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Molecular cloning of enantioselective ester hydrolase from *Bacillus pumilus* DBRL-191

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Abstract

A gene from *Bacillus pumilus* expressed under its native promoter was cloned in *Escherichia coli*. Recombinant *B. pumilus* esterase (BPE) affects the kinetic resolution of racemic mixtures such as unsubstituted and substituted 1-(phenyl)ethanols ($E \sim 33$–103), ethyl 3-hydroxy-3-phenylpropanoate ($E \sim 45$–71), *trans*-4-fluorophenyl-3-hydroxymethyl-*N*-methylpiperidine ($E \sim 10$–13) and ethyl 2-hydroxy-4-phenylbutyrate ($E \sim 7$). The enzyme is composed of a 34-amino acid signal peptide and a 181-amino acid mature protein corresponding to a molecular weight of ~19.2 kD and pI ~ 9.4. 3-D the structural model of the enzyme built by homology modelling using the atomic coordinates from the crystal structure of *B. subtilis* lipase (LipA) showed a compact minimal α/β hydrolase fold.

Keywords: Phenyl–Sepharose; Drug intermediates; Kinetic resolution; Enantioselective hydrolysis

1. Introduction

Production of enantiopure organic compounds is assuming increasing importance for chemical and pharmaceutical industries [1–7]. Enantiopure pharmaceuticals accounted for 36% of the US $410 billion market in 2001 and was expected to grow by >9% average annual growth rate between 2002 and 2005 [8]. One of the efficient options to carry out asymmetric synthesis for enantiomerically pure or enriched organic compounds is through the use of biocatalysts. Large numbers of microorganisms or the enzymes derived from them have been employed for the production of optically active compounds by means of kinetic resolution or stereo-specific chemical transformations [9–12] and efforts to find new biocatalysts and their applications continue [13,14]. Similarly, we have reported a number of enantioselective biotransformations of drugs or drug intermediates utilizing either commercial lipases/esterases or our own isolated microorganisms and enzymes derived from them [15–18]. In most cases, however, the microbial enzymes are expressed at low levels in wild isolates. Therefore, an approach which allows the production of an enantioselective enzyme for a reaction of choice at an affordable cost would be appropriate, hence cloning and over expression becomes imperative. Moderation of substrate selectivity can be achieved by subjecting the selected genes to site-specific mutagenesis or by random/directed evolution. These molecular techniques have been used to modify stereoselectivity as well as other properties of enzymes [19]. Hence, identification, cloning and hyper expression of the gene encoding

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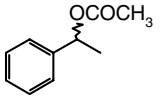
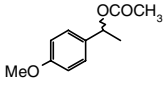
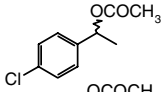
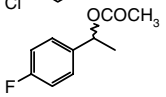
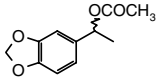
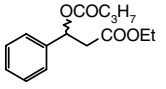
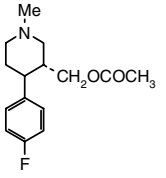
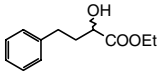
the enzyme of choice is often required. In this paper, we describe the molecular cloning, nucleotide sequencing and expression of a stereoselective esterase gene from *B. pumilus* (DBRL-191) isolated from a soil sample obtained from Northwest Himalayas and deposited in the culture collection of RRL Jammu (India). Characterization of the protein sequence, the biochemical properties, and homology modelling to elucidate the 3D-structure of *B. pumilus* esterase (BPE) are described.

2. Materials and methods

2.1. Chemicals

Prepacked phenyl–Sepharose CL-6B, Mono S columns were obtained from Amersham Pharmacia Biotech (USA). Tris base, acrylamide, SDS, bromophenol blue, protein markers for Native and SDS–PAGE, grades for polyacrylamide gels, dithiothreitol (DTT), β-mercaptoethanol, lysozyme, ampicillin, isopropyl β-D-thiogalactoside (IPTG), phenylmethylsulphonyl fluoride (PMSF) and agarose were obtained from Sigma chemical (USA), Triton X-100, *p*-nitrophenyl esters such as acetate, butyrate, palmitate, laurate, stearate, caprylate, caproate and triacetin, tributyrin, tristearin, triolein, olive oil, gum arabic, α-naphthyl acetate and fast blue RR were obtained from Himedia, Pvt. Limited, Bombay. Oligonucleotide primers, deoxynucleotides *Taq* polymerase, T7 and SP6 promoter sequences, T4-DNA ligase and restriction endonucleases were purchased from Bio Basics Inc. Canada and Amersham Pharmacia Biotech (USA). All other chemicals for growth media used for this work were obtained from Glaxo (Qualigens), New Delhi and E. Merck, Germany. HPLC grade solvents were purchased from E. Merck/Glaxo (Bombay). Chiral

Table 1
Hydrolysis of racemic acylates/esters of different substrates by native and recom. BPE[a,b]

Entry	Esterase	Structure	Subs.: biocat.	Time (h)	Conv. (%)	Subs. conc (g/L)	Conf prod.[h]	ee (%)	E[g]
1	Native		5:1	8	16	40	*R*	93[c]	32
	Recom. pure			8	17	40	*R*	94[c]	39
2	Native		5:1	8	34	40	*R*	96[c]	80
	Recom. pure			8	36	40	*R*	96[c]	100
3	Native		5:1	8	31	40	*R*	95[c]	59
	Recom. pure			8	30	40	*R*	95[c]	69
4	Native		5:1	8.0	33	40	*R*	95[c]	62
	Recom. pure			8.0	35	40	*R*	96[c]	98
5	Native		5:1	8.0	35	40	*R*	90[c]	33
	Recom. pure			8.0	33	40	*R*	92[c]	39
6	Native		1:2	10.5	41	30	*R*	91[d]	45
	Recom. pure			10.5	42	30	*R*	94[d]	70
7	Native		1:3	36.0	42	200	3*R*,4*S*	76[e]	12
	Recom. Pure			36.0	33	200	3*R*,4*S*	75[e]	10
8	Native		1:0.6	46.0	71	40	*R*[i]	72[f]	6.8
	Recom. pure			46.0	70	40	*R*[i]	68[f]	6.8

[a] Reaction temperature 25 °C.
[b] Reaction medium 0.1 M phosphate buffer pH 7.0 (substrate 1–6) pH 6.5 (substrate 7).
[c] ee% measured on Chiracel OD-H chiral column using hexane:isopropanol (90:10) mobile phase (0.6 ml/min).
[d] ee% measured on Chiracel OD-H chiral column using hexane:isopropanol (95:05) mobile phase (0.6 ml/min).
[e] ee% measured on Chiradex column using methanol:water:triethyl amine (1:1:0.01) mobile phase (0.8 ml/min).
[f] ee% determined by comparison of optical rotation with known optical value $[\alpha]_D$ −8.4 (c 1.15, EtOH, *R*-ester ee ~ 99%).
[g] E calculated according to Chen et al. [40].
[h] Absolute configuration determined on the basis of sign of specific rotation.
[i] Enriched ester.

columns (S,S)-Whelk-O1, (Lichro Cart 250-4, 5 μm), Chiradex (E-Merck) and Chiralcel OD-H (Diacel, Japan) were used to monitor the enantiomeric purity of the resolved compounds. Substrates 1–5 (Table 1) were prepared by Grignard reaction of the corresponding aldehydes in near quantitative yields. Compound 6 (Table 1) was prepared by hydrogenation of the commercially available ethyl benzoylacetate (Pd/C 5%, 50 psi) and purification by column chromatography over silica gel using 2% ethyl acetate in *n*-hexane to give racemic mixture (85% yield) and compound 8 (Table 1) was prepared from commercially available ethyl 2-oxo-4-phenylbutyrate by the procedure described for compound 6 after column chromatography over silica gel and elution with dichloromethane:ethyl acetate (4:1) to obtain racemic 8 (65% yield). The compound 7 (Table 1) was procured from Cadilla Health Care (Ahmedabad, India) as a research sample. The racemic derivatives of 7 were prepared by allowing racemic 7 to react with respective alkanoic anhydride at room temperature and after the completion of the reaction, the contents concentrated on rotavapor followed by chromatography over neutral alumina with *n*-hexane: ethyl acetate (97:3) as eluent to give respective acyl esters (90–95% yield). The chemical structures of all the synthesised compounds were characterised by spectral analysis.

2.2. Bacteria, plasmids and media

The bacterial strain *B. pumilus* (DBRL-191) was isolated from one of the selected soil collection sites in Northwestern Himalayas, India. Different microbiological methods and 16S rRNA gene typing were used to identify the strain. The DBRL-191 strain served as the source for genomic DNA. *E. coli* JM110 (Promega, Madison, USA) and *E. coli* BL21(DE3)pLacI (Novagen, USA) were used as cloning and expression hosts. Plasmid pGEM5Z (Promega, Madison, USA) and pETBlue-2 (Novagen, USA) were used as cloning and expression vectors. DBRL-191 was grown overnight at 30 °C in LB medium consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl at pH 7.2 for genomic DNA preparation. Plasmid DNAs were isolated using a nucleospin plasmid isolation kit (Qiagen). Transformation of *E. coli* with recombinant plasmids was performed by electroporation using Gene pulsar (Bio Rad).

2.3. Cloning and sequencing of the esterase gene

Genomic DNA was prepared by the method developed by Pitcher et al. [20]. Genomic DNA from DBRL-191 was completely digested with *Hin*dIII and ligated with *Hin*dIII-digested/dephosphorylated pGEM5Z vector. The ligated mixture was used to electro-transform *E. coli* (JM110). Out of about 5000 colonies, a colony forming a clearing zone on tributyrin-LB plates containing ampicillin 100 μg ml^{-1} and streptomycin 50 μg ml^{-1} was selected. Recombinant plasmid designated as pGEM-Bpest was purified from the transformant and the insert DNA sequence determined on a 310-ABI-PRISM Genetic Analyzer (Perkin–Elmer, USA) using Big Dye sequencing kit (Applied Biosystems Inc.). Gene prediction was performed by DNASTAR using Megalign software and signal peptide analysis [21] Compute pI/MW [22–24] and CLUSTAL W [25].

The nucleotide sequence of the *B. pumilus* (DBRL-191) esterase gene in pGEM-Bpest plasmid was deposited in the Genbank sequence database under the Accession Number AY494714.

2.4. Construction of expression plasmids

Primer design software in ExPASY website was used to construct primers. A pair of degenerate primers was designed carrying sites for *Nde* I at the 5′ end (immediately upstream of mature protein) of the BPE gene and *Xho* I at its 3′ end. The primers used were forward 5′ GGG CAT ATG GCT GAR CAY AAY CCN and reverse 3′ GGG CTC GAG ATT CGT ATT CTG TCC. *Pfu* polymerase was used for PCR with pGEM-Bpest DNA as the template. The PCR conditions were as follows: initial denaturation step at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, except for the final cycle where extension proceeded for 10 min. The PCR product was resolved in a 1% (w/v) agarose gel, and the 648 bp DNA fragment was recovered with GenElute gel DNA extraction kit (Sigma–Aldrich). The purified DNA fragment was ligated into *Eco* RV-cleaved pETBlue-2, according to the manufacturers recommendations, then electro-transformed into *E. coli* BL21 (DE3) pLacI and plated on LB media containing 50 μg ml^{-1} carbenicillin and 34 μg ml^{-1} chloramphenicol. The clone containing the correct insert encoding mature protein was identified by restriction enzyme analysis and denoted as pET-Bpest.

A single colony containing pET-Bpest was grown in 100 ml LB medium containing 50 μg ml^{-1} carbenicillin and 34 μg ml^{-1} chloramphenicol to an OD_{600} of 0.8. IPTG was added to the culture to 1 mM and incubation continued at 30 °C for 4–6 h. Cells were harvested by centrifugation at 5000×*g* for 10 min at 4 °C, resuspended in 50 mM Tris HCl buffer (pH 7.0) containing 1 mM DTT, 2 mM EDTA and 100 mM ammonium sulphate for 30 min, followed by disruption in MSE 20 kHz ultrasonicator at 4 °C for 5 min with intermittent cooling to release intracellular proteins. The cell-free extract was centrifuged at 15,000×*g* for 20 min to remove cell debris, and the extract assayed for enzyme activity and stored at −20 °C.

2.5. Purification of recombinant esterase

Protein was estimated spectrophotometrically by the Bradford method using Bovine serum albumin (BSA) as standard [26]. The enzyme was salted out by ammonium sulfate precipitation (25–65% saturation) and purified on FPLC (Amersham Pharmacia Biotech) using phenyl–Sepharose followed by Mono S column. Native and SDS–PAGE was performed according to the method of Laemmili [27].

2.6. Enzyme assays

Agar diffusion assay was followed using tributyrin as substrate [28]. Detection of lipase activity was performed by the Rhodamine B plate assay [29]. Esterase activity was quantitatively assayed by titrimetry, using tributyrin as substrate [30]. One unit of enzyme activity was expressed as the amount of enzyme that releases 1 μmol of titreable fatty acid per min. For the estimation of enzyme activity using different *p*-nitrophenyl esters as substrates, 20 μl enzyme was added to 880 μl of 0.1 M potassium phosphate buffer, pH 8.0 containing 0.1% (w/v) gum arabic and 0.2% (w/v) deoxycholate. After 3 min incubation at 37 °C, the reaction was initiated by adding 100 μl of substrate (8 mM) solubilised in acetonitrile. The reaction was stopped by addition of 0.5 ml 3 M HCl. The reaction mixture was centrifuged for 10 min and the supernatant mixed with 2 M NaOH and the OD_{420} recorded. Enzyme activity was also directly located on polyacrylamide gel zymogram by the method of Gabriel [31].

2.7. Enantioselectivity assay

Pure substrates thus obtained were incubated with the enzyme in sodium phosphate buffer (0.1 M) pH 7.0 and continuously stirred at ambient temperature. Progress of the enzymatic hydrolysis at various substrates (1–7; Table 1) was monitored by periodic withdrawal of an aliquot from the assay mixture, extraction with ethyl acetate (HPLC grade) and its analysis on chiral HPLC columns (Chiralcel-OD-H/Chiradex) as well by TLC run on silica gel $60F_{254}$ plates (Merck) or Aluminium oxide $60F_{254}$ (neutral) plates (Merck).

In the case of the resolved substrate 8, enantiomeric excess and absolute configuration was determined on the basis of sign of specific rotation (Perkin–Elmer 241 Polarimeter) and its comparison with reported values in the literature.

2.8. Enzymatic hydrolysis of racemic acylates of secondary alcohols and drug intermediates using native and recombinant enzyme

Two sets, each comprising of racemic acylates of substrates 1–7 (1 mmol), were suspended in sodium phosphate buffer (0.1 M) pH 7.0, and to one set was added native BPE and to the other set was added recombinant BPE at specified pH and temperature in a ratio given in Table 1. The pH of the reaction mixture was maintained by a pH stat with 0.5 N NaOH. 25 μl sample aliquots were extracted with a mixture of HPLC grade ethyl acetate (100 μl) and hexane (400 μl). The samples were centrifuged at 6000*g* and the organic layer filtered through 20 μM filter and analysed on HPLC using Chiralcel-OD-H column, with mobile phase *n*-hexane:2-propanol (90:10) at a flow rate 0.6 ml min^{-1} for chiral secondary alcohols (1–5) (Table 1). For compound 6 (Table 1) as a substrate, *n*-hexane:2-propanol (90:05) was used as the mobile phase at a flow rate of 0.8 ml min^{-1} and 25 °C using a PDA detector. For the isolation of the biotransformed products (after the completion of the enzymatic reaction) of compounds 1–6, the contents were extracted with ethyl acetate, concentrated in vacuo and the products charged on silica gel column using a gradient of *n*-hexane:ethyl acetate to separate the resolved hydrolysed product and the optically enriched acyl ester in overall yields of 70–93%. The enantiomeric excess of resolved 7 (Table 1) was determined on a Chiradex chiral column using methanol:water (50:50) as the mobile phase at flow rate 0.8 ml min^{-1}. For the isolation of transformed products after the completion of the reaction, the contents were separated by extraction with dichloromethane to remove enriched acylate and the aqueous portion was concentrated to dryness, then redissolved in dichloromethane:methanol mixture (19:1) and purified through a neutral alumina column after elution with a gradient of dichloromethane: methanol mixture to provide enriched (−)-alcohol (yields 85–90%).

2.9. Enzymatic hydrolysis of racemic ethyl 2-hydroxy-4-phenylbutyrate (8) using native and recombinant enzyme

Racemic 8 (1 mmol) in sodium phosphate buffer (0.1 M) was incubated in the presence of native and recombinant BPE separately while maintaining pH 7.0 with 0.5 M NaOH. In this case, the hydrolysis reaction was continued up to ~70% conversion (Table 1). Thereafter, the contents of the reaction were adjusted to pH 5.0 and extracted with ethyl acetate, the organic layer washed with water, dried over anhydrous sodium sulphate and concentrated to give crude bio-products which on column chromatography over silica gel and elution with a gradient of dichloromethane:ethyl acetate afforded the transformed products in overall yields of 70–73%.

The enantiomeric excess of (*R*)-ester and (*S*)-acid of 8 were determined on the basis of their optical rotation values with those reported in the literature.

2.10. Homology modelling of BPE

A molecular model was developed for BPE using the technique of comparative homology modelling with the help of Molecular Operating Environment (MOE) software. The template structure used for development of the homology model was the resolved X-ray structure of *B. subtilis* lipase LipA with Protein database entry code 1I6W [32].

3. Results and discussion

3.1. Cloning and nucleotide sequencing of BPE esterase gene

The *B. pumilus* strain DBRL 191 produces an enantioselective ester hydrolase (BPE), which is capable of enantioselectively hydrolysing the esters of chiral auxillaries, drug intermediates of fluoxetine, paroxetine (anti-depressant) and angiotensin converting enzyme inhibitor (Table 1). Since the activity of secreted ester hydrolase was not stable in the medium, probably owing to proteolytic degradation, cloning of the gene encoding BPE in a suitable host was attempted. A genomic library of DBRL-191 was constructed in *E. coli* (JM110) using pGEM5Z as the vector. Screening of the library led to the selection of a clone which after 24 h incubation formed a clear halo on LB plate due to hydrolysis of tributyrin. The transformant also produced orange fluorescence under UV on Rhodomine B plates with triolein as substrate indicating lipolytic activity of the clone. The recombinant plasmid (pGEM-Bpest) isolated from the transformant had an approximately 1.0 kb insert. Sequencing of the cloned DNA fragment indicated one major open reading frame of 648 bp, which encodes a polypeptide of 215 amino acids. BLAST [33] search showed that the recombinant enzyme displayed biochemical and structural features common to alkali-tolerant lipases belonging to the Bacilli lipase subfamily I.4 of family I with 80–95% similarity at the amino acid level [32,34,35]. The computer data analysis of the sequence suggests that BPE is a basic protein with pI $\sim$ 9.44 and a molecular mass of mature enzyme $\sim$19–20 kD, which is in agreement with the properties of alkali-tolerant lipases of Bacilli.

3.2. Expression and purification of BPE

For the expression of mature enzyme in *E. coli*, recombined expression vector pET-Bpest was transformed into *E. coli* BL21 (DE3)pLacI. The construct under the control of the T7 promoter was inducible with IPTG. Esterase activity was present in the cytosolic fraction of clone BL21/pET-Bpest. Inhibition of growth was observed for recombinant expressing the heterologous protein. Compared with the specific activity of 1.8 U mg^{-1} protein with the wild strain, the esterase expression increased by >5-fold in BL21 (DE3)pLacI (10.0 U mg^{-1} crude protein). Esterase expression was observed in the cytosolic fraction only and no inclusion bodies were found.

Mature BPE from pGEM-Bpest was purified over 850-fold to obtain a specific activity of 1533 U mg^{-1} protein (Table 2).

3.3. BPE catalysed resolution of racemic acylates/esters of some important drug intermediates/chiral auxillaries

Both native and recombinant BPE were used for the enantioselective hydrolysis of racemic esters of some important drug intermediates or auxiliaries with the purpose of detecting any significant variation in their reactivity and stereoselectivity profiles. It was observed that in the resolution of racemic alkylacyl esters of secondary alcohols (1–5 Table1), the cell-free extracts for wild and recombinant strains displayed high selectivity for acetyl derivatives (ee $\sim$ 92–97%) and moderate to low selectivity for the higher homologues furnishing *R* alcohols. The presence of different substituent groups in the phenyl ring did not influence the reactivity or stereoselectivity to any appreciable extent. On the other hand, acylates of racemic 6 (Table 1) underwent facile hydrolysis with high selectivity for butyrate as compared to acetate and propionate thus furnishing *R* enriched products (ee $\sim$ 91–95%). Although there are two hydrolysable groups present in 6 (Table 1), BPE displayed preference for acyl esters leaving the carboxy ester in-

Table 2
Purification of recombinant BPE

Fractions	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	% Yield
Cell free extract	3071	1708	1.8	1	100
25–65% ammonium sulfate precipitation.	2350	62.4	3.8	2	76.50
Phenyl–Sepharose	1482	8.9	166.5	93	48.25
Mono S	550	0.36	1533	851	17.90

Activity is expressed as unit equivalents of fatty acids generated per min from tributyrin.

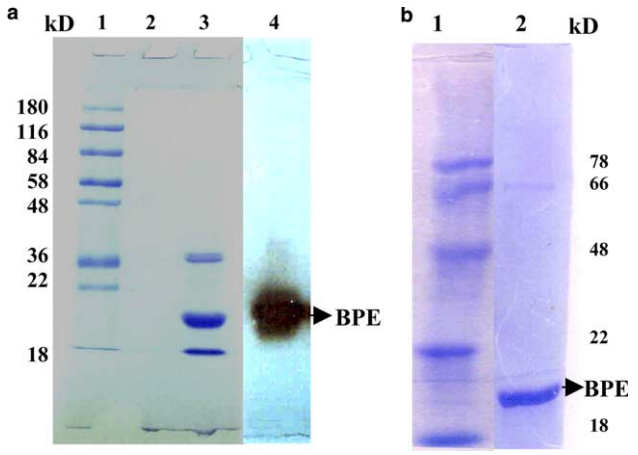


Fig. 1. (a) 13% SDS–PAGE of BPE. Lane 1, MW markers; lane 3, partially purified enzyme; lane 4, stained for activity. (b) 11% SDS–PAGE of purified BPE. Lane 1, MW markers; lane 2, purified enzyme.

tact. The enzyme also facilitated the hydrolysis of acylates of 7 (Table 1), showing preference for acetate, thus furnishing required (3*R*, 4*S*)-enantiomer of 75.6% enantiopurity. The rate of reaction as well as selectivity was poor for propionate and butyrate derivatives (ee < 10%). It should be mentioned here that only a few enzymes have reportedly displayed the capability of resolution of paroxetine intermediate 7 [36–38]. BPE also displayed the capacity to resolve racemic 8 (Table 1), displaying moderate selectivity for *S*-isomer leaving the required *R*-ester of 70–72% ee after 71–72% conversion. From these studies it is evident that there are no significant differences in activities/selectivity between native or recombinant BPE. BPE therefore offers an opportunity to develop a biocatalyst bearing broad substrate specificity with applications for industrial-scale kinetic resolutions of racemic mixtures of value. The results of these BPE-catalysed reactions are summarized in Table 1, wherein the typical selectivity data for different substrates have been included.

3.4. Characterization

SDS–PAGE analysis indicated that purified recombinant BPE is a monomer with a molecular mass of ~20 kD (Fig. 1(a) and (b)). The enzyme is optimally active at pH 9.0 and 37 °C and fairly stable up to 40 °C. BPE belongs to the family of serine hydrolases as it was completely inhibited by serine reactive agent PMSF (2 mM), which is true for most of the known microbial ester hydrolases.

3.5. Substrate specificity of BPE

The substrate specificity for the recombinant esterase was studied with *p*-nitrophenyl fatty acid esters of various chain lengths and natural triglycerides. The highest hydrolysis rates were obtained with *p*-nitrophenyl butyrate (C4) and *p*-nitrophenyl caproate (C6), which exhibited relative activities of 90% and 100%, respectively. *p*-nitrophenyl esters of lauric (C12), palmitic (C16) and stearic (C18) exhibited relative activities of 25–45%. Similarly, the highest ester hydrolytic rates were obtained with substrates like triacylglycerides with C4–C6 chain lengths. The profile of substrate specificity suggests the presence at its active site of a hydrophobic binding pocket able to accommodate optimally short- and medium-length acyl chains. However, long chain esters (C12–C18) were hydrolysed as well, albeit at a lower rate.

3.6. Structure elucidation of BPE

A three dimensional structural model of BPE was proposed by homology modelling using the atomic coordinates from the crystal structure of *B. subtilis* lipase (LipA) [32]. This structure showed a similar α/β hydrolase fold common to all ester hydrolases, but with the absence of a region of non-polar residues in the form of a lid. The active-site residues Ser77, Asp133 and His156 constitute the catalytic triad of BPE. Alignment of the secondary structure elements of BPE and LipA showed a similar structure with a single compact domain that consists of six β-sheets, surrounded by five α-helices. A superimposition of the BPE three-dimensional model with the 3D-structure of LipA from *B. subtilis* revealed a relative mean standard difference of 0.54 °A for the 181 aligned amino acid residues (data not shown). The 3D-structure of the two superimposed models appeared similar with no hydrophobic lid present and the active site solvent exposed. Ala most likely replaced Gly75 in the conserved pentapeptide G-X-S-X-G around the active site Ser77. The primary sequence showed that most of the non-homologous residues are placed away from the nucleophile Ser77 and are not clustered at one place (Fig. 2). In the core of BPE, in place of the Val96 and Leu134 present in LipA, Ile96 and Met134 residues could be located. Although LipA and BPE are of similar size and their hydrophobic surfaces around the active site are similar, significant differences in their preferences for natural and synthetic substrates and with respect to enantioselective hydrolytic profile (data not shown) might be due to the variations in amino acids at the surface of the molecules. Crystallographic and molecular-modelling studies in the literature have shown that small differences affected by a few amino acid substitutions are able to greatly affect the enzyme enantioselectivity [39]. The fact that BPE can be easily expressed and purified from *E. coli* makes the enzyme a very interesting candidate for optimisation by site-specific mutagenesis or directed evolution to produce a modified enzyme with the desired selectivity.

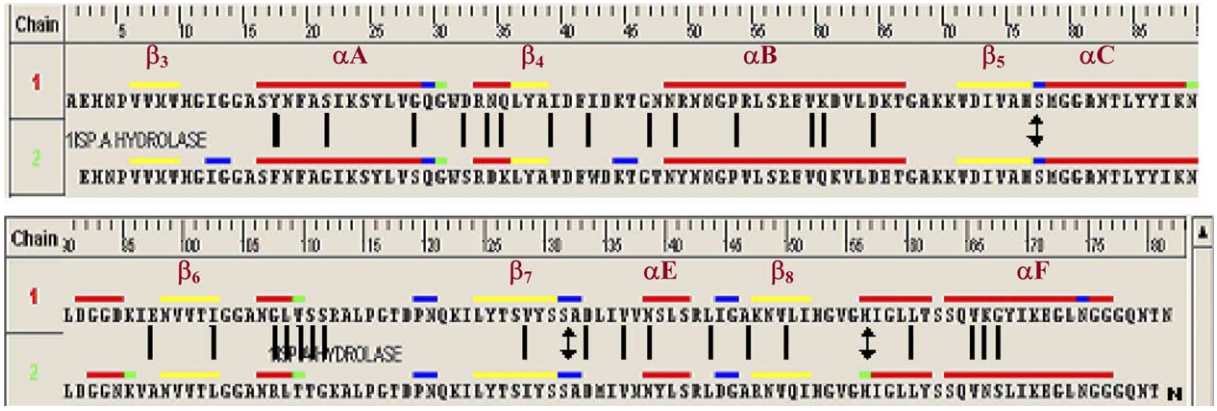


Fig. 2. Sequence alignment of *Bacillus subtilis* Lip A (upper line) and *Bacillus pumilus* (DBRL-191) esterase (lower line). Non-identical residues are boxed in black and the catalytic triad residues are highlighted in green and marked by arrows.

References

[1] Patel, R.N. (2002) Microbial enzymatic synthesis of chiral intermediates for pharmaceuticals. Enzyme Microb. Technol. 31, 804–826.

[2] Reetz, M.T. (2002) Lipases as practical biocatalysts. Curr. Opin. Chem. Biol. 6, 145–150.

[3] Gotor, V. (2002) Biocatalysis applied to the preparation of pharmaceuticals. Org. Process Res. Develop. 6, 420–426.

[4] Davis, B.G and Boyer, V. (2001) Biocatalysis and enzymes in organic synthesis. Natl. Prod. Rep. 18, 618–640.

[5] Schmid, A., Dordick, J.S., Hauer, B., Kieners, A., Wubbolts, M. and Witholt, B. (2001) Industrial biocatalysis today and tomorrow. Nature 409, 258–268.

[6] Koeller, K.M. and Wong, C.H. (2001) Enzymes for chemical synthesis. Nature 409, 232–240.

[7] Collins, A.N., Sheldrake, G.N. and Crosby, J., Eds., (1997). Chirality in Industry II: Developments in the Commercial Manufacture and Applications of optically Active Compounds. Wiley, Chichester.

[8] Rouhi, A.M. (2002) Chiral round up. Chem. Eng. News, 42–50.

[9] Burg, B. van den (2003) Extremophiles as a source of novel enzymes. Curr. Opin. Microbiol. 6, 213–218.

[10] Laumen, K., Kittelmann, M. and Ghisalba, O. (2002) Chemoenztymatic approaches for the creation of novel chiral building blocks and reagents for Pharmaceutical application. J. Mol. Catal. B: Enzymatic, 55–56.

[11] Drauz, K. and Waldman, H. (2002) Enzyme Catalysis in Organic Synthesis. Wiley-VCH Verlag GmbH, Weinheim.

[12] Faber, K. (1999) Biotransformations in Organic Chemistry. Springer-Verlag, Berlin.

[13] Asano, Y. (2002) Overview of screening for new microbial catalysts and their use in organic synthesis-selection and optimisation of biocatalysis. J. Biotechnol. 94, 65–72.

[14] Kers, J.A., Wach, M.J., Krasnoff, S.B., Widom, J., Cameron, K.D., Bukhalid, R.A., Gibson, D.M., Crane, B.R. and Loria, R. (2004) Nitration of a peptide phytotoxin by bacterial nitric oxide synthase. Nature 429, 79–82.

[15] Kapoor, M., Anand, N., Ahmad, K., Koul, S., Chimni, S.S., Taneja, S.C. and Qazi, G.N (2005) Synthesis of beta-adrenergic blockers. (*R*)-(−)-nifenalol and (S)-(+)-sotalol via a highly efficient resolution of a bromohydrin. Tetrahedron: Asymmetry 16, 717–725.

[16] Anand, N., Kapoor, M., Koul, S., Taneja, S.C., Sharma, R.L. and Qazi, G.N. (2004) Chemoenzymatic approach to optically active phenylglycidates: resolution of bromo-and iodohydrins. Tetrahedron: Asymmetry 15, 3131–3138.

[17] Koul, S., Parshad, R., Taneja, S.C. and Qazi, G.N. (2003) Enzymatic resolution of Naproxen. Tetrahedron: Asymmetry 14, 2459–2465.

[18] Maqbool, Q.A., Johri, S., Verma, L., Verma, V., Koul, S., Taneja, S.C., Parshad, R. and Qazi, G.N. (2002) Purification and characterization of novel enantioselective ester hydrolase from *Bacillus subtilis*. Biotechnol. Appl. Biochem. 36, 1–8.

[19] Liebeton, K., Zonta, A., Schimossek, K., Nardini, M., Lang, D., Dijkstra, B.W., Reetz, M.T. and Jaeger, K.E. (2000) Directed evolution of an enantioselective lipase. Chem. Biol. 7, 709–718.

[20] Pitcher, D.G., Saunders, N.A. and Owen, R.J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8, 151–156.

[21] Neilsen, H., Engelbrecht, J., Brunak, S. and Von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

[22] Bjellqvist, B., Hughes, G.J., Pasquali, C.H., Paquet, N., Ravier, F., Sanchez, J.C.H., Frutiger, S. and Hochstrasser, D.F. (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 14, 1023–1031.

[23] Bjellqvist, B., Bassa, B., Olsen, E. and Celis, J.E. (1994) Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis 15, 529–539.

[24] Wilkins, M.R., Gasteiger, E., Bairoch, A., Sanchez, J.C., Williams, K.L. and Hochstrasser, D.F. (1998) Protein identification and analysis tools in the ExPASy server In: Proteome Analysis Protocols (Link, E.J., Ed.). Humana Press, New Jersey.

[25] Thompson, J.D., Higgins, D.G., Gibson, T.J. and Clustal, W. (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

[26] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

[27] Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

[28] Lawrence, R.C., Fryer, T.F. and Reiter, B. (1967) Rapid method for the quantitative estimation of microbial lipases. Nature 213, 1264–1265.

[29] Kouker, G. and Jaeger, K.E. (1987) Specific and sensitive plate assay for bacterial lipases. Appl. Env. Microbiol. 53, 211–213.

[30] Staubmann, R., Ncube, I., Gubitz, G.M., Steiner, W. and Read, J.S. (1999) Esterase and lipase activity in *Jatropha curcas* L. seeds. J. Biotechnol. 75, 117–126.
[31] Gabriel, O. (1971) Locating enzymes on gels In: Methods in Enzymol (William, Jakoby B., Ed.), Vol. 22, pp. 578–604. Academic Press Inc., London.
[32] Pouderoyen, G., Eggert, T.E., Jaeger, K.E. and Digkstra, B.W. (2001) The crystal structure of *Bacillus subtilis* lipase: a minimal α/β hydrolase fold enzyme. J. Mol. Biol. 309, 215–226.
[33] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[34] Nthangeni, M.B., Patterton, H.G., Tonder, A., Vergeer, W.P. and Litthauer, D. (2001) Over expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on *Bacillus* lipases. Enzyme Microbiol. Technol. 28, 705–712.
[35] Kim, H.K., Choi, H.J., Kim, M.H., Sohn, C.B. and Oh, T.K. (2002) Expression and characterization of Ca^{2+}-independent lipase from *Bacillus pumilus* B26. BBA-Mol. Cell Biol. Lipids 1583, 205–212.
[36] Yu, M.S., Lantos, I., Peng, Z.Q., Yu, J. and Cacchio, T. (2000) Asymmetric synthesis of (−)-paroxetine using PLE hydrolysis. Tetrahedron Lett. 41, 5647–5651.
[37] Gonzalo, de G., Brieva, R., Sanchez, V.M., Bayod, M. and Gotor, V. (2001) Enzymatic Resolution of *trans*-4-(4′-fluorophenyl)-3-hydroxymethyl-piperidines. Key intermediates in the synthesis of (−)-paroxetine. J. Org. Chem. 66, 8947–8953.
[38] Gonzalo, de G., Brieva, R., Sanchez, V.M., Bayod, M. and Gotor, V. (2003) Enzymatic alkoxycarbonylation reactions on the intermediate in the synthesis of (−)-paroxetine,*trans*-*N*-benzyloxycarbonyl-4-(4′-fluorophenyl)-3-hydroxymethylpiperidine. Tetrahedron:Asymmetry 14, 1725–1731.
[39] Simons, J.W.F.A., Van Kampen, M.D., Ubarretxena-Belandia, I., Cox, R.C., Alves dos Santos, C.M., Egmong, M.R. and Verheji, H.M. (1999) Identification of a calcium binding site in *Staphylococcus haemolyticus* lipase: generation of calcium-independent variant. Biochemistry 38, 2–10.
[40] Chen, C.S., Fujimoto, Y., Girdaukas, G. and Sih, C.J. (1982) Quantitative analyses of biochemical kinetic resolutions of enantiomers. J. Am. Chem. Soc. 104, 7294–7299.