

Lipase of *Geotrichum candidum* Immobilized on Silica Gel

Phillip E. Sonnet*, Gerald P. McNeill and Wang Jun¹
ERRC, ARS, USDA, Philadelphia, Pennsylvania 19118

Silica gel is a useful support for the lipases of *Geotrichum candidum*. Esterification of selected fatty acids and alcohols proceeded to 85–92% conversion in hydrocarbon solvents, and the degree of esterification was increased to 96–98% by adding 4Å molecular sieves at later stages of reaction. The equilibrium ratio of ester to fatty acid (butyl oleate to oleic) was determined for the supported lipase in a number of solvents and ranged from 92:8 in hexane and isooctane to 16:84 in *t*-butanol. The essential character of the enzyme seemed unimpaired by deposition onto silica gel as judged by fatty acid selectivity and stereoselectivity.

KEY WORDS: Fatty acid selectivity, *Geotrichum candidum*, immobilized, lipase.

The lipases of the fungus *Geotrichum candidum* are the subject of intense investigation for details of structure that can explain the differing fatty acid selectivities observed in hydrolytic reactions when catalyzed by these enzymes (1–9). The preferential reaction of *cis*-9-unsaturated fatty acids, notably oleic, linoleic, linolenic and palmitoleic acids, is viewed as industrially useful. However, this selectivity is vested in one or more isozymes that are present in differing amounts in the lipases of the various strains of this fungus. Additionally, the utility of these proteins would be greatly enhanced if they could be successfully immobilized and the desired fatty acid selectivity of the enzyme is retained in the immobilized state. This report describes the preparation of a silica gel-borne *G. candidum* lipase and its catalytic activity in several organic solvents.

MATERIALS AND METHODS

Gas-liquid chromatography (GLC) was performed with a Hewlett-Packard Model 5610 instrument (Avondale, PA), a flame-ionization detector and a (nonpolar) DB1-HT capillary column (0.32 mm × 30 m) (J&W Scientific, Folsom, CA). On-column injection was employed with a helium carrier gas flow rate of 5.5 mL/min. Solvents were reagent-grade or better, and fatty acids, alcohols and other reagents were purchased either from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used directly. The 2-methyloctanoic acid had been synthesized previously for related research (10), and the silica gel employed for adsorption was 70–230 mesh, 60Å and was purchased from Aldrich Chemical Co. The enzymes used were GC-20 from Amano (Troy, VA), with a specific activity of 5.1 μmol of free fatty acid (FFA) released/min/mg of power in an olive oil assay, 28.6% protein (11), having relative activity of oleic and palmitic acids of 1.5:1, in hydrolysis of umbelliferate esters and a preparation from Biocatalysts Ltd. (Midgiamorgan, United Kingdom).

Reactions of lipase powders. Solutions that were 0.25 M in 1-butanol and oleic acid in isooctane, to which 25 mg of lipase powders was added, were shaken at 200 rpm and 30°C for varying periods of time. For “dry” reactions, the alcohol and the solvent were placed over 4Å molecular sieves for an hour; for other reactions, the solvent was shaken with buffer briefly and/or specific additions of water were made. Aliquots of the reaction mixtures were treated with ethereal diazomethane, analyzed by GLC and corrected with standards. All reactions were conducted at least twice and averaged.

Adsorption procedure. Amano GC-20 or powder from Biocatalysts Ltd. (1.0 g, 240 μmol of activity) was dissolved in 10 mL of 0.05 M TRIS, which was buffered at pH 8.0 and contained 10 mM CaCl₂. Silica gel (10.0 g) was added in portions, and the final mixture was shaken to produce a free-flowing solid. The supported enzyme was stored at 5°C.

Assays for lipase activity. The fatty acid (1.0 mmol) and alcohol (1.0–3.0 mmol) were allowed to react with 1.0 g of supported enzyme in 2.0 mL of solvent at 30°C and were shaken at 170 rpm for varying lengths of time. Reactions initiated with esters (1.0 mmol) were conducted in the same manner. Aliquots of reaction mixtures were treated with ethereal diazomethane, and the resulting mixtures were then analyzed by GLC. In reactions that were conducted with a separate aqueous phase present, a solution of Amano GC-20 powder (0.10 g) in the same buffer (2.0 mL) was substituted for the absorbed enzyme. One gram of 4Å molecular sieves was added to several reaction mixtures that had reached equilibrium and these reactions were shaken for an additional 24 h.

Assay for fatty acid selectivity. Oleic and stearic acids (0.05 mmol each) were allowed to react in isooctane (2.0 mL) that contained the alcohol (1.5 mmol) and supported enzyme (1.0 g) at 30°C and shaken at 170 rpm. Aliquots were treated with diazomethane and analyzed by GLC as described previously.

Assay for stereoselectivity. Samples of 2-methyloctanoic acid (1.0 mmol) and 1-butanol (3.0 mmol) were allowed to react in isooctane (2.0 mL) that contained the supported enzyme (1.0 g) for 24 h. The degree of conversion was determined as above by using diazomethane and GLC to characterize methyl and butyl esters. An aliquot of the reaction mixture was treated with thionyl chloride and dimethylformamide in ether (12), followed by (*S*)- α -phenylethylamine to generate GLC-separable diastereomeric amides. The enantiomeric excess in the recovered (unesterified) acid fraction was read from the ratio of the amides, and, using the degree of conversion, a relative rate of reaction for the enantiomeric acids was calculated (13).

RESULTS AND DISCUSSION

The topic of lipase immobilization has been thoroughly reviewed (14), and immobilization of *G. candidum* lipase has been accomplished by various means, such as simple adsorption onto hydrophilic and hydrophobic supports, gel entrapment and covalent binding to an insoluble car-

*To whom correspondence should be addressed at the Hides, Lipids and Wool Research Unit, ERRC, ARS, USDA, 600 East Mermaid Lane, Philadelphia, PA 19118.

¹Present address: RIDCI, Ministry of Light Industry, Taiyuan Shanxi, P.R. China.

rier *via* reaction between functional groups of the protein and reactive groups on the carrier surface. Previous research showed that deposition of *G. candidum* lipase onto an inorganic support (Celite) resulted in a material that could be used to introduce oleic acid into olive oil (15). Stearic acid was not incorporated under these conditions, suggesting that the essential features of the enzyme had been preserved.

To investigate this point further, some reported methods for immobilization (14) were reevaluated with *G. candidum* lipase from Amano Co. (GC-20). The activities of the supported lipase were variable, and sometimes it exhibited little catalytic activity at all in esterification reactions (P.E. Sonnet, and W. Jun, unpublished results). Although lipases are generally quite reactive in organic solvents, it is also understood that in esterification reactions one must strike a balance between sufficient water to retain enzyme activity while keeping water activity low enough to allow a desired degree of conversion to ester at equilibrium (16). The commercial *G. candidum* lipase preparations, when allowed to contact oleic acid and (dry) 1-butanol in (dry) isooctane, produced less than 1% butyl oleate in 16 h. In contrast, *Candida rugosa* lipase, under these same conditions, yielded 74% of the ester. The same reaction conducted in moist isooctane gave the following yields of ester: *C. rugosa*, 98%; Amano GC-20 (*G. candidum*), 67%; Biocatalysts Ltd. (*G. candidum*), 34%. Incremental addition of water to reactions of the *G. candidum* lipases produced lower yields (slower reactions). Finally, a system that was patterned after the seminal work of Okumura and co-workers (17) and contained a distinct aqueous phase gave a 92% yield of butyl oleate in 72 h from both commercial *G. candidum* lipases. The exposure of butyl oleate to these conditions led to the same ratio of materials. By contrast, simple exposure of Celite, kiesel gel and alumina to buffered solutions of *G. candidum* lipase or "drying down" onto these supports produced materials that were low in activity (18,19). Clearly, a support that would associate the enzyme with an ample supply of water should provide a recoverable material that would be capable of catalyzing esterification. This was apparently the case for the Celite-borne enzyme to which water was added conditionally (details not provided) until the support regained its activity (15).

In the present investigation, a buffered solution of GC-20 was placed onto silica gel (see the Materials and Methods section) so as to produce a free-flowing solid. We initially observed that alcohols (ethanol, 1-butanol, 1-hexanol, 1-octanol) could be routinely converted to fatty acid esters in isooctane. For example, oleates were obtained in 85–92% yields. As expected, when the esters were exposed to these conditions, they underwent partial hydrolysis (10–15%). The degree of conversion and rate of reaction were qualitatively equal to that observed in reactions that were conducted with the free enzyme dissolved in buffer, except that the ethyl ester was only produced in 75% yield.

To drive the equilibrium further toward ester formation, the support was dried at (i) *ca.* 100 Torr or (ii) at 0.1 Torr for 16 h. Although no specific water contents or activities were measured, this preliminary effort revealed that reducing the water content of the support merely slowed down reaction rates with no indication that the endpoint was shifted. The dryer the support (enzyme), the slower the

reaction; and apparently other parameters must be examined to alter the equilibrium position.

In a series of articles, Valivety and co-workers (20–23) have probed the dependence of catalytic activity of several lipases on water activity and the effects of a large number of organic solvents on equilibrium position in lipase-catalyzed esterification. Because equilibrium positions depend on the activities of all participating species in the equilibrium, the interactions of solvents with polar molecules, such as carboxylic acids and alcohols, can dramatically influence equilibrium position (20). In that approach, porcine pancreatic lipase had been placed onto Celite with about 10% by weight of water, an activity of unity was assumed for water, and esterification of 1-dodecanol with dodecanoic (lauric) acid were carried out for extended reaction times. The results were tabulated as $[\text{Ester}]/[\text{Acid}][\text{Alcohol}]$. We were particularly interested in examining the equilibrium position for esterification of less hydrophobic alcohols, such as 1-butanol. In fact, the results are qualitatively similar, and they have been expressed as mole ratio of ester to acid, which might be more directly useful (Table 1). Apparently, the system "1-butanol + oleic acid" can be pushed no further than 92% conversion without reducing water activity. To further enhance the formation of ester, 4Å molecular sieves were added to several equilibrated reaction mixtures. In each case, the endpoint was altered toward ester (96–98% in hexane, for example) (Table 1). Esterification reactions have in fact been augmented in this manner, usually by removing the water formed with the molecular sieves external to the reaction mixtures (24,25). Because the water being removed in the reactions must include water that is readily available from the support, it is likely that this protocol would soon interrupt the performance of the supported enzyme in subsequent reactions.

A major concern was that the supported enzyme would retain its ability to discriminate between selected fatty acids. The relative reactivity of oleic vs. stearic acid in esterification with 1-butanol is 10:1 for Amano lipase from *G. candidum*, and the relative reactivity of oleic vs. palmitic acid in this reaction is about 2.5:1 (26). The lipase from Biocatalysts Ltd. is considerably more selective in its reactions. The data of Table 2 indicate complete preservation of these selectivities. Additionally, any stereoselec-

TABLE 1

Equilibrium Position for the Esterification of Oleic Acid with 1-Butanol and Amano GC-20/Silica Gel at 30°C^a

Solvent	Ester/Acid	$\epsilon(T^\circ\text{C})^b$
Hexane	92:8 ^c	1.89 (20)
Isooctane	92:8 ^c	1.94 (20)
Carbon tetrachloride	88:12	2.23 (25)
<i>t</i> -Butyl methyl ether	40:60 ^d	—
3-Methyl-3-pentanol	18:82	—
<i>t</i> -Butanol	16:84	10.9 (30)

^aValues were obtained from both esterification and hydrolysis and are the average of at least two observations ($\pm 2\%$). Reactions in hydrocarbon solvents reached equilibrium within 24 h; other solvents required up to 72 h. Amano GC-20/silica gel from Amano (Troy, VA).

^bDielectric constant.

^cThe endpoint was 87% ester with the lipase dissolved in a separate aqueous phase, and 96–98% ester when the reaction was conducted with the silica gel and added molecular sieves.

^dThe endpoint was 49% ester with the lipase dissolved in a separate aqueous phase.

TABLE 2

Relative Reactivity of Oleic and Stearic Acids
in Esterifications (Isooctane)^a

	C ^b	SM ^c	P ^d
Amano GC-20/silica gel			
1-Butanol ^e	0.50	79.5:20.5	21:79
2-Methyl-1-propanol ^f	0.48	82.5:17.5	14.5:85.5
Biocatalysts Ltd./silica gel			
1-Butanol	0.14	58:42	0:100
2-Methyl-1-propanol	0.16	59.5:40.5	0:100

^aRelative reaction rates for oleic vs. palmitic acids ranged from 1.8–2.7:1 with these same alcohols. Company source as in Table 1; other silica gel from Biocatalysts Ltd. (Mid Glamorgan, United Kingdom).

^bFraction conversion \pm 2%.

^cRatio of stearic to oleic acid in unreacted acid fraction. SM, starting material.

^dRatio of stearic to oleic acid in the esterified fraction. P, product.

^eRelative reactivity of oleic to stearic = 6.9 (Ref. 13).

^fRelative reactivity of oleic to stearic = 11.1 (Ref. 13).

tivity exhibited by an enzyme would serve as a stringent test of retained structure and performance by the enzyme. For example, the stereobias in esterification of 2-methyl-octanoic acid, supported Amano GC-20 lipase favored reaction of the *S*-enantiomer (relative reactivity = 8.7) (see the Materials and Methods section) and compares well with the original lipase powder (5.3 favoring *S*-configuration).

The supported lipase was used repeatedly, and after five sequential exposures to oleic acid and 1-butanol in isooctane it retained 50% of its activity. After one week of continuous use in several particularly lethargic reactions (not described here), the recovered catalyst retained 30% of its activity in the butyl oleate synthesis. The supported enzyme has been stored for several months at 5°C with no significant loss of activity.

REFERENCES

- Veeraragavan, K., T. Colpitts and B.F. Gibbs, *Biochem. Biophys. Acta* 1044:26 (1990).
- Shimada, Y., A. Sugihara, Y. Tominaga, T. Iizumi and S. Tsunisawa, *J. Biochem.* 106:383 (1989).
- Sugihara, A., Y. Shimada and Y. Tominaga, *Ibid.* 102:426 (1990).
- Shimada, Y., A. Sugihara, T. Iizumi and Y. Tominaga, *Ibid.* 107:703 (1990).
- Sidebottom, C.M., E. Charton, P.P.J. Dunn, G. Mycock, C. Davies, J.L. Sutton, A.R. Macrae and A.R. Slabas, *European J. Biochem.* 202:485 (1991).
- Charton, E., and A.R. Macrae, *Biochem. Biophys. Acta* 1123:59 (1992).
- Schrag, J.D., Y. Li, S. Wu and M. Cygler, *J. Mol. Biol.* 220:541 (1991).
- Schrag, J.D., Y. Li, S. Wu and M. Cygler, *GBF Monogr.* 16:55 (1991).
- Jacobsen, T., and O.M. Poulsen, *Can. J. Microbiol.* 38:75 (1991).
- Sonnet, P.E., *J. Agric. Food Chem.* 41:319 (1993).
- Baillargeon, M.W., *Lipids* 25:841 (1990).
- Fieser, L.F., and M. Fieser (eds.), *Reagents for Organic Synthesis*, John Wiley and Sons, New York, 1967, pp. 286–289.
- Sih, C.J., and S.-H. Wu, *Topics in Stereochemistry* 19:63 (1989).
- Malcata, F.X., H.R. Reyes, H.S. Garica, C.G. Hill, Jr. and C.H. Amundson, *J. Am. Oil Chem. Soc.* 67:890 (1990).
- Macrae, A.R., *Ibid.* 60:291 (1983).
- Mukherjee, K.D., *Biocat.* 3:277 (1990).
- Okumura, S., M. Iwai and Y. Tsujisaki, *Biochim. Biophys. Acta* 575:156 (1979).
- Kroll, J., F.R. Hassenien, E. Glapinska and C.L. Franzke, *Die Nahrung* 24:215 (1980).
- Marlot, G., G. Langrande, C. Triantaphylides and J. Baratti, *Biotech. Lett.* 7:647 (1985).
- Valivety, R.H., G.A. Johnston, C.J. Suckling and P.J. Halling, *Biotech. Bioeng.* 38:1137 (1991).
- Valivety, R.H., P.J. Halling and A.R. Macrae, *Ibid.* 1118:218 (1992).
- Valivety, R.H., P.J. Halling, A.D. Peilow and A.R. Macrae, *Biochim. Biophys. Acta.* 1122:143 (1992).
- Valivety, R.H., P.J. Halling and A.R. Macrae, *Biotech. Lett.* 15:1133 (1993).
- Knox, T., and K.R. Cliffe, *Proc. Biochem.*, Oct., pp. 188–192, (1984).
- Ergan, F., M. Trani and G. Andre, *Biotech. Bioeng.* 35:195 (1990).
- Sonnet, P.E., T.A. Foglia and M.W. Baillargeon, *J. Am. Oil Chem. Soc.* 70:1043 (1993).

[Received April 5, 1994; accepted August 30, 1994]