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Selection of novel lipases and esterases for enantioselective biocatalysis
Dröge, Melloney Joyce

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Chapter 3

Comparison and Functional Characterisation of Three Homologous Intracellular Carboxylesterases of *Bacillus subtilis*

Melloney J. Dröge, Rein Bos and Wim J. Quax
Comparison and functional characterisation of three homologous intracellular carboxylesterases of *Bacillus subtilis*

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 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.

**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 stereospecificity, carboxylesterases appear to be attractive biocatalysts for organic chemistry (Bornscheuer, 2002a).

Many bacterial esterases have been cloned and overexpressed during the last decades in order to assess their enantioselective properties (e.g. Krebsfanger et al., 1998; Kim et al., 2003a; Quax & Broekhuizen, 1994; Petersen, et al., 2001; Talker-Huiber et al., 2003).
most attractive biocatalysts seem to originate from *Bacillus* and *Pseudomonas* species (for a review see Bornscheuer & Kazlauskas, 1999). For example, the naproxen esterase of *B. subtilis* Thai I-8 was characterised as a very efficient enantioselective biocatalysts 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 (Azzolina et al, 1994; 1995 Smeets & Kieboom, 1992) 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 (Cohen et al., 2001; Jaeger et al., 2001; Lin and Cornish, 2002; Pelletier and Sidhu, 2001), 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 alternative approach for finding alternative biocatalysts for rational drug preparation.

In *B. subtilis* 168, 9 genes have been functionally characterised as intracellular or extracellular esterases. In addition, 8 genes can be classified as potential esterases (Bischoff & Ordal, 1991; Chen et al., 1995; Dröge et al., 2001; Eder et al., 1996; Eggert et al., 2000; Henke & Bornscheuer, 2002a; Higerd & Spizizen, 1973; Kneusel et al., 1994; Kunst et al., 1997; Moore & Arnold, 1996; Nilsson et al., 1994; Quax & Broekhuizen, 1994; Riefler & Higerd, 1976). 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 (Dröge et al., 2001). 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.
Material and methods

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: 2xTY medium containing: Bactotrypton (1.6%), Bacto yeast extract (1%) and sodium chloride (0.5%); 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 MgSO₄, 0.0022 % ferric ammonium citrate, 0.1% casein hydrolysate, 0.002% l-tryptophane (Kunst & Rapoport, 1995). Antibiotic agents were used in the following concentrations: ampicillin 100 µg.ml⁻¹, kanamycin 20 µg.ml⁻¹, and chloramphenicol 5 µg.ml⁻¹.

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). ß-naphtylacetate, p-nitrophenyl esters, racemic ibuprofen methyl ester, α-methylbenzylacetate, N-acetyl phenylalanines methyl ester, α-methoxy phenylacetic acid methyl ester and ß-phenyllactic acid methyl ester were all purchased from Sigma Chem. Co. (Axel, The Netherlands).

Oligonucleotides
To construct the plasmid mentioned in table 1, the following primers were used (Life Technologies, UK): Pnapfor1: 5’-GTTACGGATCCCTCCATTGTGCTCG-3’ (BamHI); naprev1: 5’-GAGAAGCTTGAGCATATTGCAGGACTTTAT-3’ (HindIII); napfor2: 5’-CTTATTTATGCCTGATCCACATTTCATTTAACAA-3’ (KpnI). Newly created restriction sites are indicated in bold italics.

DNA techniques
Recombinant DNA techniques were performed as described by Sambrook et al. (1989). Enzymes endonucleases were from Life Technologies. Plasmid DNA was prepared as described by Birnboim & Doly (1979). DNA purification was performed by using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany).

Construction of the plasmids
The cesB gene was cloned as described by Dröge et al. (2001). The promotor of the cesA gene and the cesA gene itself (both originating from B. subtilis 168) were amplified using the primers Pnapfor1 and naprev1. All PCRs were performed using Pfu DNA polymerase
Chapter 3

(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, DNA production was finished with 10 min at 72°C. The amplified gene fragments were cloned into the KpnI and HindIII sites of pMA5, an Escherichia coli/B. subtilis shuttle vector. In this plasmid, pMA\textit{nap}, the \textit{cesA} gene became located downstream of the \textit{cesA} promoter. \textit{BamHI} digestion removed the \textit{E.coli} replicon and positioned the gene and the \textit{cesA} promoter downstream of the strong Gram positive \textit{HpaII} promoter (Brückner et al., 1984; Dartois et al., 1994; Zyprian & Matzura, 1986). The shortened plasmid was used to transform \textit{Bacillus} strain BCL1050. Restriction analysis and DNA sequencing were used to verify the sequence of the construct. The \textit{nap} gene of \textit{B. subtilis} Thai I-8 was amplified from chromosomal DNA of \textit{B. subtilis} Thai I-8 using primers \textit{nap}for2 and \textit{nap}rev1. The gene was cloned in pMA5 by replacing the RsrII and HindIII fragment of pMA\textit{nap} with the RsrII and HindIII digested PCR fragment resulting in pMA\textit{thai}.

**Enzyme purification**

To produce enzymes, \textit{B. subtilis} was grown in 2 litre shake flasks, containing 500 ml 2xTY 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. (2001). CesA and carboxylesterase NP were purified by loading the cytoplasmic fraction on 3 coupled HitrapQ columns (1.6 × 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 × 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 M 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. Protein concentration was determined by the Bradford and Lowry method (Pierce, Rockford, Illinios, USA). CesB was purified as described previously (Dröge et al., 2001).

**Electrophoresis**

SDS-PAGE was performed on a 12% separating and a 4% stacking gel (Laemmli, 1970). 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, Illinios, USA) or by Coomassie Brilliant Blue R-250 (Pierce, Rockford, Illinios, USA).
TABLE 1: Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain/plasmids</th>
<th>Genotype/properties</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> TG-1</td>
<td><em>SupE, K</em> 12 ∆(lac-pro), <em>thi, hsdD5/F</em>, <em>traD36, proAB</em>, <em>lacIq, lacZΔM15</em></td>
<td>Amersham Pharmacia Biotech, Uppsala, Sweden</td>
</tr>
<tr>
<td><em>B. subtilis</em> ThaiI-8</td>
<td></td>
<td>CBS 679.85</td>
</tr>
<tr>
<td><em>B. subtilis</em> 168</td>
<td><em>TigC2</em></td>
<td>Kunst et al., 1997</td>
</tr>
<tr>
<td><em>B. subtilis</em> 1050</td>
<td>*NprR2, aprE18, aprA3, *cesCm, <em>ΔLipA</em></td>
<td>Dartois et al, 1992/1993</td>
</tr>
<tr>
<td><em>Bs1050(pMA)</em></td>
<td><em>B. subtilis</em> 1050 transformed with pMA, a pMA5 derivative, containing the <em>HpaII and cesA</em> promoter</td>
<td>Dröge et al., 2001</td>
</tr>
<tr>
<td><em>Bs1050(pMAybfK)</em></td>
<td><em>B. subtilis</em> 1050 transformed with pMAybfK</td>
<td>Dröge et al., 2001</td>
</tr>
<tr>
<td><em>Bs1050(pMANap)</em></td>
<td><em>B. subtilis</em> 1050 transformed with pMANap</td>
<td>This work</td>
</tr>
<tr>
<td><em>Bs1050(pMAThai)</em></td>
<td><em>B. subtilis</em> 1050 transformed with pMAThai</td>
<td>Dröge et al., 2001</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td><em>Pm</em>, ColE1, <em>φ/80dlacZ Amp</em></td>
<td>Norranser et al., 1983</td>
</tr>
<tr>
<td>pMA5</td>
<td>ColE1, npB, NcoI, <em>Amp</em>, <em>Ppae</em></td>
<td>Zyprian &amp; Matzura, 1986; Brückner et al., 1984</td>
</tr>
<tr>
<td>pMAybfK</td>
<td>pMA5 derivative, containing the <em>B. subtilis</em> 168 <em>cesB</em> gene, downstream of the <em>HpaII</em> and <em>cesA</em> promoter</td>
<td>Dröge et al., 2001</td>
</tr>
<tr>
<td>pMANap</td>
<td>pMA5 derivative, containing the <em>B. subtilis</em> 168 <em>cesA</em> gene, downstream of the <em>HpaII</em> and <em>cesA</em> promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pMAThai</td>
<td>pMA5 derivative, containing the <em>B. subtilis</em> Thai I-8 <em>nap</em> gene, downstream of the <em>HpaII</em> and <em>cesA</em> promoter</td>
<td>Dröge et al., 2001</td>
</tr>
</tbody>
</table>

**Esterase activity assays**

*Naproxen methyl ester assay*

Esterase activity was determined using the naproxen methyl ester assay. 13 mg (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 (Mutsaers & Kooreman, 1991; Quax & Broekhuizen, 1994). Samples were diluted with 0.1 M MOPS buffer containing 0.2% w/v BSA to a volume of 250 µl. 10 mM 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. 750 µl substrate solution was added to the sample solution and the
final solutions were incubated in a water bath for four hours 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 SPD-M6A-Diode Array detector (Shimadzu Europe GmbH, Duisburg, Germany). The chromatographic conditions were: analytical column, LiChrospher 100 RP-18 (5 µm; LiChrocart 250-4); guard column, LiChrospher 100 RP-18 (5 µm; LiChrochart 4-4, Merck Darmstadt, Germany); eluens methanol : 10% acetic acid (90:10 v/v); isocratic flow of 0.75 ml.min⁻¹; pressure 1500 psi; injected volume 20 µl; DAD wave length 239 nm, band width 2 nm; spectrum absolute scale (mAbs) –10-1000; normalisation threshold 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.

ß-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. UV was detected at 274 nm (ß-naphtylacetate) and 239 nm (ibuprofen methyl ester), respectively.

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. 10 mM 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. 500 µl substrate solution was added to the sample solution and the final solutions were incubated in a water bath at 32°C for four hours. After incubation, 500 µl saturated NaCl solution was added and the aqueous solution was extracted twice with 1 ml ethyl acetate. GC analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a 7673 injector and a Hewlett Packard 3365 Chemstation under the following conditions: column WCOT fused-silica CP-wax 52 CB (10 m x 0.25 mm id, film thickness 0.25 µm, Chrompack International, Middelburg, The Netherlands), oven temperature programme 50-125°C at 3°C.min⁻¹; injector temperature 250°C; detector (FID) temperature 300°C; carrier gas helium; inlet pressure 5 psi; linear gas velocity 26 cm.s⁻¹; split ratio 56:1, injected volume 1 µl. The hydrolysis of butyrate and caprylate esters by the blanks was always zero, the hydrolysis of acetate esters was negligible.
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. 1 ml of substrate solution was added and the final solutions were incubated in a water bath at 32°C for four hours. The assays were performed using the HPLC system as described above for the naproxen methyl ester assay. UV absorption was detected at 254 nm.

p-Nitrophenyl ester assays

10 mM solution of the different p-nitrophenyl esters in methanol were prepared. 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 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.

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 four hours of incubation, some samples were analysed by SDS-PAGE and Coomassie staining.

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 four hours of incubation, some samples were analysed by SDS-PAGE and Coomassie staining.

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 (Chen et al. 1982; 1987). When E > 100, the enantiomeric excess, ee, was calculated. All data were the results of three experiments.
Chapter 3

Results

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 (see figure 6 Introduction and figure 1 Chapter 2). 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 (Dröge et al., 2001). The cesA gene and its promotor of B. subtilis 168 were cloned in the same plasmid. In short, the KpnI and HindIII digested PCR fragment was ligated in the pMA5. After ligation, E. coli DH5α was transformed. Digestion with BamHI 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 HpaII promotor (Brückner et al., 1984; Dartois et al., 1994; Zyprian and Matzura, 1986). 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 (Dartois et al., 1993; 1994), resulting in strain Bs1050(pMAnap). Then, the RsrII/HindIII fragment of the plasmid pMAnap was exchanged with the RsrII/HindIII fragment of the PCR amplified gene fragment of nap of B. subtilis Thai I-8. DNA sequencing confirmed that the sequences of all constructs were correct.


Expression in B. subtilis and isolation of the enzyme

Comparison of the cytoplasmic fractions of Bs1050(pMAnap) and Bs1050(pMAthai) revealed comparable overexpression levels of the esterases in the cytoplasm of B. subtilis.
Intracellular carboxylesterases of Bacillus subtilis 1050, while the expression of CesB of strain Bs1050(pMA\textit{lyfK}) 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 \textit{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 (Dröge et al., 2001). SDS-PAGE and silver staining confirmed the purity of all samples (figure 1).

**Table 2:** Purification of CesA, carboxylesterase NP and CesB from the cytoplasm of a 1l culture of \textit{B. subtilis} 1050. N.d.: not determined.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific activity (U.mg\textsuperscript{-1})</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CesA (B. subtilis 168)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonication</td>
<td>561</td>
<td>49.5</td>
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<td>100</td>
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<tr>
<td>HitrapQ</td>
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<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
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<td>28.0</td>
<td>4.21</td>
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<td>Sonication</td>
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<td>4.56</td>
<td>62</td>
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<td><strong>CesB (B. subtilis 168)</strong></td>
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<td>n.d</td>
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<td>100</td>
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<tr>
<td>Phenylsepharose</td>
<td>6</td>
<td>1.02</td>
<td>0.17</td>
<td>60</td>
</tr>
</tbody>
</table>

**Enzymatic activity of the esterases**

*Hydrolysis of the methyl ester of naproxen*

The catalytic activity towards the (S)-methyl ester of naproxen was determined (figure 2). The specific activities of CesA and carboxylesterase NP were not significantly different, 4.2 and 4.6 U.mg\textsuperscript{-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.

**Figure 2**: Hydrolysis of the (S)-naproxen methyl ester by CesA, carboxylesterase NP and CesB. The assay conditions were described in material and methods.

Then, the enantioselectivity of the three esterases towards the methyl ester of (R)-naproxen was determined and compared with the hydrolysis of the (S)-naproxen methyl ester. **Figure 3** summarises these results.

**Figure 3**: Hydrolysis of the (R) and (S)-naproxen methyl ester by CesA, carboxylesterase NP and CesB. The assay conditions were described in material and methods.
TABLE 3: Specific activities of the hydrolysis of the racemic ibuprofen methyl ester and β-naphtylacetate by CesA, carboxylesterase NP, and CesB. The assay conditions were described in material and methods.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ibuprofen methyl ester (U.mg⁻¹)</th>
<th>β-naphtylacetate (U.mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CesA (B. subtilis 168)</td>
<td>25.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Carboxylesterase NP (B. subtilis Thai I-8)</td>
<td>20.9</td>
<td>0.10</td>
</tr>
<tr>
<td>CesB (B. subtilis 168)</td>
<td>0.55</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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, resulting in a selectivity towards the (S)-naproxen methyl ester of at least 99% (carboxylesterase NP and CesA) and 85% (CesB) enantiomeric excess, ee, respectively.

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.

![FIGURE 4](image_url): Specific activities of CesA, carboxylesterase NP and CesB towards racemic esters of IPG-acetate, butyrate and caprylate. The assay conditions were described in material and methods.
Hydrolysis of 1,2-O-isopropylidenglycerol esters.

The substrate specificity and enantioselectivity of the three esterases towards IPG esters with different aliphatic side chains were determined (figure 4). Both CesA and carboxylesterase NP showed affinity towards both stereoisomers of the IPG esters, ranging from an enantioselectivity of $E = 1.6$ (IPG caprylate and acetate esters) to $E = 1.2$ (IPG butyrate esters). As reported previously, the highest activity of CesB was measured when (+)-IPG-caprylate was used as a substrate ($0.022 \text{ U.mg}^{-1}$). Surprisingly, CesB was unable to hydrolyse (-)-IPG caprylate esters. The detection limit of the GC system used was 1 ng IPG, resulting in a selectivity of CesB towards (+)-IPG-caprylate of at least 99.9% ($E > 200$) (Dröge et al., 2001). In contrast to CesA and carboxylesterase NP, CesB was unable to hydrolyse (-)- and (+)-IPG butyrate; (-) and (+)-IPG acetate were hydrolysed by CesB but no selectivity was observed ($E=1.2$).

Hydrolysis of $\alpha$-methylbenzylacetate, $N$-acetyl phenylalanines methyl ester, $\alpha$-methoxy phenylacetic acid methyl ester and $\beta$-phenyllactic acid methyl ester assay

CesB was able to hydrolyse the $\alpha$-methylbenzylacetate and $N$-acetyl phenylalanines methyl ester, whereas $\alpha$-methoxy phenylacetic acid methyl ester and $\beta$-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.

| TABLE 4: Specific activities 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 |
| $\beta$-naphthylacetat | 0.08 | 0.1 | 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 |
Intracellular carboxylesterases of Bacillus subtilis

Comparison of the enantioselective properties

The enantioselective properties of CesA, carboxylesterase NP and CesB are summarised in table 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.

Hydrolysis of p-nitrophenyl esters

The hydrolytic activity of the three esterases was studied using various p-nitrophenyl esters, varying from a C₂ to a C₁₈ alkyl chain length. The three esterases showed activity only towards short chain length esters (up till C₆). CesA and carboxylesterase NP showed maximal activity when C₆ esters were used as a substrate whereas CesB had maximum activity towards the C₈ ester. Interestingly, CesB was unable to hydrolyse the C₆ ester of p-nitrophenol (figure 5).

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 (figure 6). 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

**FIGURE 5:** Hydrolysis of p-nitrophenyl esters with different aliphatic side chain, ranging from C₂ to C₁₈ with C₂ stepwise, by CesA, carboxylesterase NP and CesB. The assay conditions were described in material and methods.
temperatures resulted in a rapid inactivation of esterase activity. Above 50ºC, no enzymatic activity could be observed. To exclude proteolytic degradation of the enzymes during the incubation at 40ºC, these samples were analysed by SDS-PAGE and Coomassie staining. Comparison with the samples incubated at 30ºC revealed that no major additional protein bands in addition to the 34 kDa band could be observed on the stained gel (figure 7).

**Figure 6:** Effect of the temperature (4, 20, 30, 40, 50 and 60ºC) on the hydrolysis of the (S)-naproxen methyl ester by CesA, carboxylesterase NP and CesB. The assay conditions were described in material and methods.

**Figure 7:** Effect of incubation at 40ºC on the stability of CesA, carboxylesterase NP and CesB.

Investigation of the influence of the pH on (S)-naproxen methyl ester hydrolysis was performed at pH 3 to 11 (figure 8). All three esterases displayed maximal activity at pH 7.5 (MOPS buffer), while the enzymes were inactive below 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 (figure 9).
In the present study, we have shown that genome analysis has provided an excellent tool for the isolation of two bacterial esterases with marked enantioselective properties. One of these esterases, CesA of *B. subtilis* 168 showed an identity of 98%, corresponding to 6 different amino acids, 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) (Azzolina et al., 1994; 1995; Mutsaers & Kooreman, 1991; Quax & Broekhuizen, 1994; Smeets & Kieboom, 1992). 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 (Smeets & Kieboom, 1992). Within the group of the 2-aryl propionates, most esters were converted with high enantioselectivity, despite their large structural variations in the substituents (Smeets & Kieboom, 1992).

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 showed rather poor enantioselectivities. For instance, some enantioselectivity towards IPG (4% ee at 20% conversion) and 1-phenylethanol esters was observed (Smeets & Kieboom, 1992). The homologous CesA of B. subtilis 168 displayed rather similar activities and enantioselectivities. We have compared the activity and enantioselectivity of both enzymes towards esters with chirality in the carboxylic acid part, such as the NSAIDs naproxen and ibuprofen, and in the alcohol part, such as IPG. Moreover, we have compared the specific activity towards typical esterase substrates, such as the p-nitrophenyl esters. 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. The obvious explanation for the observed similarities in specific activity is found mainly in the 98% identical sequence. In addition, comparison of the biochemical properties revealed that both enzymes showed maximal activity at similar pH and temperature.

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 and 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, while the activity towards the (R)-naproxen methyl esters was rather similar compared to CesA and carboxylesterase NP. As a consequence, comparison of the enantioselectivity of CesB with CesA and carboxylesterase NP towards the naproxen esters revealed quite different enantiomeric excesses (respectively, 85% and 99%). 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. CesB hydrolysed only acetate esters of (-)- and (+)-IPG and caprylate esters of (+)-IPG. Since no activity towards (-)-IPG-caprylate was measured, the selectivity for (+)-IPG-caprylate is more than 99.9%. Therefore, CesB seems to be more suitable for the kinetic resolution of substrates with chirality in the alcohol part of the ester.

Interestingly, this narrow substrate specificity of CesB is also reflected in the hydrolysis of the p-nitrophenol esters. CesA and carboxylesterase NP could hydrolyse all p-nitrophenyl esters. In contrast, CesB only hydrolysed acetate (C₂), butyrate (C₄) and caprylate (C₈) esters p-nitrophenol, while the caproate (C₆) 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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Chapter 3