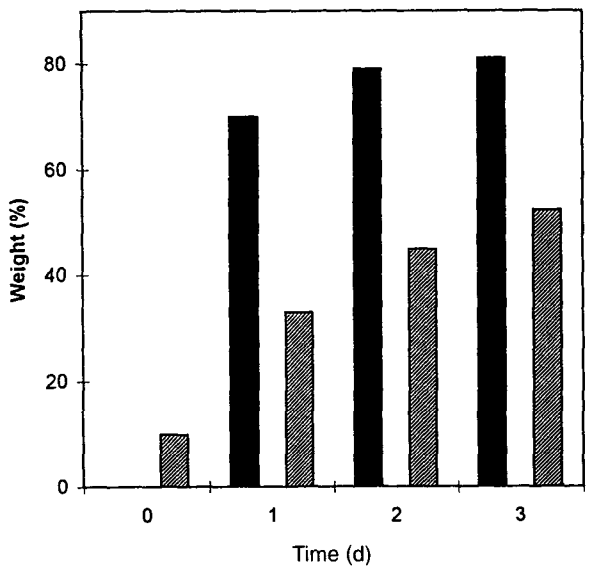

FIG. 7. Relative proportions of gorlic acid in fatty acids (hatched bar) formed by esterification of fatty acids from *Hydnocarpus wightiana* oil with *n*-butanol catalyzed by acetone powder from rape seedlings at different percentages of esterification (solid bar). Gorlic acid was present in butyl esters in trace amounts only.

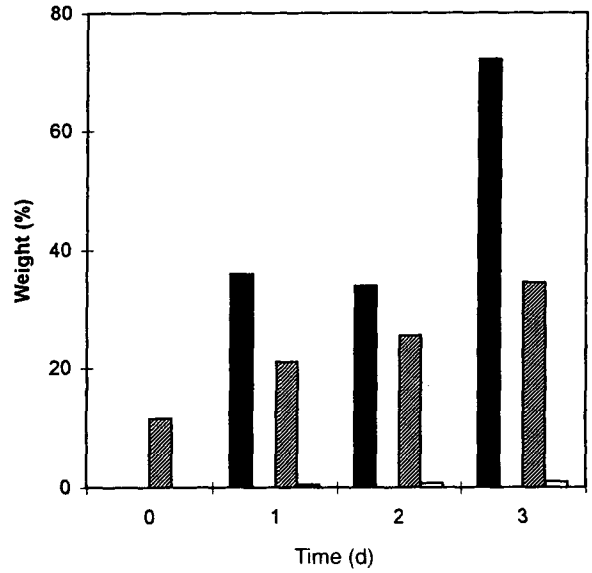

FIG. 9. Relative proportions of docosahexaenoic acid in fatty acids (hatched bar) and butyl esters (open bar) in products formed by esterification of fatty acids from hake liver oil with *n*-butanol catalyzed by acetone powder from rape seedlings at different percentages of esterification (solid bar).

with the substrate specificities reported so far for purified rapeseed lipase (15) and microbial lipases (10,16). It should be possible to utilize such substrate selectivities for the enrichment of certain fatty acids from mixtures by selective esterification, i.e., *via* kinetic resolution.

In conclusion, our results show, that an acetone powder preparation from germinating rape seedlings can be applied as biocatalyst in processes such as esterification and interesterification reactions that require a low water activity, thereby exhibiting a significantly higher activity as biocatalyst for esterification reactions. The acetone powder preparation shows essentially similar substrate selectivity as purified rapeseed lipase preparations reported so far (15). It is conceivable that the acetone powder may serve as a cheap and

easy-to-obtain catalyst for lipid biotransformations with the aim to obtain specific products of good commercial value.

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