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Enantioselective hydrolysis of alkyl esters of substituted 1-phenyl ethanols using newly isolated strain of *Arthrobacter* sp.; a comparative study with known commercial lipases

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Abstract

The enantioselective hydrolysis of alkyl esters of substituted 1-phenyl ethanol racemates has been conducted using a newly selected strain of *Arthrobacter* sp. (ABL) and its efficacy compared with commercially available enzymes such as PPL (porcine pancreatic lipase), CCL (*Candida cylindracea*) and PSL (*Pseudomonas* sp.). The ABL has demonstrated reasonable superiority over the above three known enzymes both in terms of its enantioselectivity as well as the overall rate of hydrolysis.

1. Introduction

The use of enzymes, especially hydrolases or lipases, is becoming increasingly prevalent in the quest for the resolution and synthesis of optically active molecules and auxiliaries.[1] Through stereoselective enzymatic ester hydrolysis, direct preferential access to one of the enantiomers within the racemates of a broad range of chiral molecules is possible. The list of lipases/esterases now commonly being employed for such reactions is ever increasing. Most of the known lipases/esterases, however, display wide and varying degrees of substrate specificity and hydrolytic activity, which is not only a function of the structure of the substrate but is also influenced by reaction conditions. Faster reaction rates of ester hydrolysis often proceed at the cost of selectivity. Therefore, the basic attributes of a suitable lipase/esterase should be to provide the highest selectivity for one of the enantiomers in a reasonably short time and space in molar concentrations. Although a number of such enzymes with broader substrate specificity are commercially available, only a few can meet the optimal requirements from a technical scale viewpoint. Well known among the hydrolases for bioresolution include PPL (porcine pancreatic

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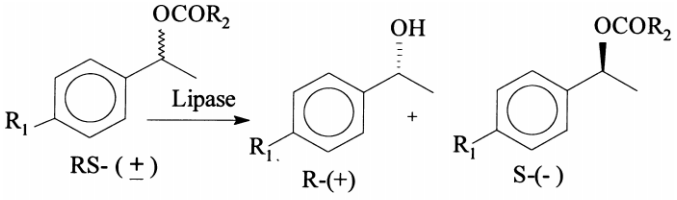
lipase), CCL (*Candida cylindracea*), PSL (*Pseudomonas* sp.), MML (*Mucor mehei*) etc. During a search for novel microbial lipases bearing above indicated attributes, a strain of *Arthrobacter* sp. was isolated by us, which under specific conditions can be made to accumulate a stereoselective lipase in reasonably large activity and may be considered as a new commercial source for such enzyme production.

A literature search for *Arthrobacter* sp. revealed only a few references regarding its use as a source of hydrolase.[2] In order to demonstrate the suitability of the *Arthrobacter* sp. (ABL) isolated by us as a potential stereoselective hydrolase source, alkyl esters of substituted 1-phenyl-ethanol were selected as substrates for reaction assay. Selection of the 1-phenyl-ethanol analogues as substrates was made due to their utility as chiral auxiliaries and synthons.[3] In the present communication the enantioselective hydrolytic activity of the *Arthrobacter* sp. (ABL) in comparison to commercial lipases, such as CCL, PPL and PSL is presented.

2. Results and discussion

A series of alkyl acylates of 1-phenyl-ethanols was prepared[4,5] for use as substrates for crude ABL enzyme preparation. The activity of the enzyme was compared vis-à-vis three commercially available crude enzymes, PSL, PPL and CCL. It was also envisaged to study the effect of small variations in the bulk of the acyl carbon chain (R_2) as well as the size of the *p*-substituents (R_1) on the efficacy of all the selected lipases.



R_1 = H, Cl, F, $-OCH_3$

R_2 = $-CH_3$, $-CH_2CH_3$, $-CH_2CH_2CH_3$

Time course enantioselective hydrolyses of all ester analogues of 1-phenyl-ethanol assayed for four selected enzymes at periodic intervals for 12 h total reaction time are compiled in Fig. 1. In general all the four enzymes showed enantiopreference for R-(+) esters as was observed from the sign of rotation of the hydrolyzed product.[6] With both ABL and PSL, acetates of *p*-substituted 1-phenyl-ethanol reacted faster than propionates and butyrates in the initial phase of the reaction. Conversely, propionates and butyrates registered a faster hydrolysis rate than acetates in case of PPL and CCL, even though their overall rate of hydrolysis remained significantly low.

Both the propionate and butyrate of 1-phenyl-ethanol demonstrated higher rates of hydrolysis than the acetate. 1-(4-Chlorophenyl)-ethanol esters registered comparatively the slowest rates of hydrolysis and even after 24 h of reaction time (data not shown) a maximum value of 50% could not be obtained even with ABL and PSL. Under the given reaction conditions, with all the esters of *p*-substituted analogues of 1-phenyl-ethanol except 1-(4-methoxyphenyl)-ethanol, enantiomeric excesses of >98% were obtained for all the enzymes. ABL was consistently superior with respect to rate of hydrolysis compared to three other selected enzymes.

Kinetic resolution of alkyl esters of 1-(4-methoxyphenyl)-ethanol showed lower enantioselectivity [as evidenced by wide variation in enantiomeric excess (ee)] than that observed for other derivatives described above. While ABL and PSL registered the highest selectivity (ee=98%) with the butylester of 1-(4-methoxyphenyl)-ethanol, PPL showed the lowest ee (73%). The other two acylates (acetate and

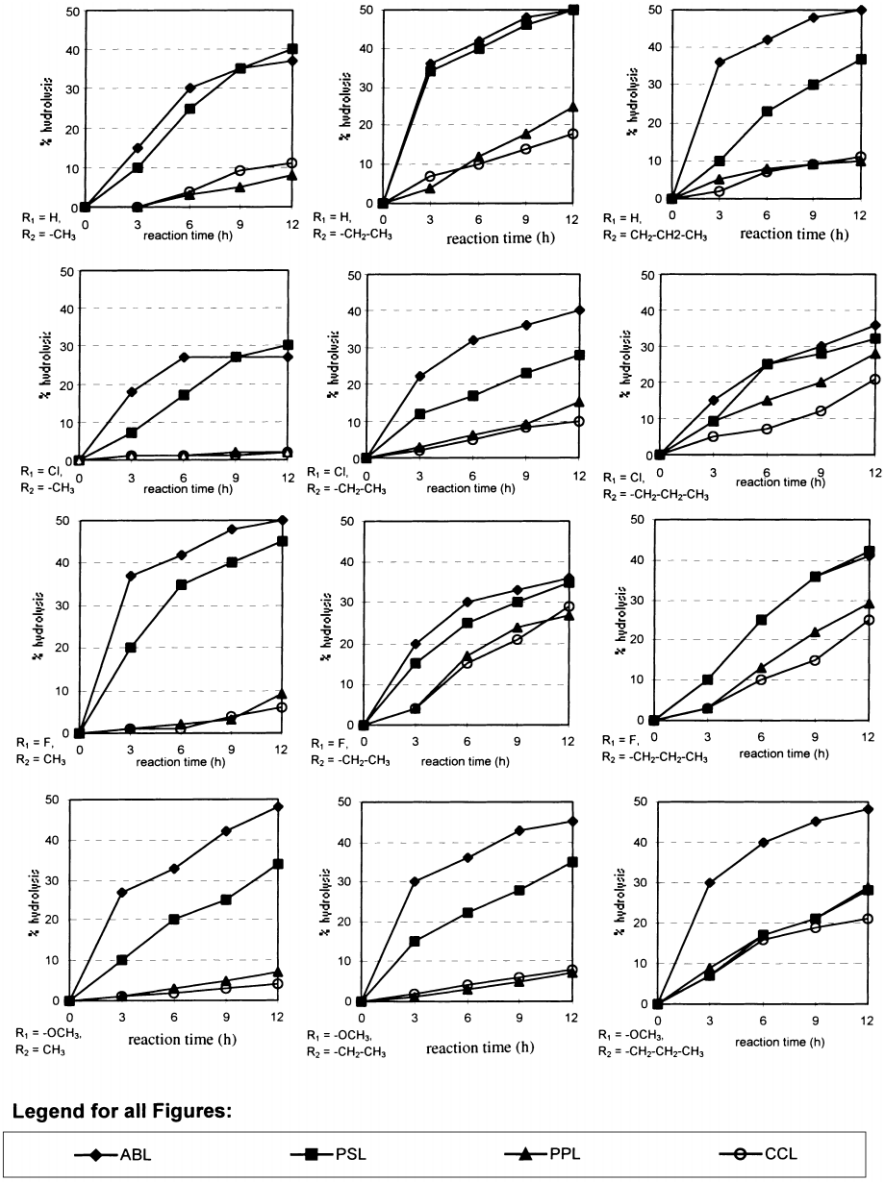


Fig. 1. Time course enantioselective hydrolysis of various ester analogues of 1-phenyl-ethanol by a new isolate of *Arthrobacter* sp. (ABL) in comparison to three known lipases (PSL, PPL and CCL)

propionate) showed moderate to high enantioselectivity (75–86%). ABL once again proved to be superior to PSL with respect to the initial rate of hydrolysis of this substrate.

3. Conclusion

We have demonstrated that *Arthrobacter* sp. lipase (ABL) in comparison to commonly used enzymes such as PSL, PPL and CCL is the fastest ester hydrolyzing lipase when assayed with the alkyl ester analogues of substituted 1-phenyl-ethanol as substrates. Within a 3–6 h reaction time, ABL reached a

plateau. PSL was the only other of the three known enzymes tested in this study capable of matching the rate of hydrolysis. PPL and CCL on the other hand recorded far slower hydrolysis rates with all the substrates assayed. In terms of enantioselectivity as depicted by final ee, ABL was comparable to the other three enzymes tested. Enantioselectivity in test substrates such as 1-phenyl-ethanol, 1-(4-fluorophenyl)-ethanol and 1-(4-chlorophenyl)-ethanol was found to be in excess of 98%. 1-(4-Methoxyphenyl)-ethanol acetate was the only substrate that recorded lower ee with both ABL and PSL, however an appreciable improvement in selectivity was achieved when propyl and butyl esters of the *p*-substituted analogue were used as the substrates. The enantioselectivity of CCL and PPL for all the ester analogues of 1-(4-methoxylphenyl)-ethanol was rather poor. In conclusion, the efficacy of ABL as a faster reacting lipase for 1-phenyl-ethanol analogues as substrates is well demonstrated, however further experiments on a wide variety of test substrates are required to widen the scope of its utility and potential for industrial use.

4. Experimental

Crude lipases: porcine pancreatic (PPL, enzyme activity 284 U/mg solid) and *Candida cylindracea* (CCL, enzyme activity 423 U/mg solid) were purchased from Sigma Chemicals, USA; *Pseudomonas* sp. (PSL, enzyme activity 700 U/mg solid) was obtained as a free gift from Amano, Japan. The procured lipases were used as such without purification. The crude lipase of *Arthrobacter* sp. (ABL, 7000 U/mg solid) was freshly prepared by the following method.

4.1. Crude enzyme preparation

An isolated strain of *Arthrobacter* sp. was grown in shaken flasks on standard LB medium for 16–18 h. Fermented broth (100 ml) was centrifuged at 10 000×*g* for 10 min at 0–4°C. The cell pellet thus obtained (1.0 g) was washed with distilled water and suspended in 100 mM phosphate buffer (5 ml) pH 7.0. The cell suspension was sonicated for 3 min at 0–4°C. The sonicated mixture was centrifuged at 10 000×*g* for 10 min at 0–4°C. For larger cell quantities (100 g cell pellet) cell disruption was made in a French press as per standard procedure. The cell-free extract thus obtained was lyophilized at −20°C and the crude enzyme thus obtained had lipase activity of 7000 U/mg solid (specific activity=25 000 U\mg protein). Lipase enzyme activity for ABL and other procured enzymes was assayed as per standard procedure using tributyrin as substrate. One unit of enzyme corresponds to hydrolysis of 1 μmol of tributyrin in 1 min. Protein estimation was made according to Lowry's method taking BSA as the reference protein.[7]

4.2. Preparation of alkyl esters of 1-phenyl-ethanol

Alkyl esters of 1-phenyl-ethanols were prepared from the corresponding secondary alcohols using alkanoic acid anhydrides in the presence of a catalytic amount of 4-dimethylaminopyridine followed by normal workup. Pure esters were obtained by column chromatography over silica gel and elution with hexane/ethyl acetate. The structures of the compounds thus synthesized were confirmed by spectral data (IR, NMR, MS) and elemental analysis. Optical purity (ee) of the products was determined by HPLC (Shimadzu, Japan) using Merck S–S Whelk chiral column: Lichro CART 250-4, 01, 5 μm, mobile phase: *n*-hexane:propan-2-ol:acetic acid (97.80:2.075:0.125), flow rate: 1 ml/min, UV 254 nm detection.

4.3. Procedure for the enzymatic hydrolysis of racemic esters

Racemic esters of 1-phenyl-ethanol and other analogues (1.0 mM) in 0.1 M phosphate buffer (5 ml) pH 7.0 at 15°C was continuously stirred in the presence of crude enzyme preparation (10 mg). The pH of the reaction mixture was maintained in a pH stat at 7.0–7.2 by adding 1 N NaOH. The samples for the analysis were drawn periodically to monitor the progress of reaction by chiral HPLC. The samples for HPLC analysis were prepared by extracting the aliquots (0.1 ml) with HPLC grade (0.5 ml) hexane, centrifuged at 10 000×*g*, filtration through 20 μm filter and injecting 5 μl hexane extract into the HPLC column. After the completion of the enzymatic reaction, the contents were extracted with chloroform or ethyl acetate, concentrated, and chromatographed down a silica gel column to obtain (*R*)-(+)-1-phenyl-ethanol and unhydrolyzed ester. The optical rotation of the alcohol isolated from each reaction mixtures was measured.

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4. Racemic 1-(4-chlorophenyl)-ethanol (II) has been synthesized from 4-chlorobenzaldehyde through its reaction with methyl magnesium iodide in dry diethyl ether. It was analyzed for C_8H_9OCl. ^1H NMR ($CDCl_3$) δ: 1.47 (3H, d, J=6.5 Hz, $–CH_3$), 4.83 (1H, q, J=6.5 Hz, CH), 7.28 (2H, d, J=8.7 Hz, Ar–H), 7.35 (2H, d, J=8.7 Hz, Ar–H); M^+ m/z 156.
5. Racemic 4-fluorophenyl-ethanol (II) has been synthesized from 4-fluorobenzaldehyde through its reaction with methyl magnesium iodide in dry diethyl ether. It was analyzed for C_8H_9OF. ^1H NMR ($CDCl_3$) δ: 1.50 (3H, d, J=6.5 Hz, $–CH_3$), 4.93 (1H, q, J=6.5 Hz, CH), 7.20 (2H, m, Ar–H), 7.35 (2H, m, Ar–H); M^+ m/z 140.
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