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Lipase from *Carica papaya* latex presents high enantioselectivity toward the resolution of prodrug (*R*,*S*)-2-bromophenylacetic acid octyl ester

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ABSTRACT

Besides the well-known papain, lipolytic activity is another interesting enzymatic activity present in latex from *Carica papaya*. This lipolytic activity is strongly attached to the latex solid phase, resulting in a naturally immobilized biocatalyst. In this work we describe the kinetic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl ester by *Carica papaya* crude latex and two partially purified latex fractions. Several parameters, such as substrate concentration and solvent effects were studied. The best results were obtained using decane as solvent with 50 mM of substrate and 50 mg/mL enzyme/reaction medium; under these conditions, a high enantioselectivity (E >200) was obtained with crude latex. A twofold increase of the initial rate maintaining E >200 was obtained using purified fractions without protease and without esterase. Lipase from *Carica papaya* latex is the most enantioselective wild-type enzyme ever described for the studied reaction.

Introduction

Therapeutic application of enantiopure drugs is highly attractive for pharmaceutical industry. Indeed, pure enantiomers have improved specificity and biological activity in comparison to racemic drugs and their use also avoid potential side effects associated to the use of racemic mixtures.[1] Nevertheless, classical methods to obtain optically enriched compounds, such as chemical synthesis and chiral chromatography, are often expensive. As an alternative, several microbial lipases were used over the last years as enantioselective biocatalysts. Indeed, these biocatalysts have the capacity to accept a large variety of substrates, display stability and performance in organic solvents, and permit to discriminate enantiomers from chiral mixtures of pharmaceutical interest, including non-steroidal anti-inflammatory drugs.[2] Amongst such chiral mixtures, 2-halogenocarboxylic acid esters are important intermediates for the synthesis of various chiral drugs such as prostacyclin and prostaglandin.[3] Therefore, the kinetic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl ester racemates by microbial lipases is of great interest.[4]

Although microbial lipases are the most studied source of lipases, such enzymes can also be found in plants, including oil seeds and laticifers (plant cells producing a milky fluid constituted by proteins, alkaloids, and so forth, commonly known as latex). *Carica papaya* latex (abbreviated here CPLtx) presents an interesting lipolytic activity, which was previously used to produce synthetic cocoa butter,[5] human milk analogs,[6] terpene,[7] biopolymers[8], and wax esters.[9]

It has been demonstrated that most of the lipolytic activity of CPLtx is highly associated to the latex solid phase, thus resulting in a stable and naturally immobilized biocatalyst.[10] Many efforts have been made in order to partially purify CPLtx lipolytic activity, through eliminating water soluble enzymes from latex (mainly proteases).[11] The resulting fraction is abbreviated here as 'CPL-p' (*Carica papaya* latex without protease). Recently, detergents were used to remove from latex part of the lipolytic activity mainly due to esterase activity.[12] This final preparation is abbreviated here as 'CPL-e' (*Carica papaya* latex without esterase). CPLtx and CPL-p have been successfully used for the racemic resolution of different non-steroidal anti-inflammatory drugs.[13]

The objective of this work was to optimize the resolution of (*R*,*S*)-2-bromophenylacetic acid esters by CPLtx and to compare the performance of CPLtx, CPL-p, and CPL-e on the enantioselective hydrolysis. The effect of substrate concentration, organic solvents, and pH was studied, using (*R*,*S*)-2-bromophenylacetic acid octyl ester as substrate (Scheme 1).

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Results and discussion

Hydrolytic activities of *C. papaya* crude and partially purified latex (CPLtx, CPL-p, and CPL-e) were evaluated and their performance on the kinetic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl esters with optimized conditions was compared.[12a]

Hydrolytic activity of CPL preparations

First, in order to characterize the enzymatic preparations, protease, esterase, and lipase activities were evaluated with specific substrates. Table 1 shows the ratio of these hydrolytic activities.

As expected, a high decrease in proteolytic activity was achieved during the preparation of CPL-p (98%) and only 0.9% protease activity remained in CPL-e. In this later, lipase is the predominant activity after removal of 43% esterase activity.

In previous works dealing with enantioresolution using *C. papaya* preparations, only CPLtx and CPL-p were tested. Here we decided to compare each partially purified fraction, including CPL-e, to discriminate which of protease, lipase, or esterase activity is responsible for the observed enantioselectivity.

Table 1
Hydrolytic activities of CPL preparations and ratio of lipase versus protease and esterase activities

Hydrolytic activity	Biocatalyst		
	CPLtx	CPL-p	CPL-e
Protease[a]	353 ± 2 U/g	7 ± 0.003 U/g	3 ± 0.001 U/g
Esterase[b]	1137 ± 37 U/g	1248 ± 68 U/g	486 ± 3 U/g
Lipase[c]	593 ± 1 U/g	753 ± 0.0 U/g	921 ± 32 U/g
Lipase/protease	1.7	108	307
Lipase/esterase	0.5	0.6	1.9

Mean and standard deviation of two independent experiments are presented.
[a] Measured by casein hydrolysis, 1 U = 1 equiv tyrosine liberated.
[b] Measured by tributyrin hydrolysis, 1 U = 1 μmol acid liberated/min.
[c] Measured by triolein hydrolysis, 1 U = 1 μmol acid liberated/min.

Kinetic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl ester using CPLtx

CPLtx's ability to discriminate between enantiomers in the racemic mixture of (*R*,*S*)-2-bromophenylacetic acid octyl esters was evaluated in a biphasic medium (water:decane 1:1). Different reaction parameters were evaluated in order to determine their effects over activity and enantioselectivity.

Unlike lipases that operate in acidic or neutral media,[14] CPLtx was previously shown to operate in basic media.[15] This constitutes a major advantage for applications as detergents, dish washing, and dry cleaning products.[16] High activity in alkaline media might also be an advantage for substrate solubility in synthesis reactions.[17] Considering the studied reaction, the highest hydrolysis activity toward the best-recognized enantiomer was indeed obtained at pH 8.5 and 9.0 (Table 2). These results are in agreement with those obtained by Abdelkafi et al. on triglycerides,[15] where the highest activity was also obtained between pH 8.5 and 9.0. pH 8.5 was thus retained for the following experiments.

At pH 8.5, CPLtx exhibited the highest initial rate (2.36 ± 0.10 μmol h^{-1} g $enzyme^{-1}$) of hydrolysis toward the preferred (*S*)-enantiomer and a very high enantioselectivity (>200), which makes this enzyme the most efficient wild-type lipase for the resolution of this racemic mixture. Indeed, reported enantioselectivity values of wild-type enzymes were much lower than those obtained using CPLtx (*E* = 72 for (*S*)-selective lipase Lip2 from *Yarrowia lipolytica* and *E* = 53 for (*R*)-selective lipase from *Burkholderia cepacia*).[4c,18] It was necessary to improve these microbial lipases' selectivity by site-directed mutagenesis in order to reach high initial rates and *E*-values >200.[4b,18]

Selection of a proper reaction medium is a key parameter to optimize any enzymatic reaction, as it can affect both the initial rate and enzyme selectivity.[19] Screening of solvents with different hydrophobicity was thus performed. The highest initial rate was obtained using decane, the most hydrophobic solvent tested, probably due to a good emulsification process where the interface water/solvent is favorable to lipase catalytic performances (Table 3).

Table 2
Effect of pH on the racemic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl ester catalyzed by CPLtx

pH	Initial reaction rate for the *S* enantiomer, r_S[a] (μmol h^{-1} g $enzyme^{-1}$)	*E* (r_S/r_R)
7.0	1.45 ± 0.04	30
8.0	2.02 ± 0.00	168
8.5	2.4 ± 0.10	>200
9.0	2.3 ± 0.10	>200

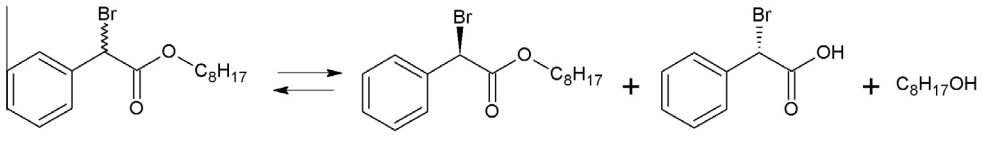
50 mM Substrate in decane, solvent:buffer 1:1, 50 mg/mL of biocatalyst, 25 °C
[a] Mean and standard deviation of two independent experiments are presented.

Previous efforts have been carried out in order to relate CPLtx catalytic performances (reaction rate and enantioselectivity toward 2-mathylalkanoic acid,[20a] propionic acid,[20b] and naproxen 2,2,2-trifluoroethyl thioester[13b]) and the properties of the solvent used. As in our case, no explicit correlation could be found between solvent properties and initial rates of reaction.

Determination of the optimal substrate concentration is essential to obtain high initial rate and *E*-value. As shown in Table 4, substrate concentration has only a slight influence on the performance of CPLtx in the studied range of concentrations. Nevertheless, over 100 mM, a slight decrease in reaction rates is observed, probably due to substrate inhibition.

Kinetic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl ester by CPL-p and CPL-e

Finally, the performances of partially purified fractions CPL-p and CPL-e were analyzed (Table 5). Results showed that the initial rate of hydrolysis of the preferred (*S*)-enantiomer increased when the partially purified fractions of latex were used, due to an enrichment of these fractions in lipolytic activity (see Table 1). Indeed, the reaction proceeded 2.1 and 2.4-fold faster when CPL-p and



(*R*,*S*) (*R*) (*S*)
1 **2** **3** **4**

Scheme 1. Hydrolysis of (*R*,*S*)-2-bromophenylacetic acid octyl ester **1** into (*R*)-2-bromophenylacetic acid octyl ester **2**, (*S*)-2-bromophenylacetic acid **3**, and octyl alcohol **4** (provided that lipase is *S* enantioselective).

Table 3
Solvent effects on the racemic resolution of *(R,S)*-2-bromophenylacetic acid octyl ester catalyzed by CPLtx

Solvent (Log *P*)[a]	Initial reaction rate *S*-enantiomer, r_S[b] (μmol h^{-1} g $enzyme^{-1}$)	*E* (r_S/r_R)
Cyclohexane (3.2)	1.2 ± 0.31	>200
Octane (4.0)	1.1 ± 0.23	>200
Heptane (4.6)	1.6 ± 0.15	>200
Decane (5.6)	2.4 ± 0.10	>200

50 mM Substrate in the solvent, solvent:buffer 1:1, 50 mg/mL of biocatalyst, 25 °C.
[a] Log*P* (solvent partition coefficient) values taken from Chemspider (www.chemsider.com).
[b] Mean and standard deviation of two independent experiments are presented.

Table 4
Effects of substrate concentration on the racemic resolution of *(R,S)*-2-bromophenylacetic acid octyl ester catalyzed by CPLtx

Substrate (mM)	Initial reaction rate *S*-enantiomer, r_S[a] (μmol h^{-1} g $enzyme^{-1}$)	*E* (r_S/r_R)
50	2.4 ± 0.10	>200
100	2.9 ± 0.10	>200
250	2.5 ± 0.10	>200

Substrate dissolved in decane, solvent:buffer 1:1, 50 mg/mL of biocatalyst, 25 °C.
[a] Mean and standard deviation of two independent experiments are presented.

Table 5
Comparison of the kinetic resolution of *(R,S)*-2-bromophenylacetic acid octyl ester catalyzed by CPLtx, CPL-p, and CPL-e

Biocatalyst	Initial reaction rate *S*-enantiomer, r_S[a] (μmol h^{-1} g $enzyme^{-1}$)	*E* (r_S/r_R)
CPLtx	2.4 ± 0.10	>200
CPL-p	5.0 ± 0.39	>200
CPL-e	5.6 ± 0.30	>200

50 mM Substrate in decane, solvent:buffer 1:1, 50 mg/mL of biocatalyst, 25 °C.
[a] Mean and standard deviation of two independent experiments are presented.

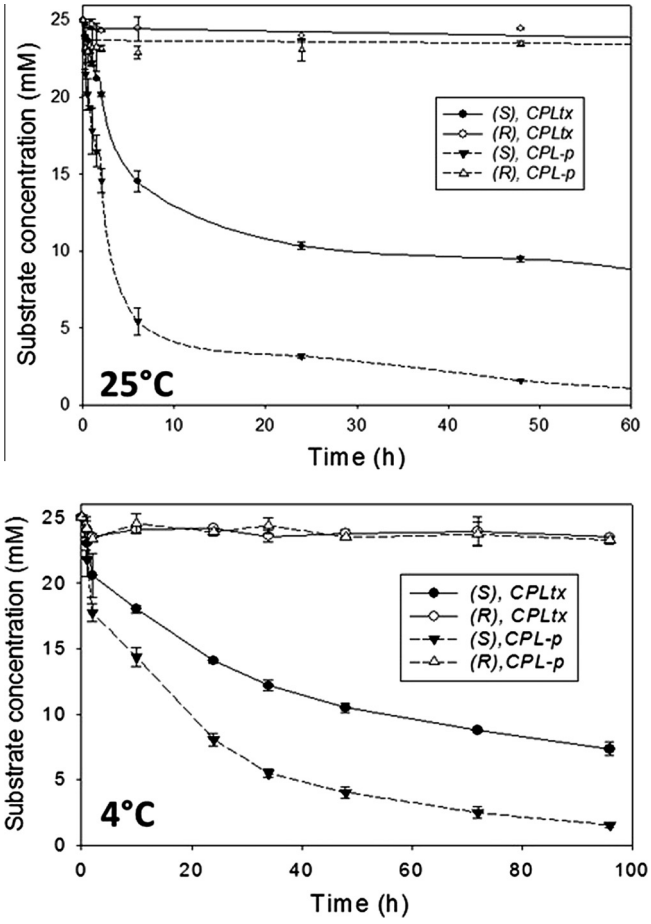


Figure 1. Time course of kinetic resolution of (*R,S*)-2-bromophenylacetic acid octyl ester catalyzed by CPLtx and CPL-p. Assays were performed in the same conditions as in Table 5. All assays were carried out in duplicate.

CPL-e were used respectively, which correlates with a 1.2 and 1.6-fold increase in the lipase activity assayed on triolein. Moreover, CPL-p and CPL-e have similar performances, which implies that the enzyme or enzymes that perform the reaction are still in the non-soluble fraction of latex. These results are consistent with the fact that soluble extracts (containing proteases and esterases) did not show any activity toward the enantiomeric resolutions tested. In the case of (*R,S*)-2,2,2-naproxen trifluoethyl thioester, Ng and Tsai also observed that specific activity was enhanced when CPL-p was used (compared to non-purified latex), while enantioselectivity was conserved.[13c]

Figure 1 shows the time course of reactions for both CPLtx and CPL-p at 4 and 25 °C. When assessed at 25 °C, CPLtx showed an important decrease in the hydrolysis rate after 6 h reaction, which was not observed with CPL-p. This is probably due to the degradation of lipolytic enzymes by proteases. In accordance, this phenomenon is less pronounced at 4 °C and for CPL-p (fraction partially purified where most of proteases have been removed).

After 48 h reaction with CPL-p at 25 °C (47.8% total conversion), enantiomeric excesses of substrate and product are 87.8% and 95.8%, respectively. At this time, 98.0% of the (*R*)-enantiomer can be recovered with a purity of 93.9%, and 93.6% with 97.9% purity for the (*S*)-enantiomer.

Interestingly, enantiomeric excess observed for CPL-e was almost the same as that observed for CPL-p; this coincided with the results obtained when initial reaction rates were evaluated with both fractions (Table 5).

Summary & conclusion

There are only few reports of microbial lipases efficient in the kinetic resolution of (*R,S*)-2-bromophenylacetic acid octyl ester. Those mainly include Lip1 and Lip3 from *Candida rugosa*, Lip2 from *Yarrowia lipolytica*, and lipase from *Burkholderia cepacia*.[4] Here we demonstrate that the catalytic performances of *Carica papaya* latex CPLtx and its partially purified fractions CPL-p and CPL-e are more efficient than those described wild-type microbial lipases. CPLtx is therefore the most enantioselective wild-type enzyme for the studied reaction.

CPLtx is an efficient and highly enantioselective biocatalyst for the kinetic resolution of (*R,S*)-2-bromophenylacetic acid octyl ester with preference for the (*S*)-enantiomer and *E* >200. In the case of the partially purified fractions CPL-p and CPL-e, a twofold increase in the initial rate was obtained, while *E* >200 was maintained. As CPL-p and CPL-e have the same performances, but the purification process for CPL-p is simpler, this is the preferred biocatalyst to continue further studies.

Experimental

The procedure for the preparation of (*R*,*S*)-2-bromophenylacetic acid octyl ester, was described previously.[3d]

CPLtx collection from fruits of 'Maradol' variety was carried out according to Rivera et al.[12a]

Partial purification of CPLtx to give a fraction without protease (CPL-p) and without esterase (CPL-e) was also carried out as described previously by Rivera et al. and lyophilized. [12a]

Proteolytic activity was measured using casein as a substrate.[21]

Esterase activity in latex was measured by hydrolysis of tributyrin while lipase activity was measured by hydrolysis of olive oil. Hydrolysis reactions were performed in a thermostated and mechanically stirred Envirogenie Scientific Industries (USA) at 30 °C containing 2.5 mL tributyrin, 1 mL 50 mM Tris–HCl, 50 mM NaCl, and 0.2% triton pH 8.5 buffer solution with the corresponding amount of enzyme (usually 50 mg). Free fatty acids released were titrated with NaOH and lipase activity was expressed as 1 U = 1 μmol free fatty acid (FFA) released per minute.

Hydrolysis of racemic substrate was carried out as described by Piamtongkam et al.[4d] with the following modifications: substrate 100 mM, buffer Tris–HCl, 100 mM NaCl and 0.2% triton TX100, pH 8.5 (of buffer solution), and 50 mg/mL of enzyme. The mixture was shaken in a Vortex Genie 2 (Brumat, France). Reactions were carried out at 25 °C. Progress of the reaction was followed at regular time intervals by taking samples after phase separation by centrifugation (50 μL diluted in 500 μL hexane).

The enantioselectivity value (*E*-value) was defined as the ratio of the initial rate of (*S*)-enantiomer production to the initial rate of (*R*)-enantiomer production ($E = r_S/r_R$). Enantiomeric excess (ee) was calculated as defined below: ees = {[R] − [S]}s/{[R] + [S]}s (s = substrate).

Initial rates were determined by linear regression from concentrations measured by HPLC as follows: the HPLC was equipped with a chiral column: Chiralpack OJ (25 cm × 4.6 mm) (Daicel Chemical Industries Ltd, Japan) connected to a UV detector (at 254 nm). A flow rate of 1.0 mL/min was used. The mobile phase was composed of a mixture of *n*-hexane/isopropanol (80:20 v/v).

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