Sugar Ester-Modified Lipase for the Esterification of Fatty Acids and Long-Chain Alcohols

Sobhi Basheer[a,*,1], Mitsutoshi Nakajima[b], and Uri Cogan[a]

[a]Department of Food Engineering and Biotechnology, Technion—Israel Institute of Technology, Haifa 32000, Israel and [b]National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305, Japan

ABSTRACT: The esterification reaction of a long-chain fatty acid and a fatty alcohol with a surfactant-modified lipase in a microaqueous *n*-hexane system was studied. Various lipases from different sources were first modified with a surfactant of the sugar ester type to improve their dispersibility in apolar organic solvents. This enzyme modification technique converted inactive crude lipases to highly active biocatalysts for the esterification of long-chain fatty acids and fatty alcohols in a microaqueous n-hexane system. The hydrophilic-lipophilic balance value and chainlength of the fatty acid residue of the fatty acid sugar ester, used for modifying the lipases, significantly influenced the amount of precipitated lipase that was dissolved in the aqueous solution, the protein content of the lipase-surfactant complex and its esterification activity.
JAOCS 73, 1475–1479 (1996).

KEY WORDS: Esterification, fatty acids, fatty alcohols, HLB, hydrophilic–lipophilic balance, long-chain fatty acid esters, microaqueous organic media, surfactant-modified lipase.

The use of enzymes, in particular lipases, in microaqueous organic media has become an active field of research during the past 10 years. This technique has promising industrial applications for the synthesis of a wide range of water-insoluble organic compounds (1,2). Because enzymes function naturally in aqueous solutions, different approaches have been applied to promote the reversed reaction of the natural hydrolytic action of lipases in microaqueous organic media. Basically, three approaches have been applied for modifying hydrolytic enzymes for use in microaqueous apolar organic solutions: (i) Powder enzymes or enzymes immobilized on insoluble matrices have been vigorously stirred with substrates dissolved in apolar organic solutions or in solvent-free systems (3,4); (ii) Enzymes have been dissolved in water pools confined in reverse micelles (5,6); (iii) Enzymes have covalently been attached to hydrophobic residues by alkylation or amidation to produce enzyme complexes with good solubility in apolar organic solvents (7,8).

In many research studies, the use of chemically modified lipases has resulted in a substantial enhancement of reaction rates compared to those obtained with suspended powder enzymes. This difference in enzyme activities is mainly attributed to the heterogeneity of the reaction system when insoluble powder enzymes are dispersed in hydrophobic organic solvents, compared to the homogeneity of the system when chemically modified lipases are employed (9). However, the complexity of preparing chemically modified lipases and perhaps a potential loss of the catalytic function of the biocatalysts have so far limited their application in organic synthesis. Although the use of water-soluble enzymes, confined in reverse micelles, has recently been a fascinating area of research, it also entails some limitations in synthesis applications because water is produced in the reaction system. A new approach to effectively use lipases in microaqueous organic solution has been developed by Okahata and Ijiro (10). Various lipases modified with a surfactant have been prepared. These enzyme complexes are completely soluble in most hydrophobic organic solutions and show high catalytic activity in organic synthesis.

Following our previous work, surfactant-modified lipase complexes have also been prepared for the interesterification of triglycerides and fatty acids (11–13). Lipases modified with sorbitan monostearate as a surfactant gave lipase–surfactant complexes of high interesterification activity. The prepared enzyme–surfactant complexes have shown good dispersibility in a microaqueous *n*-hexane system, and their solubilities depend on the water content of the reaction system. Surfactant-modified lipases have also been prepared by another group (14). These investigators described the preparation method of lipases, modified with synthetic surfactants, as biocatalysts acting on various substrates. We now report the preparation procedure of lipases modified with sugar ester surfactants. The esterification of lauric acid and dodecanol has been chosen as a reaction model system, and a number of lipases from different sources have been tested for the esterification reaction. The influence of hydrophilic–lipophilic balance (HLB) value and the chainlength of the fatty acid residue of the surfactant on the biocatalyst esterification activity, as well as on the protein content of the lipase–surfactant complex have also been studied.

*To whom correspondence should be addressed.

[1]Present address: The Galilee Society, R&D Regional Center, P.O. Box 536, Elabon 16972, Israel.

MATERIALS AND METHODS

Materials. Lyophilized lipase preparations with 1,3-positional specificity, SP 523 (4000 KLU/g), SP 524 (2500 KLU/g) and SP 525 (150 KLU/g), and nonspecific lipase powder, SP 526 (25 KLU), all selected from fungi and their genetic codings cloned to the host organism *Aspergillus oryzae,* were provided by Novo Nordisk A/S (Denmark). Lipozyme 10.000 L (10,000 LU/g), produced from genetically cloned *Mucor miehei* with 1,3-positional specificity, was also provided by Novo Nordisk A/S. Powder lipase F, derived from *Rhizopus* was supplied by Amano Pharmaceutical Co. (Nagoya, Japan), and lipase Saiken 100, produced from *Rhizopus japonicus*, was provided by Nagase, Biochemicals Ltd. (Osaka, Japan). Powder lipases type II and type VII, derived from porcine pancreas and from *Candida cylindracea*, respectively, both containing lactose as an extender, were purchased from Sigma (St. Louis, MO). All fatty acids and fatty alcohols with better than 99% purity were from Fluka (Switzerland). *n*-Hexane, acetone, and ethyl alcohol, all of analytical grade, were obtained from Bio Lab. (Israel). Sucrose stearate esters (mono-, di-, and tristearate esters) of different HLB value were from Kao Pure Chemicals Ind. (Tokyo, Japan). Sorbitan mono-stearate,-palmitate and -laurate, *tris*(hydroxymethyl)-aminomethane (*tris*) and bovine serum albumin (98–99%) were purchased from Sigma.

Lipase modification. The lipase modification technique described in our previous study was followed (11): crude lipase (total protein content 300 mg) was dissolved in 1L tris buffer, adjusted to pH 5. The enzyme solution was magnetically stirred at 10°C, and a solution of 0.75 g sugar ester, dissolved in 20 mL ethanol, was added dropwise to the stirred enzyme solution. The resultant colloidal dispersion was sonicated for 10 min and then stirred for 2 h at 10°C. The produced precipitate was collected by centrifugation at 12,000 rpm and 4°C in a Sorvall Centrifuge, model RC-5B (Newtown, CT), followed by overnight freezing at –20°C and subsequent freeze-drying. The water content of the modified lipases after freeze-drying was less than 0.05 mg per mg biocatalyst. The protein content of the crude and modified lipases was determined according to Lowry's method (15) with bovine serum albumin as a standard. Throughout this study, the measured protein content, either of the crude lipases or of the enzyme–surfactant complexes, was considered to be the lipase content. The modification technique was carried out in duplicate for each experiment.

Esterification reaction. A typical esterification reaction system consisted of 5 mL *n*-hexane containing 0.5 mol/L lauric acid and 0.5 mol/L dodecyl alcohol in a screw-cap glass bottle, to which 10 mg modified lipase was added. The reaction system was magnetically stirred, and the temperature was controlled at 40°C. In all experiments, the initial water content in the reaction system did not exceed 30 mg/L. Samples were taken periodically, filtered through a disposable microfiltration filter (0.45 μm; Schlecher & Schuell, Germany), and then mixed with *n*-hexane that contained palmitic acid as an internal standard. Reaction rates were determined from the concentration of fatty acid, dodecyl alcohol or their ester in the first 30 min of the esterification reaction. In all experiments, *n*-hexane before use was dried over molecular sieves 5Å, so that the total content of the water in the reaction system was brought to less than 30 mg/L. Unless stated otherwise, all experiments were carried out under the above conditions. Each esterification reaction was carried out in duplicate.

Analytical methods. The course of the esterification reaction was followed by determining the concentrations of fatty acids, fatty alcohol, and their esters with time in a gas chromatograph, HP-5890, equipped with a flame-ionization detector. A capillary column, RTX-65TG, 0.53 mm i.d. × 30 m, 0.1 μm film thickness (RESTEK Corp., PA), was used under the following separation conditions: Injector and detector temperatures were maintained at 300°C, initial column temperature 80°C, followed by a 1 min isotherm; thereafter, the oven temperature was raised at a rate of 20°C/min to 270°C. Reaction samples (50 μL) before analysis were mixed with similar volumes of palmitic acid in *n*-hexane (10 mg/mL) as an internal standard.

RESULTS AND DISCUSSION

Effect of lipase source on the protein content of the lipase–surfactant complex and on its esterification activity. The various lipases used produced insoluble complexes with the added surfactant, sorbitan monostearate, in the aqueous system. However, the weight and protein content of the precipitated lipase–surfactant complex depended on the source of the enzyme (Table 1). Because the protein concentration in all experiments was equal, the results suggest that various lipases exhibit different interactions with sorbitan monostearate, which leads to the formation of the insoluble complexes. The surfactant used in the study contains primarily sorbitan monostearate with minor quantities of sorbitan di-

TABLE 1
Protein Content of Crude Lipases and the Amount of Precipitated Lipase Complexes and Their Protein Content After Modification[a]

Lipase	Protein content (%) (crude lipase)	Precipitate (g)	Protein content (%) (modified lipase)
Blank	—	0.37	0
Saiken 100	10.0	0.37	3.5
Lipase F	31.1	0.05	6.0
Lipozyme 10.000 L	11.6	0.32	1.1
Lipase type II	25.3	0.36	14.3
Lipase type VII	11.7	0.24	5.4
SP 523	67.7	0.08	7.3
SP 524	50.6	0.17	4.5
SP 525	66.8	0.21	9.2
SP 526	14.1	0.09	8.5

[a]Experimental conditions: crude lipase (total protein content in all experiments was 300 mg/L) dissolved in 0.5 L tris buffer solution of pH 5. Sorbitan monostearate dissolved in ethanol (0.38 g/10 mL) was added to the enzyme solution. The modification procedure is described in the Materials and Methods section. The blank experiment did not contain crude lipase.

and tri-stearate, free stearic acid, and free sorbitol. The nature of interaction between the lipase and the components of the surfactant is not yet clear to us. However, Okahata and Ijiro (10) propose that hydrogen bridges between free amino groups of the enzyme and free hydroxyl groups of the surfactant are the main force that leads to the formation of the lipase–surfactant complex. The blank experiment, where no enzyme was present in the solution, showed that the sorbitan monostearate can be totally recovered from the solution at pH 5. The lipases Saiken 100, type II, type VII, SP 524 and SP 525 yielded reasonable amounts of precipitated protein from the solution. The lipases F, lipozyme 10.000 L, SP 523 and SP 526 apparently formed water-soluble enzyme-surfactant complexes or a stable emulsion system, and therefore, yielded low amounts of precipitate. The protein content of the modified lipases was in the range 1.1% (wt) for Lipozyme 10.000 L up to 14.3% (wt) for lipase type II.

Table 2 shows the esterification reaction rate, r_i, for modified lipases in microaqueous *n*-hexane system at an initial total water content of less than 30 mg/L. The esterification reaction rates were calculated per mg biocatalyst and per mg protein. The blank experiment was carried out under the same reaction conditions, but no biocatalyst was present in the system. It has been reported that the nonenzymatic esterification reaction, in general, accounts for not more than 1% of total conversion (16). The results show that among the modified lipases, lipase SP 524, Saiken 100, SP 525, lipase F and lipase type II exhibited the highest esterification activities calculated per mg protein.

The esterification activities of the crude lipases without surfactant in the reaction medium were tested under the same reaction conditions. The results revealed that progress of the esterification reaction with time, when crude lipases were used, was slow and did not exceed 2% conversion during 8 h of reaction. The addition of water to the reaction system significantly enhanced the activity of some crude lipases. However, this effect was not studied here.

TABLE 2
Esterification Rates, r_i, Achieved by Various Modified Lipases[a]

Modified lipase	r_i^b	r_i^c
Blank	0.10	—
Saiken 100	1.30	37.1
Lipase F	1.30	21.7
Lipase type II	0.23	20.9
Lipase type VII	0.21	1.5
Lipozyme 10.000L	0.55	10.2
SP 523	0.20	2.7
SP 524	2.86	63.6
SP 525	3.40	37.0
SP 526	0.34	4.0

[a]Reaction conditions: *n*-hexane (5 mL) with 0.5 mol/L lauric acid and 0.5 mol/L dodecyl alcohol and 10 mg lipase Saiken 100 modified with sorbitan monostearate ester. The reaction solution was stirred magnetically and thermostated at 40°C.
[b]Esterification rate (μmol/min · mg biocatalyst).
[c]Esterification rate (μmol/min · mg protein).

Effect of fatty acid residue chainlength of sorbitan monoesters on the modified lipase activity. The amount of precipitate of the lipase–surfactant complex and its protein content were influenced by the chainlength of the fatty acid residue of the sorbitan ester used in the modification process (Table 3). The enzyme preparation was a yellowish solid when sorbitan stearate or sorbitan palmitate were used as surfactants, and a brownish viscous preparation when sorbitan laurate was used in the modification procedure. The results in Table 3 show that higher amounts of precipitate were obtained when the fatty acid chainlength of the surfactant was longer, in the region of C12–C18. This observation can be explained by the higher solubility in water of the surfactants with short-chain fatty acid residues compared to that of sorbitan esters with long-chain fatty acid residues. On the contrary, the results show that the protein content of lipase complexes, formed with short-chain fatty acid sorbitan ester, was higher than that of lipase complexes prepared with long-chain fatty acid sorbitan ester. This is mainly because of the low solubility of long-chain fatty acid (C16 and C18) sorbitan esters, which precipitate immediately after addition to the enzyme buffer solution. Sorbitan laurate ester has higher solubility in buffer solutions, and therefore, the degree of the interaction between the dissolved surfactant and the enzyme is high, leading to the formation of agglomerates with higher levels of enzyme.

Figure 1 shows the progress with time of the esterification reaction for lipase Saiken 100, modified with sorbitan esters of different fatty acid residues. Saiken 100, modified with sorbitan stearate, gave the highest esterification activity, although the protein content of the lipase–surfactant complex was the lowest among the tested modified lipases. The esterification activity of the lipase modified with sorbitan palmitate was also high, and that of the lipase modified with sorbitan laurate was relatively low. The maximum conversion of fatty acid to its corresponding ester, obtained after 24 h reaction with lipase Saiken 100 modified with sorbitan stearate, sorbitan palmitate and sorbitan laurate, without removal of water from the reaction system, was 96, 87, and 53%, respectively. The reaction yields after 2 h for lipases modified with sorbitan stearate, sorbitan palmitate, and sorbitan laurate were 75.4, 68.6, and 11.5 mmol ester/mg enzyme, respectively. This esterification activity exceeded other reported activities measured for different preparations of lipases (9,16,17).

Effect of HLB of sucrose esters on the modification process and on the activity of the modified lipase. Table 4 shows the

TABLE 3
Effect of Fatty Acyl Chainlength of Various Sorbitan Mono-Esters on the Amount of the Precipitated Lipase Saiken 100 Complex and on Its Protein Content[a]

Sorbitan ester	Precipitate (g)	Protein content (%)
Sorbitan monolaurate	0.20	8.7
Sorbitan monopalmitate	0.35	3.5
Sorbitan monostearate	0.37	3.5

[a]Modification procedure was as in Table 1.

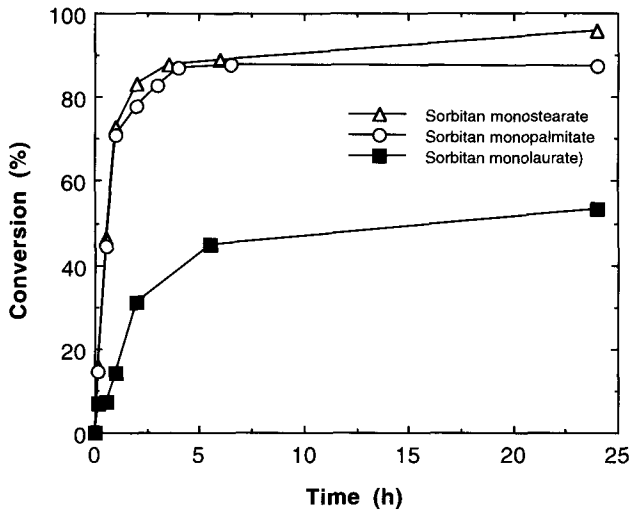

FIG. 1. Effect of fatty acid residue chainlength of sorbitan ester-modified lipase Saiken 100 on conversion of the esterification reaction. Reaction conditions: *n*-hexane (20 mL) with 0.25 mol/L lauric acid and 0.25 mol/L dodecyl alcohol and 80 mg sorbitan mono fatty acid ester-modified lipase Saiken 100. The reaction solution was stirred magnetically and thermostated at 40°C.

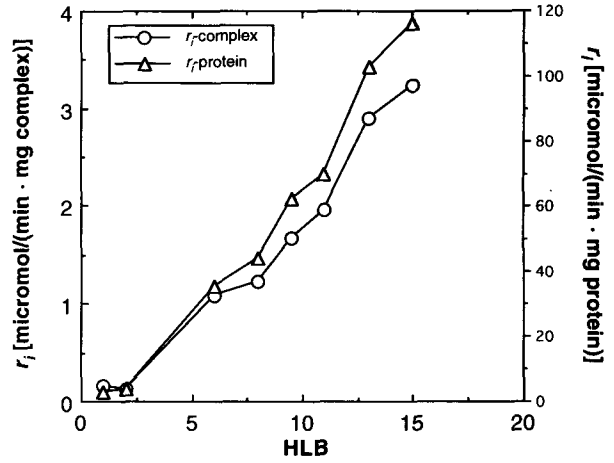

FIG. 2. Effect of hydrophilic–lipophilic balance (HLB) value of sucrose stearate esters on the rate of the esterification reaction after 30 min. Reaction conditions: *n*-hexane (5 mL) with 0.5 mol/L lauric acid and 0.5 mol/L dodecyl alcohol and 10 mg lipase Saiken 100 modified with sucrose stearate ester of different HLB values. The reaction solution was stirred magnetically and thermostated at 40°C.

effect of HLB value of various sucrose stearate esters on the weight of the precipitated enzyme–surfactant complex and on its protein content for lipase Saiken 100 as the enzyme source. Despite the increased solubility of the fatty acid sucrose esters in buffer solutions with the increase of HLB value, increasing the latter in the range of 1–15 resulted in an increase in the weight of the precipitate. This result indicates that the interaction between the enzyme and fatty acid sugar ester was favored with the increase of the HLB value of the surfactant in the range of 1–15. Nonetheless, because of the high solubility of sucrose stearate ester with an HLB value of 19, the weight of the enzyme precipitate formed after the modification process with that surfactant was low.

The protein content of the enzyme–surfactant complexes decreased with an increase in the HLB in the range of 1–8 and became constant at HLB values above 8 (Table 4). These results suggest that sucrose ester surfactants of different HLB values have different modes of interactions with the enzyme. Hydrophobic interactions between the hydrophobic tails of the surfactant and hydrophobic domains of the enzyme may play a significant role besides the possible role of polar interactions between the surfactant and the enzyme, as reported by Okahata and Ijiro (10), in determining the activity of the enzyme–surfactant complex.

When the esterification activity of the various lipase–surfactant complexes was evaluated, a low activity was obtained for the preparations that contained sucrose stearate esters of low HLB values of 1–2 (Fig. 2). The esterification activity increased steeply and quite linearly in the range of HLB values of 2–16. These results suggest that the HLB value of the surfactant used for modifying the lipase is a crucial factor in determining the activity of the modified enzyme.

In conclusion, the employed lipase modification technique has converted lipases to active biocatalysts for esterification reactions in microaqueous hydrophobic organic media. The method enabled the use of lipase for obtaining high esterification conversions, reaching above 95%, with no need for water removal from the reaction system. Furthermore, the modification technique exhibits potential for enzymatic synthesis of water-insoluble ingredients because of its general simplicity. In particular, employing food-grade surfactants for activating lipases may prove to be a powerful preparative tool for the synthesis of food-grade ingredients. Further work is in progress to evaluate sugar ester-modified lipases for the synthesis of food-grade specialty fats and oils.

TABLE 4
Effect of HLB Value of Sucrose Stearate Esters on the Amount of Precipitated Surfactant–Lipase Saiken 100 Complex and on Its Protein Content[a]

Sucrose stearate[b]	HLB	Precipitate (g)	Protein content (%)
F-10	1	0.08	5.7
F-20	2	0.08	3.5
F-50	6	0.09	3.1
F-70	8	0.10	2.8
F-90	9.5	0.14	2.7
F-110	11	0.20	2.8
F-140	13	0.24	2.8
F-160	15	0.27	2.8
SS	19	0.02	2.8

[a]Modification procedure was as in Table 1; HLB, hydrophilic–lipophilic balance.
[b]Manufacturing notation by Kao Pure Chemicals (Tokyo, Japan).

ACKNOWLEDGMENT

The first author gratefully acknowledges receipt of the Lady Davis Fellowship.

REFERENCES

1. Macrae, A.R., Lipase-Catalyzed Interesterification of Oils and Fats, J. *Am. Oil Chem. Soc. 60*:291–294 (1983).
2. Mustranta, A., P. Forssel, and K. Poutanen, Application of Immobilized Lipases to Transesterification and Esterification Reactions in Organic Systems, *Enzyme Microb. Technol. 15*:133–139 (1993).
3. Malcata, F.X., H.R. Reyes, H.S. Garcia, C.G. Hill, and C.H. Amundson Jr., Immobilized Lipase Reactors for Modification of Fats and Oils, *J. Am. Oil Chem. Soc. 67*:890–910 (1990).
4. Sonnet, P.E., G.P. McNeill, and W.Jun, Lipase of *Geotrichum candidum* Immobilized on Silica Gel, *Ibid. 71*:1421–1423 (1994).
5. Luisi, P.L, Enzymes Hosted in Reverse Micelles in Hydrocarbon Solutions, *Angew. Chem. Int. Ed. Engl. 24*:439–450 (1985).
6. Rees, G.D., and B.H. Robinson, Esterification Reactions Catalyzed by *Chromobacterium viscosum* Lipase in CTAB-Based Microemulsion Systems, *Biotechnol. Bioeng. 45*:344–355 (1995).
7. Baillargeon, M.W., and P.E. Sonnet, Polyethylene Glycol Modification of *Candidum rugosa* Lipase, *J. Am. Oil Chem. Soc. 65*:1812–1815 (1988).
8. Basri, M., K. Ampon, W.M Z. Yunus, C.N.A. Razak, and A.B. Salleh, Amidation of Lipase with Hydrophobic Imidoesters, *Ibid. 69*:579–583 (1992).
9. Ito, Y., H. Fujii, and Y. Imanishi, Modification of Lipase with Various Synthetic Polymers and Their Catalytic Activities in Organic Solvents, *Biotechnol. Prog. 10*:398–402 (1994).
10. Okahata, Y., and K. Ijiro, A Lipid-Coated Lipase as a New Catalyst for Triglycerides Synthesis in Organic Solvents, *J. Chem. Soc., Chem. Commun.*, 1392–1394 (1988).
11. Basheer, S., K. Mogi, and M. Nakajima, Surfactant-Coated Lipase for the Catalysis of the Interesterification of Triglycerides and Fatty Acids, *Biotechnol. Bioeng. 45*:187–195 (1995).
12. Basheer, S., J.B. Snape, K. Mogi, and M. Nakajima, Transesterification Kinetics of Triglycerides for a Modified Lipase in *n*-Hexane, *J. Am. Oil Chem. Soc. 72*:231–237 (1995).
13. Basheer, S., K. Mogi, and M. Nakajima, Interesterification Kinetics of Triglycerides and Fatty Acids with Modified Lipase in *n*-Hexane, *Ibid. 72*:511–518 (1995).
14. Goto, M., N. Kamiya, M. Miyata, and F. Nakashio, Enzymatic Esterification by Surfactant-Coated Lipase in Organic Media, *Biotechnol. Prog. 10*:263–268 (1994).
15. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.Y.J. Randall, Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*:265–268 (1951).
16. Trani, M., F. Ergan, and G. André, Lipase-Catalyzed Production of Wax Esters, *J. Am. Oil Chem. Soc. 68*:20–22 (1991).
17. Boyer, J.L., B. Gilot, and R. Guiraud, Heterogeneous Enzymatic Esterification: Analysis of the Effect of Water, *Appl. Microbiol. Biotechnol. 3*:372–376 (1990).

[Received November 21, 1995; accepted June 30, 1996]