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Enantioselective esterification of (*RS*)-2-(4-chlorophenoxy)-propionic acid via *Carica papaya* lipase in organic solvents

Yu-Chi Cheng and Shau-Wei Tsai*

Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan

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Abstract—*Carica papaya* lipase (CPL) stored in the crude papain is found to be enantioselective for the kinetic resolution of (*RS*)-2-(4-chlorophenoxy)propionic acid via esterification in anhydrous organic solvents. Of the alcohols screened, trimethylsilylmethanol acted as the best acyl acceptor and gave the highest enzyme activity and enantioselectivity in anhydrous cyclohexane. The kinetic analysis at temperatures between 20 and 60 °C indicated that CPL is thermally stable, giving a high *E* value of 113 at 20 °C. A change of cyclohexane to other hydrophobic solvents resulted in better lipase performances. In comparison with the performances of other crude *Candida rugosa* lipases, *Carica papaya* lipase is more active, enantioselective and thermally stable.

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) have been employed as versatile biocatalysts for the kinetic resolution of a variety of racemic drugs, agrochemicals or their intermediates via hydrolysis or synthesis.[1,2] Most commercial lipases are produced from animals or microorganisms.[3–6] Although plant lipases seem to be very attractive owing to their low cost, ease of purification and being widely available from the natural sources, the low levels of the lipase content in the post-germination seed, bran part of the grain or wheat germ have limited their extensive use in pilot or large scale applications.[7,8] Recently, this drawback has been overcome as lipases from Caricaceae and Euphorbiaceae latex are available in large quantities and can be employed as promising catalysts for lipid bioconversions.[8–10] The spray-dried *Carica papaya* latex, with the commercial name papain, contains many cysteine thiol-proteases such as papain (EC 3.4.22.2) and chymopapain and others such as lysozyme and lipase.[11] The lipase activity, which is stored in the non-water-soluble fraction of the latex, suggests that the lipase is naturally bound and immobilized to the non-soluble matrix.

In contrast to the high enantioselectivity towards alcohols and amines, most lipases show low to moderate enantioselectivity towards carboxylic acids. Nevertheless, this is not the case for the *Candida rugosa* lipase (CRL), which possesses high enantioselectivity for α-substituted propionic acids such as 2-arylpropionic acids and 2-aryloxypropionic acids, although purification or modification of the isoenzymes from the crude preparation is generally imperative.[12,13] Therefore, a continuous study on screening or selecting novel lipases having high enantioselectivity, activity and stability for chiral acids by using conventional or molecular procedures is an absolute prerequisite for developing competitive biotransformation processes.[14,15]

2-Phenoxypropionic acids and their esters, with the (*R*)-enantiomers being biologically active, are widely used as herbicides. Moreover, (*R*)-2-(4-chlorophenoxy)propionic acid can lower the level of serum cholesterol and prevents platelet aggregation; however, the (*S*)-antipode can cause a side effect, which inhibits the chloride channel in muscles.[16] A literature survey has indicated that low to moderate enantioselectivity can be obtained when CRL is used as the biocatalyst for the esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid in nonpolar solvents.[17,18] Improvements in lipase activity and enantioselectivity were also reported if the lipase after purification or modification with 2-propanol, carbon tetrachloride as solvent or organosilicon alcohols as acyl acceptors is employed.[12,15,19,20]

This report is aimed at exploring *Carica papain* lipase (CPL) as a potential enantioselective biocatalyst for the

* Corresponding author. Tel.: +886-6-2757575x62647; fax: +886-6-2344496; e-mail: t62647@mail.ncku.edu.tw

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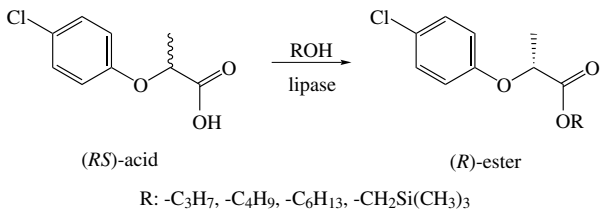
kinetic resolution of α-substituted carboxylic acids. Esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid in organic solvents is employed as the model system (Scheme 1) for studying the effects of alcohol, temperature and organic solvents on the lipase performance. Results of the enzyme activity and enantioselectivity are also compared with those for CRL.



Scheme 1.

2. Results and discussion

2.1. Effects of alcohol

With an alcohol as the acyl acceptor and CPL as the biocatalyst in anhydrous cyclohexane at 45 °C, the time-course conversions of (*R*)-acid (X_R) and (*S*)-acid (X_S) were obtained (data not shown), from which the initial rates for both substrates (V_R and V_S), enantiomeric ratio (*E* value) and the time-course enantiomeric excesses for (*R*)-ester (ee_P, defined as $[(X_R)-(X_S)]/[(X_R)+(X_S)]$) could be estimated. Some results are represented in Table 1, indicating that CPL is similar to CRL, which possesses an (*R*)-stereospecificity in hydrophobic solvents.[18] An increase of the chain length of the primary aliphatic alcohol from C3 to C6 resulted in an enhancement of the initial rate for each substrate and led to a decrease of the *E* value from 43 to 11.[21] On the contrary, when CRL was the biocatalyst in water-saturated benzene at 30 °C, the initial rates V_R and V_S decreased with an increase of alcohol chain length, yet the *E* value increased from 1.8, 3.5 to 7 for *n*-propanol, *n*-butanol and *n*-hexanol, respectively.[17] Improvements of the *E* value of 50 for purified CRL in *n*-heptane at 30 °C and that of 73 for crude CRL in carbon tetrachloride at 50 °C were also obtained.[16,20] However, CPL has potential as an enantioselective biocatalyst as the purified CRL is expensive and carbon tetrachloride is not environmental friendly.

Table 1. Effects of alcohol on CPL performances for the esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid at 45 °C

Alcohol	*n*-Propanol	*n*-Butanol	*n*-Hexanol	Trimethylsilylmethanol
Time (h)	3.6	3.7	5.4	5.5
X_R (%)	33.9	52.9	74.1	79.4
X_S (%)	1.1	2.2	8.5	2.3
Ee_p (%)	93.6	92.0	79.3	94.4
V_R ($\times 10^2$ mM h^{-1})	8.8	14.3	15.8	17.5
V_S ($\times 10^3$ mM h^{-1})	2.1	4.8	14.3	3.1
E	43	29	11	57

Conditions: 1.8 mM of (*RS*)-2-(4-chlorophenoxy)propionic acid, 15 mM of alcohol and 1 mg mL^{-1} of crude CPL in 3 mL of anhydrous cyclohexane.

An improvement of lipase activity and enantioselectivity is shown in Table 1 when trimethylsilylmethanol is the acyl acceptor. Similar results have been found when (*RS*)-2-(4-chlorophenoxy)propionic acid and (*RS*)-naproxen are kinetically resolved via CRL-catalyzed esterification.[17,22] Although specific characteristics of the silicon atom such as molecular recognition for the lipase, the low electronegativity and large atom radius compared with the carbon atom have been applied for elucidating this specificity, any direct evidence or proof by using molecular modelling has yet to be found. Based on the fast and highly stereoselective reaction, trimethylsilylmethanol was selected as the best alcohol in the following experiments.

2.2. Effects of temperature

Figure 1 illustrates the temperature effects on the time-course conversions of the (*R*)- and (*S*)-acid, from which the initial rates, *E* value and the time-course ee_P at various temperature were estimated and represented in Table 2. High lipase stereoselectivity of $E = 113$ at 20 °C was obtained. As expected, an increase in temperature resulted in an enhancement of initial rates V_R and V_S, while the *E* value decreased. Moreover, good linear relationships relating $\ln(V_S)$ and $\ln(V_R)$ with the inverse of absolute temperature (i.e. Arrhenius equation) were found for the range of 20–60 °C, indicating that CPL was thermally stable at 60 °C. However, the lipase could deactivate at a temperature higher than 60 °C as the conversion X_R reaches a plateau at the reaction time greater than 16 h.

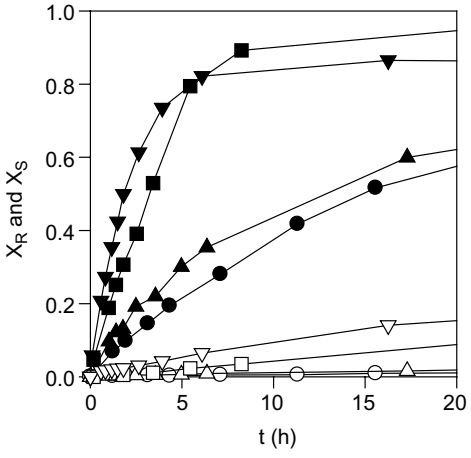


Figure 1. Temperature effects on time-course conversions of (*R*)-acid (solid) and (*S*)-acid (empty) at 60 °C (▼, ▽), 45 °C (■, □), 35 °C (▲, △) and 20 °C (●, ○). Conditions: 1.8 mM of (*RS*)-2-(4-chlorophenoxy)propionic acid, 15 mM of trimethylsilyl methanol and 1 mg mL^{-1} of crude CPL in 3 mL of anhydrous cyclohexane.

Thermodynamic analysis can be employed to estimate the difference in activation enthalpy (i.e. $\Delta\Delta H =$

Table 2. Temperature effects on CPL performances for esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid

Temp (°C)	20	35	45	60
Time (h)	15.6	17.3	8.3	6.1
X_R (%)	59.8	75.4	89.2	95.0
X_S (%)	1.0	2.0	3.5	7.5
Ee_p (%)	96.5	94.7	92.5	85.4
V_R ($\times 10^2$ mM h^{-1})	5.1	7.9	17.5	41.0
V_S ($\times 10^4$ mM h^{-1})	4.5	9.2	30.6	92.7
E	113	86	57	44

Conditions: 1.8 mM of (*RS*)-2-(4-chlorophenoxy)propionic acid, 15 mM of trimethylsilylmethanol and 1 mg mL^{-1} of crude CPL in 3 mL of anhydrous cyclohexane.

$\Delta H_S - \Delta H_R$) and that in activation entropy (i.e. $\Delta\Delta S = \Delta S_S - \Delta S_R$) for the transient states of the (*S*)- and (*R*)-acid. With the liner relationship $\ln(E) = 2381.9/T - 3.37$ ($r^2 = 0.97$), we obtained $\Delta\Delta H = -19.8$ kJ/mol and $\Delta\Delta S = -28.0$ J/mol in cyclohexane. This implies that the racemic temperature of the system is 433.8 °C at which CPL will lose the enantioselectivity. A linear relationship between $\Delta\Delta S$ and ΔH has been found many times in enzymatic reaction systems.[23,24] Thus, it would be interesting to find if the so-called enthalpy–entropy compensation exists for CPL when solvent, acid and reaction type vary.

2.3. Effects of anhydrous solvent

Table 3 shows the effects of solvent hydrophobicity in terms of log *P* (*P* as the partitioning coefficient of the solvent between *n*-octanol and water) on the lipase activity and enantioselectivity. No correlations relating the initial rates V_R and V_S, and hence the *E* value, with the log *P* value were found. Changing cyclohexane to another solvent improved the enzyme enantioselectivity but not the activity. Crude CRL has been employed in the kinetic resolution of menthol, 2-arylpropionic acids and 2-chloropropionic acid, where the lipase activity increases when increasing the log *P* value of the solvent.[25–28] However, the *E* value may also increase, for example, for menthol and 2-arylpropionic acids, or decreases for 2-chloropropionic acid.[25–27] Therefore in order to give insight into how solvent molecules interact with the transient states of both substrates, kinetic and thermodynamic analysis for all the solvents in Table 3 were carried out in our laboratory.

Table 3. Solvent effects on CPL performances for the esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid at 45 °C

Solvent* (log *P*)	A (3.0)	B (3.2)	C (3.5)	D (4.5)	E (5.6)
Time (h)	4.5	5.5	4.5	9.5	12.9
X_R (%)	55.1	79.4	45.1	21.3	40.4
X_S (%)	1.3	2.3	2.6	2.7	1.9
Ee_p (%)	94.5	94.4	89.1	77.5	91.0
V_R ($\times 10^2$ mM h^{-1})	15.7	17.5	10.3	2.6	4.3
V_S ($\times 10^3$ mM h^{-1})	2.1	3.4	1.2	0.3	0.7
E	74	57	87	87	63

* Symbols A, B, C, D and E represent carbon tetrachloride, cyclohexane, *n*-hexane, isooctane and *n*-decane, respectively. Conditions: 1.8 mM of (*RS*)-2-(4-chlorophenoxy)propionic acid, 15 mM of trimethylsilylmethanol and 1 mg mL^{-1} of crude CPL in 3 mL of anhydrous solvent.

2.4. Comparisons with CRL

Similar results were seen in Table 2 for CPL that were obtained in Table 4, when CPL (Lipase MY) was the biocatalyst. In general, CRL was less enantioselective and active for the (*R*)-acid in cyclohexane at any specific temperature. Moreover, a maximum initial rate V_R occurred at 35 °C, implying that CRL is also less thermally stable in comparison with CPL. The good thermal stability of CPL was attributed to the binding and immobilization of the enzyme to the non-soluble matrix of the latex. Moreover, a good linear relationship between $\ln(V_S)$ and the inverse of the absolute temperature (i.e. Arrhenius equation) was found for the range of 20–60 °C. This implies that the enzyme conformation change is negligible for the unfavorable (*S*)-substrate and gives further reduction of *E* values at higher temperatures.

Table 4. Temperature effects on CRL performances for esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid

Temp (°C)	20	35	45	60
Time (h)	6.7	6.5	6.2	6.4
X_R (%)	41.1	39.8	41.3	21.1
X_S (%)	1.6	1.5	1.8	2.6
Ee_p (%)	96.3	92.8	91.7	77.9
V_R ($\times 10^2$ mM h^{-1})	6.1	6.7	5.2	3.6
V_S ($\times 10^4$ mM h^{-1})	9.0	16.2	23.0	32.4
E	68	41	23	11

Conditions: 1.8 mM of (*RS*)-2-(4-chlorophenoxy)propionic acid, 15 mM of trimethylsilylmethanol and 1 mg mL^{-1} of crude CRL in 3 mL of anhydrous cyclohexane.

3. Conclusions

The kinetic resolution of (*RS*)-2-(4-chlorophenoxy)propionic acid in anhydrous organic solvents via esterification was employed as the model system for exploring *Carica papaya* lipase as a potential enantioselective biocatalyst. The chain length of the primary alcohol had profound effects on the lipase activity and enantioselectivity in cyclohexane: shorter-chain alcohols served as good substrates for the kinetic resolution of the acid, although the reaction rate was moderate. When trimethylsilylmethanol was used as the acyl acceptor, further improvements in the lipase performances were found, yielding a very high enantioselectivity of $E = 113$ at 20 °C. Changing cyclohexane to other hydrophobic solvents also gave better lipase performances, yet no correlations between the enzyme activity and enantioselectivity with the log *P* of the solvent could be found. A comparison of lipase performances for CPL and CRL indicated that the former had better lipase activity

and enantioselectivity as well as thermal stability for producing the desired (*R*)-ester at any specific temperature.

4. Experimental

4.1. General remarks for analytical procedure

The esterification of (*RS*)-2-(4-chlorophenoxy)propionic acid with various alcohols was monitored by HPLC using a chiral column (Chiralcel OD-H, Daicel Chemical Industries) capable of separating the internal standard of acetophenone, (*R*)- and (*S*)-ester with the retention time and composition of mobile phase given in Table 5. A flow rate of 1.0 mL min^{-1} with UV detection at 270 nm was used for quantification at the column temperature of 25 °C.

Table 5. Retention time and composition of mobile phase

Alcohol	*n*-Hexane:isopropanol:acetic acid (v/v)	Retention time (min)		
		Acetophenone	(*R*)-Ester	(*S*)-Ester
n-Propyl	97:3:1	8.75	6.7	7.8
n-Butyl	100:0.6:0	12.3	8.8	11.1
n-Hexyl	100:0:0	21.1	13.3	17.8
Trimethylsilylmethyl	100:0:0	21.1	12.4	16.1

4.2. General procedure for enantioselective esterification in organic solvents

(*RS*)-2-(4-Chlorophenoxy)propionic acid, alcohols and organic solvents at the highest purity are commercially available. A *Candida rugosa* lipase (Lipase MY, 30 units/mg of solid) was provided by Meito Sangyo (Tokyo, Japan). The crude papain (P-3375, Sigma) was dissolved at first in deionized water at 4 °C, centrifuged to collect the precipitate and then lyophilized in vacuum to obtain the crude *Carica papaya* lipase (CPL). To 3 mL of anhydrous cyclohexane were added 15 mM of alcohol, 1.8 mM of (*RS*)-2-(4-chlorophenoxy)propionic acid and 3 mg of CPL at 45 °C. Samples were withdrawn and injected onto the HPLC at different time intervals, from which conversions, enantiomeric excesses for the (*R*)-ester and enantiomeric ratios (i.e. *E* value, defined as the ratio of initial rates for both substrates) were determined. Similar experiments were carried out by changing the solvent, temperature or Lipase MY.

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