Comparative Study of Commercially Available Lipases in Hydrolysis Reaction of Phosphatidylcholine

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ABSTRACT: Nineteen commercial lipase preparations were compared with respect to their ability to hydrolyze phosphatidylcholine (PC) in a reverse micellar system. In terms of the source organism, lipases from *Mucor* or *Rhizopus* species showed comparatively high reactivity, while those from *Aspergillus* and *Humicola* sp. exhibited little PC hydrolysis activity. Almost no hydrolysis reaction was observed by positionally nonspecific lipases derived from *Candida* sp. The results suggest that particular attention should be paid to the source organism in selecting lipases for use in the enzymatic conversion of phospholipids.

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KEY WORDS: Fungi, hydrolysis, lipase, phosphatidylcholine, positional specificity, reverse micelle, source organism.

Phospholipids, which are important structural components of cell membranes, have recently been receiving increasing attention in the pharmaceutical, food, cosmetics, and other industries because of their inherent physiological activities (1–4), as well as for use as natural emulsifiers (5). Phospholipids that exist in nature contain several fatty acids, the proportions depending on the source. For some practical applications, it is desirable to obtain phospholipids with specific fatty acids, which has prompted much research into changing the fatty acid composition of phospholipids by enzymatic conversion methods, such as hydrolysis (6–14), esterification (6,13–16), or transesterification (6,10,14,16–22).

Enzymes are attractive catalysts for these transformations because of the mild reaction conditions employed and their high stereochemical or positional specificities. Lipases are frequently used in such reactions because phospholipids are generally included within their substrate range, and they are commercially available from a wide range of organisms. In selecting an enzyme to catalyze a reaction, it is important to choose one with a good level of reactivity for the target substrate. Although lipases from many sources have appeared in the literature on phospholipid modification research, including those from *Mucor miehei* (Lipozyme) (9–11,14,16–18,22), *M. javanicus* (19), *Candida cylindracea* (*rugosa*) (11–13, 17–20), *C. antartica* (11), *Candida* sp. (14), *Humicola lanuginosa* (12), *Rhizopus delemar* (6,12,13,19,20), *R. niveus* (12), *R. javanicus* (12), *R. arrhizus* (17,18), *Rhizopus* sp. (19), *Penicillium cyclopium* (10,12), *Aspergillus niger* (10), *Pseudomonas* sp.(12,13), potato tubers (17), porcine pancreas (17), and calf pregastric (12), the reaction conditions used in these studies have varied widely, making it difficult to compare the various lipases in terms of their reactivities for phospholipids. We therefore attempted in the present work to compare a broad range of commercially available lipase preparations under identical reaction conditions. Hydrolysis of phosphatidylcholine (PC) in a reverse micellar system with sodium bis(2-ethylhexyl) sulfosuccinate (AOT) was chosen as the reaction for evaluating the reactivities of the lipases, our reasoning being that, because enzymes added to this system would be dissolved in the reverse micelles and adsorb onto the hydrophobic interface, they would be fully activated by what is known as "interfacial activation" (23), unlike in microaqueous organic media in which transesterification occurs (24).

The performances of nineteen commercial lipases in hydrolyzing PC in this reverse micellar system are compared with respect to their source organism and positional specificity.

MATERIALS AND METHODS

Materials. Lipases from *R. delemar* (6000 U/mg) and *C. cylindracea* (10,000 U/mg) were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Lipozyme 10.000L (10 U/mg), SP388 (20 U/mg), SP398 (50 U/mg), SP523 (4000 U/mg), SP524 (2500 U/mg), SP525 (150 U/mg), SP526 (25 U/mg), Paratase A (0.75 U/mg), Paratase M (1 U/mg), and the immobilized lipases Lipozyme IM20 [25 Batch Interesterification Units (BIU)/g] and SP435 (7 U/mg) were generous gifts from Novo Nordisk (Bagsvaerd, Denmark). Lipase A6 (60 U/mg), Lipase AY30 (30 U/mg), Lipase F (150 U/mg), Lipase M10 (10 U/mg), Newlase F (30 U/mg), and Pancreatin F (0.75 U/mg) were kindly supplied by Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). The source organisms of all these lipases are listed in Table 1. Phospholipase C (PLC) (Type III, from *Bacillus cereus*), which was used to determine the positional specificity of the above lipases for PC, was obtained from Sigma Chemical Company (St. Louis, MO). Egg

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TABLE 1
Comparison of Commercially Available Lipases in Hydrolysis of Phosphatidylcholine (PC)

Enzyme	Supplier[a]	Source organism	Degree of hydrolysis [%] (at 24 h)	Degree of hydrolysis [%] (at 48 h)	Positional specificity (for PC)	Positional specificity (for triglycerides)
Lipase M10	A	*Mucor javanicus*	100[b]		*sn*-1 Specific	1,3-Specific
Lipozyme IM20	N	*M. miehei*	100[c]		*sn*-1 Specific	1,3-Specific
Pancreatin F	A	Hog pancreas	100		*sn*-2 Specific	1,3-Specific
SP388	N	*M. miehei*[d]	74.1	89.0	*sn*-1 Specific	1,3-Specific
Lipozyme 10.000L	N	*M. miehei*	77.1	94.0	*sn*-1 Specific	1,3-Specific
SP524	N	*Mucor* sp.[d]	53.0	84.0	*sn*-1 Specific	1,3-Specific
Lipase F	A	*Rhizopus* sp.	48.7	62.7	*sn*-1 Specific	1,3-Specific
Lipase (from *R. delemar*)	S	*R. delemar*	42.9	46.0	*sn*-1 Specific	1,3-Specific
Newlase F	A	*R. delemar*	21.6	35.5	*sn*-1 Specific	1,3-Specific
SP435	N	*Candida antartica*[d]	4.1	7.2	*sn*-1 Specific	Nonspecific
Paratase A	N	*Aspergillus* sp.	2.9	7.2	*sn*-1 Specific	1,3-Specific
Lipase A6	A	*A. niger*	1.3	2.6		1,3-Specific
SP523	N	*Humicola* sp.[d]	0.0	11.0	*sn*-1 Specific	1,3-Specific
SP398	N	*Humicola* sp.	0.0	6.0	*sn*-1 Specific	1,3-Specific
Paratase M	N	*M. miehei*	0.0	1.0		1,3-Specific
SP525	N	*Candida* sp.[d]	0.0	0.0		Nonspecific
SP526	N	*Candida* sp.[d]	0.0	0.0		Unknown[e]
Lipase AY30	A	*C. cylindracea*	0.0	0.0		Nonspecific
Lipase (from *C. cylindracea)*	S	*C. cylindracea*	0.0	0.0		Nonspecific

[a]Abbreviations: A, Amano Pharmaceutical; N, Novo Nordisk; S, Seikagaku Kogyo.
[b]Complete hydrolysis was established in 1.5-h reaction.
[c]4 BIU of the enzyme was used.
[d]These lipases were prepared using recombinant DNA technology: the genetic coding for the lipase was transferred from the source organism to a host, *Aspergillus oryzae.*
[e]No information was found in the brochure obtained from the supplier.

yolk phosphatidylcholine (PC), used as the substrate, was purchased from Asahi Chemical Industry (Osaka, Japan). All other chemicals were of analytical grade and were obtained from commercial sources.

Hydrolysis reaction and analysis. A reaction mixture consisting of 5 mL isooctane, containing PC (50 mg, 65 μmol) and AOT (50 mg), 1 mL buffer (0.1 M borate-HCl with 1 mM $CaCl_2$, pH 7.5), and 250 U lipase, unless otherwise stated, was stirred magnetically in sealed screw-capped tubes at 30°C. Periodically, 100-μL aliquots were withdrawn and mixed with 100 μL $CHCl_3$/MeOH (2:1, vol/vol) and 100 μL H_2O. After centrifugation, the organic layer was applied onto a silica gel rod (SIII; Iatron Laboratories, Tokyo, Japan) and developed by $CHCl_3$/MeOH/H_2O (20:10:1, vol/vol/vol). The amounts of PC and lysophosphatidylcholine (LPC) in the hydrolysates were determined by an Iatroscan TH-10 TLC analyzer, equipped with an SIC chromatorecorder II (Iatron Laboratories) by using the relationship between the peak area ratio and the molar ratio, obtained in advance. The degree of PC hydrolysis was calculated according to Equation 1:

$$\text{degree of hydrolysis (\%)} = \text{LPC}/(\text{LPC} + \text{remaining PC}) \times 100 \quad [1]$$

Determination of positional specificity. To investigate the positional specificities of the lipases for PC, the LPC produced was hydrolyzed to monoglycerides (MG) with PLC as follows (1-MG and 2-MG are formed from 1-LPC and 2-LPC, respectively). After the hydrolysis reaction, 1 mL of the reaction mixture was added to 2 mL $CHCl_3$/MeOH (2:1, vol/vol) to stop the reaction, and the solvent was then removed by evaporation. The residue was dissolved into 1 mL diethyl ether, and dispersed in 200 μL of 20 mM Tris-HCl buffer (pH 7.5) that contained 10 mM $CaCl_2$, followed by incubation with 2.5 U PLC at 30°C until the LPC was completely hydrolyzed. The upper layer was then developed by $CHCl_3$/acetone (9:1, vol/vol) on an HBO_3-coated silica gel rod capable of separating MG isomers. The resulting MG isomers were qualitatively identified from their respective R_f values with the same analytical devices described in the *Hydrolysis reaction and analysis* section.

To confirm the positional specificity of one of the lipases, Pancreatin F, against triglycerides, we also carried out the hydrolysis reaction with olive oil, and identified the structural isomers of the hydrolyzed products (diglycerides and MG). The reaction procedure was the same as that described for the hydrolysis of PC, expect that olive oil was used in place of PC. After allowing the reaction to proceed for over 4 h, the organic layer was directly analyzed on an HBO_3-coated silica gel rod in a similar manner to that described above.

RESULTS AND DISCUSSION

The degrees of hydrolysis by the 19 lipases after 24- and 48-h reactions are given in Table 1, together with the names of the suppliers, source organisms, and information on the positional specificity of the lipases for triglycerides, obtained

from the respective suppliers. The positional specificities of these lipases for PC, obtained in this work, are also shown. The commercially available lipases tested could be classified into three groups. The first group (type 1), Lipozyme IM 20, Lipase M10, and Pancreatin F, had high hydrolysis activity for PC, hydrolyzing it completely within 24 h. Of the three type-1 lipases, that obtained from *M. javanicus* (Lipase M10) was the most active, the PC being completely hydrolyzed after only 1.5-h reaction. Lipozyme IM20 and Lipase M10 showed *sn*-1 positional specificity for PC, which is the same as that for triglycerides. Pancreatin F, however, attacked the *sn*-2 position of PC, which is different from its specificity for triglycerides. Pancreatin F is derived from hog pancreas, in which another lipolytic enzyme, phospholipase A_2 (PLA_2), capable of attacking the *sn*-2 position of phospholipids, is known to occur (6,16,22,25). It is thus thought that Pancreatin F may consist of a mixture of lipases and PLA_2. To verify this, Pancreatin F was employed in a hydrolysis reaction with olive oil. In this situation, the enzyme exhibited 1, 3-positional specificity for olive oil, indicating that it is obviously a mixture of the two enzymes, and that the PLA_2 in Pancreatin F dominantly hydrolyze PC because of their superiority over lipases in PC hydrolysis.

The second group (type 2), consisting of SP388, SP524, Lipozyme 10.000L, Lipase F, Newlase F and Lipase from *R. delemar*, showed moderate activity in PC hydrolysis; the degree of hydrolysis increased gradually with reaction time, but complete hydrolysis was not attained during 48-h reaction. To determine whether the hydrolysis reactions of these lipases ceased while incomplete or whether it was only that the reaction rates were slower than those of the type-1 lipases, similar hydrolysis reactions were performed with an increased amount of enzyme from 250 to 1000 U (for SP388, SP524, Lipozyme 10.000L, and Lipase F) or 2000 U (for Newlase F, and Lipase from *R. delemar*). As a result, all type-2 lipases completely hydrolyzed PC, suggesting that type-1 and type-2 lipases differ only in their reaction rates. The positional specificities of all type-2 lipases for PC were the same as those for triglycerides.

Lipases of the third group (type 3), which consisted of SP398, SP435, SP523, SP525, SP526, Paratase A, Paratase M, Lipase A6, Lipase AY30, and Lipase from *C. cylindracea*, showed little or no hydrolysis of PC, and almost no improvement was realized when the amount of enzyme added was increased in the same way as for the type-2 lipases.

The lipases belonging to type 1 are from *Mucor* species, while those of type 2 are from *Mucor* or *Rhizopus* sp. Lipases derived from *Aspergillus* and *Humicola* sp. (type 3) had little PC hydrolysis activity, in spite of being derived from fungi and/or showing 1,3-positional specificity for triglycerides like the lipases of type 1 and 2. It is also interesting that all positionally nonspecific lipases, derived from *Candida* sp. as tested in this study, were of type 3 and showed almost no PC hydrolysis activity. These results suggest that lipases from *Mucor* or *Rhizopus* sp. should preferably be selected for use in the enzymatic conversion of phospholipids.

Svensson *et al.* (17) compared the reactivities of commercial lipases toward phospholipids in a transesterification reaction, paying special attention to their source organisms, and found a tendency similar to that observed in the present study. However, several other researchers have reported appreciable activities for lipases from *Candida* sp. (11,12,14,21), *A. niger* (10) or *Humicola* sp. (12) in the hydrolysis or transesterification of phospholipids. The discrepancy between their results and ours is probably due to differences in the reaction conditions employed [i.e., hydrolysis or transesterification reaction with limited water (11,12,14)] and enzyme purity [for instance, *A. niger* seems to have the potential to produce PLA_1 or PLA_2 (10)], and/or modification of lipases by polyethylene glycol (21).

Although not all lipase preparations that are commercially available worldwide were examined in this work, we believe that the results presented here provide information that will be useful in selecting lipases suitable for use in the enzymatic conversion of phospholipids.

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REFERENCES

1. Bendich, A., L.J. Machlin, O. Scandurra, G.W. Burton, and D.D.M. Wayner, The Antioxidant Role of Vitamin C, *Adv. Free Radicals Biol. Med. 2*:419–444 (1986).
2. Hallman, M., and L. Gluck, Phosphatidylglycerol in Lung Surfactant III. Possible Modifier of Surfactant Fraction, *J. Lipid Res. 17*:257–262 (1976).
3. Hanada, E., and K. Suzuki, Activation of Human Brain Galactosylceramidase by Phosphatidylserine, *Biochim. Biophys. Acta 575*:410–420 (1979).
4. Nishizawa, Y., The Molecular Heterogeneity of Protein Kinase C and Its Implications for Cellular Regulation, *Nature 334*:661–665 (1988).
5. Rydhag, L., and I. Wilton, The Function of Phospholipids of Soybean Lecithin in Emulsions, *J. Am. Oil Chem. Soc. 58*:830–837 (1981).
6. Holmberg, K., and C. Eriksson, Lipase and Phospholipase Catalyzed Transformations in Microemulsions, *Indian J. Chem. 31B*:886–890 (1992).
7. Mason, J.T., A.V. Broccoli, and C. Huang, A Method for Synthesis of Isomerically Pure Saturated Mixed-Chain Phosphatidylcholines, *Anal. Biochem. 113*:96–101 (1981).
8. Aura, A.M., P. Forssell, A. Mustranta, T. Suortt, and K. Poutanen, Enzymatic Hydrolysis of Oat and Soya Lecithin: Effect on Functional Properties, *J. Am. Oil Chem. Soc. 71*:887–891 (1994).
9. Haas, M.J., D.J. Cichowicz, J. Phillips, and R. Moreau, The Hydrolysis of Phosphatidylcholine by an Immobilized Lipase: Optimization of Hydrolysis in Organic Solvents, *Ibid. 70*:111–117 (1993).
10. Mustranta, A., P. Forssell, A.M. Aura, T. Suortti, and K. Poutanen, Modification of Phospholipids with Lipases and Phospholipases, *Biocatalysis 9*:181–194 (1994).
11. Haas, M.J., D.J. Cichowicz, W. Jun, and K. Scott, The Enzymatic Hydrolysis of Triglyceride–Phospholipid Mixtures in an Organic Solvent, *J. Am. Oil Chem. Soc. 72*:519–525 (1995).

12. Haas, M.J., K. Scott, W. Jun, and G. Janssen, Enzymatic Phosphatidylcholine Hydrolysis in Organic Solvents: An Examination of Selected Commercially Available Lipases, *Ibid. 71*:483–490 (1994).
13. Yamaoka, M., J. Fortkamp, M. Morr, and R.D. Schmid, The Effect of 2,4,6,8-Tetramethyldecanoic Acid on the Hydrolysis and Acylation of Phospholipid by Lipase in Isooctane, *Ibid. 69*:826–828 (1992).
14. Mutua, L.N., and C.C. Akoh, Lipase-Catalyzed Modification of Phospholipids: Incorporation of n-3 Fatty Acids into Biosurfactants, *Ibid. 70*:125–128 (1993).
15. Pernas, P., J.L. Olivier, M.D. Legoy, and G. Bereziat, Phospholipid Synthesis by Extracellular Phospholipase A_2 in Organic Solvents, *Biochem. Biophys. Res. Com. 168*:644–650 (1990).
16. Hosokawa, M., K. Takahashi, Y. Kikuchi, and M. Hatano, Preparation of Therapeutic Phospholipids Through Porcine Pancreatic Phospholipase A_2-Mediated Esterification and Lipozyme-Mediated Acidolysis, *J. Am. Oil Chem. Soc. 72*:1287–1291 (1995).
17. Svensson, I., P. Adlercreutz, and B. Mattiasson, Interesterification of Phosphatidylcholine with Lipases in Organic Media, *Appl. Microbiol. Biotechnol. 33*:255–258 (1990).
18. Svensson, I., P. Adlercreutz, and B. Mattiasson, Lipase-Catalyzed Transesterification of Phosphatidylcholine at Controlled Water Activity, *J. Am. Oil Chem. Soc. 69*:986–991 (1992).
19. Yagi, T., T. Nakanishi, Y. Yoshizawa, and F. Fukui, The Enzymatic Acyl Exchange of Phospholipids with Lipases, *J. Ferment. Bioeng. 69*:23–25 (1990).
20. Totani, Y., and S. Hara, Preparation of Polyunsaturated Phospholipids by Lipase-Catalyzed Transesterification, *J. Am. Oil Chem. Soc. 68*:848–851 (1991).
21. Yoshimoto, T., M. Nakata, S. Yamaguchi, T. Funada, Y. Saito, and Y. Inada, Synthesis of Eicosapentaenoyl Phosphatidylcholines by Polyethylene Glycol-Modified Lipase in Benzene, *Biotechnol. Lett. 8*:771–776 (1986).
22. Aura, A.M., P. Forssell, A. Mustranta, and K. Poutaren, Transesterification of Soy Lecithin by Lipase and Phospholipase, *J. Am. Oil Chem. Soc. 72*:1375–1379 (1995).
23. Martinek K., N.L. Klyachko, A.V. Kabanov, Y.L. Khmelnitsky, and A.V. Levashov, Micellar Enzymology: Its Relation to Membranology, *Biochim. Biophys. Acta 981*:161–172 (1989).
24. Louwrier, A., G.J. Drtina, and A.M. Klibanov, On the Issue of Interfacial Activation of Lipase in Nonaqueous Media, *Biotechnol. Bioeng. 50*:1–5 (1996).
25. Blain, J.A., J.D.E. Pattterson, C.E. Shaw, and M.W. Akhtar, Study of Bound Phospholipase Activites of Fungal Mycelia Using an Organic Solvent System, *Lipids 11*:553–560 (1976).

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