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Comparison and functional characterisation of three homologous intracellular carboxylesterases of *Bacillus subtilis*

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Abstract

Enzymatic hydrolysis of racemic mixtures may provide an attractive method for the enantiopure production of chiral pharmaceuticals. For example, the carboxylesterase NP of *Bacillus subtilis* Thai I-8 is an excellent biocatalyst in the kinetic resolution of NSAID esters, such as naproxen and ibuprofen methyl esters. Two homologues of this enzyme were identified when the genome sequence of *B. subtilis* 168 was revealed in 1997. We characterised one of the homologous, YbfK, as a very enantioselective 1,2-*O*-isopropylidene-*sn*-glycerol caprylate esterase, while only modest enantioselectivity towards the naproxen ester was observed. The other homologue, the carboxylesterase NA has not been characterised yet. The purpose of the present study was to fully characterise these three highly homologous esterases with respect to their applicability towards the enantiospecific hydrolysis of a wide range of compounds. The esterase genes were cloned and expressed in *B. subtilis* using a combination of two strong promotors in a multi-copy vector. After purification of the enzymes from the cytoplasm of *B. subtilis*, the biochemical and enantioselective properties of the enzymes were determined. Although all carboxylesterases have similar physico-chemical properties, comparison of their specific activities and enantioselectivities towards several compounds revealed rather different substrate specificities. We conclude that carboxylesterase NP and carboxylesterase NA are particularly suited for the enzymatic conversion of naproxen esters, while YbfK offers enantiopure (+)-IPG from its caprylate ester. Given the carboxylesterase activities of the esterases it has been proposed to rename the *nap* gene of *B. subtilis* 168 into *cesA* and the *ybfK* gene into *cesB*.

Keywords: *Bacillus subtilis* 168; Carboxylesterase; YbfK; 1,2-*O*-isopropylideneglycerol; Enantioselective production

1. Introduction

Carboxylesterases (E.C. 3.1.1.1.) represent a diverse group of hydrolytic enzymes catalysing the cleavage and formation of ester bonds. In spite of their distribution throughout humans, animals, plants and microorganisms, their physiological functions remain to be elucidated. Nevertheless, due to their high stability, their activity in organic solvents, and their high regio- and stereo-specificity, carboxylesterases appear to be attractive biocatalysts for organic chemistry [1].

Many bacterial esterases have been cloned and overexpressed during the last decades in order to assess their enantioselective properties (e.g. [2–6]). The most attractive biocatalysts seem to originate from *Bacillus* and *Pseudomonas* species (for a review see [7]). For example, the naproxen esterase of *Bacillus subtilis* Thai I-8 was characterised as a very efficient enantioselective biocatalyst for the kinetic resolution of non steroidal anti-inflammatory drug (NSAID) esters, such as the naproxen and ibuprofen methyl ester. Its modest selectivity towards chiral alcohols such as the interesting chiral intermediate 1,2-*O*-isopropylideneglycerol [8–10] is in sharp contrast with its enantioselective properties towards chiral carboxylic acids.

Although many well-established methods are available today for the directed evolution of the enantioselective properties of a potential biocatalyst [11–19], the search for homologous and paralogous genes in the sequence information derived from several genome projects, for example the genome project of *B. subtilis* 168, offers an attractive

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alternative approach for finding alternative biocatalysts for rational drug preparation.

In *B. subtilis* 168, nine genes have been functionally characterised as intracellular or extracellular esterases. In addition, eight genes can be classified as potential esterases [4,20–30]. Two of these esterases of *B. subtilis* 168, carboxylesterase NA (encoded by the *nap* gene) and YbfK (*ybfK* gene) showed a high homology towards the carboxylesterase NP of *B. subtilis* Thai I-8 (98 and 64% identity on protein level, respectively). Given the carboxylesterase activities of the esterases it has been proposed to rename the *nap* gene of *B. subtilis* 168 into *cesA* and the *ybfK* gene into *cesB*. Recently, the CesB protein was characterised as a very enantioselective 1,2-*O*-isopropylideneglycerol esterase, while only modest enantioselectivity towards naproxen esters was observed [22]. In contrast, CesA has not been characterised yet.

In this paper, we describe the production of these three highly homologous carboxylesterases in *B. subtilis*, and their biochemical characterisation. A pH and temperature dependency profile was established for all three enzymes. In particular, we have determined the specific activity of the three enzymes towards several chiral compounds, with chirality residing in both the carboxylic acid part as well as the alcohol part of the ester, in order to investigate the applicability of these enzymes for kinetic resolution experiments.

2. Experimental

2.1. Plasmids, bacterial strains and media

The plasmids and bacterial strains that were used in the present study are listed in Table 1. The following media were used: (1) 2 × TY medium containing bactotryptone (1.6%), bacto yeast extract (1%) and sodium chloride (0.5%); (2) medium to prepare *B. subtilis* competent cells containing 100 mM potassium phosphate buffer, pH 7, 1% glucose, 0.4% potassium L-glutamate, 3 mM trisodium citrate, 3 mM $MgSO_4$, 0.0022% ferric ammonium citrate, 0.1% casein hydrolysate and 0.002% L-tryptophan [31]. Antibiotic agents were used in the following concentrations: ampicillin 100 μg ml^{-1}, kanamycin 20 μg ml^{-1}, and chloramphenicol 5 μg ml^{-1}.

2.2. Chemicals

The methyl ester of (*S*)-naproxen was provided by Prof. H.V. Wikström (Department of Medicinal Chemistry, University of Groningen, Groningen, The Netherlands). (−)-IPG-acetate, (−)-IPG-butyrate, (−)-IPG-caprylate, (+)-IPG-acetate, (+)-IPG-butyrate and (+)-IPG-caprylate were kindly provided by M.T. Reetz (Max-Planck Institut für Kohlenforschung, Mülheim, Germany). β-Naphtylacetate, *p*-nitrophenyl esters, racemic ibuprofen methyl ester, α-methylbenzylacetate, *N*-acetyl phenylalanines methyl ester, α-methoxy phenylacetic acid methyl ester and β-phenyllactic acid methyl ester were all purchased from Sigma Chem. Co. (Axel, The Netherlands).

2.3. Oligonucleotides

To construct the plasmid mentioned in Table 1, the following primers were used (Life Technologies, UK): P*nap*for1: 5′-GTTAC*GGATCC*CTCCATTGTGCTCG-3′ (*Bam*HI); *nap*-rev1: 5′-GAG***AAGCTT***GAAGCATATTGCAGGACTTTAT-3′ (*Hin*dIII); *nap*for2: 5′-CTTATTTATGCT***GGTACC***CA-CATTCATTTAAACAA-3′ (*Kpn*I). Newly created restriction sites are indicated in bold italics.

Table 1
Bacterial strains and plasmids

	Genotype/properties	Reference/source
Strains		
E. coli TG-1	*Sup*E, K 12 Δ(lac-pro), *thi*, *hsd*D5/F′, *tra*D36, *pro*AB, *laq*Iq, *lacZΔ-M15*	Amersham Pharmacia Biotech, Uppsala, Sweden
B. subtilis ThaiI-8		CBS 679.85
B. subtils 168	*Trp*C2	Kunst et al., 1997
B. subtilis 1050	*Npr*R2, *npr*E18, *apr*A3, *est*::Cm, Δ*Lip*A	Dartois et al., 1992, 1993
Bs1050(pMA)	*B. subtilis* 1050 transformed with pMA, a pMA5 derivative, containing the *Hpa*II and *cesA* promotor	Dröge et al., 2001
Bs1050(pMA*ybfK*)	*B. subtilis* 1050 transformed with pMA*ybfK*	Dröge et al., 2001
Bs1050(pMA*nap*)	*B. subtilis* 1050 transformed with pMA*nap*	This work
Bs1050(pMA*thai*)	*B. subtilis* 1050 transformed with pMA*thai*	Dröge et al., 2001
Plasmids		
pUC18	P_{lac}, ColE1, ϕ/fi80dlacZ Amp^r	Norrander et al., 1983
pMA5	ColE1, *rep*B, Neo^r, Amp^r, $P_{hp\ aII}$	Zyprian and Matzura, 1986; Brückner et al., 1984
pMA*ybfK*	pMA5 derivative, containing the *B. subtilis* 168 *cesB* gene, downstream of the *Hpa*II and *cesA* promotor	Dröge et al., 2001
pMA*nap*	pMA5 derivative, containing the *B. subtilis* 168 *cesA* gene, downstream of the *Hpa*II and *cesA* promotor	This work
pMA*thai*	pMA5 derivative, containing the *B. subtilis* Thai I-8 *nap* gene, downstream of the *Hpa*II and *cesA* promotor	Dröge et al., 2001

2.4. DNA techniques

Recombinant DNA techniques were performed as described by Sambrook et al. [32]. Enzymes endonucleases were purchased from Life Technologies. Plasmid DNA was prepared as described by Birnboim and Doly [33]. DNA purification was performed by using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany).

2.5. Construction of the plasmids

The *cesB* gene was cloned as described previously by Dröge et al. [22]. The promotor of the *cesA* gene and the *cesA* gene itself (both originating from *B. subtilis* 168) were amplified using the primers P*nap*for1 and *nap*rev1. All PCRs were performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The PCR protocol was as follows: 4 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 1 min 50 °C and 1 min at 72 °C. At the end of the protocol, DNA production was finished with 10 min heating at 72 °C. The amplified gene fragments were cloned into the *Kpn*I and *Hin*dIII sites of pMA5, an *Escherichia coli*/*B. subtilis* shuttle vector. In this plasmid, pMA*nap*, the *cesA* gene became located downstream of the *cesA* promotor. *Bam*HI digestion removed the *E.coli* replicon and positioned the gene and the *cesA* promotor downstream of the strong Gram positive *Hpa*II promotor [34–36]. The shortened plasmid was used to transform *Bacillus* strain BCL1050. Restriction analysis and DNA sequencing were used to verify the sequence of the construct. The *nap* gene of *B. subtilis* Thai I-8 was amplified from chromosomal DNA of *B. subtilis* Thai I-8 using primers *nap*for2 and *nap*rev1. The gene was cloned in pMA5 by replacing the *Rsr*II and *Hin*dIII fragment of pMA*nap* with the *Rsr*II and *Hin*dIII digested PCR fragment resulting in pMA*thai*.

2.6. Enzyme purification

To produce enzymes, *B. subtilis* was grown in 2 l shake flasks, containing 500 ml 2 × TY medium, at 37 °C at 300 rpm with good aeration for 16 h. After harvesting the cells, the cytoplasmic fraction was isolated as described by Dröge et al. [22]. CesA and carboxylesterase NP were purified by loading the cytoplasmic fraction on three coupled HitrapQ columns (1.6 cm × 2.5 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with 10 mM Tris–HCl, pH 8, containing 1 mM EDTA, using a flow of 5 ml min^{-1}. Elution was performed with a linear gradient from 0 to 1 M of sodium chloride in 10 mM Tris–HCl, pH 8, containing 1 mM EDTA. The collected fractions (10 ml) were screened for the presence of CesA or carboxylesterase NP using a SDS-PAGE and the naproxen methyl ester assay. The fractions containing carboxylesterase were pooled and 0.5 mM ammonium sulphate was added. This solution was loaded on a MT20 column (15 mm × 110 mm; Bio-Rad, Hercules, CA, USA) packed with 20 ml phenylsepharose HP (Amersham Pharmacia Biotech, Uppsala, Sweden), and equilibrated with 10 mM phosphate buffer, pH 8, containing 0.5 M ammonium sulphate. Elution was performed using a combination of a stepwise and linear gradient from 0.5 to 0 M ammonium phosphate in 10 mM phosphate buffer, pH 8. Fractions (10 ml) were screened for activity and the carboxylesterase containing fractions were pooled and stored at −20 °C. The protein concentration was determined both by the Bradford and Lowry method (Pierce, Rockford, Illinios, USA). CesB was purified as described previously [22].

2.7. Electrophoresis

SDS-PAGE was performed on a 12% separating and a 4% stacking gel [37]. Molecular mass markers were purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA). Proteins were stained by the silver staining procedure of Pierce (Pierce, Rockford, IL, USA) or by Coomassie Brilliant Blue R-250 staining (Pierce, Rockford, IL, USA).

2.8. Esterase activity assays

2.8.1. Naproxen methyl ester assay

Esterase activity was determined using the naproxen methyl ester assay. Thirteen milligrams (*S*)- or (*R*)-naproxen methyl ester was dissolved in 10 ml 14.3% (w/v) Tween 80 in 0.07 M MOPS buffer, pH 7.5, at 60 °C in an ultrasonic bath (60 min). The solution was diluted to 50 ml with 0.07 M MOPS buffer, pH 7 [4,38]. Samples were diluted with 0.1 M MOPS buffer containing 0.2% (w/v) BSA to a volume of 250 μl. Ten millimolars phosphate buffer, pH 8, was diluted correspondingly and was used as a reference. The sample solution and the substrate solutions were preincubated at 32 °C in a water bath. Seven hundred and fifty microlitres substrate solution was added to the sample solution and the final solutions were incubated in a water bath for 4 h at 32 °C. The samples were analysed by HPLC. HPLC was performed using an Isco pump 2350, an Isco gradient mixer 2360 (ISCO Inc., Lincoln, NE, USA), a Kontron autosampler 360 (Kontron Instruments SpA, Milan, Italy), and a Shimadzu SPDM6A-Diode Array detector (Shimadzu Europe GmbH, Duisburg, Germany). The chromatographic conditions used were as follows: an analytical column (LiChrospher 100 RP-18, 5 μm; LiChrocart 250-4), a guard column (LiChrospher 100 RP-18, 5 μm; LiChrochart 4-4, Merck Darmstadt, Germany), an eluens consisting of methanol:10% acetic acid (90:10, v/v), an isocratic flow of 0.75 ml min^{-1} with a pressure of 1500 psi, an injected volume of 20 μl, a DAD wave length of 239 nm, a band width of 2 nm, a spectrum absolute scale (mAbs) of −10 to 1000, and a normalisation threshold of 10 mABS. The capacity factor (k') for naproxen and the methyl ester of naproxen was 1.43 and 1.82, respectively. The hydrolysis by the blanks was always zero.

2.8.2. β-Naphtylacetate and ibuprofen methyl ester assay

Both substrates were dissolved in 10 ml 14.3% (w/v) Tween 80 in 0.07 M MOPS buffer, pH 7.5. Naphtylacetate was dissolved at 60 °C in an ultrasonic bath (60 min). The solutions were diluted to 50 ml with 0.07 M MOPS buffer, pH 7.5. The assay was performed as described above. The conversion of the ester was determined using HPLC analysis as described for the naproxen methyl ester assay above. UV absorption was detected at wavelengths of 274 nm (β-naphtylacetate) and 239 nm (ibuprofen methyl ester), respectively.

2.8.3. 1,2-O-isopropylideneglycerol (IPG) ester assay

The esters of IPG were dissolved in 10 ml 14.3% (w/v) Tween 80 in 0.07 M MOPS buffer, pH 7.5 and diluted to 50 ml with 0.07 M MOPS buffer, pH 7.5. Samples were diluted with 0.1 M MOPS buffer containing 0.2% (w/v) BSA to a volume of 150 μl. Ten millimolars phosphate buffer, pH 8, was diluted correspondingly and was used as a reference. The sample solution and the substrate solutions were preincubated at 32 °C in a water bath. Five hundred microlitres substrate solution was added to the sample solution and the final solutions were incubated in a water bath at 32 °C for 4 h. After incubation, 500 μl saturated NaCl solution was added and the aqueous solution was extracted twice with 1 ml ethylacetate. GC analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a 7673 injector and a Hewllet Packard 3365 Chemstation. The chromatographic conditions used were as follows: a WCOT fused-silica CP-wax 52 CB column (10 m × 0.25 mm i.d., film thickness 0.25 μm, Chrompack International, Middelburg, The Netherlands), an oven temperature programme of 50–125 °C at 3 °C min^{-1}, an injector temperature of 250 °C, a detector (FID) temperature of 300 °C, the carrier gas was helium, an inlet pressure of 5 psi, a linear gas velocity of 26 cm s^{-1}, a split ratio of 56:1 and an injected volume of 1 μl.

Chiral GC analysis was performed on the same Hewlett Packard 5890 series II gas chromatograph. The used chromatographic conditions were as follows [39]: a WCOT fused silica heptakis(6-*O*-*t*butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin column (50% in OV-1701 (w/w), 25 m × 0.25 mm i.d., film thickness 0.25 μm, Prof. W.A. König, Institut für Organische Chemie, Universität Hamburg, Germany), an oven temperature programme of 10 min at 90 °C, 90–125 °C at 3 °C min^{-1}, 5 min at 125 °C, an injector temperature of 250 °C, a detector (FID) temperature of 300 °C, the carrier gas was helium, an inlet pressure of 17 psi, a linear gas velocity of 40 cm s^{-1}, a split ratio of 11:1, and an injected volume of 1 μl (1 psi = 6894.76 Pa). A different oven temperature programme was applied to separate the enantiomers of IPG-caprylate. The programme consisted of 10 min at 90 °C, 90–150 °C at 3 °C min^{-1}, 15 min at 150 °C. The capacity factor (k') was calculated using the formula $k' = (T_r - T_0)/T_0$. T_r confers to the retention time (min) of the compound and T_0 the retention time of methane (the void volume), which was 1.0505 min [39].

The hydrolysis of butyrate and caprylate esters by the blanks was always zero, while the hydrolysis of acetate esters was negligible.

2.8.4. α-Methylbenzylacetate, N-acetyl phenylalanines methyl ester, α-methoxy phenylacetic acid methyl ester and β-phenyllactic acid methyl ester assay

All substrates were diluted to 10 mM in 10 mM Tris–HCl buffer, pH 7.5, according to the manufacturer's instructions. Samples were diluted with 10 mM Tris–HCl buffer, pH 7.5, to a volume of 100 μl. One millilitre of substrate solution was added and the final solutions were incubated in a water bath at 32 °C for 4 h. The assays were performed using the HPLC system as described above for the naproxen methyl ester assay. UV absorption was detected at a wavelength of 254 nm.

2.8.5. p-Nitrophenyl ester assays

Ten millimolar solutions of the different *p*-nitrophenyl esters in methanol were prepared. An amount of 0.5 mM *p*-nitrophenylcaprylate (50 μl) was added to 900 μl assay buffer, containing 50 mM phosphate buffer (pH 8), 0.36% Triton X100 (v/v) and 0.1% gum arabic. Samples were diluted with assay buffer to a volume of 50 μl and added to the substrate solution. The absorbance was measured at a wavelength of 410 nm. Concentrations were calculated using a molar extinction coefficient of 15,000 M^{-1} cm^{-1}. Corrections were made for spontaneous hydrolysis of the substrate.

2.9. pH optimum

The pH optimum was determined using the naproxen methyl ester assay as described above. The activity of the enzymes at different pH was assessed using (*S*)-naproxen methyl ester solutions dissolved in: 0.07 M glycine HCl buffer (pH 3), 0.07 M potassium phosphate buffer (pH 4), 0.07 M potassium phosphate buffer (pH 6), 0.07 M Tris–HCl buffer (pH 7), 0.07 M MOPS buffer (pH 7.5), 0.07 M Tris–HCl buffer (pH 9), and 0.07 M glycine buffer (pH 11), respectively. In addition, after 4 h of incubation, some samples were analysed by SDS-PAGE and Coomassie staining.

2.10. Temperature optimum

The temperature optimum was determined using the naproxen methyl ester assay as described above. Enzymatic activity was determined by incubating the enzyme reaction at different temperatures of 4, 20, 30, 40, 50, and 60 °C, respectively. In addition, after 4 h of incubation, some samples were analysed by SDS-PAGE and Coomassie staining.

2.11. Data analysis

One unit (U) is defined as the amount of enzyme that hydrolyses 1 μmol of substrate ester per minute. Enantiomeric ratios, *E*, were defined as the ability of the enzyme to distinguish between enantiomers [40,41]. When $E > 100$, the enantiomeric excess, ee, was calculated. All data were the results of three experiments.

3. Results

3.1. Cloning of the carboxylesterase genes

After the elucidation of the genome sequence of *B. subtilis* 168, it became clear that this organism contained two genes with a high homology to the carboxylesterase NP of *B. subtilis* Thai I-8. These proteins, CesA (encoded by the *cesA* gene) and CesB (encoded by the *cesB* gene) were, respectively, 98 and 64% identical on protein level (Fig. 1). The characteristic pentapeptide for most lipases and esterases, Gly-Xaa-Ser-Xaa-Gly, was present in all three carboxylesterases.

CesB was cloned in pMA5 an *E. coli*/*B. subtilis* shuttle vector as described previously [22]. The *cesA* gene and its promotor of *B. subtilis* 168 were cloned in the same plasmid. In short, the *Kpn*I and *Hin*dIII digested PCR fragment was ligated in the pMA5. After ligation, *E. coli* DH5α was transformed. Digestion with *Bam*HI and subsequent self-ligation of the resulting vector removed the *E. coli* replicon and positioned the *cesA* gene and its promotor region downstream of the strong Gram positive *Hpa*II promotor [34–36]. Both promotors are expressed constitutively in *B. subtilis*. The shortened plasmid was used to transform the lipase A and esterase A negative *B. subtilis* strain 1050 [35,42], resulting in strain Bs1050(pMA*nap*). Then, the *Rsr*II/*Hin*dIII fragment of the plasmid pMA*nap* was exchanged with the *Rsr*II/*Hin*dIII fragment of the PCR amplified gene fragment of *nap* of *B. subtilis*

Fig. 1. Comparison of the sequences of carboxylesterase NP (*B. subtilis* Thai I-8), CesA (*B. subtilis* 168) and CesB (*B. subtilis* 168). Carboxylesterase NP (*B. subtilis* Thai I-8) upper lane, CesA (*B. subtilis* 168) middle lane and CesB (*B. subtilis* 168) lower lane. The matching amino acids are in black, the Gly-Xaa-Ser-Xaa-Gly motif is underlined.

Table 2
Purification of CesA, carboxylesterase NP and CesB from the cytoplasm of a 1 l culture of *B. subtilis* 1050

Purification step	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Purification factor (%)	Yield (%)
CesA (*B. subtilis* 168)					
Sonication	561	49.5	0.27	1	100
HiTrapQ	47.5	n.d.	n.d.	n.d.	n.d.
Phenylsepharose	19.1	28.0	4.21	15.5	57
Carboxylesterase NP (*B. subtilis* Thai I-8)					
Sonication	557	12.4	0.66	1	100
HiTrapQ	60	10.1	1.47	2.22	81
Phenylsepharose	8	6.3	4.56	6.90	62
CesB (*B. subtilis* 168)					
Sonication	330	n.d.	n.d.	n.d.	n.d.
HiTrapQ	14	1.7	0.12	1	100
Phenylsepharose	6	1.02	0.17	1.42	60

n.d.: not determined.

Thai I-8. DNA sequencing confirmed that the sequences of all constructs were correct.

3.2. Expression in *B. subtilis* and isolation of the enzyme

Comparison of the cytoplasmic fractions of Bs1050 (pMA*nap*) and Bs1050(pMA*thai*) revealed comparable overexpression levels of the esterases in the cytoplasm of *B. subtilis* 1050, while the expression of CesB of strain Bs1050(pMA*ybfK*) was somewhat lower (data not shown). CesA and carboxylesterase NP were purified using a two-step purification protocol (Table 2). Firstly, anion exchange chromatography at pH 8 was performed. Although many proteins present in the cytoplasm of *B. subtilis* bound to a HitrapQ column at pH 8, CesA and carboxylesterase NP were detected in the flow-through. Surprisingly, SDS-PAGE and Coomassie staining revealed that the flow-through fraction contained almost purified carboxylesterase. Afterwards, a second chromatography step based on hydrophobic interaction was performed to remove the remaining contaminating proteins. CesA and carboxylesterase NP bound strongly to a phenylsepharose column and eluted during the isocratic flow with a phosphate buffer without ammonium sulphate. CesB was purified as described previously [22]. SDS-PAGE and silver staining confirmed the purity of all samples (Fig. 2).

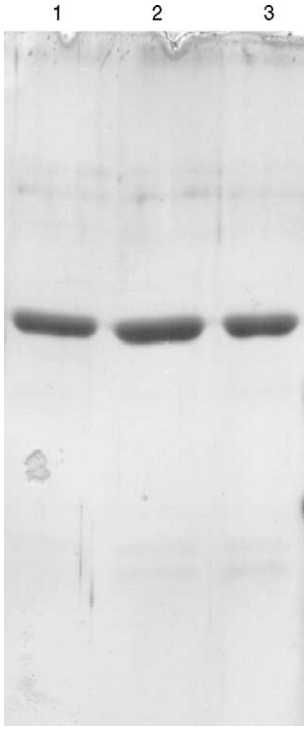

Fig. 2. (A) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, silver staining. Lane 1: purified CesA of *B. subtilis* 168; lane 2: purified carboxylesterase NP of *B. subtilis* Thai I-8; lane 3: purified CesB of *B. subtilis* 168.

3.3. Enzymatic activity of the esterases

3.3.1. Hydrolysis of the methyl ester of naproxen

The catalytic activity towards the (*S*)-methyl ester of naproxen was determined (Table 3) using a non chiral HPLC method. The specific activities of CesA and carboxylesterase NP were not significantly different, 4.2 and 4.6 U mg^{-1}, respectively. Comparison of the specific activities of CesB and CesA and carboxylesterase NP revealed an approximate 25-fold lower specific activity of CesB towards the naproxen ester (e.g. the specific activity was 0.17 U mg^{-1} for CesB). It should be noted that the production of (*S*)-naproxen by CesB is still far above the detection limit of the HPLC system and for this, it can be attributed to enzymatic activity of CesB. Moreover, the hydrolysis of the substrate by the blanks was always below detection.

Then, the activity of the three esterases towards the methyl ester of (*R*)-naproxen was determined and compared with the hydrolysis of the (*S*)-naproxen methyl ester. Table 3 summarises these results. The three esterases displayed similar specific activities towards the (*R*)-naproxen methyl ester, ranging from a specific activity of 0.019 U mg^{-1} (CesB) to 0.022 U mg^{-1} (CesA). It should be mentioned that the production of (*R*)-naproxen by the blanks was always below detection. Therefore, the specific activities towards the (*R*)-naproxen methyl ester can be attributed to enzymatic activity.

Consequently, the enantioselectivity of these enzymes towards the racemic naproxen methyl ester can be calculated from the activities towards both substrates. This results in an apparent selectivity towards the (*S*)-naproxen methyl ester

Table 3
Specific activities (U mg^{-1}) of CesA, carboxylesterase NP and CesB

Substrate	CesA (*B. subtilis* 168)	Carboxylesterase NP (*B. subtilis* Thai I-8)	CesB (*B. subtilis* 168)
Rac-ibuprofen methyl ester	25.4	20.9	0.55
(*S*)-Naproxen methylester	4.23	4.60	0.17
β-Naphtylacetate	0.08	0.10	0.01
(*R*)-Naproxen methyl ester	0.04	0.04	0.03
(+)-IPG-C8	0.051	0.036	0.022
(−)-IPG-C8	0.031	0.023	Below detection
(+)-IPG-C4	0.011	0.008	Below detection
(−)-IPG-C4	0.014	0.010	Below detection
(+)-IPG-C2	0.004	0.004	0.002
(−)-IPG-C2	0.004	0.002	0.002

Table 4
Enantioselective properties of CesA, carboxylesterase NP and CesB towards IPG esters

Enzyme	IPG-C2		IPG-C4		IPG-C8	
	E-value	ee (%)	*E*-value	ee (%)	*E*-value	ee (%)
CesA (*B. subtilis* 168)	1.0	2.0	1.3	2.3	1.9	26.1
Carboxylesterase NP (*B. subtilis* Thai I-8)	1.1	2.6	1.1	3.6	1.9	24.1
CesB (*B. subtilis* 168)	1.3	10.1	1*	1*	>200	>99.9

* 1: below detection (0.6 ng IPG).

of at least 99% (carboxylesterase NP and CesA) and 85% (CesB) enantiomeric excess, ee, respectively.

3.3.2. *Ibuprofen methyl ester and* β*-naphtylacetate and assay*

The specific activities of the esterases towards the ibuprofen methyl ester and β-naphtylacetate are summarised in Table 3. Comparison of the specific activities of CesA, carboxylesterase NP and CesB revealed an almost 40-fold lower specific activity of CesB towards the racemic ibuprofen ester. Furthermore, all three esterases have a low specific activity towards β-naphtylacetate.

3.3.3. *Hydrolysis of 1,2-O-isopropylideneglycerol esters*

The substrate specificity and enantioselectivity of the three esterases towards IPG esters with different aliphatic side chains were determined (Table 4) using non chiral GC analysis. Both CesA and carboxylesterase NP showed affinity towards both stereoisomers of the IPG esters, ranging from a specific activity 0.051 U mg^{-1} (IPG caprylate esters) to 0.002 U mg^{-1} (IPG acetate esters). As reported previously, the highest activity of CesB was measured when (+)-IPG-caprylate was used as a substrate (0.022 U mg^{-1}). Surprisingly, CesB was unable to hydrolyse (−)-IPG caprylate esters. In contrast to CesA and carboxylesterase NP, CesB was unable to hydrolyse (−)- and (+)-IPG butyrate. (−)- and (+)-IPG acetate were hydrolysed by CesB.

The enantioselectivity towards the IPG was determined in a kinetic resolution experiment using a chiral GC method. Table 4 summarises these enantioselectivities. Both CesA and carboxylesterase NP showed only modest enantioselectivities towards the IPG esters, ranging from an *E*-value of 1.3 (CesA, IPG butyrate esters) to 1.0 (CesA, IPG acetate esters). As CesB was unable to hydrolyse (*R*)-IPG caprylate esters (conversion below detection), the estimated *E*-value of CesB towards IPG-caprylate was >200 (corresponding to an estimated enantioselectivity of at least 99.9%). Additionally, CesB was unable to hydrolyse (*R*)- and (*S*)-IPG butyrate, and (*R*)- and (*S*)-IPG acetate were hydrolysed but only modest enantioselectivities were observed (*E*-value is 1.9).

3.3.4. *Hydrolysis of* α*-methylbenzylacetate, N-acetyl phenylalanines methyl ester,* α*-methoxy phenylacetic acid methyl ester and* β*-phenyllactic acid methyl ester assay*

CesB was able to hydrolyse the α-methylbenzylacetate and *N*-acetyl phenylalanines methyl ester, whereas α-methoxy phenylacetic acid methyl ester and β-phenyllactic acid methyl ester were not hydrolysed at all. However, very low specific activities were observed (data not shown). CesA and carboxylesterase NP only hydrolysed the *N*-acetyl phenylalanines methyl ester with a comparable specific activity compared to CesB.

3.3.5. *Comparison of the specific activities and enantioselective properties*

The specific activities and enantioselective properties of CesA, carboxylesterase NP and CesB are summarised in Tables 3 and 4. Comparison of the specific activities of CesB with CesA and carboxylesterase NP towards chiral carboxylic acid esters (e.g. (*S*)-naproxen methyl ester; (*R*)-naproxen methyl ester; and ibuprofen methyl ester) revealed relatively lower specific activities for CesB. In contrast, similar specific activities were observed when chiral alcohol esters were used as a substrate ((+)-IPG-caprylate and (−)- and (+)-IPG acetate). Most interestingly, CesA and carboxylesterase NP showed highest enantioselectivity towards the production of the chiral carboxylic acid naproxen, whereas CesB displayed excellent enantioselective properties towards the chiral alcohol IPG.

3.3.6. *Hydrolysis of p-nitrophenyl esters*

The hydrolytic activity of the three esterases was studied using various *p*-nitrophenyl esters, varying from a C_2 to a C_{18} alkyl chain length. The three esterases showed activity only towards short chain length esters (up till C_8). CesA and carboxylesterase NP showed maximal activity when C_6 esters were used as a substrate whereas CesB had maximum activity towards the C_8 ester. Interestingly, CesB was unable to hydrolyse the C_6 ester of *p*-nitrophenol (Fig. 3).

3.4. *Biochemical characteristics*

The hydrolysis of the (*S*)-naproxen methyl ester was studied at different temperatures in order to determine the influence of the temperature on the enzymatic reaction (Fig. 4). Maximal activities were observed at 30 °C for CesA and carboxylesterase NP, while maximal activity and stability of CesB was observed at 40 °C. Incubation at higher temperatures resulted in a rapid inactivation of esterase activity. Above 50 °C, no enzymatic activity could be observed. To exclude proteolytic degradation of the enzymes during the incubation at 40 °C, these samples were analysed by SDS-PAGE and Coomassie staining. Comparison with the samples

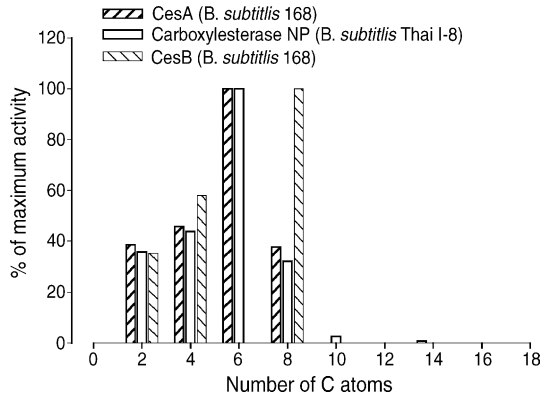

Fig. 3. Hydrolysis of *p*-nitrophenyl esters with different aliphatic side chain, ranging from C_2 to C_{18} with C_2 stepwise, by CesA, carboxylesterase NP and CesB. The assay conditions were described in material and methods.

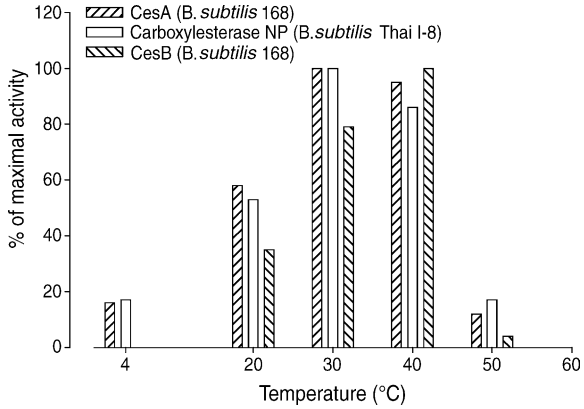

Fig. 4. Effect of the temperature (4, 20, 30, 40, 50 and 60 °C) on the hydrolysis of the (*S*)-naproxen methyl ester by CesA, carboxylesterase NP and CesB. The assay conditions were described in material and methods.

incubated at 30 °C revealed that no major additional protein bands in addition to the 34 kDa band could be observed on the stained gel. Investigation of the influence of the pH on (*S*)-naproxen methyl ester hydrolysis was performed at pH 3–11 (Fig. 5). All three esterases displayed maximal activity at pH 7.5 (MOPS buffer), while the enzymes were inactive below

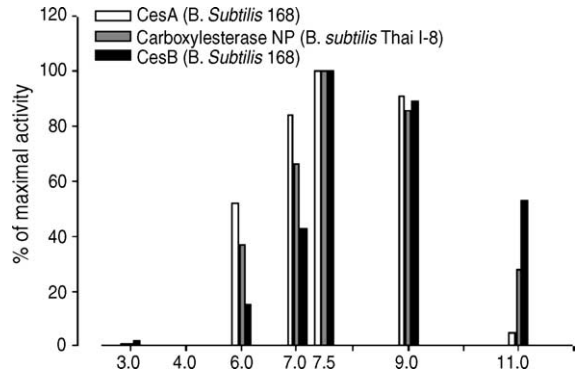

Fig. 5. Effect of pH (3, 4, 6, 7, 7.5, 9, 11) on the hydrolysis of the (*S*)-naproxen methyl ester by CesA and carboxylesterase NP and CesB. The assay conditions were described in material and methods.

pH 6. Compared to CesA and carboxylesterase NP, CesB showed a relatively high activity at pH 11 (53; 28 and 5% of maximal activity for CesB, carboxylesterase NP, CesA). Analysis of the samples incubated at pH 7.5, 9 and 11 using SDS-PAGE, revealed that the enzymes were not degraded.

4. Discussion

Today, more than 50% of the organic pharmaceuticals are chiral [43]. Since chiral compounds represent more than 50% of the world-wide most frequently prescribed drugs, the interest in the preparation and isolation of chiral drugs has increased dramatically. However, although it is commonly accepted that the specific effect of a drug is caused by just one enantiomer out of a racemic mixture of compounds, both enantiomers are still frequently applied as it has been difficult to properly separate them up till now [43,44]. In recent years, the use of enzymes for preparation of optically enriched compounds has become an alternative to chemical synthesis. Bacterial enzymes, like lipases and esterases, are capable of enantioselective hydrolysis and esterification in an environmental friendly and cheap process. The advantageous characteristics of esterases reside in their stability, their activity in organic solvents, the fact that they do not require cofactors, and their high regio- and stereo-specificity. These properties make esterases attractive biocatalysts for organic chemistry [7].

Over the past years, many esterases have been cloned and overexpressed. A large number of bacterial esterases have been described as well (e.g. [1,4,21,45–50]). However, only a few of them are useful biocatalyst in kinetic resolution experiments. In the present study, we have shown that genome analysis has provided an excellent tool for the isolation of two novel bacterial esterases with marked enantioselective properties. One of these esterases, CesA of *B. subtilis* 168 showed an identity of 98% to the carboxylesterase NP of *B. subtilis* Thai I-8. This Thai I-8 esterase was characterised in 1994 as an effective biocatalyst in the racemic resolution of propionate esters with an aromatic ring containing a 2-substituent, such as 2-arylpropionates, 2-(aryloxy)propionates and *N*-arylalanine esters (>99% ee) [4,8–10,38]. Relatively slow hydrolysis and poor enantioselectivities were observed when methyl acetate, 2-substituted butyrates, 2-substituted pentanoates, 2-substituted 2-phenylacetates or amino acid esters were used as a substrate [10]. The activity and enantioselectivity towards substrates with chirality residing in the alcohol part of the ester were also investigated, but the majority of these substrates, such as IPG and 1-phenylethanol esters, showed rather poor enantioselectivities [10]. In this study we have compared the activities and enantioselectivities of the homologous CesA of *B. subtilis* 168 with the carboxylesterase NP. No marked differences were observed between these two enzymes and the highest enantioselectivities were obtained using substrates with chirality residing in the carboxylic acid part of

the ester, such as the NSAIDs naproxen and ibuprofen. The obvious explanation for the observed similarities in specific activity is found mainly in the 98% identical sequence.

The other esterase CesB, was identified as a paralogue of carboxylesterase NP. We have previously reported the isolation and cloning of this intracellular esterase of *B. subtilis* 168 in order to determine whether the 36% difference in amino acid sequence resulted in altered stereospecific characteristics towards IPG esters. Comparison of the specific activities and enantioselective properties of CesB with CesA and carboxylesterase NP showed in fact some striking differences. First of all, the catalytic activity and enantioselectivity of CesB towards substrates with chirality in the carboxylic acid part of the esters was relatively low. For example, the specific activity towards the methyl ester of (*S*)-naproxen was approximately 25-fold lower. In line with these results, the catalytic activity of CesB towards another 2-aryl-propionate ester, the ibuprofen methyl ester, was almost 40-fold lower compared to CesA and carboxylesterase NP. Secondly, CesB combined a very narrow substrate specificity towards IPG esters with markedly enantioselective properties, whereas CesA and carboxylesterase NP could hydrolyse all IPG esters, although without enantioselectivity. Due to this enantioselectivity, CesB can be used for chiral resolution of IPG-caprylate. Although the specific activity of YbfK towards (*S*)-IPG-caprylate is almost 200 times lower than the specific activity of carboxylesterase NP towards (*S*)-naproxen methyl ester, it could be sufficient for the development of a (*S*)-IPG production process. Compared to other studies, the amount of IPG produced is likely to be enough for large-scale production in a bioreactor using immobilised CesB [51]. Interestingly, this narrow substrate specificity of CesB is also reflected in the hydrolysis of the *p*-nitrophenol esters since CesB only hydrolysed acetate (C_2), butyrate (C_4) and caprylate (C_8) esters *p*-nitrophenol, while the caproate (C_6) ester was not hydrolysed at all.

Although comparison of the specific activities and enantioselectivities of CesB with CesA and carboxylesterase NP towards several chiral ester substrates revealed some more marked differences, their biochemical characteristics appear to be strikingly similar. All three enzymes displayed maximal activities between 30 and 40 °C. Incubation at higher temperatures resulted in a rapid inactivation of enzymatic activity. Investigation of the pH dependency revealed a maximal activity at pH 7.5. A major difference, however, relative to CesA and carboxylesterase NP, was the high activity of CesB at pH 11.

In conclusion, we have identified three homologous intracellular carboxylesterases from *B. subtilis*. It should be noted that a classical enzyme screening programme would probably never have identified CesA nor CesB, since wild type cells show no activity towards (*S*)-naproxen and IPG esters. Comparison of the enantioselective properties towards several esters compounds revealed that rather similar physico-chemical properties (pH and temperature optimum) can be associated with rather different enantioselectivities. CesA and carboxylesterase NP seem to be more suitable for the enantioselective production of chiral carboxylic acid such as the NSAIDs ibuprofen and naproxen, whereas CesB can be applied for the enantioselective production of the chiral alcohol IPG.

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