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Journal of Molecular Catalysis B: Enzymatic 19–20 (2002) 237–245

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Cloning, expression and characterization of a new 2-Cl-propionic acid ester hydrolase from *B. subtilis*

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Received 30 September 2001; received in revised form 6 May 2002; accepted 20 May 2002

Abstract

Applying an agar plate assay, a novel gene encoding an esterase from *Bacillus subtilis* Est4B was cloned and highly overexpressed in *E. coli*. The protein sequence revealed high homology to the *srf*D gene, which encodes the fourth open reading frame, a putative thioesterase gene from the surfactin synthetase gene cluster. It was almost identical to an unpublished sequence from a putative surfactin synthetase cluster from *B. subtilis* A13. The enzyme (Est4B1) was produced in *E. coli*, purified to homogeneity and crystallized.

Computational prediction of the protein fold showed high structural similarity of Est4B1 to haloperoxidases and dehalogenases and much lower similarity to lipases and esterases. However, by primary sequence analysis we found a typical esterase/thioesterase/lipase motif. Biocatalytic activity on several model esterase substrates was detected and quantified. This is the first time enzymatic activity could be shown for this type of independent putative thioesterases and this enzyme may serve as a model protein to solve the structure of this type of hydrolytic enzymes, which is commonly found in gene clusters of non-ribosomal peptide synthetases.

Keywords: *Bacillus subtilis*; Est4B1; *E. coli*

1. Introduction

Hydrolytic enzymes are synthetically useful for producing enantio pure compounds [1,2]. Especially, lipases have been used successfully in industrial scale [3] for several reasons.

1. They are available in large quantities and therefore cost effective.
2. Many lipases are very stable under process conditions.
3. High *E*-values for the hydrolysis and transesterification can be achieved by lipase catalyzed biotransformations [4].

However, many substrates remain to be hardly convertable by lipases or at least react very slowly [5–9].

Therefore, there is an ongoing big demand for new esterases for applications in fine chemical synthesis. We have chosen a recombinant approach to develop a kit of diverse esterases, to have well characterized clones available which show at least some of the

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1381-1177/02/$ – see front matter
PII: S1381-1177(02)00173-X

desired properties for bioconversions of the very wide spectrum of organic ester compounds. Many examples have demonstrated that such clones can be further improved fast and efficiently by laboratory evolution [10,11]. Production of useful enzymes can be scaled up easily by overexpression in *E. coli*. This approach, using heterologous overexpression of esterase genes, also gives access to new esterases which show only low expression levels in its native host organisms.

Several esterase genes from *Rhodococcus* and *Burkholderia* have already been cloned and characterized in our laboratory [12–16]. Here, we describe a new esterase from the strain *Bacillus subtilis* Est4B, which has already been described by our group to produce esterases [17].

2. Materials and methods

2.1. Bacterial strains and media

B. subtilis strains Est4A, Est4B, Est4C and Est4D were isolated from tributyrin emulsion agar plate and further cultivated on Luria Broth (LB) agar plates as described in this work. *E. coli* sure and the cloning vector pBluescript SK(−) from Stratagene (La Jolla, CA, USA) were used for standard cloning experiments. *E. coli* BL21 (DE3) from Stratagene was used as a host for enzyme production. The vector pMS470Δ8 for heterologous enzyme expression was obtained as a gift from E. Lanka [18].

E. coli cells were grown routinely at 37 °C in LB media, containing 100 mg/l ampicillin. For the preparation of tributyrin emulsion agar plates, 5–7 g/l were added to the molten agar and emulsified by sonication with an ultrathurax homogenizer prior to pouring onto the plates.

2.2. Construction and screening of a genomic library from B. subtilis Est4B

Genomic DNA from *B. subtilis* Est4B was isolated from cells grown overnight at 37 °C in LB using the Qiagen (Qiagen, Hilden, Germany) genomic kit (tip 500). Ten micrograms of genomic DNA was partially digested with *Sau*3AI (Roche, Mannheim, Germany). The fragments were size selected by agarose gel electrophoresis and isolation of fragments from 4 to 8 kb from the gel using the Qiaex DNA elution kit (Qiagen). The genomic DNA fragments were ligated into the *Bam*HI site of pBluescript SK(−). The ligation mix was directly transformed into *E. coli* sure without prior amplification of the library.

Active clones were identified with a filter paper colony esterase assay as described earlier [13,14]. 2-Cl-propionic acid-2-naphthyl ester was used as a substrate for screening the library.

2.3. Sequence analysis and construction of the expression clone

Plasmid DNA was purified by using a plasmid DNA isolation kit (Qiagen) and the insert was sequenced using an ABI 373A automated sequencer and the Dye Deoxy Terminator Sequencing Kits from Perkin-Elmer Biosystems (California, USA). DNA and protein sequences were analyzed with the GCG program package (Genetics Computer Group, Madison, WI, USA). Structural classification of the protein was done by using the 3D-PSSM program ([30], http://www.bmm.icnet.uk/∼3dpssm/).

PCR primers with restriction sites for *Nde*I and *Sph*I on 5′ extensions of the oligonucleotides (B3ADstart: 5′GAACACACATATGGTCCAGCTC3′, B3ADend: 5′AGCGCATGCTGTCTGTCATATC3′) were synthesized. These primers were used to introduce appropriate restriction sites by PCR at the 5′ and 3′ ends of the gene coding for protein Est4B1. The reaction was performed in a total volume of 50 μl, using 10 ng of template (pTB3), 5 μl dNTPs (1 mM each), 200 ng of each primer, 5 μl PCR buffer (10× Dynazyme) and 2 U of Dynazyme DNA polymerase (Finnzyme, Espoo, Finland) for 6 cycles at 94 °C (1 min), 38 °C (2 min) and 72 °C (2 min), followed by 23 cycles at 94 °C (1 min), 65 °C (2 min) and 72 °C (2 min). The PCR product was purified employing the Qiaquick PCR purification kit (Qiagen) and cut with *Nde*I and *Sph*I followed by ligation with the expression plasmid pMS470Δ8. This vector is based on the strongly regulated *tac*-promoter and allows insertion of the gene at the start ATG downstream of a highly efficient Shine Dalgarno sequence originating from the T7 gene 10. The resulting construct was named pMS470BS1.

2.4. Protein production and purification

A preculture of *E. coli* BL21 (pMS470BS1) was made by shaking the cells in LB supplemented with 100 mg/l ampicillin overnight at 37 °C. This overnight culture was used to inoculate 100 ml of LB in a 300 ml Erlenmeyer flask and shaken at 37 °C until the culture reached the mid-log phase. Finally, the main culture (300 ml in 1 l flasks) was started by inoculation with 10 ml of the second preculture. For induction of the expression, lactose was added from a 1 M stock to a final concentration of 10 mM immediately after inoculation. The cells were cultivated for further 12–14 h at 37 °C and then harvested by centrifugation. The cell pellet was resuspended in 20 ml of Tris buffer (0.1 M, pH 7.5) and frozen after addition of 1 U of benzonase per flask. The cell suspension was thawed and lysed by sonication (Branson Sonifier 250, pulsed sonication for 6 min). The cell debries were centrifuged at 100.000 g for 1 h. The supernatant containing the soluble protein Est4B1 was frozen in aliquots at −18 °C.

The protein was purified using a two-step procedure. The enzyme was loaded onto a 15 ml QFF column (Amersham Pharmacia, Uppsala, Sweden) and eluted with a step gradient from buffer A (10 mM Tris–HCl, pH 7.5) to buffer B (10 mM Tris–HCl, pH 7.5, 2 M NaCl). The majority of the enzyme was eluted at a holding phase for 15 ml at a concentration of 8.5% buffer B and a following flat gradient from 8.5% of buffer B up to 13% for 30 ml. The fractions containing the enzyme were pooled together and concentrated to 5–8 ml by ultrafiltration using Centricon YM-10 centrifugal devices (Millipore, Bedford, USA). Further purification was done by gel filtration using a Pharmacia Sephacryl S-100 HR in XK 26/70 column at a flow rate of 4 ml/min.

2.5. Protein crystallization

An enzyme solution of a concentration of 6.5 mg/ml was used for crystallization trials. Setups were made using the hanging drop vapor diffusion method, in 24-well Linbro culture plates with drops on siliconized glass cover slides. Wells were sealed with high-vacuum laboratory grease. Drops contained 5 μl protein solution and 5 μl precipitant solution from the corresponding 750 μl reservoir. The temperature was 20 °C. A standard, coarse matrix crystallization screen from Hampton was used. Platelet crystals appeared from the following conditions: 28% PEG 4000, 0.2 M NaAc, 0.1 M Tris–Cl pH 7.5.

2.6. Enzyme assays

Esterase activity was measured photometrically in Tris–HCl buffer (100 mM, pH 7.0) using *ortho*- and *para*-nitrophenyl butyrate (4 mM in DMSO) as substrates. The amount of nitrophenol released during the enzymatic reaction was determined photometrically at 405 nm at room temperature. One unit of activity is defined as the amount of enzyme releasing 1 μmol/min of alcohol under assay conditions. Under these conditions, the extinction coefficients for *ortho*- and for *para*-nitrophenol are 2.42 or 9.6 ml μmol/cm, respectively.

For determination of free SH-groups after hydrolysis of CoA esters a photometric assay as described by Cho and Cronan Jr. [19] was used. Enzyme preparations purified by anion exchange chromatography were used for thioesterase assays and alkalimetric titrations.

Esterase activity of Est4B1 on tributyrin and 2-Cl-propionic acid methyl ester was determined by alkalimetric titration applying an autotitrator Mettler DL 21 from Mettler Toledo (Switzerland) in 50 ml of 20 mM NaH_2PO_4 with 100 μl of substrate and 0.75 ml of Est4B1 lysate (corresponding to 56 U esterase, determined using *para*-nitrophenyl butyrate) at pH 7.5 and 30 °C.

Thin-layer chromatography (TLC) screening reactions were performed by suspending 100 μl of crude bacterial lysate of the overexpressing cultures in 0.5 ml buffer (0.1 M NaH_2PO_4, pH 7.3). After adding substrate (∼20 μl), the mixture was agitated on a rotary shaker (150 rpm) at 25 °C. Samples were taken after 2 and 4 h and 1 day (20–24 h) and directly analyzed without any work up by TLC using Merck Silica gel 60 F_{254}. For substrates octanyl-2-acetate and 2-Cl-propionic acid-2-naphthyl ester an eluent composition of petroleum ether/ethyl acetate (3:1) was used and products were visualized by spraying with vanillin/H_2SO_4 (conc.). Substrates mandelic acid ethyl ester and 3-hydroxy butanoic acid ethyl ester were run by petroleum ether/ethyl acetate (1:1). Compounds were visualized by UV-detection

or spraying with cer ammonium molybdate, respectively.

All substrates were obtained from Sigma (St. Louis, MO, USA) with the exception of 2-Cl-propionic acid-2-naphthyl ester and octanyl-2-acetate, which were obtained as a gift from Michael Schmidt from the institute of organic chemistry (TU-Graz).

3. Results and discussion

3.1. Isolation and characterization of the strain

Big halos have been detected on contaminated tributyrin emulsion agar plates. These plates originated from an insufficiently cleaned ultrathurax homogenizer that was used for homogenization of plant material before. A colony forming a big halo was isolated and restreaked three times to ensure a pure culture. The halo forming activity was seen when the strain was grown at room temperature, 30, 37, and at 50 °C. This strain was named Est4 and sent to the "Deutsche Stammsammlung fuer Mikroorganismen und Zellkulturen (DSMZ)" in Braunschweig, Germany for characterization. They isolated four morphologically different variants from this strain, and they have been named Est4A, Est4B, Est4C and Est4D. Est4A and Est4B have been chosen for further characterizations. Both strains have been identified as *B. subtilis* strains and the only difference between the two was that variant Est4A produced lecithinase, what was not found for Est4B. After receiving the separated variants from DSMZ *B. subtilis* Est4A was recultured on LB agar plates, and again several morphological variants were observed. It seems that these variants are originating from different morphological forms of this strain rather than to be contaminating bacteria of another species. Under the light microscope, the different variants showed the same shape but different mobility. It has also been demonstrated that Est4A, Est4B, Est4C, and Est4D show a different esterase and lipase profile [17]. Esterase activity could be found in all four variants, but variants Est4A and Est4C have been shown to have additional lipase activities with different stereopreference. We decided to take the esterase producer *B. subtilis* Est4B for construction of gene libraries in *E. coli* for cloning of esterase genes, since it lacked lipase activity.

3.2. Isolation and sequencing of the esterase clone pTB3

Applying an agar plate assay, the gene library, constructed from partially digested genomic DNA from *B. subtilis* Est4B was screened for clones hydrolyzing 2-Cl-propionic acid-2-naphthyl ester. A clone named *E.coli* (pTB3) was isolated and the insert of the plasmid pTB3 was sequenced. The sequence is shown in Fig. 1. Due to a short deletion at the 3′ end of the insert and some bases of the vector DNA, this end of the *Sau*3AI digested genomic DNA insert was not correctly ligated with the cloning vector pBluescript SK(−).

3.3. Sequence analysis of the open reading frames of pTB3

There are two truncated proteins and one full-length protein encoded by the *B. subtilis* DNA fragment of pTB3 (Fig. 2). The partial ORF1, which codes for the C-terminus of a protein showed high amino acid sequence similarity to integrated thioesterases of surfactin synthetase gene clusters. Ninety-eight percent amino acid sequence identity was found to the putative surfactin synthetase protein SrfC from *B. subtilis* A13. The sequence of this highly homologous protein is deposited at the NCBI database under the accession number AF233756. The Srf3 protein from *B. subtilis* (accession number Q08787) showed an identity of 75% and was described to be essential for the non-ribosomal peptide synthesis of the surfactant surfactin [20]. Surfactin is a bacterial cyclic lipopeptide showing a hexapeptide with an LLDLLDL chiral sequence, linked via a lactone bond, to a ß-hydroxy fatty acid with 13–15 C atoms and shows exceptional surfactant activity and also antibacterial, antiviral and antitumoral properties [21]. Many other known integrated thioesterase domains such as tyrocidine synthetase, fengycin synthetase, bacitracin synthetase, and lichenysin synthetase are described [22–24] and showed amino acid similarities between 27 and 51%. These integrated thioesterase domains are involved in elongation and macrocyclization during peptide synthesis. Trauger et al. [25] demonstrated that an excised carboxy-terminal thioesterase (TE) domain of tyrocidine synthetase can catalyze macrocyclization independently of its upstream domains. When the

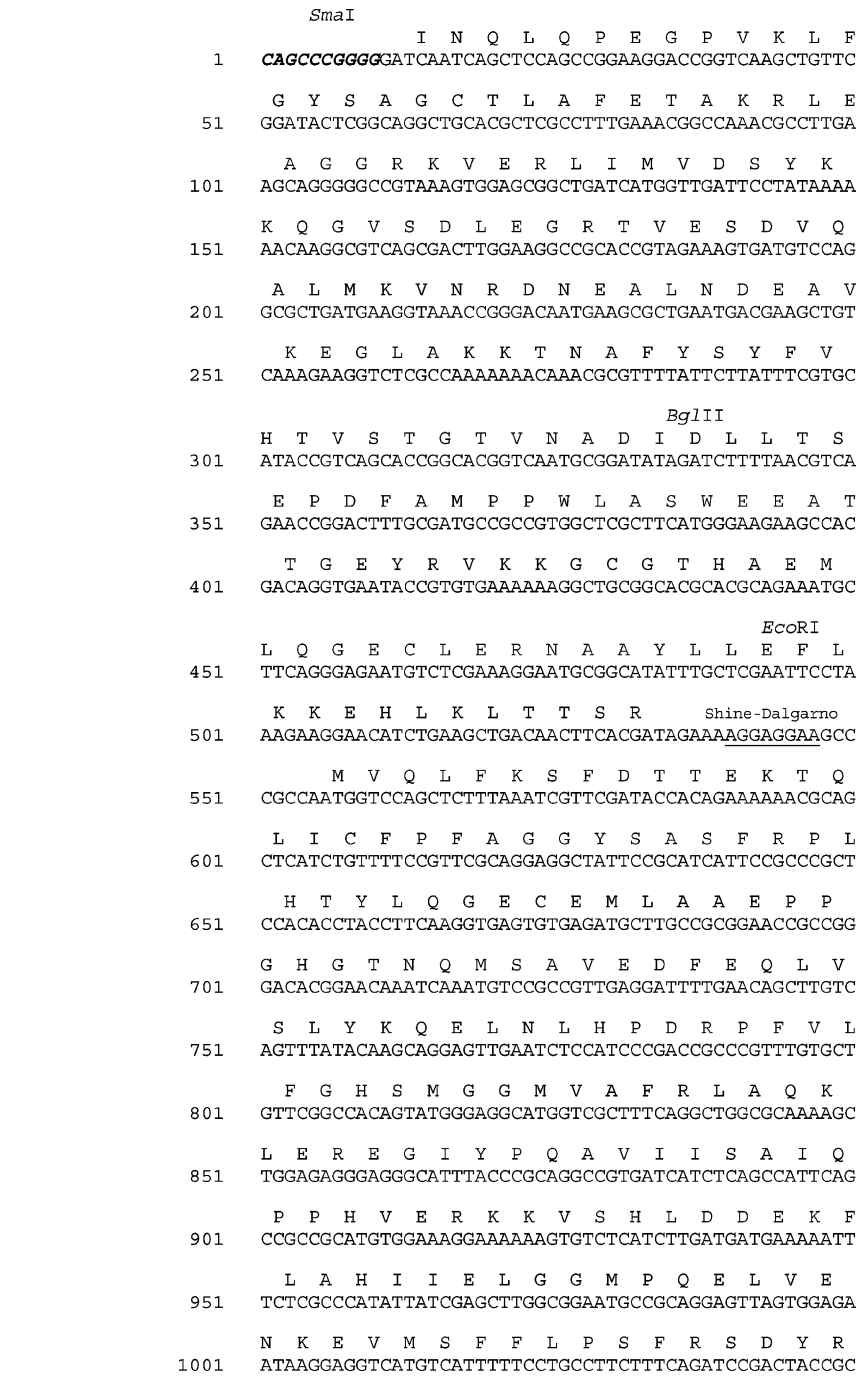

Fig. 1. Complete nucleotide sequence of the genomic DNA from *B. subtilis* including the regions coding for one full-length (*est*4B1) and two truncated open reading frames (*orf*1 and *orf*3). The putative Shine Dalgarno Sequence of gene *est*4B1 is underlined. Sequences originating from the cloning vector pBluescript SK(−) are in bold and italic.

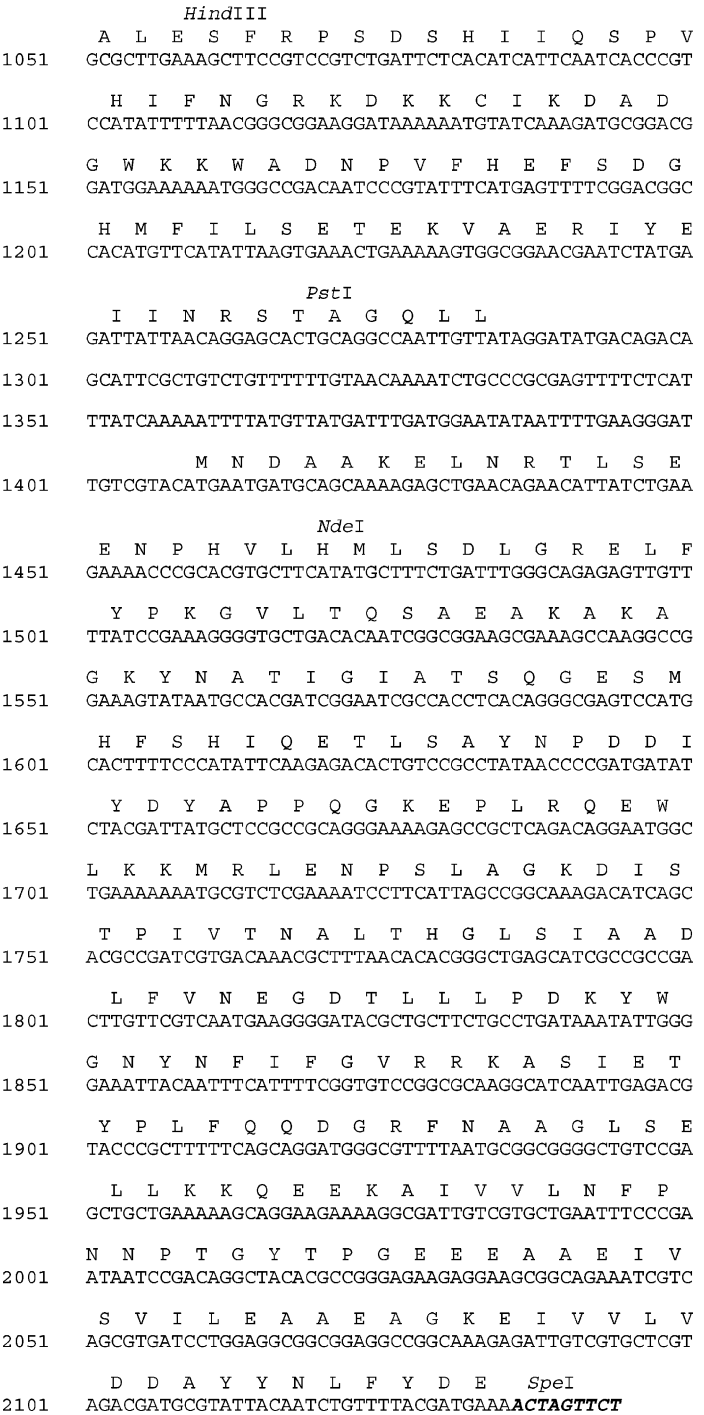

Fig. 1. (*Continued*).

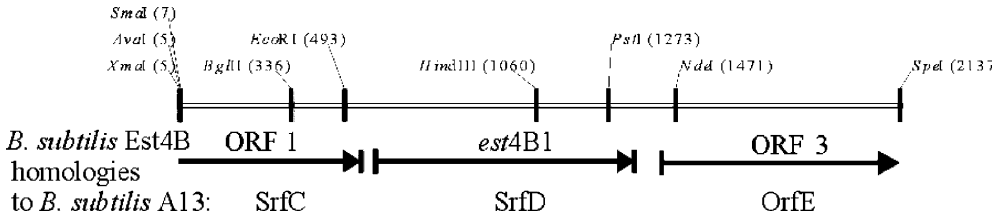

Fig. 2. The cloned DNA fragment codes for one full-length protein (Est4B1) and two truncated proteins (ORF1, ORF3). The amino acid sequence showed high similarity to putative proteins from *B. subtilis* A13 (SrfC, SrfD and ORFE).

deoxyerythronlide B synthetase TE domain was overexpressed it showed hydrolytic activity, but no cyclization of synthetic acyl-thioester substrates was observed [26,27]. This observation could be explained by a high specificity of the enzyme to its natural substrate.

The ORF2 coded for 243 amino acids and was named est4B1. It showed 99% amino acid sequence identity to the putative independent thioesterase from *B. subtilis* A13 (accession number AAF87217) and 79% identity to surfactin synthetase subunit 4 from *B. subtilis* ATCC 21332, which is also known as cold shock protein CSI16 (accession number Q08788). The function of this protein in not known yet. However, there are some speculations that it could be involved in termination of the peptide synthesis or in liberation of mischarged molecules [28]. That deletion of the *srf*4 gene is not deleterious to surfactin production has been reported by Cosmina et al. [29]. Disruption of the *srf*4 gene resulted in wild-type level production of surfactin. In contrast to that, Schneider and Marahiel [28] reported that there was no alteration of the amino acid composition or the ß-hydroxy fatty acid moiety, but deletion of the Srf1–3-TE (SrfA-C-TE) domain or of Srf4 (SrfD) led to a reduction in surfactin production of 97 or 84%, respectively. Deletion of both domains resulted in total loss of surfactin production. An enzymatic function of Srf4 has not been shown yet. The sequence of the homologous protein from *B. subtilis* A13 is deposited in the NCBI database, but no description about the function of this protein was found. A structural similarity search for a structural classification of the protein was done using the 3D-PSSM web server V2.5.6 [30]. Interestingly, the predicted structure of Est4B1 showed much higher structural similarities to enzymes of the α–β fold hydrolase family other than esterases and lipases. The highest similarity was predicted to haloperoxidase L (*Streptomyces lividans*), chloroperoxidase F (*Pseudomonas fluorescens*), bromoperoxidase A2 (*Streptomyces aureofaciens*), proline iminopeptidase (*Seratia marescens*), haloalkane dehalogenase (*Xanthobacter autotrophicus*) and hydroxynitrile lyase (*Hevea brasiliensis*). This leaded to a speculation that halogenated ester compounds and esters with nitrogen containing groups are substrates, which should be well accepted by Est4B1.

The truncated ORF3 is different from the downstream region of the functionally described surfactin synthetase gene cluster from *B. subtilis* ATCC 21332, but highly homologous again to the putative aspartate aminotransferase from *B. subtilis* A13 (accession number AAF87218). These amino acid sequences were 98% identical. Fifty-eight percent amino acid sequence identity were found to an enzyme from *Bacillus cirrulans*, which displays aspartate aminotransferase activity [31].

3.4. Overexpression, purification and crystallization of the protein Est4B1 (ORF2)

*Nde*I and *Sph*I restriction sites were introduced by PCR at the start and the end of the full-length reading frame. The PCR product was cloned into the expression plasmid pMS470Δ8 behind the strongly regulated *tac*-promoter and transformed into *E. coli*. Clones showing esterase activity were identified using the agar plate assay as described above. Plasmid DNA from one positive clone was isolated and sequenced to verify the DNA sequence after PCR.

Induction by lactose resulted in high-level production of the esterase Est4B1 (Fig. 3). The expression was extremely strong in comparison to other esterases produced in our laboratory. This also indicated that the codons used in this *B. subtilis* gene are well suited

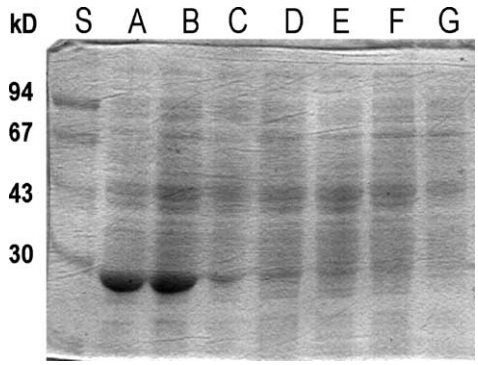

Fig. 3. Heterologous overexpression of Est4B1 using *E. coli* BL21 (pMS470BS1) at 37 °C. Expression was induced by addition of lactose to a final concentration of 10 mM at different times: (A) induction immediately after inoculation; (B) 4 h, (C) 6 h, (D) 8 h, (E) 10 h, (F) 12 h after inoculation. (G) *E. coli* BL21 pBluescript SK(−) was used as a negative control.

for high-level expression in *E. coli*. Therefore, in another work we applied this codon usage to modify the first 150 codons of the *est*C gene from *Burkholderia gladioli* NCPPB1891 to get high overexpression in *E. coli* [13]. By this strategy, it was possible to reduce the high GC content of this gene, to resolve high-melting RNA structures and to introduce restriction sites by PCR thereby making high-level heterologous expression of that *Burkholderia* esterase feasible.

Three further clones, containing an insert, but showing no esterase activity on the agar plate assay were sequenced. One of these clones, named Est4B1,6 showed a mutation of the putative active serine residue in the typical lipase/esterase motif GHSMG to glycine and two additional silent mutations. The expression level of the serine mutant was the same as with the wild-type clone.

Est4B1 was purified close to homogeneity using a two-step purification strategy as described in Section 2 (data not shown). The molecular weight of the enzyme determined by MALDI was 27 445. However, the calculated average molecular weight (calculated by the program peptide-mass at the expasy server (http://ca.expasy.org/cgi-bin/peptide-mass.pl)) based on its amino acid sequence was 27 760. This small difference could perhaps arise partly from post-translational N-terminal processing of the first amino acids, but also from small inaccuracies from the mass spectrometry of this large molecule. The purified enzyme has been crystallized and single crystals could be achieved. This provides an initial step to solve the first 3D protein structure of this class of hydrolytic enzymes and to establish CLECS.

3.5. Hydrolytic activity of Est4B1

Positive signals of the clone *E. coli* (pTB3) in the agar plate assays for hydrolysis of 2-Cl-propionic acid-2-naphtyl ester indicated some esterase activity of Est4B1. Crude lysates of *E. coli* BL21 (DE3) overexpressing the gene coding for Est4B1 were used for further analysis of the esterase activity of this protein. Several esters were evaluated to check the substrate specificity of Est4B1 (Table 1). Hydrolytic activity was analyzed by thin-layer chromatography (TLC). All results were compared with the activity of the same *E. coli* strain without the expression plasmid. After 20 h most of 2-Cl-propionic acid-2-naphtyl ester had been hydrolyzed by Est4B1, whereas only 10–20% of 3-hydroxy butyrate was cleaved after that time. No turnover of mandelic acid ethyl ester and octanyl-2-acetate could be observed.

The hydrolytic turnover of 2-Cl-propionic acid methyl ester was quantified by alkalimetric titration. The activity of Est4B1 on this substrate was 0.5 U/mg of protein. The pH optimum for hydrolysis was found at pH 8. The activity was strongly reduced at a pH lower than 6.5. The protein precipitated already under weakly acidic conditions. The hydrolytic activity on 3-hydroxy butyrate was lower than 0.01 U/mg. No activity could be detected upon titration using tributyrine as a substrate.

Table 1
Substrates of Est4B1 analyzed by thin-layer chromatography (TLC), titrimetric or spectrophotometric determination of hydrolytic activity

Substrate	Activity	Method
2-Cl-propionic acid-2-naphthyl ester	+	TLC
3-Hydroxy-butyric acid ethyl ester	+/−	TLC, titrimetric
Mandelic acid ethyl ester	−	TLC
Octanyl-2-acetate	−	TLC
2-Cl-propionic acid methyl ester	+	Titrimetric
Tributyrine	−	Titrimetric
4-Nitrophenyl butyrate	+	Photometric
2-Nitrophenyl butyrate	+/−	Photometric
Fatty acid CoA esters	−	Photometric, titrimetric

(+): good activity; (+/−): weak activity; (−): no activity.

The photometric assays using 4-nitrophenyl butyrate resulted in a specific activity of 0.3 U/mg, but less than 0.01 U/mg for hydrolysis of 2-nitrophenyl butyrate. A set of fatty acid thioesterase substrates (propionyl CoA, DL-ß-hydroxybutyryl CoA, DL-ß-hydroxyglutaryl CoA, *n*-decanoyl CoA and myristoyl CoA) has also been analyzed photometrically for hydrolysis by Est4B1. No activity was seen on any of these carboxylic acid CoA esters.

This is the first time that enzymatic activity was shown for a protein of this putative independent thioesterase family from non-ribosomal peptide synthetases using a thiotemplate mechanism. The activity on substrates which are described in this study was not extraordinary high, but very specific. One could speculate about applications of this esterase in processes where there is a demand for hydrolysis at specific sites with a substrate containing several putative hydrolyzable side groups.

Many examples have shown that activity, stability and even selectivity can be further improved using the new methods of protein engineering by directed evolution [10,11].

We hope that our understanding of the biochemical properties of esterases, a very diverse group of enzymes, will help us to be able to adjust these biocatalysts to the demand for large scale applications and scale up its production within a minimum of time.

Acknowledgements

We would like to thank Dr. Fritz Andreae from piCHEM Research in Graz for the MALDI experiments, Dr. Michael Schmidt from the institute of organic chemistry for kindly providing the substrates and E.T. Farinas and U. Schwaneberg (Caltech, CA) for critical review of the manuscript. This work was supported by the SFB Biokatalyse project F0101.

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