0273-2289/96/5902—0145$08.50

Purification and Partial Characterization of *Rhizomucor miehei* Lipase for Ester Synthesis

XIAO YAN WU, SANNA JÄÄSKELÄINEN, AND YU-YEN LINKO*

Laboratory of Biotechnology and Food Engineering, Department of Chemical Engineering, Helsinki University of Technology, FIN-02150 Espoo, Finland

Received February 14, 1995; Accepted May 16, 1995

ABSTRACT

A commercial *Rhizomucor miehei* lipase was purified by ammonium sulfate precipitation. Phenyl Sepharose 6 Fast Flow hydrophobic interaction chromatography, and DEAE Sepharose Fast Flow anion-exchange chromatography. The recovery of lipase activity was 32% with a 42-fold purification. The molecular size of the purified enzyme was 31,600 Dalton and the pI 3.8. The enzyme was stable for at least 24 h within a pH range of 7.0–10.0, and 96.8% of the enzyme activity remained when kept at 30°C for 24 h. Further, about 10–30% of the lipase activity was inhibited by K^+, Li^+, Ni^+, Co^{2+}, Zn^{2+}, Mg^{2+}, Sn^{2+}, Cu^{2+}, Ba^{2+}, Ca^{2+}, and Fe^{2+} ions and by SDS, but EDTA had no effect. Under the experimental conditions, the optimum temperature for the hydrolysis of olive oil was 50°C (pH 8.0), and for the synthesis of 1-butyl oleate, 37°C. It was concluded that hydrolytic activity of lipase alone is not a sufficient criterion for its synthetic potential. The optimal molar ratio of oleic acid and 1-butanol was 2:1 for 1-butyl oleate synthesis. The 1-butyl oleate yield was unaffected by purification of the enzyme after 12 h.

Index Entries: *Rhizomucor miehei* lipase; purification; characterization; hydrolytic activity; synthetic activity; 1-butyl oleate.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are enzymes that hydrolyze the ester bonds of acylglycerols. The interest in lipases for food and detergent uses to produce higher-quality products, and as digestive and diagnostic enzymes has markedly increased in recent years. Consequently, there is a need for improved isolation and purification procedures, since the availability of purified lipases enables one to obtain thorough knowledge of the properties of the enzyme and a better understanding of the mechanism of lipase-catalyzed hydrolysis and synthesis. Prior work on the purification of lipases from different microbial, animal, and plant sources has been comprehensively reviewed by Taipa et al. (*1*). In particular, research on microbial lipases has substantially progressed from both the academic and industrial viewpoints mainly because of the increased availability and stability of the commmercially available enzymes. The purification and characterization of fungal lipases from *Aspergillus* (*2,3*), *Geotrichum* (*4*), *Mucor* (*5,6*), *Penicillium* (*7*), and *Thermomyces* (*8*) species have been reported. Different microbial strains produce enzymes of varying properties, such as the hydrolytic and synthetic activity, pH and thermal stability, activator requirement, and so forth. Two forms (A and B) of purified lipases have been reported from fermentation with a selected strain of *Rhizomucor miehei* (*9,10*). The two forms were obtained with two independent purification methods. Form A was obtained by DE-cellulose 52 anion-exchange chromatography elution by a decreasing pH gradient from 7.0 to 3.5, Con A-Sepharose affinity chromatography, and TSK-Phenyl-5W hydrophobic interaction chromatography, and form B by DEAE-Sepharose anion-exchange chromatography elution using a decreasing pH gradient from 7.0 to 3.0, Phenyl-Sepharose hydrophobic interaction chromatography, and TSK G3000 SW gel filtration. The two forms have a high degree of antigenic identity, similar pH-activity profiles with tributyroylglycerol as substrate, and the same molecular size, but differ in their affinity toward Con A and in isoelectric points (pI 3.9 for A and 4.3 for B). Such purification methods will somewhat affect the lipase activity by decreasing pH during elution. However, to our knowledge, no information has been published on the effect of purification of commercial *R. miehei* lipase on its synthetic activity.

In the present work, a commercial *R. miehei* lipase was purified by a relatively simple and efficient method, partially characterized, and used for 1-butyl oleate synthesis as the example process in investigating the ester synthesis activity.

MATERIALS AND METHODS

Materials

R. miehei lipase used in the present study was purchased from Biocatalysts Ltd. (Batch No. 5944064; Wales, UK). Oleic acid was obtained from Sigma (St. Louis, MO), and 1-butanol from E. Merck (Darmstadt, Germany).

BioPilot System from Pharmacia (Uppsala, Sweden) equipped with a fraction collector FRAC-200 was used for column chromatography and collection of the enzyme. Phenyl Sepharose 6 Fast Flow (high-substitution) hydrophobic interaction medium and DEAE Sepharose Fast Flow anion exchanger were also obtained from Pharmacia.

Bio-Rad Mini-Protean II Dual Slab Cell was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Low-range molecular-size prestained SDS-PAGE protein standards were from Bio-Rad Laboratories (Hercules, CA). Multiphor II Electrophoresis System, Ampholine PAGplate (pH 3.5–9.5) and Broad pI Calibration Kit used in isoelectric focusing were obtained from Pharmacia.

Lipase Activity Assays

Lipolytic (hydrolytic) activity was determined according to Sigma Technical Bulletin No. 800, using 50% olive oil emulsion (Sigma) as substrate at 37°C and pH 7.0 for 30 min. Free fatty acids liberated were titrated with 0.05*M* sodium hydroxide using phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of fatty acid/min at 37°C and pH 7.0.

Synthetic activity was determined as described previously (*11*) and reported as a yield (%) of 1-butyl oleate at a certain time.

Activities were given as the average of duplicate determinations.

Protein Measurement

The protein quantity was determined according to Lowry et al. (*12*), using bovine serum albumin (Sigma) as standard.

Purification of the Enzyme

All of the following procedures were carried out at 4–8°C.

Enzyme Pretreatment

R. miehei lipase (2.0 g) was dissolved in 100 mL distilled water by stirring for half an hour, and the enzyme solution was centrifuged at 7.6 × 1000*g* for 20 min. Ammonium sulfate was added to the supernatant to 70% saturation, and the solution was stirred for 1 h. The resulting precipitate was collected by centrifugation at 17.0 × 1000*g* for 15 min and dissolved in 35 mL of 0.6*M* ammonium sulfate in 0.05*M* sodium phosphate buffer (pH 7.0).

Hydrophobic Interaction Chromatography

The pretreated enzyme solution was loaded onto a Phenyl Sepharose 6 Fast Flow (high-sub.) column (2.6 × 32 cm) equilibrated with 0.6*M* ammonium sulfate in 0.05*M* sodium phosphate buffer (pH 7.0) and followed by elution with a linear gradient of ammonium sulfate from 0.6 to 0*M*, in distilled water at a flow rate of 10 mL/min. Ten-milliliter fractions

were collected by means of a fraction collector FRAC-200. The fractions containing lipase activity were pooled for the subsequent ion-exchange chromatography.

DEAE Sepharose Fast Flow Chromatography

The pooled lipase fractions from hydrophobic interaction chromatography described above were applied to a DEAE Sepharose Fast Flow column (2.6 × 26 cm) previously equilibrated with 0.05*M* sodium phosphate buffer (pH 7.0). The unadsorbed materials were washed out thoroughly using the equilibration buffer. The lipase active fractions were obtained by elution with 0.25*M* sodium chloride in 0.05*M* sodium phosphate buffer (pH 7.0), at a constant flow rate of 10 mL/min.

Molecular-Size Determination

SDS-PAGE was carried out by Laemmli discontinuous polyacrylamide gel (*13*). The separation gel contained 12% polyacrylamide. Proteins were stained with Coomassie brilliant blue R-250 and silver stain (Bio-Rad). The molecular size of the purified enzyme was estimated using Bio-Rad low-range molecular-size prestained protein SDS-PAGE standards containing lysozyme (18,500 Dalton), soybean trypsin inhibitor (27,500 Dalton), carbonic anhydrase (32,500 Dalton), ovalbumin (49,500 Dalton), bovine serum albumin (80,000 Dalton), and phosphorylase (106,000 Dalton) as reference proteins.

Isoelectric Point Determination

The isoelectric point of the purified lipase was determined by isoelectric focusing performed on Multiphor II apparatus using Ampholine PAGplate precast polyacrylamide gel as described by the manufacturer (Pharmacia). The broad pI calibration kit (pH 3.0–10.0) containing amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-acidic band (6.85), horse myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), and trypsinogen (9.30) was used as reference proteins.

Hydrolysis Experiments

The temperature and pH optima, as well as the effects of a number of inhibitors, were investigated in covered 25-mL Erlenmeyer flasks containing 3 mL of 50% olive oil emulsion, 2.5 mL deionized water, 1 mL Mcilvaine buffer (pH 7.0) or 1 mL other buffer for investigating pH optima (citric acid-sodium citrate, pH 3.0–6.0; imidazole-HCl, pH 6.0–8.0; Tris-HCl, pH 8.0–9.0 and sodium carbonate-sodium hydrogen carbonate, pH 9.0–10.0) and lipase (2–20 U). Standard assay method for lipase activity was employed, except for the special conditions investigated.

Ester Synthesis Experiments

Ester syntheses were carried out in 35-mL screw-capped flasks at 37°C. The reaction mixtures containing 0.7–1.4 mmol of oleic acid and 1.4–0.7 mmol of 1-butanol in molar ratios of 0.50, 0.83, 0.91, 1.0, 1.1, 1.2, and 2.0, deionized water (18.1%, w/w), and lipase (53 U) were magnetically stirred at 150 rpm. Samples were taken at certain time intervals, the contents were mixed with a blend of diethylether and ethanol (1:1, v/v), and the residual free acid was titrated with 0.1*M* sodium hydroxide. The yield of ester was calculated on the basis of the limiting substrate (*11*).

RESULTS AND DISCUSSION

Enzyme Purification

Figure 1A shows the elution profile of *R. miehei* lipase from Phenyl Sepharose 6 Fast Flow hydrophobic interaction chromatography. The lipase activity peak was obtained when the concentration of ammonium sulfate was lower than 0.1*M*. After that, two other peaks having no lipase activity were washed out from the column with distilled water. Active fractions (10 mL each) were combined and applied into DEAE-Sepharose Fast Flow ion exchanger. The elution profile of ion-exchange chromatography is given in Fig. 1B. Lipase was eluted as a single peak of activity by 0.25*M* sodium chloride in 0.05*M* sodium phosphate buffer (pH 7.0). Active fractions (10 mL each) were pooled for further studying. The column was then washed with a linear gradient sodium chloride of 0.25–1.0*M* in the same buffer to remove the proteins remaining in the column.

The purification procedures and results from the different steps are given in Table 1. The enzyme was purified 42-fold with an activity yield of 32%. The purity of the enzyme was examined by SDS-PAGE (Fig. 2) and isoelectric focusing (Fig. 3), both showing only one band. Only one lipase active peak was obtained and purified further. By using different separation and purification procedures, Huge-Jensen et al. (*9*) were able to isolate two forms of extracellular lipase from a mutant strain of *R. miehei*. Of these, the active form B was obtained from the form A at a low pH (*9*), and appeared to be less glycosylated (*10*). The apparent molecular size of the lipase purified in the present work by SDS-PAGE was 31,600 Dalton (Fig. 2). This agrees well with the 32,000 Dalton previously reported for both A and B forms obtained after reduction (*10*).

Isoelectric Point

The isoelectric point of the purified enzyme was estimated by isoelectric focusing on Ampholine PAGplate precast polyacrylamide gel using broad pI calibration kit as marker proteins. The result is presented in Fig. 3.

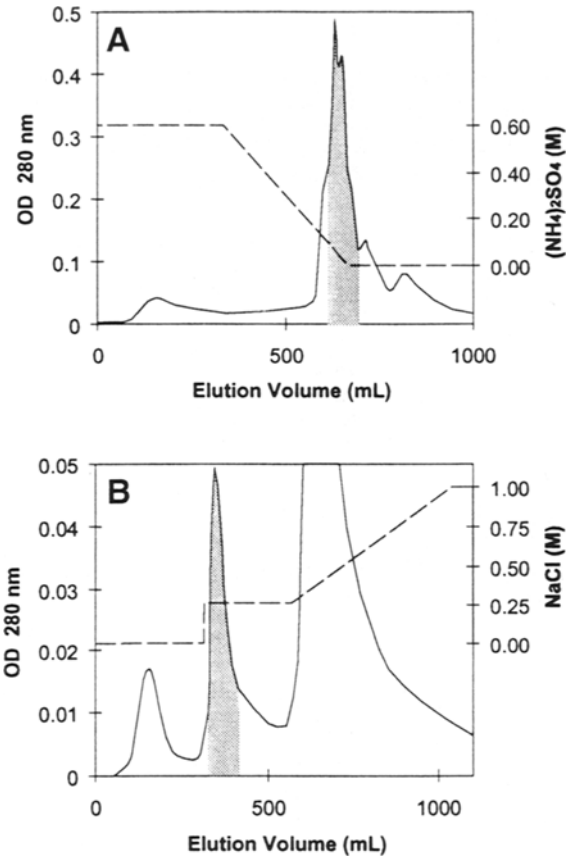

Fig. 1. Elution profile of *R. miehei* lipase on Phenyl Sepharose 6 Fast Flow (**A**) and on DEAE Sepharose Fast Flow (**B**). Shaded area indicates lipolytically active fractions.

Table 1
Summary of Purification of Lipase from *R. miehei*

Purification step	Total protein, mg	Total activity,[a] U	Specific activity,[a] U/mg	Purification factor	Yield, %
Crude powder	460	12000	26	1.0	100
Ammonium sulfate	110	8400	77	3.0	76
Phenyl Sepharose 6 Fast Flow	37	7800	210	8.0	65
DEAE Sepharose Fast Flow	3.6	3900	1080	42	32

[a]Hydrolytic activity.

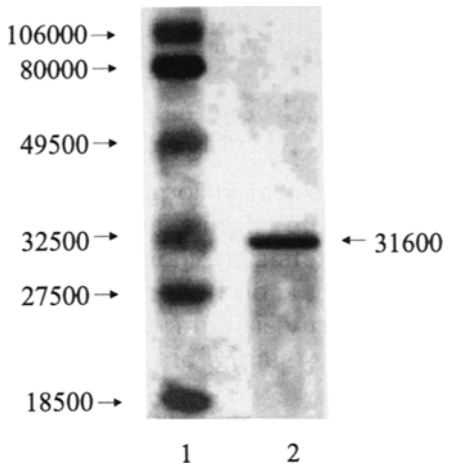

Fig. 2. SDS-PAGE of purified *R. miehei* lipase. Column 1, 12.5 μg of the mixture of molecular-size standard proteins; column 2, 2.5 μg of the purified lipase. Gel was stained with both Coomassie brilliant blue R-250 and silver stain.

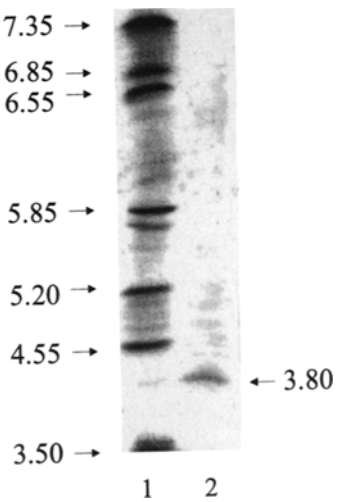

Fig. 3. Isoelectric focusing of the purified lipase. Column 1, 60 μg of the mixture of isoelectric point standard proteins; column 2, 10 μg of the purified lipase. Gel was stained with Coomassie brilliant blue R-250.

The isoelectric point of the purified enzyme was calculated to be 3.8, which is consistent with the result obtained from chromatofocusing, which showed only one peak at pH 4.2–3.7 (data not shown). The pI value of the purified lipase was close to the value of 3.9 reported for A form (*9*).

Effect of Temperature on the Activity and Stability

The hydrolytic and synthetic activities of the enzyme were determined at various temperatures from 30 to 70°C. The optimum temperature for

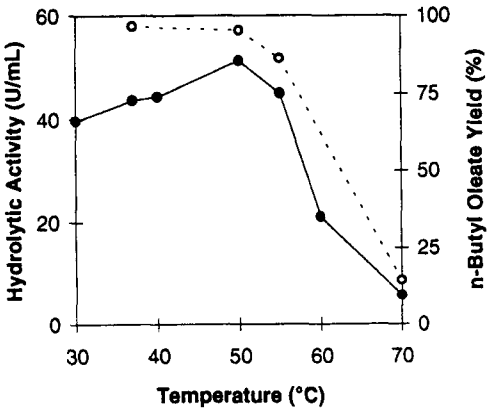

Fig. 4. Effect of temperature on the lipase hydrolytic activity (●) and synthetic activity (○).

the hydrolytic activity of the enzyme under the experimental conditions was found to be 50°C and for synthetic activity 37°C (Fig. 4). Clearly, the measurement of hydrolytic activities alone cannot be used as the sole criterion in determining the suitability of lipases for ester syntheses. While the present work was in progress, Robinson et al. (*14*) also reported similar conclusions by comparing synthetic and hydrolytic activities of six different lipases. Of them, *Pseudomonas fluorescens* lipase showed the highest lactone synthetic activity, but low hydrolytic activity, whereas *Chromobacterium viscosum* lipase had the highest hydrolytic activity, but a low synthetic activity. Figure 4 also shows that both hydrolytic and synthetic activities decreased sharply at temperatures above 50°C. The measurement of the enzyme activity was carried out by the standard assay method, except for the temperature. The hydrolytic activity was expressed as U/mL, and the synthetic activity was calculated as the yield (%) of ester in 12 h.

The thermal stability of the enzyme was investigated by incubating the purified enzyme solution in a water bath at various temperatures from 30 to 60°C for 24 h. The residual activity was measured and expressed as the percentage of the activity of the nonincubated enzyme. The result is presented in Fig. 5. After 24 h, 97% of the hydrolytic activity remained for the enzyme incubated at 30°C, but there was only 67% of the hydrolytic activity left for the enzyme incubated at 40°C. When the enzyme was incubated at temperatures above 55°C for 24 h, almost all of the activity was lost. When the lyophilized purified enzyme was kept at 4°C for 30 d, only about 10% of the activity was lost. An immobilized form of *R. miehei* lipase has been shown to have an activity half-life exceeding 100 d at 60°C (*15*).

Effect of pH on the Activity and Stability

The effect of pH on the hydrolytic activity was determined in different buffer systems with a pH of 3.0–10.0. The lipase activity was measured by the standard method, except for the pH, and expressed as the percentage

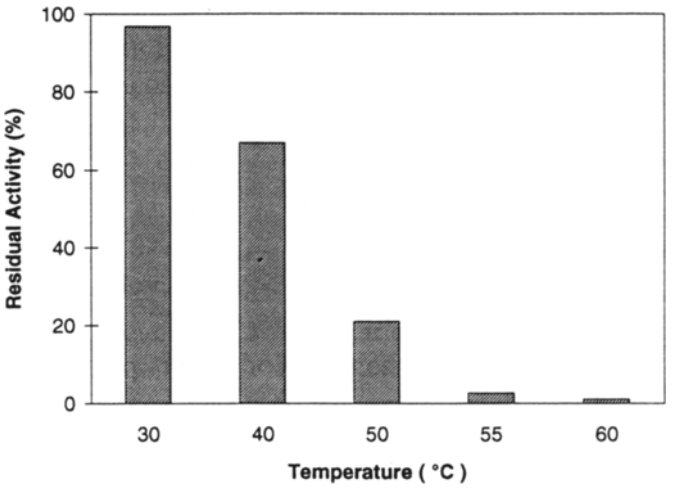

Fig. 5. Effect of temperature on the lipase stability.

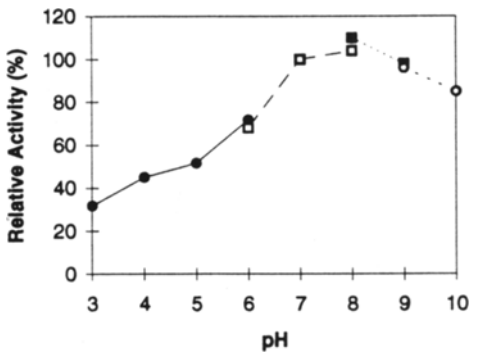

Fig. 6. Effect of pH on the lipase activity. ● 0.1*M* citric acid-sodium citrate buffer; □ 0.1*M* imidazole-HCl buffer; ■ 0.1*M* Tris-HCl buffer; ○ 0.1*M* sodium carbonate-sodium hydrogen carbonate buffer.

of the activity measured at pH 7.0 with 0.1*M* imidazole-HCl as buffer. The optimum pH for the enzyme was 8.0 with olive oil as the substrate, and relative activities (at pH 8.0) of 104% in imidazole-HCl buffer system and 110% in Tris-HCl buffer system, as indicated in Fig. 6. Under acidic conditions, the hydrolytic activity was lower than that under alkaline conditions. For example, the relative activity was 32% at pH 3.0, and 88% at pH 10.0. Huge-Jensen et al. (*9*) reported an optimum pH of 7.0 for the two forms (A and B) of *R. miehei* lipase, using tributyroylglycerol as a substrate, with similar pH-activity profiles for both.

To investigate the enzyme stability at varying pHs, the purified enzyme was exposed to different buffers of pH 3.0–10.0 for 24 h at 4°C. The remaining activity was expressed as the percentage of the activity of the untreated enzyme solution at pH 7.0. The enzyme was relatively stable for at least

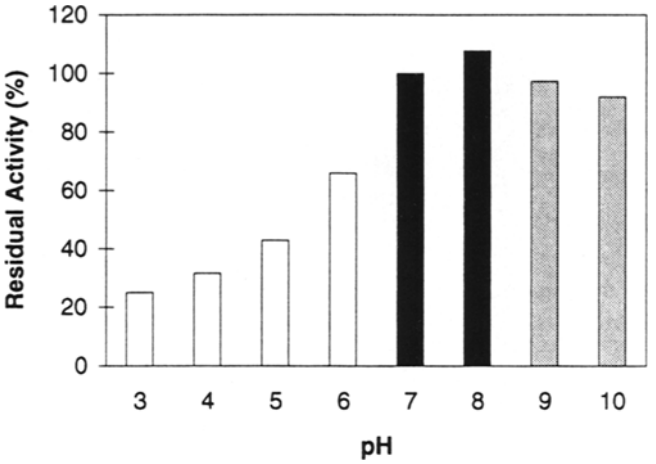

Fig. 7. Effect of pH on lipase stability. □ 0.1*M* citric acid-sodium citrate buffer; ■ 0.1*M* imidazole-HCl buffer; ▨ 0.1*M* sodium carbonate-sodium hydrogen carbonate buffer.

24 h within a pH range of 7–10 and the optimal stability was at pH 8.0 as indicated in Fig. 7. It could be concluded that pH 8.0 was optimal both from the point of view of activity and stability. When the enzyme was kept under acidic conditions (pH 3.0–6.0) for 24 h, the activity decreased by more than 35%.

Effect of Metal Ions and Other Reagents on the Activity

After DEAE Sepharose Fast Flow chromatography, the enzyme solution was dialyzed against distilled water overnight at 4°C.

The obtained enzyme solution was incubated in the presence of 1 m*M* of various metal ions (as chlorides) or a number of other reagents for 30 min at 30°C. The hydrolytic activity was then measured and reported as the percentage of the enzymes without metal ion or other reagent treatment. The results are given in Table 2. All the metal ions used in the present study and SDS decreased the hydrolytic activity by about 10–30%. EDTA had no effect.

Optimization of Substrate Molar Ratio for Lipase-Catalyzed Synthesis of 1-Butyl Oleate

Lipase-catalyzed ester synthesis is a reversible reaction. In order to drive the reaction toward synthesis, one should remove the water formed in the reaction or increase the molar ratio of either substrate. However, initial water content also plays an important role in 1-butyl oleate synthesis (*11,16*). *R. miehei* lipase exhibits little 1-butyl oleate synthesis activity with 3.2% water or less, but with 14% water, a yield of 90% is obtained in 24 h (*17*). Optimal substrate molar ratio, and the quantity of added water

Table 2
Effects of Metal Ions
and Other Reagents on the Lipase Activity

Metal ions or other reagents 1 m*M*	Relative activity, %
None	100
K^+	86
Ni^+	73
Li^+	84
Co^{2+}	72
Zn^{2+}	71
Mg^{2+}	73
Sn^{2+}	76
Cu^{2+}	91
Ba^{2+}	73
Ca^{2+}	72
Fe^{2+}	70
EDTA	99
SDS	91

Table 3
Comparison of Optimal Conditions and Yields
in 1-Butyl Oleate Syntheses with Various Lipases

Lipases	Enzyme, mg	Molar ratio of oleic acid and 1-butanol	Water, %, w/w	Yield, 2 h	6 h	12 h	24 h	30 h
R. miehei	9.0 (53 U)[a]	2.0	18.1	95.7	**97.2**[b]	96.5	94.0	95.0
C. rugosa	8.0 (980 U)	2.0	3.2	93.1	87.6	90.4	94.4	**97.3**
Porcine pancr.	30.0 (115 U)	1.1	3.4	19.5	54.0	77.0	88.1	**93.6**
P. fluorescens	10.5 (123 U)	1.0	3.2	65.2	88.9	94.0	91.0	**96.5**

[a]Hydrolytic activity is given in parentheses.
[b]Highest activity is given in bold.

and enzyme for 1-butyl oleate syntheses with various commercial available lipases are given in Table 3. Figure 8 further illustrates the effect of the molar ratio of oleic acid and 1-butanol for ester synthesis with 53 U of *R. miehei* lipase and 18.1% (w/w) of water. With an excess of oleic acid (Fig. 8A, substrate molar ratio of 2:1), the highest 1-butyl oleate yield of about 97% was obtained in 6 h, and with 1-butanol excess (Fig. 8B, molar ratio of 1:2), about 93% in 30 h. Ester yields obtained at different reaction times with a number of commercially available lipases under the optimal conditions are given in Table 3. *R. miehei* lipase exhibited the fastest rate of

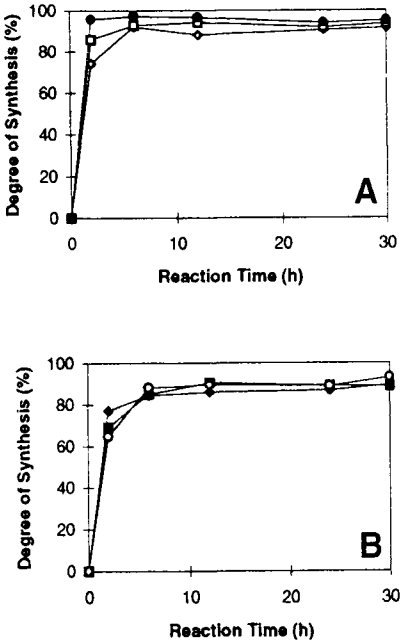

Fig. 8. Ester synthesis with an oleic acid excess (**A**), oleic acid: 1-butanol molar ratio = 1.1 (◇); 1.2 (□) and 2.0 (●), and with 1-butanol excess (**B**), oleic acid: 1-butanol = 0.9 (◆); 0.83 (■) and 0.50 (○).

synthesis and the highest ester yield of about 96% in 2 h and 97% in 6 h, whereas Porcine pancreatic lipase needed a much longer reaction time with only 54% of ester yield in 6 h and 94% in 30 h. The widely different hydrolytic activities for the different enzymes given in Table 3 were chosen in order to obtain approximately the same order of magnitude of ester synthesis activity. One can clearly see the marked variations in the relationships of hydrolytic to synthetic activity with the different enzymes under the experimental conditions used.

Kinetics of 1-Butyl Oleate Biosynthesis with Commercial and Purified *R. miehei* Lipases

The purified lipase solution after DEAE Sepharose chromatography was concentrated and desalted by using an ultrafiltration membrane with a cutoff of 10,000 (PM 10, Amicon). The concentrated lipase was lyophilized and the obtained powder was used for ester synthesis. Figure 9 shows the results of 1-butyl oleate synthesis using the optimal oleic acid and 1-butanol molar ratio of 2:1, and either commercial or purified *R. miehei* lipases as biocatalyst. The initial rate of the esterification was markedly reduced when the purified lipase was used as the catalyst. For example, the yield of 1-butyl oleate was 96% in 2 h with the commercial lipase, but $< 64\%$ with the purified lipase with the same hydrolytic activity. A lag time in heptyl oleate synthesis has been reported by Sanchez-Montero et al.

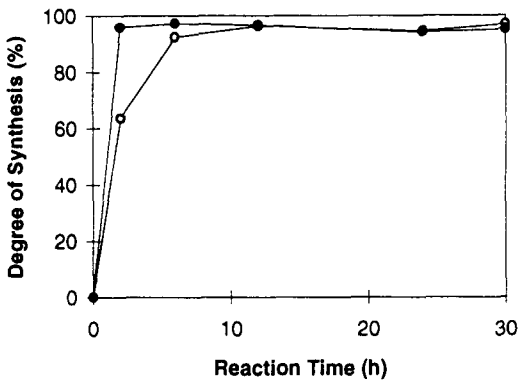

Fig. 9. 1-Butyl oleate synthesis using lipase as biocatalyst; ● commercial lipase; ○ purified lipase.

(*18*) when dialyzed *Candida rugosa* lipase (partially purified) is used as biocatalyst. They concluded that the reason was insufficient available water around the enzyme molecules for the lipase to be fully active, but that lactose present in the crude lipase could improve the synthesis by acting as a water "reservoir." Because in the present work there always was a large quantity (18.1%) of initial water present during the ester synthesis, the explanation given by Sanchez-Montero et al. (*18*), who added no excess water to the reaction mixture in addition to that present in the enzyme preparation after equilibrating to a water activity of 0.44, is not the probable cause in this case. However, it is likely that the changes in the microenvironment of the enzyme as suggested by Sanchez-Montero et al. (*18*) can markedly affect the initial synthesis activity. As the ester synthesis progressed for about 6 h, the ester yield was no more affected by the initial purity of the enzyme. The 1-butyl oleate yield of 96% in 12 h obtained with purified lipase was nearly equal to the about 97% obtained with the original commercial lipase preparation under comparable conditions. It can be concluded that although the purification procedure resulted in a decrease in the initial rate of ester synthesis with *R. miehei* lipase, it had little effect on the final 1-butyl oleate yield.

REFERENCES

1. Taipa, M. A., Aires-Barros, M. R., and Cabral, J. M. S. (1992), *J. Biotechnol.* **26,** 111–142.
2. Tombs, M. P. and Blake, G. G. (1982), *Biochem. Biophys. Acta* **700,** 81–89.
3. Sugihara, A., Shimada, Y., and Tominaga, Y. (1988), *Agric. Biol. Chem.* **52,** 1591,1592.
4. Veeraragavan, K., Colpitts, T., and Gibbs, B. F. (1990), *Biochem. Biophys. Acta* **1044,** 23–26.
5. Nagaoka, K. and Yamada, Y. (1973), *Agric. Biol. Chem.* **37,** 2791–2796.
6. Ishihara, H., Okuyama, H., Ikezawa, H., and Tejima, S. (1975), *Biochem. Biophys. Acta* **388,** 413–422.

7. Isobe, K. and Nokihara, K. (1991), in *Lipases: Structure, Mechanism and Genetic Engineering*, vol. 16, Alberghina, L., Schmid, R. D., and Verger, R. eds., GBF Monographs, VCH, Weinheim, pp. 345–348.
8. Omar, I. C., Nishio, N., and Nagai, S. (1987), *Agric. Biol. Chem.* **51,** 2145–2151.
9. Huge-Jensen, B., Galluzzo, D. R., and Jensen, R. G. (1987), *Lipids* **22,** 559–565.
10. Boel, E., Huge-Jensen, B., Christensen, M., Thim, L., and Fill, N. P. (1988), *Lipids* **23,** 701–706.
11. Linko, Y. Y. and Yu, H. C. (1992), *Ann. NY Acad. Sci.* **672,** 492–496.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
13. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
14. Robinson, G. K., Alston, M. J., Knowles, C. J., Cheetham, P. S. J., and Motion, K. R. (1994), *Enzyme Microb. Technol.* **16,** 855–863.
15. Eigtved, P. (1989), US Patent No. 4,798,793.
16. Leitgeb, M. and Knez, Z. (1995), *J. Am. Oil Chem. Soc.* **67,** 775–778.
17. Linko, Y.-Y., Rantanen, O., Yu, H. C., and Linko, P. (1992), in *Biocatalysis in Non-Conventional Media, Progress in Biotechnology*, vol. 8, Tramper, J., Vermue, M. H., Beeftink, H. H., and von Stockar, U., eds., Elsevier Science Publishers B.V., Amsterdam, pp. 601–608.
18. Sanchez-Montero, J. M., Hamon, V., Thomas, D., and Legoy, M. D. (1991), *Biochem. Biophys. Acta* **1078,** 345–350.