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New efficient lipase from *Yarrowia lipolytica* for the resolution of 2-bromo-arylacetic acid esters

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Abstract—A new extracellular lipase (Lip2p) from the yeast *Yarrowia lipolytica* was used for the resolution of 2-bromo-arylacetic acid esters, an important class of chemical intermediates for the pharmaceutical industry. Its efficiency for the transesterification of racemic mixtures with 1-octanol in *n*-octane was compared with the most efficient lipases described to date, lipases from *Burkholderia cepacia* and *Rhizomucor miehei*. Resolution of 2-bromo-*p*-tolylacetic acid ethyl ester catalyzed by *Y. lipolytica* lipase showed an enantiopreference of 28, almost equal to that obtained with *B. cepacia* lipase ($E = 30$). Moreover, *Y. lipolytica* lipase presents a higher catalytic activity and an (*S*)-enantiopreference, while *B. cepacia* lipase is (*R*)-enantiomer selective. The most interesting result is that *Y. lipolytica* lipase has until now been the only enzyme able to catalyze the resolution of 2-bromo-*o*-tolylacetic acid ethyl ester ($E = 27$).

1. Introduction

The need for enantiomerically pure compounds, especially in the pharmaceutical industry, has grown since legislation required investigation on the pharmacological effect of each enantiomer. The market for drugs sold in their single-enantiomer form was worth $160 billion worldwide in 2002.[1]

Since classical ways to obtain pure enantiomers, that is, chemical asymmetric synthesis, stereoselective crystallization and chiral chromatography, are often expensive, enzymatic procedures have become attractive. In this field, lipases are the most frequently used catalysts. The reasons for this interest lie in their high stability, their non-requirement of cofactors, their synthetic activity in organic solvents and mainly in the wide range of substrate specificities. They are also very capable of catalyzing reactions on non-natural substrates.

Enantiopure carboxylic acids are important building blocks for the synthesis of many pharmaceuticals, pesticides and natural compounds such as pheromones. The most studied compounds are 2-arylpropionic acids, which have the properties of non-steroid anti-inflammatory drugs (Ibuprofen, Naproxen, Ketoprofen, etc.).[2–5] The resolution of another important group of molecules, 2-haloarylacetic acids, has been sadly neglected.[6–12] These compounds are important intermediates found in the synthetic pathways of a number of drugs, such as prostaglandin, prostacyclin, semi-synthetic penicillin and thiazolium salts. In particular, ethyl ester derivatives of 2-bromo-*o*-tolylacetic acid are used as precursors for the synthesis of analgesics and non-peptide angiotensin II-receptor antagonists. Recently, it has been demonstrated that lipases from *Burkholderia cepacia* and *Rhizomucor miehei* are the most efficient for the resolution of these molecules.[6,7] Using free *B. cepacia* lipase, *E*-values higher than 50, with an (*R*)-enantiopreference, can be achieved during transesterification in *n*-octane of 2-bromo-*m*/*p*-tolyl- or 2-bromophenylacetic acid esters. Unfortunately, a low enantioselectivity was observed ($E = 6$) with the *ortho* derivatives. For the resolution from this last substrate, the best enzyme was the lipase for *R. miehei* immobilized on polypropylene ($E = 11.3$).[6] However, this enantioselectivity degree is not acceptable for industrial purposes and the need for a more selective catalyst is crucial.

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Herein, a new lipase is evaluated for the resolution of 2-bromoarylacetic acids. This lipase, *LIP2p* is produced by the non-pathogenic lipolytic yeast, *Yarrowia lipolytica*, and has recently been characterized.[13] It is a secreted lipase with an apparent molecular mass of 38.5 kDa. The yeast *Y. lipolytica* was proposed as an alternative host organism for heterologous protein production.[14,15] It combines the facility of single cell use, high secretion abilities and tools for post-translational modifications.[14] Strains and vectors for protein expression and secretion have been developed.[16,17] The host strain was made auxotrophe for uracile while protease-encoding genes were deleted. A gene amplification system was developed for efficient lipase expression in *Y. lipolytica*.[14] The oleic acid-inducible *POX2* promotor was used to drive the transcription of the *LIP2* gene. Protein secretion is directed by the wild-type lipase targeting sequence. After *Not*I digestion of plasmid JMP6, the bacterial part was eliminated and only the expression cassette was integrated within the genome. A mutant *URA3* allele, *ura3d4*, was used for the selection for multicopy integration into the genome. In this way, multicopy integrants containing 3–16 copies of the lipase gene were obtained.[14,16] The extracellular lipase represents the main enzyme in the fermentation broth.

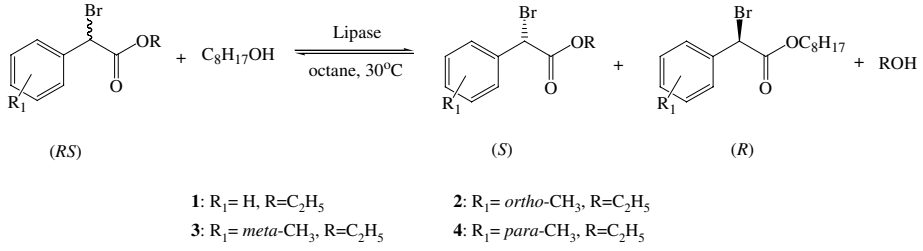
The objective of this work was to compare performances obtained with this lipase to those obtained with the two most efficient lipases already described in the literature, lipases from *B. cepacia* and *R. miehei*. Comparisons were based both on activity and enantioselectivity.

2. Results and discussion

The performance of *Y. lipolytica*'s lipase enantioselectivity towards the esters of 2-bromo-phenyl and tolyl acetic acids was studied during the transesterification in *n*-octane using 1-octanol as the alcohol (Scheme 1). In a previous paper,[7] it was demonstrated that the presence and position of the methyl substitution on the aromatic ring were crucial for the enzyme enantioselectivity. For this reason, four substrates were tested, the 2-bromo-phenylacetic acid ethyl ester (substrate 1) and the 2-bromo-*o*/*m*/*p*-tolylacetic acid ethyl esters (substrates 2–4) (Table 1). Activity and enantioselectivity efficiency of *Y. lipolytica*'s lipase were compared with those obtained with the lipases from *B. cepacia* (*Bcl*) and *R. miehei* (*Rml*), the most efficient lipases described to date.[6,7]

2.1. Lipase activity

Whatever the acyl donor, the enzyme from *Y. lipolytica* was more active than the two other enzymes (Table 1). For the non-substituted substrate, this increase in activity was roughly 10-fold. It was highest for the *para*-substituted substrate (69 and 26 times higher when



Scheme 1.

Table 1. The enantioselectivity of free *Y. lipolytica*, *B. cepacia* and *R. miehei* lipases in the transesterification between substrates **1**–**4** and 1-octanol in *n*-octane

Substrate	Lipase[a]	Initial rate (μmol min^{-1} g^{-1})	% Conv.	E[b] (−)[c]	%Ee$_p$
1	*Y. lipolytica*	21.0	45 (48 h)	5.1 (*S*)	50
	B. cepacia	3.2	37 (22 h)	69.0 (*R*)	93
	R. miehei	2.1	58 (22 h)	1.4 (*S*)	10
2	*Y. lipolytica*	2.6	38 (56 h)	27.0 (*S*)	84
	B. cepacia	0.1		2.1 (*R*)	
	R. miehei	0.23		2.5 (*S*)	
3	*Y. lipolytica*	50.0	52 (11 h)	1.1 (*R*)	
	B. cepacia	2.0	46 (120 h)	59.0 (*R*)	87
	R. miehei	2.5	55 (72 h)	1.9 (*S*)	9
4	*Y. lipolytica*	40.0	46 (20 h)	28.0 (*S*)	82
	B. cepacia	0.6	30 (43 h)	30 (*R*)	92
	R. miehei	1.5	56 (18 h)	3 (*S*)	34

[a] *Y. lipolytica* lipase (1 mg/mL), *B. cepacia* lipase (5 mg/mL), *R. miehei* lipase (5 mg/mL).
[b] E = ratio of initial rates.
[c] Fast reacting enantiomer.

Table 2. The protein content and different activities (*p*NPB, tributyrin and triolein hydrolysis, esterification of oleic acid with ethanol in *n*-hexane) of free *Y. lipolytica*, *B. cepacia* and *R. miehei* lipases

	Y. lipolytica	*B. cepacia*	*R. miehei*
Protein content (%) [a]	52	12	15
*p*NPB hydrolysis ($\mu mol\,min^{-1}\,mg^{-1}$)	41	50	4.4
Tributyrin hydrolysis ($\mu mol\,min^{-1}\,mg^{-1}$)	177	287	429
Triolein hydrolysis ($\mu mol\,min^{-1}\,mg^{-1}$)	1140	209	274
Oleic acid esterification ($\mu mol\,min^{-1}\,mg^{-1}$)	1.16	0.45	0.28

[a] Determined by the micro-Bradford method using BSA as standard.

compared with *Bcl* and *Rml*, respectively). For the *ortho*-substituted substrate, the increases were 26- and 11-fold compared with *Bcl* and *Rml*, respectively. However, the methyl group at the *ortho*-position of the aromatic ring inhibits the kinetics whatever the enzyme used, with the most effective enzyme being the *Y. lipolytica* enzyme. This effect can be attributed to the *ortho* substitution steric hindrance. Reasons for the kinetic efficiency of the lipase from *Y. lipolytica* can be found either in better enzyme preparation purity or in higher intrinsic catalytic activity. The protein content of the *Y. lipolytica* enzyme was three times higher than for the two other enzymes (Table 2). This was confirmed by SDS polyacrylamide gel electrophoresis (data not shown). The three enzyme preparation activities were tested towards the hydrolysis of *para* nitro phenyl butyrate, tributyrin and triolein, and, during a synthesis, the esterification of oleic acid with ethanol in *n*-hexane (Table 2). *Y. lipolytica* enzyme preparation appeared the most active during hydrolysis of triolein (5.5- and 4.2-times higher compared with *Bcl* and *Rml*, respectively) and during the esterification (2.6- and 4.1-times higher when compared with *Bcl* and *Rml*, respectively). If these activities are expressed versus the protein contents, they are globally the same. On the contrary, its activity during the hydrolysis of tributyrin is lower (1.6- and 2.4-times lower compared with *Bcl* and *Rml*, respectively). It is a remarkable fact to have an activity higher on triolein than on tributyrin. *Y. lipolytica* lipase presents a high specificity for long chain glycerides, which underlines its high level of lipase character. Taking into account enzyme purities, *Y. lipolytica* lipase is about 3- to 20-fold more active than *B. cepacia* lipase depending on the substrate.

2.2. Lipase enantioselectivity

From the point of view of selectivity, *Y. lipolytica* and *R. miehei* lipases give an opposite enantiopreference to the lipase from *B. cepacia*. In fact, the (*S*)-enantiomer is preferred by these two lipases, whereas the lipase from *B. cepacia* prefers the (*R*)-enantiomer. This is the result of the differences in the structure of their active sites. The binding pocket of the *B. cepacia* lipase is a 17 Å long funnel, whereas the active site of *R. miehei* lipase is at the surface of the protein protected by a lid. The three dimensional structure of the *Y. lipolytica* lipase is unknown. However, one of closest lipases at the level of the primary structure is the *R. miehei* lipase (30% of amino acids in common after Blast alignment). It can be assumed that both active sites are similar, thus explaining the same enantiopreference, but sufficiently different to each other to explain difference in enantioselectivity.

Table 3. Effect of temperature on *Y. lipolytica* lipase activity and enantioselectivity

T (°C)	Initial rate ($mmol\,min^{-1}\,g^{-1}$)	*E*
20	1.18	33
30	2.56	27
40	3.13	23
50	4.67	16

The lipase from *Y. lipolytica* gave no enantioselectivity for 2-bromo-phenylacetic acid ethyl ester and 2-bromo-*m*-tolylacetic acid ethyl ester ($E = 5$ and 1, respectively). For the 2-bromo-*p*-tolylacetic acid ethyl ester, *Y. lipolytica* lipase gave an enantioselectivity similar to the one obtained with *B. cepacia* lipase ($E = 28$). The most interesting result concerned the 2-bromo-*o*-tolylacetic acid ethyl ester. Indeed, no free enzyme was able to efficiently resolve this racemic mixture.[6] *Y. lipolytica* lipase gave an enantioselectivity of 27. This result is better than that observed with the lipase from *R. miehei* immobilized on polypropylene ($E = 11$), which was the best result found in the literature.[6]

For the most interesting substrate, the 2-bromo-*o*-tolylacetic acid ethyl ester, the influence of the temperature was studied (Table 3). As usually observed with an increase in temperature, activity was higher and enantioselectivity lower.

3. Conclusion

Herein, it has been demonstrated that the lipase from *Y. lipolytica* is a very active and selective catalyst in the transesterification of 2-bromo-phenyl and 2-bromo-tolyl acetic acid ethyl esters. The most interesting result is that this lipase is the first described lipase able to resolve the racemic mixture of 2-bromo-*o*-tolyl acetic acid ethyl ester ($E = 27$), an industrial pharmaceutical building block. However, this enantioselectivity value is still not high enough. In order to understand and improve the enantioselectivity, it would be necessary to obtain the three-dimensional structure of this protein. While waiting for crystallographic data, Lip2p 3D structure is being modelled by homology modelling using the *R. miehei* lipase as a template. The final objective will be to design variants of the enzyme by site-directed mutagenesis.

4. Materials and methods

4.1. Biological reagents

Commercial lipases were purchased from Roche Diagnostics (Germany). Chirazyme L-9, lyo., (free lipase from *R. miehei*); Chirazyme L-1, lyo., (free lipase from *B. cepacia*).

Lipase Lip2p from *Y. lipolytica* was produced on a mineral medium. The strain JMY329 used in this study contained multiple integrated copies of the *LIP2* gene (~16 copies) in the genome. It was obtained by gene amplification as previously described.[17] Cells were eliminated by centrifugation. The enzyme was precipitated by the addition of an equivolume of cold acetone (−20 °C). Centrifugation enabled the enzyme to be recovered. The enzyme was used after lyophilization.

4.2. Chemical reagents

All reagents were of commercial quality and purchased from Sigma/Aldrich. *n*-Octane was dried over molecular sieves (3 Å) before use.

4.3. General procedure for the preparation of 2-bromo carboxylic acid esters

The procedure for the preparation of (±)-2-bromo phenylacetic acid ethyl and octyl ester, (±)-2-bromo-*o*/*p*/*m*-tolylacetic acid ethyl and octyl ester has been described in a previous paper.[7]

4.4. Procedure for the enzymatic transesterification

Transesterification was carried out in 5 mL tubes containing the ester (50 mM) and octanol (150 mM) in dried octane. The temperature was maintained at 30 °C. The mixture was shaken at 1250 rpm. Addition of the free enzyme marked the zero time of the reaction. At regular time intervals the progress of the reaction was followed by taking samples (100 μL diluted in 1 mL of a mixture hexane/isopropanol 99.8:0.2, v/v).

4.5. HPLC analysis

The HPLC device 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 for (±)-2-bromo phenyl ethyl and octyl acetate and (±)-2-bromo-*p*-tolyl ethyl and octyl acetate and 98/2 v/v for (±)-2-bromo-*o*/*m*-tolyl ethyl and octyl acetate].

4.6. Determination of the enantiomeric excess (ee), conversion and enantioselectivity (*E*)

From HPLC results, the enantiomeric excess (ee) was calculated as defined below: ees = $\{[R] - [S]\}$s/$\{[R] + [S]\}$s (s = substrate) and the conversion: $C = 1 - [(R - S)_t/(R - S)_{t=0}]*100$.

The enantioselectivity value was the ratio of the initial rates of the two enantiomers.

4.7. Determination of the protein content of enzyme preparation

The enzyme solution was prepared in a phosphate buffer 0.05 M pH 7 at 100, 25 and 25 mg/L for *R. miehei*, *Y. lipolytica* and *B. cepacia*, respectively. The protein content was determined with the micro-Bradford method with BSA as standard.

4.8. Determination of enzyme activities

Triolein hydrolysis: The enzyme solution was prepared in a phosphate buffer 0.05 M pH 7 at 2, 0.5 and 0.6 g/L for *R. miehei*, *Y. lipolytica* and *B. cepacia*, respectively. The reaction mixture contained 5 mL of olive oil emulsion (Sigma, 800b), 2 mL of trizma buffer (Sigma, 800b) and 20 μL of enzyme solution. Hydrolysis was run for 20 min at 37 °C. The reaction was stopped by the addition of a mixture of acetone/ethanol (v/v) containing three drops of thymolphthalein (0.9% (w/v) in ethanol). The titration was realized using a 0.05 M sodium hydroxide solution.

p-Nitro phenol butyrate hydrolysis: The enzyme solution was prepared in a phosphate buffer 0.05 M pH 7 at 20, 5 and 6 mg/L for *R. miehei*, *Y. lipolytica* and *B. cepacia*, respectively. The reaction mixture contained 175 μL of a phosphate buffer 0.05 M pH 6.8, 5 μL of a 10 mM *p*-nitro phenol butyrate in 2-methyl 2-butanol, 20 μL of enzyme solution. Activity was measured following absorbance at 405 nm.

Esterification: 200 mM oleic acid and 300 mM ethanol were dissolved in *n*-hexane. The enzyme (10–30 mg) was then added. The reaction mixture was incubated at 40 °C and agitated with magnetic stirring. For each sample, *n*-hexane was evaporated and the solutes redissolved in the HPLC solvent. Oleic acid and ethyl oleate concentrations were determined by an HPLC system equipped with a Kontron 420 pump, a Varian R14 refractive index monitor (Varian Associates, Orsay, France) and a Spherisorb ODS2 C18, 5 μm column (250 × 4.0 mm). Elution was conducted at 50 °C with methanol/acetic acid (99.7:0.3, v/v) at a flow rate of 1 mL/min.

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