KOR. JOUR. MICROBIOL June, 1992 p. 171~176 Vol. 30, No. 3

Lipolytic Properties of *Candida Cylindracea* Lipase Toward Triacylglycerols with Different Fatty Acyl Chains

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Lipolytic characteristics of *candia cylindracea* lipase was studied by various triacylglycerols with different fatty acyl chains as substrate. The substrate was emulsified with gum arabic and the rate of hydrolysis was determined by pH stat method. The effects of gum concentration, pH, temperature, and Ca^{2+} ion on the enzyme activities were examined. The results show that the effect of these factors are markedly depending on the structural nature of substrates. The triolein was the best substrate among tested. Present study demonstrates that for characterization of lipolytic enzymes, it is critically important to select proper substrate and activator.

KEY WORDS □ ***Candida cylindracea* Lipase, Hydrolysis of Triacylglycerol, Lipolytic Properties.**

Generally lipases (EC 3.1.1.3) catalyze the hydrolysis of ester bonds in triacylglycerols to yield free fatty acids, di- and monoacylglycerols, and glycerols (5). Some lipases from *Rhizopus arrhizus*, *Rhizopus delemar*, and *Asperillus niger* have a marked specificity for the external of α-position of triacylglycerols (2, 13). The lipase from yeast *Candida cylindracea* has no positional specificity, however it is known to be particularly active on long chain triacylglycerols (3) like the lipases from *Geotrichum candidum* and *Penycillium cyclopium*. The *Candida cylindracea* enzyme also catalyzes the hydrolysis of simple synthetic esters such as fatty acid methyl esters and p-nitrophenyl acetate (6). Additionally, this lipase has been shown to catalyze the synthesis of esters in non-aqueous media. Several biotechnological applications have been investigated in interesterification (1), ester synthesis (7), and stereo selective hydrolysis of esters (7, 10).

One of the characteristic features of lipolytic enzymes is their activation by interfaces (17). As the reaction of lipase proceeds in a heterogeneous system, its activity toward substrate depend on the physical properties of lipid-water interface. The optimum pH for the hydrolysis of olive oil catalyzed by *Candida cylindracea* lipase depended on the assay methods which were using polyvinylalcohol-emulsified system or a shaken system without emulsifier (15). Therefore it is desirable to define and select carefully the physical state of lipid substrate in order to have a clearcut result. In this report, lipolytic capability of *Candida cylindracea* lipase was studied with triacylglycerols emulsified with gum. The triacylglycerols empolyed here have different lengths of fatty acyl groups containing saturated or unsaturated hydrocarbon chains. The results revealed that some characteristics of the enzyme such as optimum temperature and pH depended largely on the structural nature of the substrates. For comparison, kinetic parameters of the different substrates were obtained under the defined assay conditions.

MATERIALS AND METHODS

Materials

Lipase from *Candida cylindracea*(Type VII, 700-1500 U/mg solid) was obtained from Sigma. This lipase was partially purified by ion exchange chromatography (DEAE-cellulose) as described by Brahimi-Horn(6). The major single peak was used as the enzyme source. Tributyrin(C 4:0), tristearin (C 18:0), triarachidin(C 20:0), triolein(C 18:1, [cis]-9), and trieicosenoin(C 20:1, [cis]-11) were also purchased from Sigma. Gum arabic with average molecular weight of 250 K dalton was purchased from Fluka. All other chemicals were reagent grade commercially available.

Preparation of Substrates

The substrate emulsion was prepared by ultrasonication of triacylglycerols for about 3 minutes in a 2 mM Tris-HCl buffer(pH 7.0) containing 2 mM $CaCl_2$, 150 mM NaCl and 0.1% gum arabic. In this reaction mixture, the molar ratio of gum arabic to substrate was 1:60 for tributyrin and 1:130 for tristearin, triarachidin, triolein, and trieicosenoin. Sonications with microtip (S&M VC 500 sonicator) were carried

out at room temperature for tributyrin and triolein, at 63°C for tristearin and triarachidine, and in ice bath for trieicosenoin. In the pH dependence experiment, 2 mM Tris-maleate buffer was used instead of 2 mM Tris-HCl buffer.

Kinetics of lipolysis

The lipase activity was determined by pH stat titration of liberated fatty acids with a autotitroprocessor(Metrohm, 670 Titroprocessor) (4). NaOH solution of 0.0144 M was used as titrant. Aproximately 2.5-5.0 m*l* of substrate emulsion was preincubated for 3 minutes at 37°C and in order to measure the rate of autohydrolysis, autotitration was carried out for another 3 minutes before addition of lipase. Then 0.1-0.4 mg of lipase was added and autotitration was started at the pH 7.0 for 3 minutes. The rate of hydrolysis was calculated from the slope of titration curve and the rate of autohydrolysis was subtracted. Activity is defined as μmols of fatty acids liberated per mg protein per minute.

RESULTS AND DISCUSSION

Effect of gum in the lipolytic activity

The *Candida cylindracea* lipase activity toward various triacylglycerols were examined under the assay condition of gum emulsification. The effect of gum on the lipolytic activities are summarized in Fig. 1. The lipase activities toward tristearin and triarachidin which contained saturated fatty acids were activated by gum and showed optimum gum concentraions, however the toward triolein and trieicosenoin which contained unsaturated fatty acids were not activated but rather inhibited by gum. The optimum molar ratios of gum to triacylglycerols that contain saturated fatty acyl moiety were 1:2600 for tributyrin(data not shown), 1:350 for tristearin and 1:150 for triarachidin. The ratio revealed that when the chain length of fatty acid increased, the molar ratio decreased accordingly. This means that longer fatty acyl group requires more amount of gum for the optimal activity. Two factors can be accounted for the effect of gum on the lipase action. One is the affinity of the enzyme for the emulsified substrate droplets and the other is the ability of the enzyme to hydrolyze the substrate lipid in emulsion particle (11). Therefore, in the case of triacylglycerols with saturated fatty acids, it could be postulated that the binding affinity of lipase to the interface is activated by gum or that triacylglycerol molecules in the emulsion with gum are favorably oriented to the lipase compared to those without gum. In the case of triacylglycerols with unsaturated fatty acids, the inhibitory effect of gum could be said opposite to what observed with saturated fatty acid. For routine assay, the amount of gum in the reaction mixture was maintained at 0.1% by weight. This

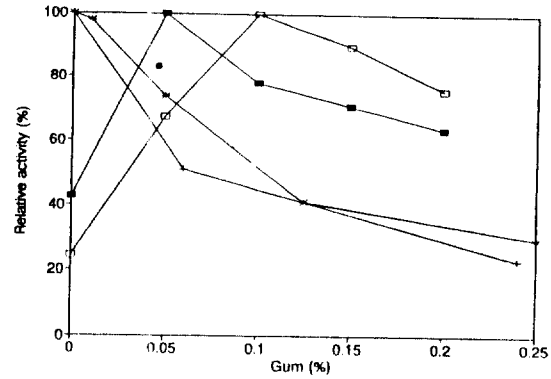


Fig. 1. *Effects of gum arabic on the activities of Candida cylindracea lipase toward tristearin(■), triarachidin(□), triolein(*), and trieicosenoin(+). Substrate concentrations in the hydrolysis of tristearin, triarachidin, triolein and trieicosenoin were 0.68, 0.6, 1.2 and 1.2 mM, respectively. Activities were expressed as relative activity (%) of highest value(0.15 μmol/mg/min for tristearin, 0.088 μmol/mg/min for triarachidin, 7.83 μmol/mg/min for triolein, and 0.56 μmol/mg/min for trieicosenoin) in each curve.*

was necessary to provide a good dispersed reaction mixture and to achieve a reproducible result, although most of substrates at this gum concentration exhibit inhibitory effect in the activities except the triarachidin which showed maximum activity at this condition. However the triarachidin showed the lowest observed activity among the substrates we examined. The enzyme properties derived from the gum emulsified system were expressed by relative activities, but the observed activities were included in the legend of each figure when necessary.

Effects of different fatty acyl groups

The pH profiles of the hydrolysis of triacylglycerols were examined (Fig. 2). The profiles of triacylglycerols with saturated fatty acids showed broad peak at basic pH and those of unsaturated fatty acids showed sharp peak at pH 7.5 for triolein and pH 7.7 for trieicosenoin. Therefore, it indicates that the effects of chain length on the pH pattern of the hydrolysis of triacylglycerols are different in detail, but the optimal pHs are similar to each other except the tristearin. The pH optimum of tristearin is shifted to more basic than the other substrates.

Fig. 3 shows the effect of temperature on the hydrolysis of triacylglycerols. The optimum temperatures or triacylglycerols which contain saturated fatty acyl moiety were higher compared to those which contain unsaturated fatty acyl moiety of same chain length. The optimum temperatures observed for tributyrin, tristearin,

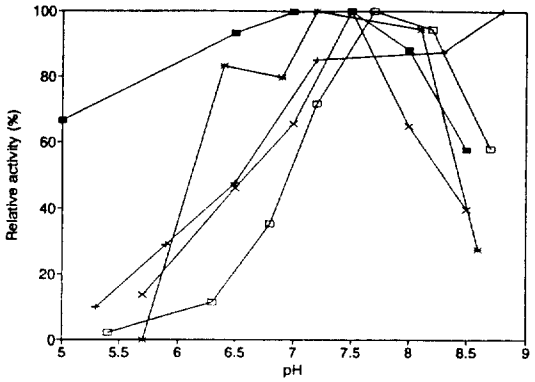


Fig. 2. *pH profiles of hydrolysis of triacylglycerols: tributyrin(■); tristearin(+); triarachidin(*); triolein(□); trieicosenoin(×). Activities were expressed as relative activity(%) of highest value (10.4 μmol/mg/min for tributyrin, 0.423 μmol/mg/min for tristearin, 0.097 μmol/mg/min for triarachidin, 4.85 μmol/mg/min for triolein, and 0.53 μmol/mg/min for trieicosenoin) in each curve.*

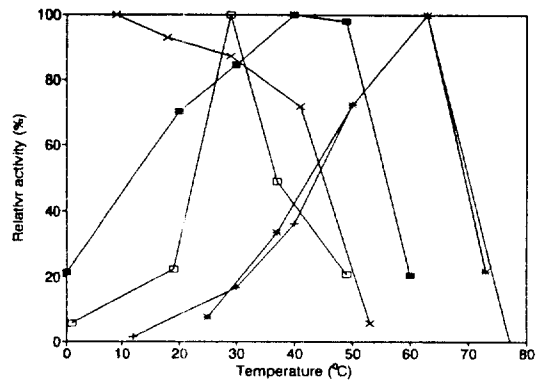


Fig. 3. *Effects of temperature on the hydrolysis of triacylglycerols: tributyrin(■); tristearin(+); triarachidin(*); triolein(□); trieicosenoin(×). Activities were expressed as relative activity of highest value(10.5 μmol/mg/min for tributyrin, 0.508 μmol/mg/min for tristearin, 0.21 μmol/mg/min for triarachidin, 6.90 μmol/mg/min for triolein, and 0.26 μmol/mg/min for trieicosenoin) in each curve*

and triarachidin were 40°C, 63°C and 63°C, respectively and the optimum temperature for triolein was 29°C. For the hydrolysis of trieiconsenoin, the lipase activity increased with decreasing temperature down to 6°C and below this temperature the lipase activity toward trieiconsenoin could not be measured. The optimum temperature of 63°C for tristearin and triarachidin was similar to the result of Sugiura and Isobe (14). They reported that the rates of *Chromobacterium viscosum* lipase catalyzed hydrolysis of triacylglycerols having saturated fatty acids increased with increasing reaction temperature until 60°C, especially for triacylglycerols of long-chain fatty acids. These findings can be explained as following points of view. In the case of triacylglycerols with saturated fatty acids, the substrate molecules in the emulsion with gum are highly packed for their long hydrophobic chains and then hardly accessible to the enzyme at relatively low temperature. Therefore it is required a relatively high temperature to become movable and accessible to the enzyme. In the case of triacylglycerols with unsaturated fatty acids, even at relatively low temperature, the substrate molecules in the emulsion could be movable and then accessible easily to the enzyme. In other words, the differences of the activities could be originated from the different physical states of lipid-water interface of emulsions because of the differences in the fatty acyl groups of triacylglycerols.

Due to the difference in unsaturation of fatty

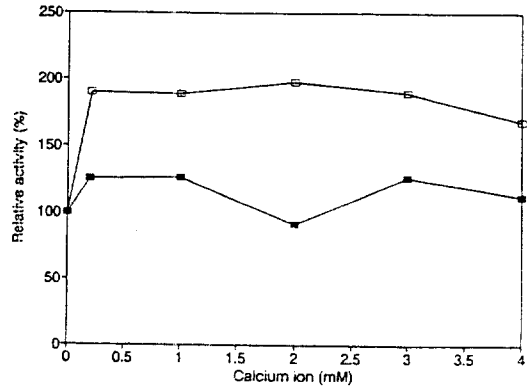


Fig. 4. *Effects of calcium ion on the lipase activities toward tristearin(■) and triolein(□). Activities were expressed as relative activity(%) of the activity of tristearin(0.211 μmol/mg/min) and triolein(1.66 μmol/mg/min) without calcium ion.*

acyl chains, the following two effects were also observed. One is the effect of calcium ion on the hydrolysis of triolein and tristearin (Fig. 4). The hydrolysis of triolein was activated 1.9 fold by addition of less than 1 mM calcium ion, whereas the hydrolysis of tristearin was not affected by calcium ion. The other one is the effects of NaCl on the hydrolysis of triolein, tristearin, and triarachidin (Fig. 5). The hydrolysis of triolein was not affected by NaCl but those of tristearin and triarachidin were inhibited upto 50% at 150 mM NaCl.

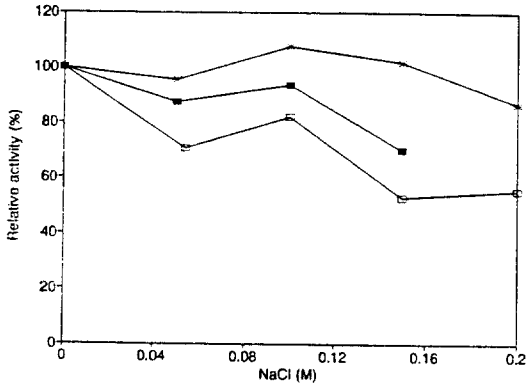
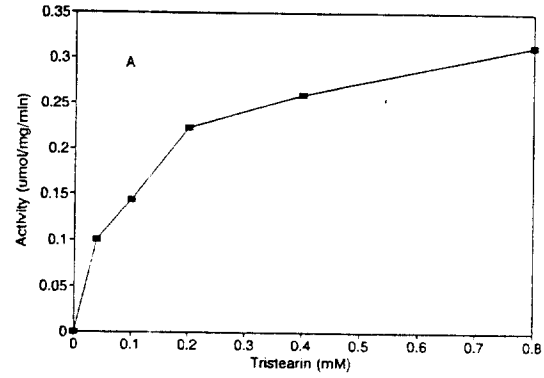


Fig. 5. *Effects of NaCl on the lipase activities toward tristearin(■), triarachidin(□) and triolein(*). Activities were expressed as relative activity(%) of tristearin(0.414 μmol/mg/min), triarachidin (0.270 μmol/mg/min), and triolein(3.72 μmol /mg/min) without any added NaCl.*



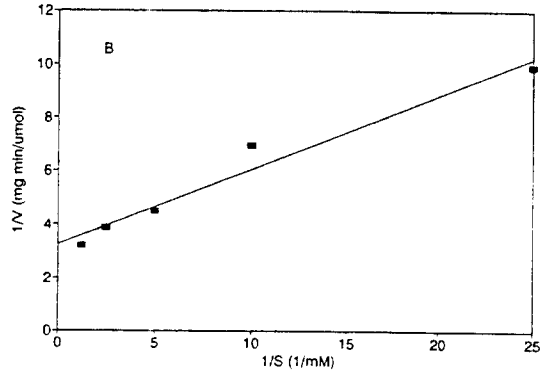


Fig. 6. *Candida cylindracea lipase activity toward tristearin. A: Substrate dependence. B: Lineweaver-Burk plot.*

Kinetic parameters

A Kinteic study of the *Candida cylimdracea* lipase toward the series of triacylglycerols were carried out in the presence of 0.1% gum at pH 7.0 and 37°C. Fig. 6A shows substrate dependence of the enzyme catalyzed hydrolysis of tristearin. It shows ideal Michaelis-Menten curve. Lineweaver-Burk plot of data of Fig. 6A was plotted in Fig. 6B. From its slope and intercept, K_m and V_{max} values were obtained as 1.3 mM and 13 μ mol/mg/min, respectively. Kinetic parameters of the other triacylglycerols were collected in Table 1.

The K_m values were decreased dramatically when the length of fatty acyl chain increased from C_4 to C_{18} or C_{20}, indicating better binding for the long chain triglycerides than the one with short chain. However the V_{max} value of tributyrine was much higher than that of the long chain substrates. This means that the hydrolysis product butyrate of triburtyrine is a good leaving group although the tributyrine bind poorly to the enzyme. In other word, the long chain triglycerides bind tightly to the enzyme but the end products, long fatty acids, are poor leaving groups. The V_{max} values also reveal another interesting point. That is the significant differences of V_{max} values when ccompared the saturated triglycerides to the unsaturated one with same length of fatty acyl chains. The hydrolysis rates of unsaturated substrates are several fold (3~10) faster than that of the saturated one. This observation can be explained again that the unsaturated fatty acids are better leaving groups than the saturated fatty acids. All of these findings can be summed up by the ratio of V_{max}/K_m. This ratio is considered a parameter for enzyme efficiency to a specific substrate. It turns out to be that the triolein shows the largest ratio. This implies the triolein is the best substrate for the enzyme among those tested. This substrate

Table 1. *Kinetic parameters of Candida cylindracea lipase catalyzed hydrolysis of triacylglycerols*

	Saturated Acyl Chain			Unsaturated Acyl Chain	
	Tributyrin ($C_{4:0}$)	Tristearin ($C_{18:0}$)	Triarachidin ($C_{20:0}$)	Triolein ($C_{18:1}$)	Trieicosenoin ($C_{20:1}$)
K_m(mM)	1.3	0.087	0.16	0.23	0.094
V_{max}(μ mol/min/mg)	13	0.31	0.14	3.0	0.47
V_{max}/K_m	10	3.6	0.88	13	5.0

specificity is comparable to the result that oleic chain was liberated before stearic acid when intact olive oil and cocoa butter were used as substrate (3). Here we ought to point out that the V_{max}/K_m values in Table 1 show rather lower limit values. Since the V_{max} of each substrate is not obtained in optimal condition owing to the defined uniform conditions employed for the enzyme kinetics. However the ratio would be expected to increase if the condition were reoptimized for each substrate, particularly for triolein and trieicosenoin because of the shifted optimal temperature and gum concentration. Nevertheless the order of the V_{max}/K_m determined in standard conditions would not be altered by the reoptimization.

Enzymatic activation of many lipolytic enzymes has been shown to affected by various surface active reagents (8, 12, 16). These phenomena suggest that the rates of enzymic hydrolysis are largely dependent on the physical state of the lipid substrates. The present results also show that the hydrolytic activity of *Candida cylindracea* lipase is affected by gum concentration as well as the structural nature of the substrates. Thereby the apparent characteristics of the lipase such as optima of temperature and pH may be different from each other depending on what kind of substrate we are employing. Therefore it is advisable to be take care of this subtle relationship between enzyme activity and substrate when characterizing a lipolytic enzyme.

ACKNOWLEDGEMENT

This research was supported by a grant (89-05-02-03) from Korean Science and Engineering Foundation. We express our appreciation to Prof. J. Shin for the use of autotitroprocessor.

REFERENCES

1. **Bello, M., D. Thomas and M.D. Legoy**, 1987. Interesterification and synthesis by *Candida cylindracea* lipase in microemulsions. *Biochem. Biophys. Res. Comm.*, **146**, 361-367.
2. **Benzonana, G.**, 1974. Some properties of an exocellular lipase from *Phizopus arrhizus*. *Lipids.*, **9**, 166-172.
3. **Benzonana, G. and S. Esposito**, 1971. On the positional and chain specificities of *Candida cylindracea* lipase. *Biochim. Biophys. Acta.*, **231**, 15-22.
4. **Borgstrom, B.**, 1975. On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts. *J. Lipid Res.*, **16**, 411-417.
5. **Borgstrom, B. and H.L. Brockman**, 1984. Lipases, pp. 527, Elsevier, Amsterdam.
6. **Brahimi-Horn, M.-C., M.L. Guglilmino, L. Elling and L.G. Sparrow**, 1990. The esterase profile of a lipase from *Candida cylindracea*. *Biochim. Biophys. Acta.* **1042**, 51-54.
7. **Cambou, B. and A.M. Klibanov**, 1984. Lipase-catalyzed production of optically active acids *via* asymmetric hydrolysis of esters. *Apply. Biochem. Biotechnol.*, **9**. 255-260.
8. **Choi, M.**, 1983. Kinetic behavior of solubilized microsomal cholesterol ester hydrolase of rate brain. *Korean Biochem. J.*, **16**, 280-287.
9. **Cillies, B., H. Yamazaki and D.W. Armstrong**, 1987. in Biocatalysis in organicc media (Laane, C., J. Tramper andd M.D. Lilly, Eds) pp. 227-231, Elsvier, Amsterdam.
10. **Dahod, S.K. and P. Siuta-Mangano**, 1987. Carbon tetrachloride-promoted stereo selective hydrolysis of methyl-2-chloropropionate by lipase. *Biotech. Bioeng.*, **30**, 995-999.
11. **Deckelbaum, R.J., J.A. Hamilton, A. Moscor, C.B. Olivecrona, E. Butbul, Y.A. Carpentier, A. Gutman and T. Olivecrona**, 1990. Medium-chain versus long-chain triacylglycerol emulsion hydrolysis by lipoprotein lipase and hepatic lipase: Implications for the mechanisms of lipase action. *Biochemistry*, **29**, 1136-1142.
12. **Jung, K., E. Koh and M. Choi**, 1989. Catalytic properties of phospholipase D using phosphatidic acid as an activator. *Bull. Korean Chem. Soc.*, **10**, 595-600.
13. **Okumura, S., M. Iwai and Y. Tsujisak**, 1976. Positional specificities of four kinds of microbial lipases. *Agr. Biol. Chem.*, **40**, 655-660.
14. **Sugiura, M. and M. Isobe**, 1975. Studies on the lipase of *Chromobacterium viscosum*. IV. Substrate specificity of a low molecular weight lipase. *Chem. Pharm Bull.*, **23**, 1226-1230.
15. **Tomizua, N., Y. Ota and K. Yamada**, 1966. Studies on lipase from *Candida cylindracea* Part II. Amino acid composition, carbohydrate component and some physical properties. *Agr. Biol. Chem.*, **30**, 1090-1096.
16. **Verger, R.**, 1980. Enzyme kinetics of lipolysis. *Methods Enzymol.*, **64**, 341-392.
17. **Verger, R. and G.H. De Haas**, 1976. Interfacial enzyme kinetics of lipolysis. *Ann. Rev. Biophys. Bioeng.*, **5**, 77-117.

(Received March 9, 1992)
(Accepted April 21, 1992)

초 록: 여러가지 지방 아실기를 갖는 트리아실그리세롤에 대한 *Candida cylindracea* 리파제의 지방분해 성질

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Candida cylindracea 리파제의 지방질 분해 성질을 여러가지 아실 지방산을 갖는 각종 트리아실그리세롤을 기질로 하여 연구하였다. 이들 기질은 아라빅 감으로 균질화 시켰으며, 반응속도는 pH stat 적정법을 이용하였다. 이 효소의 겉보기 활동도를 감의 농도, pH, 온도, 및 Ca^{2+} 이온영향 등에 대해 조사하였다. 결과는 지방산 구조에 따라 이들 요인들이 크게 영향을 받고 있음이 드러났다. 검토된 기질중 triolein이 가장 좋은 기질로 판단되었다. 또 이들 결과는 지방질 가수분해 효소특성 연구에는 적절한 기질과 활성화 인자 선택이 매우 중요함을 말해주고 있다.