Triacylglycerol Lipase from Rape (*Brassica napus* L.) Suitable for Biotechnological Purposes

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ABSTRACT

Triacylglycerol lipase (EC 3.1.1.3) from rape (*Brassica napus* L. cv Ceres) is quite easily prepared from the 100,000×*g* supernatant of cotyledon homogenates. The lipase is present in a high-molecular-mass fraction (>1.5×10^6 dalton by gel filtration), but it can be rapidly extracted from the 100,000×*g* supernatant by precipitation with polyethyleneglycol 8000 (4%, w/v) and $MgCl_2$ (40 m*M*) giving about a 10-fold purification. After delipidation, the lipase has an M_r of about 300,000. It hydrolyzes triacylglycerols to fatty acids and glycerol, although the fatty acids from the *sn*-1 or -3 positions are hydrolyzed first to yield 1,2(2,3)-diaclyglycerols. Lipase immobilized onto Celite by precipitation with acetone at −20°C catalyzes the esterification of oleic acid with butanol dissolved in hexane.

Index Entries: Lipase from *Brassica napus;* immobilized enzyme; esterification.

INTRODUCTION

The use of immobilized enzymes to catalyze reactions under non-aqueous conditions is a rapidly expanding field (*1*). Lipases have found many industrial and technical applications in catalyzing esterification and

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transesterification reactions of triacylglycerols and other lipids in the presence of only small amounts of water as well as hydrolysis in aqueous media (*2,3*). A wide variety of other applications of lipases, such as the resolution of stereoisomers by enantioselective hydrolysis or esterification, have also been developed (*4*).

To date, the majority of lipases used in reactions of biotechnological interest are isolated from fungi such as *Candida* or *Rhizopus* spp. (*5*). The possibility of immobilizing lipases isolated from higher plants and using them under nonaqueous conditions has not been investigated, though the use of acid lipase from castor bean in catalyzing esterification reactions received early attention (*6*). Lipases are present at high activity in reserve tissues of many young oilseed plants, with some plants containing two or more triacylglycerol lipases (*7*). It has been found that, in the hydrolytic reaction, lipases from different plant species display quite different specificities with regard to fatty acids esterified in the triacylglycerols (*8*). However, studies with antibodies raised against castor bean lipase show that lipases from a wide range of oilseed plants might be structurally similar (*9*). It has been suggested that lipases from plants might provide a wide diversity of substrate specificities for use in biotechnology (*8,10*).

In this study we report that lipase isolated from the cotyledons of rape seedlings can be immobilized onto Celite and used to catalyze the esterification of oleic acid to butanol in the presence of hexane as solvent.

EXPERIMENTAL

Material

Seeds of rape (*Brassica napus* L. cv Ceres) were germinated at 25°C in the dark on moist filter paper. Biochemicals were purchased from Sigma (Deisenhofen, FRG) and radiochemicals from Amersham (Braunschweig, FRG). All solvents were distilled before use. Silica Gel H was from Merck (Darmstadt, FRG).

Methods

Homogenization and Polyethyleneglycol Precipitation

Cotyledons (10 g) from 6-d-old rape seedlings were homogenized with mortar and pestle in 30 mL of a medium containing 150 m*M* Tricine-NaOH (pH 7.5), 0.4*M* sucrose, 2 m*M* DTT, 10 m*M* KCl, 1 m*M* $MgCl_2$, and 0.5 m*M* phenylmethylsuphonylfluoride (PMSF). The homogenate was filtered through two layers of Miracloth and centrifuged at 20,000×*g* for 15 min, the fat layer was removed, and the filtration and centrifugation steps were repeated. The 20,000×*g* supernatant was diluted with 5 vol of sucrose-free homogenization medium (including 50 m*M* $MgCl_2$) and cen-

Table 1
Precipitation of Lipase from 100,000×*g* Supernatant of Rape Cotyledon Homogenate[a]

Experiment	Specific activity, μmol-mg^{-1} protein-min^{-1}	Lipase activity precipitated, %
0% PEG 1 m*M* $MgCl_2$	0	0
4% PEG 1 m*M* $MgCl_2$	4.9	8
8% PEG 1 m*M* $MgCl_2$	6.0	26
0% PEG 40 m*M* $MgCl_2$	23.5	20
4% PEG 40 m*M* $MgCl_2$	8.7	96

[a]Different proportions (w/v) of solid PEG 8000 and 2*M* $MgCl_2$ were added to aliquots of the 100,000×*g* supernatant, stirred on ice for 10 min, and centrifuged at 10,000×*g* for 10 min. The pellets were resuspended in 20 m*M* Tris-HCl (pH 8.1) containing 0.5 m*M* $MgCl_2$, 0.5 m*M* EDTA, 5 m*M* β-mercaptoethanol, and 0.5 m*M* PMSF.

trifuged at 100,000×*g* for 60 min ($MgCl_2$, 50 m*M*, was omitted from the dilution buffer for the experiments shown in Table 1). Solid polyethyleneglycol (PEG) 8000 was added to the supernatant (4%, w/v) and stirred on ice for 10 min. The precipitate was removed by centrifugation (10,000×*g* for 10 min) and resuspended in 10 mL buffer containing 20 m*M* Tris-HCl (pH 8.1), 0.5 m*M* $MgCl_2$, 0.5 m*M* EDTA, 5 m*M* β-mercaptoethanol, and 0.5 m*M* PMSF. Solid deoxycholic acid (1%, w/v) was added and the mixture stirred for 1 h at 4°C in order to solubilize the lipase. Immobilization was carried out in a manner similar to that of Wisdom et al. (*11*) by addition of the solubilized lipase to 1 g of Celite followed by slow dilution with 90 mL of acetone at −20°C. After allowing the mixture to stand for 30 min at −20°C, the acetone was decanted and the remaining acetone removed under vacuum in a desiccator at 4°C.

Gel Filtration Chromatography

Sephacryl S-300 columns were used for gel filtration chromatography. The PEG precipitate was treated with pure acetone at −20°C before being resuspended in elution buffer containing 1% deoxycholate but in the absence of NaCl. The lipase was dialyzed against column elution buffer. The dialyzed lipase was concentrated three fold with an Amicon ultrafiltration cell 8010 (30,000 kdalton cutoff) and loaded onto the Sephacryl column. The elution buffer contained 20 m*M* Tris-HCl (pH, 8.1), 5 m*M* β-mercaptoethanol, 0.5 m*M* EDTA, and 100 m*M* NaCl. The flow rate of elution was 10 mL/h, and 3-mL fractions were collected.

Lipase/Esterase Activity

Lipase activity was measured according to Belfrage and Vaughn (*12*) in a medium containing 50 m*M* Bis-Tris-Propane (pH 7.5), 2 m*M* DTT, 2 m*M* $CaCl_2$, 0.1% (w/v) deoxycholic acid, and 4 m*M* [^{3}H]trioleoylglycerol. The [^{3}H]trioleoylglycerol (75 μCi/mmol) emulsion was prepared as a 100 m*M* stock in 5% gum arabic by sonication for 60 s at 20 W with an ultrasonic probe. The final volume of the assay mixture was 100 μL. Reaction mixtures were incubated for 15 min at 30°C in a gyratory shaker (300 rev/min). Positional specificity of hydrolysis was measured as in the radioactive assay, except that 10 m*M* triacylglycerol was used with 10 m*M* $CaCl_2$. Substrates were 1,3-dipalmitoyl-2-oleoylglycerol (POP) and 1,3-dioleoyl-2-palmitoylglycerol (OPO). Reactions were terminated by the addition of CH_2Cl_2:MeOH (2:1, v/v). Heptadecanoic acid was included as an internal standard. Fatty acids were separated from substrates by thin-layer chromatography (TLC), converted to methyl esters, and quantified by gas chromatography (GC) as described in Analytical Methods, below. Hydrolysis of *p*-nitrophenylacetate (pNPA) was measured spectrophotometrically at 405 nm in Bis-Tris-Propane Buffer (pH 7.5) containing 2 m*M* DTT and 1.2 m*M* pNPA in 4% acetonitrile.

Esterification Catalyzed by Immobilized Rape Lipase

Esterification reactions were carried out in sealed vials at 30°C. The reaction mixture contained 0.05*M* butanol, 0.025*M* [^{14}C]oleic acid (4.5 μCi/mmol), and 30 mg immobilized lipase in 1.5 mL hexane (water saturated). The lipids in 10-μL aliqouts of the reaction mixture were separated by TLC, the fractions stained with I_2 vapor, and those corresponding to butyl oleate and oleic acid were scraped into scintillation vials and assayed for radioactivity in an LKB 1214 liquid scintillation spectrometer.

Analytical Methods

Protein was assayed according to the method of Markwell et al. (*13*). Lipids were extracted from the PEG pellet with CH_2Cl_2/MeOH according to the method of Folch et al. (*14*), and a known aliquot of heptadecanoic acid was added as an internal standard. The lipids were converted to methyl esters and analyzed by GC on Silar 5CP: total lipids were fractionated into classes by TLC on Silica Gel H, and each fraction was converted to methyl esters and quantitated by GC using methylheptadecanoate as an internal standard (*15*).

RESULTS AND DISCUSSION

Characteristics of Rape Lipase

Differential centrifugation of rape cotyledon homogenate yields about 60% of the lipase activity in the 100,000 × *g* supernatant (*16*). An aliquot of

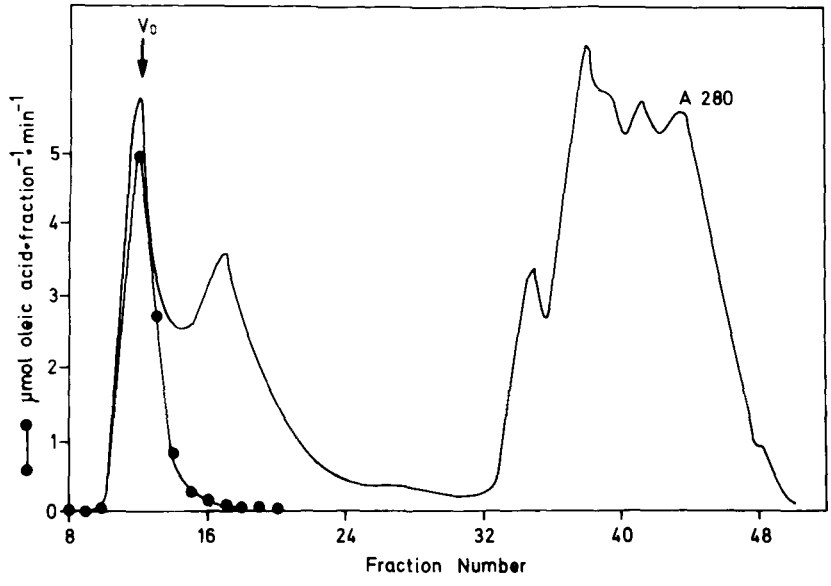

Fig. 1. Elution of lipase from Sephacryl S-300 gel filtration column. Ultrafiltration was used to concentrate 100,000×*g* supernatant fivefold, and 3 mL of the concentrate was loaded onto a 60×1.6 cm column and eluted at 10 mL/h. Fractions (3 mL) were collected, and lipase activity, measured as µmol oleic acid liberated from trioleoylglycerol-fraction^{-1}·min^{-1} (●——●), was plotted.

this supernatant was concentrated threefold by ultrafiltration and the proteins separated by gel filtration (Fig. 1). Lipase activity eluted exclusively in fractions with a very high molecular weight (i.e., $>1.5\times10^6$). These fractions were found to contain lipids in a ratio of about 0.4 to 1 with protein (w/w). The bulk of the protein eluted from the column had much lower molecular weights, but lipase activity was not detected in those fractions.

The lipase could be obtained much more rapidly from the 100,000×*g* supernatant by precipitation with PEG 8000 (Table 1). In the presence of low $MgCl_2$ concentrations, little lipase activity was precipitated at either 4 or 8% (w/v) PEG. In the presence of 40 m*M* $MgCl_2$, however, almost all of the lipase activity was precipitated by 4% PEG and recovered by centrifugation, giving about a 10-fold purification. This PEG pellet was used as the source of lipase for immobilization onto Celite. The lipid/protein ratio in the PEG precipitate was 0.48:1 (±0.13, $n=4$), showing that the aggregates were composed mainly of protein with some lipid. Analysis of lipid classes showed that 50% of the extractable lipid in the PEG precipitate was fatty acid, 20% was phospholipid, and the rest was comprised of mono-, di- and triacylglycerols.

Lipase/Esterase Activity in PEG Precipitate

Since the immobilized lipase was used to catalyze esterification reactions under low water conditions, knowledge of the activity of nonspecific

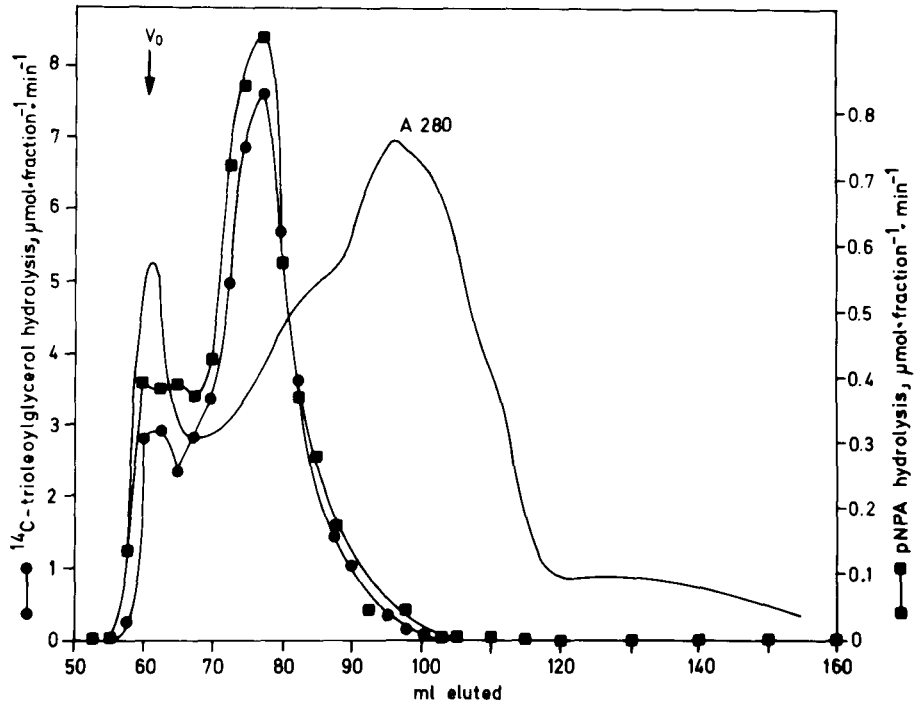

Fig. 2. Elution of lipase/esterase from Sephacryl S-300 gel filtration column. PEG precipitated lipase was delipidated and dialyzed as described in Methods. The column dimensions were 80×1.6 cm. Flow rate was 10 mL/h and 3 mL fractions were collected.

esterase in the PEG precipitate was required. This was estimated using pNPA as substrate. The lipase preparation catalyzed the hydrolysis of pNPA, although the specific activity was much lower (1.0 μmol·mg^{-1} protein·min^{-1}) than that observed in trioleoylglycerol hydrolysis (15.9 μmol·mg^{-1} protein·min^{-1}). It was not possible to separate the pNPA hydrolysis and trioleoylglycerol hydrolysis activities by gel filtration. Delipidated PEG precipitate was passed through a Sephacryl S-300 gel filtration column. The elution profiles of hydrolytic activity toward trioleoylglycerol and pNPA were found to coincide almost exactly (Fig. 2). Again, the specific activity of pNPA hydrolysis was an order of magnitude lower than that of trioleoylglycerol hydrolysis. Although lipases act primarily on water-insoluble ester substrates, they are often found to catalyze the hydrolysis of "esterase" substrates (*17*). It is likely that the small amount of soluble ester hydrolase activity detected in this lipase preparation is in fact being catalyzed by the lipase, although this cannot be confirmed until the rape lipase has been purified to homogeneity.

Molecular Weight of Native Lipase

The gel filtration of delipidated PEG precipitate also yielded information about the molecular weight of the native enzyme. Calibration of the

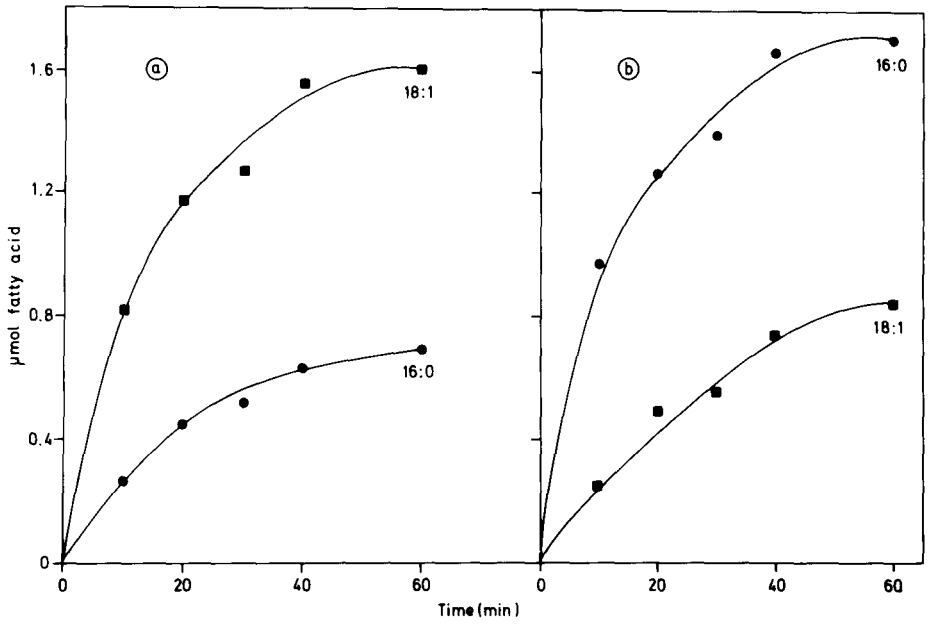

Fig. 3. Positional specificity of rape lipase. Kinetics of release of oleic acid (18:1, ■) and palmitic acid (16:0, ●) by hydrolysis of POP (a) and OPO (b).

column with standard proteins gave an estimated M_r of 300,000. In a recent abstract, a figure of 250,000 was reported for rape lipase (*18*). These estimates are quite close to that of the corn lipase (M_r = 260,000), which is the only other plant lipase that has been examined in this regard (*19*). Recent cross-reaction studies using an antibody to castor bean lipase indicate that the subunit size of both rape and corn lipases is about 62,000 (*9*).

Positional Specificity in Hydrolysis of Triacylglycerols

Both nonspecific lipases and those specific for the *sn*-1 and *sn*-3 positions of triacylglycerols are used to catalyze hydrolysis, esterification, and interesterification reactions of fats and other lipids (*2*). In order to evaluate the possible applications of the rape lipase preparation in such reactions, we assessed its positional specificity in hydrolysis using the triacylglycerols POP and OPO. Fig. 3 shows that fatty acids esterified to both the primary and secondary hydroxyl groups were hydrolyzed at high rates, although the first-order rate constant for the hydrolysis of the fatty acid esterified to the secondary hydroxyl group was only about 60% of that esterified to the primary hydroxyl groups. Only 1,2(2,3)-diacylglycerols were found, however, and 1,3-diacyglycerols could not be detected. Therefore, the first fatty acids hydrolyzed from triacylglycerols are those esterified at the primary hydroxyl groups. There was no accumulation of di- or monoacylglycerols in the reaction mixture, their concentrations remaining very low throughout the reaction, at about 1–2% of the level of

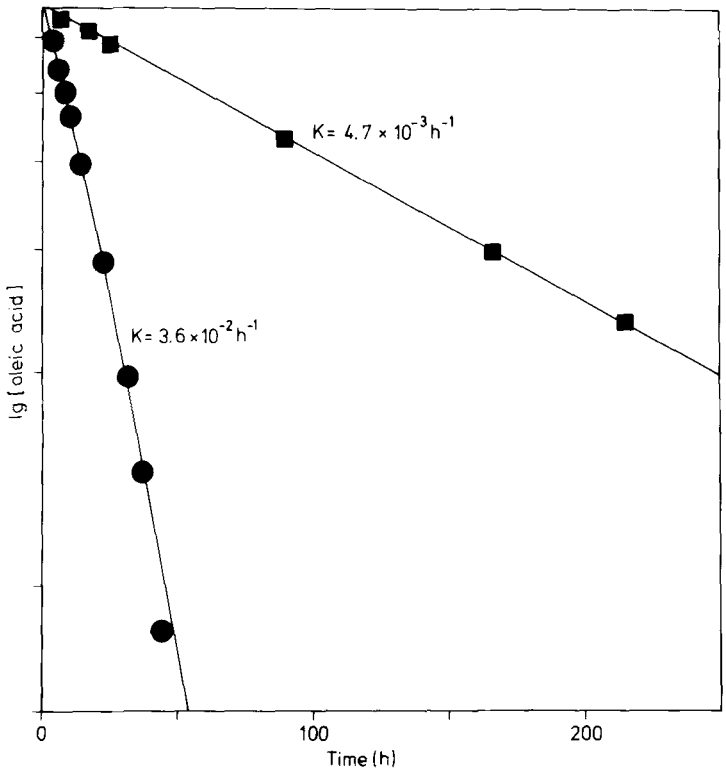

Fig. 4. Kinetics of esterification of oleic acid to butanol given as a semilog plot. Experiments were carried out at 30°C (●) and −22°C (■).

released fatty acids. In contrast, hydrolysis of triacylglycerol by *sn*-1,3-specific lipases such as pancreatic lipase leads to a large accumulation of monoacylglycerols (*19*). It was found that the monoacylglycerols formed by the rape lipase contained fatty acids from the *sn*-1,3 and *sn*-2 positions, indicating that fatty acids were cleaved from both the *sn*-1,3 and the *sn*-2 positions of 1,2(2,3)-diacylglycerols. The rape lipase is apparently similar to those lipases from other oilseed plants, i.e., castor bean (*20*) and corn (*19*), and fungal lipases, such as those from *Geotrichum candidum* and *Penicillium sp.* (*5*), which hydrolyze acyl groups from all three ester bonds of acylglycerols.

Esterification of [^{14}C]oleic Acid with Butanol

In order to establish whether the immobilized rape lipase was active in catalyzing esterification reactions in the presence of organic solvents, the esterification of [^{14}C]oleic acid with butanol was measured (Fig. 4). The rape lipase catalyzed this esterification efficiently, and the reaction went to 99% of completion. The semilog plot shows that the kinetics were

of first order, indicating that the concentrations used were well below the K_m of the lipase. The K_m(app) for oleic acid was estimated to be about 0.15*M* (data not shown). Esterification was almost undetectable in the absence of active lipase. Centrifugation of aliquots from the reaction mixture to remove the lipase immobilized on Celite was essential. When aliquots from reaction mixtures were kept at −20°C prior to analysis without removal of the enzyme, quite a large amount of esterification occurred during storage. Esterification reactions were therefore carried out at −22°C, and a surprisingly high activity of the lipase was found, even at such a low temperature. The kinetics were again of first order, and the rate constant was nearly 14% of that of the reaction at +30°C (Fig. 4). After 400 h of reaction at −22°C, more than 80% of the substrate had been esterified.

In summary, lipases from a higher plant will catalyze esterification reactions in hexane. Lipase is readily extracted from rape seedlings and immobilized onto Celite. Application of such lipase preparations in other synthetic reactions of biotechnological interest can be envisaged.

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