0273-2289/91/2702—0123$2.00

Synthetic Reactions Catalyzed by Immobilized Lipase from Oilseed Rape (*Brassica napus* L.)

MATTHEW J. HILLS,** IRMGARD KIEWITT, AND KUMAR D. MUKHERJEE*

Bundesanstalt für Fettforschung, Institut für Biochemie und Technologie, H.P. Kaufmann-Institut, Piusallee 68, D-4400 Münster, FRG

Received January 2, 1990; Accepted March 9, 1990

ABSTRACT

Lipase from rape (*Brassica napus* L.), immobilized onto celite, catalyzes esterification and transesterification reactions in hexane. The activity of the lipase is stimulated up to 35 fold by the addition of water (1.3% w/v). The activity of the lipase in hydrolysis is about 8 times higher than in the esterification reactions in hexane. Interesterification reactions between mono- and diacylglycerols and transesterification reactions of mono- and diacylglycerols with alcohols were also catalyzed at relatively high rates but transesterification/esterification of these acylglycerols with fatty acids was comparatively slow. In transesterification reactions, triacylglycerols reacted rather slowly.

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

INTRODUCTION

Most of the lipases used as catalysts for esterification and transesterification reactions in organic solvents are produced from fungi, yeasts and porcine pancreas (*1*). Another source of lipase, not investigated until recently, is from cotyledons or endosperm of young

*Author to whom all correspondence and reprint requests should be addressed.
**Present address: John Innes Centre for Plant Science Research, Cambridge Laboratories, Colney Lane, Norwich, NR4 7UJ, England, UK.

oilseed plants (*2–5*). Oilseed lipases from different plants were shown to have quite different specificities with regard to acyl moieties in the hydrolysis of triacylglycerols (*6*) and it was suggested that these lipases might show such specificities in catalyzing reactions under low water conditions (*7*). Oilseeds are a promising source of lipase since seedlings of these plants are very easy to grow on moist paper in the dark for 4–6 d. Large quantities can be grown without the equipment required for growing microbial cultures. The immobilized lipase from rape used in the experiments described here is relatively easy to prepare and this was recently described (*3–5*).

The rape lipase is active in catalyzing esterifications between butanol and a wide range of fatty acids; however, it has been found to discriminate strongly against gamma-linolenic acid in such reactions. We have used this unusual characteristic for the kinetic resolution of gamma-linolenic acid from evening primrose oil fatty acids. Gamma-linolenic acid was concentrated from 9.5 mol% in the original mixture to as much as 82 mol% by preferential esterification of all the other fatty acids with butanol (*8*). In this paper, we describe some further characteristics of the rape lipase in synthetic reactions.

EXPERIMENTAL

Enzyme Preparation

Lipase partially purified from oilseed rape cotyledons was immobilized on to celite as described previously (*4,5*).

Preparation of Substrates

^{14}C-Monooleoylglycerols and ^{14}C-dioleoylglycerols were prepared by reacting [1-^{14}C]oleic acid (Amersham, specific activity 1.86 μCi/μmol) with glycerol using Lipase G (Amano Pharmaceuticals) as previously described (*9,10*). The products were purified by TLC on Silica Gel H. The chromatograms were developed twice up to 2 cm from the origin with diethyl ether and finally up to 19 cm with hexane/diethyl ether/acetic acid (70:30:1 v/v). Radioactivity was monitored by a Berthold-Radio-TLC scanner and the fractions of mono- and dioleoylglycerols scraped and eluted with water-saturated diethyl ether.

Esterification Reactions

^{14}C–Oleic acid (25 mM, specific activity 13 nCi/μmol) and butanol (50 mM) were esterified by 30 mg immobilized rape lipase in 1.5 mL hexane (water saturated) in sealed vials at 30°C with stirring. Reaction rates were measured as reported previously (*5*).

Transesterification Reactions

Either ^{14}C-monooleoylglycerols (specific activity 22 nCi/μmol), ^{14}C-dioleoylglycerols (specific activity 30 nCi/μmol) or ^{14}C-trioleoylglycerol (25 nCi/μmol) at 25 m*M* in 1 mL hexane (water saturated) were incubated with 20 mg celite-lipase at 30°C in the presence of butanol (100 m*M*). Aliquots were removed and the extent of interesterification determined from the distribution of ^{14}C-oleoyl moieties in the products analyzed by radio-TLC. For acidolysis reactions, ^{14}C-oleic acid (specific activity 13 nCi/μmol; 25 *m*M) replaced butanol and unlabeled acylglycerols were used. Again products were analyzed by radio-TLC methods (*9,10*).

Transesterification of unlabeled oleoyl moieties from dioleoylglycerols (25 m*M*) to a mixture of alcohols at 20 m*M* each was carried out as described for esterification reactions. The mixture contained methanol, ethanol, propanol, butanol, hexanol, octanol, dodecanol, tetradecanol and hexadecanol. After termination of the reaction, the esters were separated from the oleic acid by TLC with hexane/diethyl ether/acetic acid (70:30:1 v/v) as solvent. The esters were eluted and analyzed by GC as previously described (*9*).

Hydrolytic Reaction

The hydrolytic activity of the celite-lipase was measured after extracting the lipase from the celite into an aqueous medium using ^{3}H-trioleoylglycerol as substrate (*5*).

RESULTS AND DISCUSSION

Stimulation of Lipase Activity by Water

Rape lipase catalyzes the esterification of oleic acid with butanol and in the concentration range used (0–25 m*M*), the kinetics are of first order (*5*). We have recently found that the immobilized rape lipase is strongly activated by the addition of a small amount of water. In the absence of added water, esterification proceeds at a relatively slow rate, but if a small volume of water is included (1.3%, v/v) esterification activity is increased by up to 35 fold depending on the preparation (Table 1). Enzymes (including lipases) acting in organic solvents require a small amount of water for catalytic activity (*11,12*) and from these data it appears that rape lipase is no exception. Since the lipase was immobilized by acetone precipitation followed by solvent evaporation under vacuum (*5*), it is likely that water, essential for catalytic activity, was also removed. However, it is the activity of water in a reaction mixture rather than its concentration by volume that affects the efficiency of enzymes. Since this varies depending on the substrate concentration, the volume of water required for optimal activity also changes.

Table 1
Stimulation of Esterification Activity
of Rape Lipase by Addition of a Small Amount of Water
and Comparison of Esterification and Hydrolytic Activities[a]

Lipase prep.	Rate of esterification no added H_2O	Rate of esterification $+H_2O$[b]	Stimulation by H_2O	Rate of hydrolysis[c]
1.	0.3	7.0±0.6	23	59± 3.6 (9%)
2.	0.2	7.3±0.1	36	106±10.0 (7%)
3.	0.4	13.6±1.5	34	118±11.0 (12%)

[a]Batches of celite-immobilized lipase were prepared in same manner. Esterification of oleic acid with butanol and hydrolysis of trioleoylglycerol: Initial rates given in $\mu mol \cdot g^{-1}$ celite lipase$\cdot min^{-1}$.
[b]The water added (1.25% v/v) contained Bis-Tris-Propane buffer (20 m*M* at pH 7.5).
[c]Figures in parentheses show the rate of esterification in the presence of added water as percentage of the rate of hydrolysis.

Lipase Stability

After 5 mo storage at 4°C, the activity of the immobilized lipase in catalyzing the esterification of oleic acid with butanol was almost 100% of the original activity.

Comparison of Esterification and Hydrolytic Reactions

The rate of esterification of oleic acid and butanol in hexane under optimal conditions was up to 12% that of the hydrolysis of trioleoylglycerol in an aqueous reaction mixture (Table 1). This shows that the lipase retains a fair proportion of its activity in the organic solvent and is comparable with the relative activity of other enzymes, such as alcohol dehydrogenase and polyphenol oxidase under nonaqueous conditions (*11*).

Transesterification Reactions

Interesterification reactions between ^{14}C-labeled monooleoylglycerols, transesterification of ^{14}C-labeled mono-, di-, and trioleoylglycerols with butanol (alcoholysis), and transesterification of unlabeled acylglycerols with ^{14}C-oleic acid (acidolysis) catalyzed by the rape lipase were assessed.

When a solution of ^{14}C-monooleoylglycerols was incubated with lipase, the transfer of ^{14}C-oleoyl moieties from one monooleoylglycerol molecule to another yielding ^{14}C-dioleoylglycerols was observed (Fig. 1). At equilibrium, 36% of the ^{14}C-oleoyl moieties were present in dioleoylglycerols and the majority of that in the 1,3-isomer. The ratio between 1,3- and 1,2-dioleoylglycerols was about 6 to 1. This is in agreement with the preference of the rape lipase for acyl moieties at the *sn*-1,3 positions of

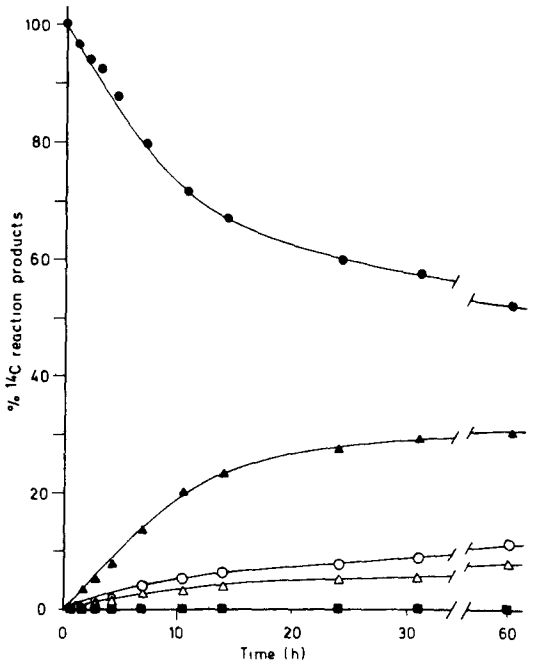

Fig. 1. Time course of formation of radioactively labeled products upon incubation of ^{14}C-monooleoylglycerols with immobilized rape lipase in the presence of hexane. (●) monooleoylglycerols, (▲) 1,3-dioleoylglycerol, (△) 1,2(2,3)-dioleoylglycerols, (■) trioleoylglycerol, (○) oleic acid.

acylglycerols (*5*). No label could be detected in trioleoylglycerol although theoretically about 6% trioleoylglycerol would be expected at equilibrium. In a control experiment, unlabeled monooleoylglycerols were reacted with ^{14}C-oleic acid in the presence of rape lipase. Only about 1% of the label was incorporated into acylglycerols showing that esterification/acidolysis of acylglycerols is very slow in comparison to the transfer of oleoyl moieties between acylglycerols. The fact that ^{14}C-oleic acid is esterified very slowly to monoacylglycerols indicates that during the acyltransfer reaction between monooleoylglycerols, oleic acid is probably not released into the solvent, but remains bound to the lipase before being transfered to another molecule of monooleoylglycerol.

The rate of alcoholysis of acylglycerols with butanol was also found to be stimulated many fold by the addition of a small quantity of water (1.3% w/v). Diacylglycerols were the preferred substrate for the enzyme. Initial rates of alcoholysis of mono-, di-, and triacylglycerols by butanol was 0.3, 2.5, and 0.1 μmol·g^{-1}celite lipase·min^{-1}, respectively. Therefore, in the best case, the fastest rate of alcoholysis appears to be about 20–35% that of the rate of esterification.

The effect of alcohol chain length on the rate of alcoholysis was measured with an equimolar mixture of alcohols and dioleoylglycerol, since

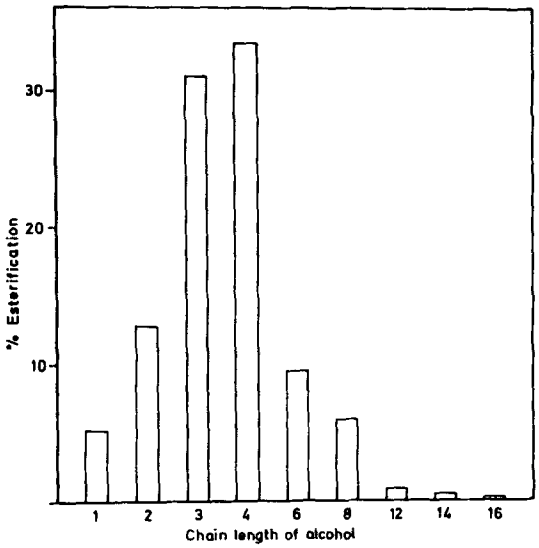

Fig. 2. Transesterification of oleoyl moieties from dioleoylglycerols (25 m*M*) to a mixture of alcohols of various chain lengths (20 m*M* each) catalyzed by immobilized rape lipase. Details in Experimental.

this was found to be the acylglycerol most efficiently used in this reaction. All of the alcohols acted as substrate in the transesterification with *n*-propanol and *n*-butanol being esterified most rapidly (Fig. 2). The longer chain alcohols reacted very slowly in competition with the shorter chain alcohols.

Rates of acidolysis/esterification of mono- and dioleoylglycerols with oleic acid were only about 5% of the rates of the respective alcoholysis reactions. Acidolysis of triacylglycerols was only barely detectable. The relatively low activity of the lipase towards triacylglycerols in hexane solution suggests that it does not have enough conformational flexibility to bind such a large substrate in organic solvents. It may be possible to improve reaction rates with triacylglycerols by the use of a suitable substrate analogue during immobilization. Such an approach gave a 100 times increase in activity for subtilisin in organic solvents (*13*).

ACKNOWLEDGMENTS

This work was funded by a research grant provided by the Bundesministerium für Ernährung, Landwirtschaft und Forsten, Bonn, FRG.

REFERENCES

1. Mukherjee, K. D. (1990), *Biocatalysis* **3**, 277.
2. Hassanien, F. R. and Mukherjee, K. D. (1986), *J. Amer. Oil Chem. Soc.* **63**, 893.

3. Hills, M. J. and O'Sullivan, J. N. (1989), *Biochem. Soc. Trans.* **17,** 480.
4. Hills, M. J., Kiewitt, I., and Mukherjee, K. D. (1989), *Biochem. Soc. Trans.* **17,** 478.
5. Hills, M. J. and Mukherjee, K. D. (1989), *Appl. Biochem. Biotechnol.* in press.
6. Lin, Y. H., Yu, C., and Huang, A. H. C. (1986), *Arch. Biochem. Biophys.* **244,** 346.
7. Huang, A. H. C., Lin, Y., and Wang, S. (1988), *J. Amer. Oil Chem. Soc.* **65,** 897.
8. Hills, M. J., Kiewitt, I., and Mukherjee, K. D. (1989), *Biotechnol. Lett.* **11,** 629.
9. Schuch, R. and Mukherjee, K. D. (1989), *J. Chromatogr.* **450,** 448.
10. Schuch, R. and Mukherjee, K. D. (1989), *Appl. Microbiol. Biotechnol.* **30,** 332.
11. Zaks, A. and Klibanov, A. M. (1988), *J. Biol. Chem.* **263,** 8017.
12. Zaks, A. and Klibanov, A. M. (1985), *Proc. Natl. Acad. Sci. USA* **82,** 192.
13. Russel, A. J. and Klibanov, A. M. (1988), *J. Biol. Chem.* **263,** 11624.