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THE IMMOBILIZATION OF *CANDIDA CYLINDRACEA* LIPASE ON PVC, CHITIN AND AGAROSE[1,2]

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Abstract

Candida cylindracea lipase was covalently coupled to PVC, chitin and agarose, which are abundant in Taiwan, by several different methods. The agarose-dodecylene-diamine-glutaraldehyde (A-DDA-GA) system showed the highest relative loading enzyme activity, 118 mg soluble lipase per gram support. The chitosan-carbodiimide glutaraldehyde (CN-EDC-GA) system immobilized 67 mg soluble lipase per gram support. The optimal pH of immobilized lipase was 8.5, which was one pH unit higher than that of soluble lipase. The opitmal temperatures were in the range between 52-64°C. The CN-EDC-GA system was the highest (64°C), which was 27°C higher than soluble lipase. The CN-EDC-GA system also had the best thermal stability (the half life at 55°C was 29 h) and operational stability at higher temperature (the half life at 40°C was 495 h). However, the PVC-ethylenediamine-GA system appeared to have the best stability at lower temperature, the projected half life at 20°C from Arrhenius plot was 31,802 h.

Key words: *Candida cylindracea* lipase; PVC; chitin; agarose; immobilization.

Introduction

Lipase from *Candida cylindracea* is a nonspecific lipolytic enzyme (Benzonana and Esposito, 1971) which is suitable for the production of fatty acid (Linfield *et al*, 1984; Posorske, 1984) and triglyceride analysis (Schifreem *et al*, 1979; Hercules and Sheehan, 1978). For industrial and clinical analysis with enzymes, the immobilization of enzyme to solid support offered several advantages including repeated

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use of enzyme, improved stability, use in continuous operation and automatic control *etc.* One of the major concern in the choice of enzyme support is the availability and cost. Polyvinyl chloride (PVC), agarose and chitin are the three abundant materials produced in Taiwan which are suitable supports for enzyme immobilization. In the present work, we use various methods for immobilizing the lipase from *C. cylindracea* to these supports and study some kinetic properties of these immobilized enzymes.

Materials and Methods

Chemicals

Lipase (from *C. cylindracea*, 1,400-2,800 units/mg), chitin, chitosan, 12-aminododecyl-agarose, gum arabic, ethylenediamine, hexamethylenediamine, 1,12-dodecanediamine, glutaraldehyde, [1-ethyl-3-(3-dimethylaminopropyl)] carbodiimide (EDC), *p*-toluene sulfonyl chloride (tosyl chloride), 6-aminohexanoic acid, olive oil and tributyrin were obtained from Sigma Chemical Co. PVC (food grade, monomer <4 ppm) was obtained from Taiwan Plastic Co. Sodium methoxide, titrisol (0.1 N NaOH) and methanol were purchased from E. Merck Co.

Immobilization of Lipase on PVC

Lipase was immobilized on PVC powder (approx. 200 mesh) as described in the previous reports (Cheng and Shaw, 1980; Cheng and Shaw, 1981; Shaw and Cheng, 1983). PVC powder (4 g), 20 ml of methanol, 5 g of sodium methoxide and 20 g hexamethylenediamine (or 12 ml of ethylenediamine or 30 g of 1,12-dodecanediamine) was placed in a round bottom flask subsequently. The reaction mixture was refluxed in a water bath at 80°C for 6 hours. Then it was filtered after cooling and washed with distilled water several times. The alkylamino derivatives of PVC (0.8 g) were suspended in 20 ml of 0.1 M potassium phosphate buffer (pH 8.0). Glutaraldehyde (0.94 g) were then added at 4°C and stirred for 12 hours. Then the activated PVC derivatives were filtered and washed with distilled water.

The activated PVC derivatives were suspended in 30 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 72 mg of lipase were added with stirring at 4°C. After 16 h of reaction, the immobilized lipases were washed with the same phosphate buffer until no enzyme was detected in the filtrate. The immobilized enzymes were stored at 4°C until use.

Immobilization of Lipase on Chitin

Tosyl chloride (2.4 g) was dissolved in 12 ml of acetone. Then 0.8 g of dry chitin (approximately 40 mesh) and 4 ml of pyridine were added successively. The mixture was stirred at room temperature for one hour and then filtered. The following

solutions (100 ml each) were used to wash derivatives in order: 70:30; 50:50; and 30:70 of acetone: water (v/v); and 1 mM HCl. 6-Amino-hexanoic acid (1.08 g) was dissolved in 30 ml of 0.1 M sodium bicarbonate buffer (pH 10.7) and added to 1.5 g of tosylated chitin. The reaction was continued for 12 hours at 4°C with continuous stirring and then filtered. It was washed several times with distilled water.

Lipase (0.75 g in 4 ml of 0.1 M potassium phosphate buffer, pH 8.2) was coupled to this derivative by adding 0.08 g of EDC. The coupling proceeded with stirring for 2 hours at 4°C. The immobilized lipase was washed several times with the same phosphate buffer until no enzyme activity was detected in the filtrate.

Immobilization of Lipase on Agarose

Wet 12-aminododecyl agarose (1.5 g) was washed with distilled water several times, then suspended in 20 ml of 0.1 M potassium phosphate buffer (pH 8.2), and then used for coupling lipase by glutaraldehyde crosslinking as described for alkylamino derivative of PVC.

Immobilization of Lipase on Chitosan by Multiple Attachment

Chitosan (1.5 g) was dissolved in 500 ml of 0.1 N acetic acid, followed by addition of 15 g of NaCl. The pH was adjusted to 6 with NaOH. The solution was centifuged to removed the precipitate. To the supernatant, 0.75 g of lipase in 4 ml water was added. After adding 0.08 g of EDC, the coupling reaction proceeded for 2 hours at 4°C with stirring. Then 0.6 ml of 50% glutaraldehyde (adjusted to pH 6.0 by NaOH) was added. After stirring for 30 min, 26 ml of 1 M phosphate buffer (pH 8.0) was added to precipitate the immobilized lipase. It was washed with the same buffer, dried and ground to 20-40 mesh.

Enzyme Assay

The lipase activity was assayed by autotitration pH stat according to Worthington Enzyme Manual (1977). The reaction mixture contained 2 ml of 0.5% albumin, 5 ml of substrate emulsion (prepared by homogenizing 8.25 g gum arabic, 90 ml distilled water and 10 ml olive oil or 2.2 ml tributyrin), 5 ml of glass distilled water, 2 ml of 3 M NaCl, 1 ml of 0.075 M $CaCl_2$ and 1 ml of lipase. The release of fatty acid was titrated with 0.05 N NaOH at pH 8.0 and 37°C with Radiometer TTT 80 autotitration apparatus. Blank rate was determined by omitting the enzyme in the reaction mixture.

In the case of measuring operational stability, the activity of lipase was measured according to Linfield *et al* (1984). 3.2 ml of olive oil was mixed with 3.5 ml of 0.1 M potassium phosphate buffer (pH 5.4) and proper amount of immobilized lipase. After shaking at 40°C and 250 rpm for 12 hours, the enzyme was removed by filtration and the filtrate was titrated at pH 10 to determine the total fatty acid

generated. New batch of reaction mixture was added to the filtered lipase to start another reaction cycle. From the fatty acid released per 12 hours in each reaction cycle, we can calculate the residual enzyme activity as a function of time and thus the denaturation rate. Under the operation condition, there was no significant change in pH.

Results

The Effect of Immobilization Methods on the Activity of Lipase

As shown in Table 1, the relative loading enzyme activities of lipase were affected by different immobilization methods. The spacer chain length appeared to have effect on immobilized enzyme activity. The PVC-HMDA-GA system had the highest loading activity, with PVC as support. Since agarose had much higher enzyme coupling capacity (191 mg protein per gram dry weight of agarose), the A-DDA-GA system had the highest relative loading enzyme activity, 118 mg soluble enzyme equivalent of olive oil hydrolysis activity per gram of support.

Table 1. *Effect of immobilization methods on the lipase activity*

The enzymes were assayed at 37°C, pH 8.0, with tributyrin (4.08%, v/v) and olive oil (18.5%) as substrates.

Immobilization system	Water content (%)	Enzyme coupled (mg/g, D. W.[b]	Relative loading enzyme activity (mg sol. enz./g support)	
			Olive oil	Tributyrin
PVC-EDA-GA[a]	70.1	15.2	9.5	10.3
PVC-HMDA-GA	72.5	22.8	18.1	19.4
PVC-DDA-GA	69.7	16.2	11.8	12.6
C-TS-AHA-EDC	78.9	92.0	68.1	83.3
CN-EDC-GA	20.1	92.5	66.8	83.9
A-DDA-GA	94.0	190.8	117.7	131.3

[a] Abbreviations: EDA: ethylenediamine; GA: glutaraldehyde; A: agarose; DDA: dodecylenediamine; C: chitin; TS: toluenesulfonyl chloride; AHA: 6-aminohexanoic acid; EDC: 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide; CN: chitosan; PVC: polyvinyl chloride.

[b] D. W.: dry weight.

Some Factors Influencing the Enzyme Immobilization

The activity of immobilized lipase was affected by the conditions of immobilization process. It was found that the lipase activity of immobilized enzyme increased when the enzyme was immobilized in the presence of NaCl or $CaCl_2$. As shown in Table 2, the immobilized enzyme showed 9% and 33% increase in relative loading

enzyme activity for PVC-HMDA-GA and C-TS-AHA-EDC systems respectively when the lipase was in the presence of 0.3 M NaCl during immobilization. The increase of relative activity suggested that NaCl and $CaCl_2$ had stabilizing effect on lipase similar to pancreatic lipase (Schandle and Pittner, 1984).

Table 2. *Effects of salts on the immobilization of lipase*

Immobilization system	Salt	Conc. (M)	Enzyme coupled (mg/g)	Relative[a] activity (%)	Relative loading enzyme activity (mg sol. enz./g support)
PVC-HMDA-GA[b]	NaCl	0.3	9.7	12.5	1.2
		0.1	7.9	13.0	1.0
	$CaCl_2$	0.03	8.2	12.2	1.0
		0.03	6.6	12.0	0.8
		0.003	6.5	11.9	0.8
	none		8.3	10.7	0.9
C-TS-AHA-EDC	NaCl	0.3	19.3	33.9	6.5
		0.1	19.6	33.0	6.5
	$CaCl_2$	0.03	19.2	31.4	6.1
		0.03	20.2	33.4	6.7
		0.003	20.9	30.4	6.4
	none		19.2	31.0	6.0

[a] The enzymes were assayed at 37°C, pH 8.0 with tributyrin as substrate.
[b] Abbreviations are the same as in Table 1.

The concentrations of reagents used also affected the final enzyme activity. As shown in Fig. 1 and Fig. 2, the optimal concentrations of EDC and glutaraldehyde were 0.025% and 0.02% respectively for the immobilization of lipase on chitosan by multiple attachment method.

Optimal Temperature and pH

As shown in Fig. 3 and Fig. 4, the optimal pH of all immobilized enzyme except A-DDA-GA was 1.0 pH unit higher (pH 8.5) than soluble lipase (pH 7.5) with tributyrin as substrate. This deviation was possibly due to the steric hindrance of the support which obstructed the diffusion of proton to the immediate vicinity of immobilized enzyme.

As shown in Fig. 5 and Fig. 6, the optimal temperature of lipase increased as a result of immobilization. The CN-EDG-GA lipase had the highest optimal temperature (64°C), which was 27°C higher than soluble lipase (37°C). The chain length between the support and lipase seemed to have no effect on optimal temperature since the PVC-EDC-GA, PVC-HMDA-GA and PVC-DDA-GA lipase had the same

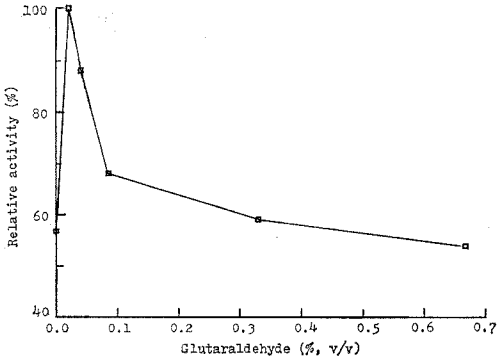

Fig. 1. Effect of amount of glutaraldehyde added on relative loading enzyme activity of CN-EDC-GA lipase.

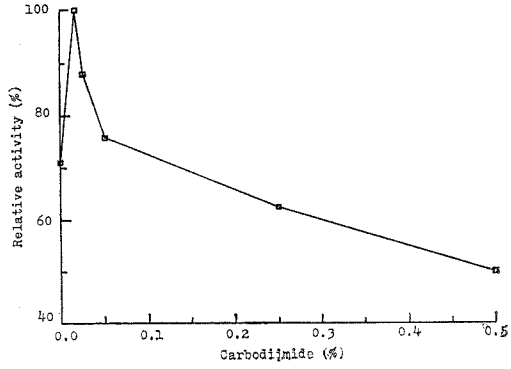

Fig. 2. Effect of amount of carbodiimide added on relative loading enzyme activity of CN-EDC-GA lipase.

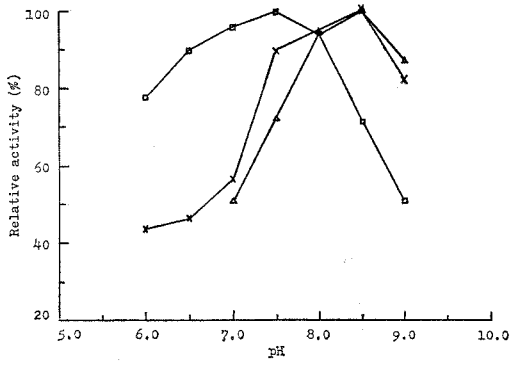

Fig. 3. pH behavior for various lipase. The enzymes were assayed at 37°C with tributyrin as substrate.
△-△: CN-EDC-GA;
×-×: PVC-EDA-GA;
□-□: soluble.

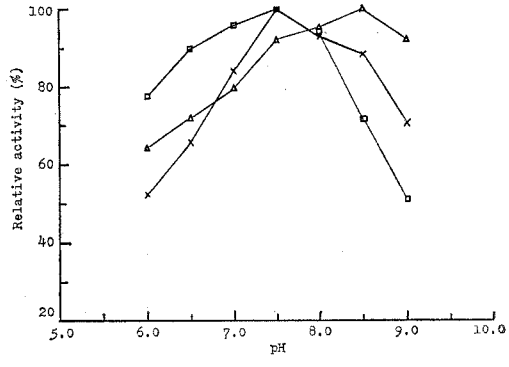

Fig. 4. pH behavior for various lipase. The enzymes were assayed at 37°C with tributyrin as substrate.
△-△: C-TS-AHA-EDC;
×-×: A-DDA-GA;
□-□: soluble.

optimal temperature (50°C). However, immobilized lipase of longer chain length (PVC-DDA-GA) appeared to be more sensitive to temperature change.

Thermostability and Operational Stability

The thermal denaturation of immobilized lipase followed first order reaction. From the equation $\mathrm{Ln}\ E_t/E_0 = -k \cdot t$, where E_0 and E_t represent the initial enzyme activity and residual activity at time t respectively, the apparent denaturation rate constant k could be obtained from the slope of $\mathrm{Ln}\ E_t/E_0$ vs. t plot. It was found that the denaturation rate decreased greatly as a result of immobilization. At 50°C, the denaturation rate constant of soluble lipase was 0.49 h^{-1}. From the data in

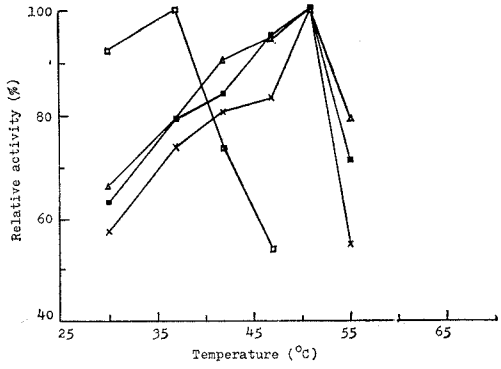

Fig. 5. Temperature behavior for various lipase. The enzymes were assayed at pH 8.0 with tributyrin as substrate.
△-△: PVC-EDC-GA;
■-■: PVC-HMDA-GA;
×-×: PVC-DDA-GA;
□-□: soluble.

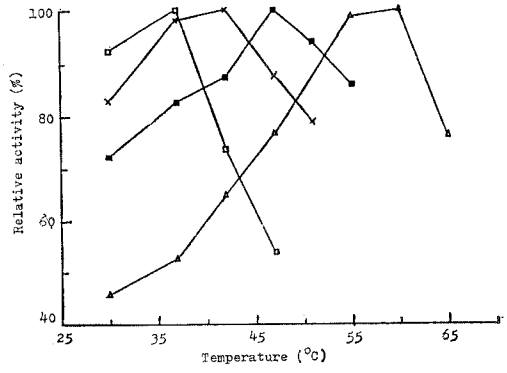

Fig. 6. Temperature behavior for various lipase. The enzymes were assayed at pH 8.0 with tributyrin as substrate.
△-△: CN-EDC-GA;
■-■: C-TS-AHA-EDC;
×-×: A-DDA-GA;
□-□: soluble.

Table 3, the thermostability of the immobilized enzyme increased 45, 33, 29, 15, 35, and 15 times over soluble lipase for PVC-EDA-GA, PVC-HMDA-GA, PVC-DDA-GA, C-TS-AHA-EDC, CN-EDC-GA, A-DDA-GA, respectively.

Since the denaturation rate constants at various temperature fitted Arrhenius equation Ln k=A−E_a/R×1/T, where A, E_a, R and T represent constant, activation energy, gas constant and absolute temperature respectively, the denaturation half lives of the immobilized enzymes could be predicted at other temperature. As shown in Table 3, the PVC-EDC-GA had the highest stability at 20°C with projected half life of 31,802 h and the CN-EDC-GA lipase had the best stability at 60°C with projected half life of 15 h. When the immobilized lipase was batch operated with 3.5 ml of 0.1 M potassium phosphate buffer (pH 5.4) and 3.2 ml of olive oil at 40°C in rotary shaker (250 rpm) and changed substrate at 12 h/cycle, the CN-EDC-GA system showed the best stability with denaturation half life of 41.7 cycle, which corresponded to 500 h or 21 days.

Discussion

PVC, agarose and chitin are the three abundant materials in Taiwan which are suitable for enzyme immobilization. Comparing relative loading activity per gram support under 37°C and pH 8.0, the agarose appeared to be the best, followed by chitin and PVC (Table 1). However, the optimal temperature, optimal pH, thermostability and operational stability of immobilized enzyme depended on the immobilization methods and reaction conditions. It is more practical to choose the proper

Table 3. *Denaturation of immobilized lipase at various temperature*

The enzymes were assayed at 37°C, pH 8.0 with tributyrin as substrate.

System[a]	Operation temp. (°C)	Denaturation rate constant k (hour^{-1})	Fitted arrhenius equation $Y=b+mX$	E_a (K cal/mole)	Projected denaturation half life (hours)	
					20°C	60°C
PVC-EDA-GA	45	5.2×10^{-3}	$Y=20.08-7,113.7X$	32.5	31,801.6	13.4
	50	1.1×10^{-2}				
	55	2.5×10^{-2}				
PVC-HMDA-GA	45	5.9×10^{-3}	$Y=19.81-7,000.3X$	32.0	8,434.2	11.4
	50	1.5×10^{-2}				
	55	2.7×10^{-2}				
PVC-DDA-GA	45	6.4×10^{-3}	$Y=20.24-7,128.1X$	32.5	8,418.4	10.1
	50	1.7×10^{-2}				
	55	3.1×10^{-2}				
C-TS-AHA-EDC	40	5.8×10^{-3}	$Y=21.74-7,504.6X$	34.3	5,114.9	1.5
	45	1.4×10^{-2}				
	50	3.2×10^{-2}				
CN-EDC-GA	45	6.3×10^{-3}	$Y=16.61-5,977.4X$	27.3	4,239.6	15.0
	50	1.4×10^{-2}				
	55	2.4×10^{-2}				
A-DDA-GA	40	6.3×10^{-3}	$Y=21.02-7,257.4X$	33.1	3,932.5	4.2
	45	1.8×10^{-2}				
	50	3.3×10^{-2}				

[a] Abbreviations are the same as in Table 1.

Table 4. *Operation stabilities of immobilized lipase at 40°C*

The enzymes were assayed at 40°C, pH 5.4 under high olive oil condition.

System[a]	Denaturation rate constant k (h^{-1})	Denaturation half life (cycle/12 h)
PVC-EDA-GA	2.1×10^{-3}	27.2
PVC-HMDA-GA	2.9×10^{-3}	19.9
PVC-DDA-GA	4.2×10^{-3}	13.9
C-TS-AHA-EDC	3.8×10^{-3}	15.3
CN-EDC-GA	1.4×10^{-3}	41.7
A-DDA-GA	8.0×10^{-3}	7.3

[a] Abbreviations are the same as in Table 1.

immobilization system by the performance of the immobilized enzyme at the intended operation condition. For example, the multiple attachment of lipase on chitosan greatly enchanced the stability of enzyme against thermal denaturation. Therefore, chitin is the most economical support at high temperature. Chitin can

be produced from the agarowaste crab shell. It was estimated that the annual production of crab shell in Taiwan was approximately 500 tons, which corresponded to 10-100 tons of chitin (Chang, 1982). Chitosan can be produced from chitin by alkaline deacetylation (Knorr, 1984). Most of the previous utilization of chitin as enzyme immobilization support was simply coupling the enzyme to chitosan by glutaraldehyde crosslinking (Riccardo and Muzzarelli, 1980). In the present work, we coupled the enzyme either to the hydroxyl group of chitin (C-TS-AHA-EDC) or to the solubilized chitosan followed by glutaraldehyde crosslinking (CN-EDC-GA). Both methods should increase the amount of enzyme coupled. In the latter method, since enzyme was fixed to the chitosan by multiple point attachment in a complementary fashion, the enzyme conformation was stabilized against thermal denaturation which caused conformational unfolding (Klibanov, 1983). This method appears to have good potential in industrial application.

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以幾丁質、聚氯乙烯及瓊脂醣固定化 *Candida cylindracea* 脂肪酶

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Candida cylindracea 脂肪酶被共價鍵結於三種臺灣盛產之不溶性擔體，即幾丁質、聚氯乙烯及瓊脂醣。在數種不同結合方法中，以 Agarose-dodecylenediamine-glutaraldehyde (A-DDA-GA) 系統具有最高之相對結合酵素活性，每克擔體結合相當 118 mg 溶性脂肪酶活性。Chitosan-carbodiimide-glutaraldehyde (CN-EDC-GA) 系統次之，每克結合相當於 67 mg 溶性脂肪酶活性。固定化脂肪酶之最適反應 pH 在 8.5，比溶性脂肪酶高出 1 個 pH 單位。其最適反應溫度在 52-64°C 範圍，其中以 CN-EDC-GA 系統爲最高 (64°C)，比溶性酵素高出27°C。CN-EDC-GA 系統也具有最高之耐熱性 (其在55°C 之變性半衰期爲29小時) 及操作穩定性 (在 40°C 下 batch-type 操作之半衰期爲 495 小時)。但在較低溫下，經由 Arrhenius 作圖法預測在 20°C 下之脂肪酶變性半衰期則以 PVC-ethylenediamine-GA 系統爲最高 (31,802 小時)。