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ORIGINAL PAPER

A cold-adapted esterase of a novel marine isolate, *Pseudoalteromonas arctica*: gene cloning, enzyme purification and characterization

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Abstract A gene encoding an esterase (*estO*) was identified and sequenced from a gene library screen of the psychrotolerant bacterium *Pseudoalteromonas arctica*. Analysis of the 1,203 bp coding region revealed that the deduced peptide sequence is composed of 400 amino acids with a predicted molecular mass of 44.1 kDa. EstO contains a N-terminal esterase domain and an additional OsmC domain at the C-terminus (osmotically induced family of proteins). The highly conserved five-residue motif typical for all α/β hydrolases (G × S × G) was detected from position 104 to 108 together with a putative catalytic triad consisting of Ser^{106}, Asp^{196}, and His^{225}. Sequence comparison showed that EstO exhibits 90% amino acid identity with hypothetical proteins containing similar esterase and OsmC domains but only around 10% identity to the amino acid sequences of known esterases. EstO variants with and without the OsmC domain were produced and purified as His-tag fusion proteins in *E. coli*. EstO displayed an optimum pH of 7.5 and optimum temperature of 25°C with more than 50% retained activity at the freezing point of water. The thermostability of EstO (50% activity after 5 h at 40°C) dramatically increased in the truncated variant (50% activity after 2.5 h at 90°C). Furthermore, the esterase displays broad substrate specificity for esters of short-chain fatty acids (C_2–C_8).

Keywords Esterase · Psychrophilic enzyme · *Pseudoalteromonas arctica* · OsmC

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Introduction

Esterases (EC 3.1.1.1) belong to the class of hydrolases, catalyzing the formation and cleavage of ester bonds of an extensive spectrum of substrates. Bacteria produce different classes of lipolytic enzymes, including carboxylesterases, which hydrolyze small ester-containing molecules at least partly soluble in water, true lipases (EC 3.1.1.3), which display maximal activity toward water-insoluble long-chain triglycerides, and various types of phospholipases (Arpigny and Jaeger 1999). The mechanism for ester hydrolysis and formation is essentially the same for lipases and esterases (Bornscheuer 2002). In addition to esters and triglycerides, lipolytic enzymes can hydrolyze peptides, amides, and halides. The fact that hydrolases, which show esterase activity are also able to hydrolyze non-ester bonds, raises interesting questions on the terminology and classification of these enzymes (Junge and Krisch 1973). Historically, hydrolases have been classified according to their known substrate specificity. In general, an esterase is specific for either the alcohol or the acid moiety of the substrate but not for both. In the year 1972, Whitaker proposed a classification scheme for esterases, based on the specificity for the acid moiety of the substrate (Whitaker 1972). In the post-genomic era, the increasing number of published sequences completed the classification of enzymes solely based on their function. The deposition of a huge number of sequence information from cloned esterases and

lipases has been used to identify possible sequence specific motifs, which led to the division of eight families of lipases and esterases in bacteria (Arpigny and Jaeger 1999). The members of the α/β hydrolase superfamily share a characteristic G × S × G consensus sequence, called the nucleophilic elbow (Ollis et al. 1992; Nardini and Dijkstra 1999). The above-mentioned serine residue embedded in this motif constitutes a catalytic triad with an aspartic acid and a histidine residue that are placed in this specific order (Ser-Asp-His) in the polypeptide chain. Recently, several other sequence motifs have also been found within this superfamily (Shaw et al. 2002). Usually the canonical α/β hydrolase motif is composed of five α-helices and eight β-sheets, but newly described variations exhibit additional inserted secondary structure elements mostly after strand β_6 (Siew et al. 2005).

Due to their broad substrate specificity, esterases are used in versatile industrial applications including their utilization as additives in laundry detergents and catalysts for organic syntheses of unstable compounds, especially at low temperatures (Bornscheuer 2002; Panda and Gowrishankar 2005). The demand of active biocatalysts under extreme conditions (low or high temperatures, acidic or basic solutions or high salt contents) increases in the industry. Therefore, the isolation of biotechnologically relevant enzymes from extremophilic microbes has become a challenging task in recent years (Burton et al. 2002; Salameh and Wiegel 2007; Hess et al. 2008; Rao et al. 2009; Royter et al. 2009). Indeed, cold-active enzymes have generated considerable interest, since they have potential to improve the efficiency of industrial processes and offer possible economic benefits through energy saving (Gerday et al. 2000; Cavicchioli et al. 2002; Feller and Gerday 2003). Psychrophilic and ectothermic organisms constantly living in a low temperature environment exhibit enzymes adapted to the accordant conditions in the environment, which often show higher catalytic activity at low and moderate temperatures and lower thermostability than their mesophilic and thermophilic counterparts (Arpigny et al. 1993; Rentier-Delrue et al. 1993; Davail et al. 1994). The high catalytic activity at low temperatures in combination with the low thermostability can be favorable in their applications as well (Margesin and Shiner 1994). So far, only a few cold-active esterases and lipases from psychrophilic microorganisms have been cloned and characterized (Choo et al. 1998; Suzuki et al. 2002, 2003; Ferrer et al. 2004; Kulakova et al. 2004; Zimmer et al. 2006; Cieslinski et al. 2007; de Pascale et al. 2008; Roh and Villatte 2008; Heath et al. 2009).

In a previous report, we have described the psychrotolerant marine isolate *Pseudoalteromonas arctica* from Spitzbergen in the Arctic, which grew optimally at 10–15°C promising an ideal model to investigate cold-adapted enzymes for diverse applications (Al Khudary et al. 2008). In the present study, we focused our attention on properties of the cold-active esterase encoding gene *estO*. Sequence characteristics of the deduced amino acid residues revealed that this protein does not belong to any of the eight families of classified lypolytic enzymes according to Arpigny and Jaeger (1999). Interestingly, the corresponding enzyme exhibits an additional putative C-terminal domain, which displays homology to the osmotically induced protein OsmC from *Escherichia coli*, which strongly influenced the estereolytic activity of purified EstO (Gutierrez and Devedjian 1991). Comparative biochemical characterizations studies of the esterase with and without the OsmC domain revealed a dramatic difference in enzymatic behavior.

Materials and methods

Bacterial strains

The psychrotolerant bacterium *Pseudoalteromonas arctica* was isolated from sea ice samples taken from Spitzbergen, Norway and was deposited in DSMZ (18437) and LMG (23753) (Al Khudary et al. 2008). *Escherichia coli* strains XL1-Blue MRF', XLOLR (both Stratagene), and Nova-Blue Singles (Novagen) were used as the bacterial hosts for plasmid amplification and propagation. Cloning experiments were performed under standard conditions (Sambrook et al. 2001). *E. coli* strain Tuner™ (DE3) pLacI was used for heterologous expression of the *estO* gene.

Gene library construction

The λ-Express Predigested Vector and ZAP Express Predigested Gigapack cloning kits (*Bam*HI/CIAP-treated) were used for the construction of a *P. arctica* λ-phage gene library as described by the manufacturers (Stratagene). In brief, chromosomal DNA of *P. arctica* was isolated by utilizing the Genomic DNA Isolation Kit (Qiagen). The genomic DNA was partially digested by *Sau*3A (New England Biolabs, Inc.). Fragments were separated by agarose gel electrophoresis and 6–10 kb fragments were isolated by electroelution and phenol/chloroform extraction. Obtained DNA fragments were ligated into the λ-ZAP express vector containing the phagemid pBK-CMV. After packaging of the ligation products, the primary phage library was amplified in *E. coli* XL1-Blue MRF'. After amplification, phagemid pBK-CMV harboring the insert DNA was excised using helper phage ExAssist and was finally transfected and stably established as plasmids in *E. coli* XLOLR cells.

Screening for recombinant esterase clones

Screening for esterase active *E. coli* XLOLR clones harboring pBK-CMV derivatives was performed on solid LB medium supplemented with 50 μg/ml kanamycin, 1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) and 1% glyceryl tributyrate (Sigma). Incubation of screening plates was carried out at 20°C for 3–4 days, and activity was observed when clearing zones (diffusion halos) around the active clones were seen as a result of tributyrin degradation.

Sequence analysis

Plasmids from selected clones were isolated using the NucleoSpin plasmid isolation kit (Macherey–Nagel GmbH & Co. KG). The DNA sequence of inserts was analyzed on an ABI automatic sequencer with the primer-walking technique starting with standard T7 and T3 oligonucleotides that annealed with pBK-CMV. To obtain the complete sequence of the *estO*-containing insert on plasmid pBK-CMV-EstO, primer walking was performed using oligonucleotides F1 (GTGATAAACCTAAACCTTCA), R1 (ATCTGCAAGCCAAGTATGAT), F2 (GGATAATG TAGGATACGTAAC) and R2 (CCGCAGATACTAAA TCTTGAATGTT). The determined open reading frame of *estO* from *P. arctica* was analyzed with Vector NTI software (Invitrogen). Sequence data from esterases of other organisms were obtained from the NCBI online database by performing BLAST analyses (Altschul et al. 1990). Reference amino acid sequences utilized in phylogenetic analysis were retrieved from NCBI database and aligned with the selected genes using CLUSTAL W (1.83) software. Signal sequence prediction was carried out using SignalP 3.0 online software, and secondary structure predictions were performed with the PSIPRED server (McGuffin et al. 2000; Emanuelsson et al. 2007).

Cloning of *estO* from *P. arctica*

To express the esterase from *P. arctica* in a heterologous system in *E. coli*, the *estO* ORF was amplified with the Expand HiFi PCR kit (Roche Diagnostics) using primers EstO_for (*CATATG*CGACAAAAAGTATCTTTTAAAA, *Nde*I restriction site is given in italics) and EstO_rev (*CTCGAG*TTCTACCAGTTCACTTACAATAACT, *Xho*I restricion site is indicated in italics) with genomic DNA as template. This PCR approach resulted in a 1,209-bp fragment with *Nde*I and *Xho*I restriction sites at the beginning and end of the amplicon, respectively. The polymerase chain reaction (PCR) was performed in the temperature gradient thermoblock PCR system (Biometra) with the following temperature profile: 94°C for 5 min and 30 cycles of 94°C for 1 min, 59 ± 5°C for 1 min and 68°C for 2 min, and then 68°C for 15 min. The subcloning of the PCR-amplified fragment was carried out by using Acceptor vector kit (Novagen) with the expression vector pETBlue-1 and competent *E. coli* NovaBlue Singles cells. Screening for positive clones was performed on solid LB medium containing 50 μg/mL carbenicillin, 15 μg/mL tetracycline, 70 μg/mL X-gal, and 80 μM IPTG. After performing activity assay on LB plates, *Nde*I/*Xho*I double digestion resulted in a 1,203-bp fragment, which was extracted from a 1% agarose gel using NucleoSpin Extract kit (Macherey–Nagel GmbH & Co. KG) and ligated into the single *Nde*I and *Xho*I restriction sites of vector pET24b (Novagen), resulting in plasmid pET24b-EstO. The pET24b vector carries an N-terminal T7-Tag® sequence plus an optional C-terminal His-Tag® sequence. The esterase *estO* gene of *P. arctica* was ligated in pET24b vector in frame with the C-terminal His-Tag® coding sequence. Similarly, a truncated *estO* variant was amplified using primer pair EstO_for/EstΔOsmC_rev (*CTCGAG*GTACTTAACATAA CGGTTTG, *Xho*I restriction site is given in italics) resulting in a 759-bp amplicon, encoding for the N-terminal part of EstO without the C-terminally located OsmC domain. Ligation of the construct in pET24b vector resulted in plasmid pET24b-EstOΔOsmC.

Heterologous expression and purification of the recombinant esterase

To produce the EstO-His fusion constructs, plasmids pET24b-EstO and pET24b-EstOΔOsmC were transformed into *E. coli* Tuner™ (DE3) pLacI Competent Cells (Novagen). Expression of *estO* and *estOΔosmC* open reading frames, respectively, was induced with 2 mM IPTG after $A_{600} = 0.6$–0.8 was reached. Thereafter, transformants were grown with constant shaking in 1 L liquid LB medium at 30°C for 24 h. Protein extraction was performed from 3 g *E. coli* Tuner™ (DE3) pLacI wet weight suspended in 15 ml Tris HCl buffer (25 mM, pH 7.0). Complete cell disruption was achieved by using the French press (Spin Aminco, Spectronic Instruments, 3 times at 2,500 psi), and finally centrifugation (12,000*g*) at 4°C for 20 min resulted in the removal of cell debris. Subsequently, a 1.5-ml Ni–NTA superflow column (Qiagen) was equilibrated with Tris–HCl buffer (25 mM, pH 7.0) and loaded with 1 ml sample of crude extract from the soluble fraction. This was followed by a first washing step with Tris–HCl buffer (25 mM, pH 7.0) and a second washing step with Tris HCl buffer (25 mM, pH 7.0) containing 25 mM of imidazole. Elution was done with Tris–HCl buffer (25 mM, pH 7.0) supplemented with 250 mM imidazole. The eluted esterase (bearing a C-terminal His-Tag) was then dialyzed overnight at 4°C against Tris–HCl buffer (25 mM, pH 7.0) containing 100 mM NaCl and

5 mM EDTA. The purity of the obtained recombinant esterase was analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) according to Laemmli (1970).

Enzyme assays

In vitro activity of *P. arctica* esterase EstO produced in *E. coli* Tuner™ (DE3) pLacI was measured using two different spectrophotometric assays. Protein concentrations in the extracts were determined in all experiments by the Bradford (1976) method. In the first assay (A), cleavage of *p*-nitrophenyl benzoate (*p*-NPB, Sigma) was determined at 20°C in Tris–HCl buffer (25 mM, pH 7.0), according to Winkler and Stuckmann (1979). In brief, a buffered *p*-NPB emulsion (10 ml of ethanol containing 37 mg *p*-NPB was mixed with 90 ml Tris–HCl buffer (25 mM, pH 7.0) containing 100 mg gum arabic) was sonicated for 2 min at room temperature before the reaction was started by the addition of crude enzyme extract in a final volume of 1 ml. After 30 min of incubation at different temperatures, the reaction was stopped by cooling the mixture on ice for 2–5 min followed by the addition of 100 µl of 25% Na_2CO_3. The probes were centrifuged for 2 min at 13,000 rpm, and then the optical density was measured at 410 nm against a blank control, which has been taken immediately after enzyme addition. In this assay, one unit of esterase activity is defined as the amount of enzyme, which is required to release 1 µmol *p*-nitrophenol per minute under test conditions. The activity toward other *p*-NP esters was measured in the same manner, by using 1 mM of each substrate. Under the tested conditions, the extinction coefficient ε_{410} for *p*-nitrophenol is 6.52×10^6 cm^2 mol^{-1}. In the second assay (B), triacylglycerols and other substrates different from *p*-NP esters were tested as described previously (Schmidt-Dannert et al. 1994). The hydrolytic activity of the esterase was measured by a spectrophotometric analysis using the formation of copper soaps for the detection of free fatty acids. Enzyme reaction was carried out under shaking for 12 h at 20°C. The blank probe absorption, which was taken immediately after the start of the reaction, was measured at 430 nm. One unit of esterase activity is defined as the amount of enzyme needed to liberate 1 µmol of free fatty acid per minute under standard conditions. All experiments were performed at least in triplicate.

Substrate specificity

The enzyme specificity was studied with *p*-NP esters of varying acyl chain lengths from C_2 to C_{18} as described in assay A. The following substrates were tested toward the length of different acyl chains of *p*-NP esters: *p*-NP acetate ($C_{2:0}$), *p*-NP butyrate ($C_{4:0}$), *p*-NP caproate ($C_{6:0}$), *p*-NP caprylate ($C_{8:0}$), *p*-NP laurate ($C_{12:0}$), *p*-NP myristate ($C_{14:0}$), *p*-NP palmitate ($C_{16:0}$), and *p*-NP stearate ($C_{18:0}$). Further substrates tested were: *p*-NP 1-naphtoate, *p*-NP 2-naphtoate, *p*-NP benzoate, 2-(4-isobutylphenyl)-*N*-(*p*-NP) propanamide, *p*-NP 2-phenylpropanoate, *p*-NP 3-phenylbutanoate, *p*-NP cyclohexanoate, *p*-NP 2-(6-methoxynaphthalen-2-yl) propanoate, *p*-NP 2-(6-methoxynaphtalen-2-yl) propanoate and *p*-NP 2-(4-isobutylphenyl) propanoate. Furthermore, the esterase activity was tested with the following compounds as described in assay B: tripalmitin ($C_{16:0}$), tristearin ($_{C18:0}$), triolein ($C_{18:1}$), olive oil, ethyl caproate, ethyl caprylate, ethyl nonanoate, ethyl laurate, methyl octanoate, vinyl acetate, and vinyl butyrate.

Enzymatic characterization

Studies on the influence of the pH and temperature were carried out with the purified enzyme EstO from *P. arctica*, produced in *E. coli* Tuner™ (DE3) pLacI. To evaluate the pH optimum, the esterase activity was measured at a pH range from 4.0 to 13.0 in universal buffer with *p*-NPB as substrate (Britton and Robinson 1931). Incubation took place in every single experiment at a constant temperature of 20°C. In a further approach, pH stability of the enzyme was tested by pre-incubation of the purified esterase for 30 min at 20°C in universal buffer (pH 4.0–13.0) without substrate. Moreover, temperature optimum and thermostability was tested for EstO. To determine the former, reaction samples were incubated at temperatures from 0 to 40°C in Tris–HCl buffer (25 mM, pH 7.0) for 30 min. Thermostability of the esterase was investigated by incubation of the enzyme without substrate at temperatures from 4 to 90°C and pH 7.0. Samples were collected at various time intervals (from 10 min to 24 h) and clarified by centrifugation before the activity assay was performed as described in assay A. Kinetic studies were performed with the recombinant enzyme (2 U/mg final concentration). In order to calculate the Michaelis–Menten constant (K_M) and the maximum velocity for the reaction (V_{max}), the enzyme activity was tested as described in assay A with various concentrations of *p*-NPB (0.05–2 mM). The reaction sample was incubated at 20°C for 30 min in Tris–HCl buffer (25 mM, pH 7.0).

Inhibitory and activating compounds

The effects of various substances on esterase activity was examined using assay A after pre-incubation of the purified esterase with these reagents in different concentrations at 20°C for 2 h. For examination of the enzyme residual activity, 100 µl of preincubation mixture was mixed with 900 µl of substrate in Tris–HCl buffer (25 mM, pH 7.0). The reaction was carried out for 20 min at 20°C. The

enzyme activity without additional compounds was defined as 100%. The following compounds were used to examine the putative effects on esterase activity: salts containing ions Na^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Sr^{2+}, Al^{3+}, Fe^{2+} or Cr^{3+}, respectively, were tested with a concentration of 10 mM in the incubation mixture. The chemical compounds β-mercaptoethanol, dithiothreitol (DTT), *p*-chloro-mercuri-benzoate (PCMB), 2-iodoacetate, guanidine hydrochloride, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), and Pefabloc (Roche) were applied with a concentration of 10 mM, and inhibitory properties of 10% (v/v) of sodium dodecyl sulfate (SDS) and Tween 20 were tested in this study.

Sequence accession number

The nucleotide sequence of *P. arctica estO* was deposited in the EMBL database under accession number FN666254.

Results

Identification of a novel esterase from *Pseudoalteromonas arctica*

Ten thousand phagemid clones obtained from a λZAP (Stratagene) genome bank based on the genomic sequence of the gammaproteobacterium *Pseudoalteromonas arctica* were screened for esterase activity on solid LB plates supplemented with kanamycin (50 μg/ml), IPTG (1 mM), and glyceryl tributyrate (1%). Plasmid DNA from a clone showing diffusion halos on plates was isolated, and an insert of about 4 kbp was sequenced using primer-walking technique, starting with standard oligonucleotides T7 and T3. Two forward and reverse walking steps with four newly designed primers (F1, R1, F2, and R2) were necessary to determine the complete inserted sequence of the plasmid pBK-CMV-EstO. A BLASTX analysis revealed the presence of two open reading frames. The 1,203 bp gene encodes a protein of 400 amino acids with a high degree of sequence similarity to putative bacterial OsmC-like proteins from *Shewanella woodyi* ATCC 51908 (Accession number YP_001762454; 62% identity in 400 aa overlap) or *Catenulispora acidiphila* DSM 44928 (YP_003117689; 54% identity in 400 aa overlap) and to a predicted hydrolase of the α/β type from *Microscilla marina* ATCC 23134 (ZP_01692024; 49% identity in 400 aa overlap). Utilization of the EBI Pro Scan software approved the presence of the OsmC (osmotic shock response protein) domain at the C-terminal end of the protein, combined with an additional putative esterase domain at the N-terminus (Fig. 1a). Interestingly, the putative protein of *P. arctica* showed no significant similarity to identified enzymes from other bacteria, belonging to the eight families (I–VIII) of prokaryotic lipolytic enzymes (Arpigny and Jaeger 1999). The classification according to Arpigny and Jaeger (1999) is based on the peptide sequences of lipases and esterases. At the amino acid level, the predicted protein from *P. arctica* shares less than 10% similarity with any known esterase. The highest identities were obtained from alignments with a putative polyurethane esterase of the soil bacterium *Delftia acidovorans* (BAA76305; 10%), a heat stable lipolytic enzyme (AAC67392; 9%) isolated from *Sulfolobus acidocaldarius* and with an already characterized ethanol-induced esterase (BAA25795, 9%) from *Acetobacter pasteurianus* (Arpigny et al. 1998; Kashima et al. 1999). However, owing to the domain prediction and the activity of the gene library clone, the open reading frame was designated *estO*.

Furthermore, the deduced peptide sequence of *estO* encodes a protein with a predicted molecular mass of 44.1 kDa and an isoelectrical point p*I* of 6.23. A hypothetical Shine–Dalgarno sequence with a weak motif (AAGG) was found to be located at 7–10 nucleotides upstream from the predicted translational start codon (Ma et al. 2002). The OsmC domain is about 429 bp in length (Fig. 1b), and encodes a putative protein domain of 142 amino acids with a predicted molecular mass of 16 kDa and a theoretical p*I* of 5.97 seperated by a linker of 8 aa from the esterase domain. No signal sequence was detected with the SignalP 3.0 Server (Emanuelsson et al. 2007). The highly conserved five-residue motif typical of all α/β hydrolases (G × S × G) was identified at position 104–108. A putative catalytic triad consisting of one serine, one aspartic acid, and one histidine acid residue was demonstrated to be located at positions 106, 196 and 225, respectively (Fig. 1b).

Heterologous expression and purification of the recombinant *P. arctica* esterase

To characterize and purify the putative esterase EstO from *P. arctica*, the complete *estO* open reading frame was ligated into expression vector pET24b. The C-terminally His-tagged version was produced in *E. coli* Tuner™ (DE3) pLacI cells carrying plasmid pET24b-EstO as described in "Materials and methods". Primary transformants were tested for esterase activity in plate assays on solid LB medium supplemented with kanamycin, IPTG and 1% tributyrin at 30°C. Optimal expression conditions for the production were determined by measuring the enzyme activity (U/mg) of the crude extract in an assay (A) using *p*-NPB as substrate. The highest specific activity was reached after 24 h of growth in liquid LB medium containing kanamycin (50 μg/ml) at 30°C, after 2 mM IPTG induction without the addition of co-factors.

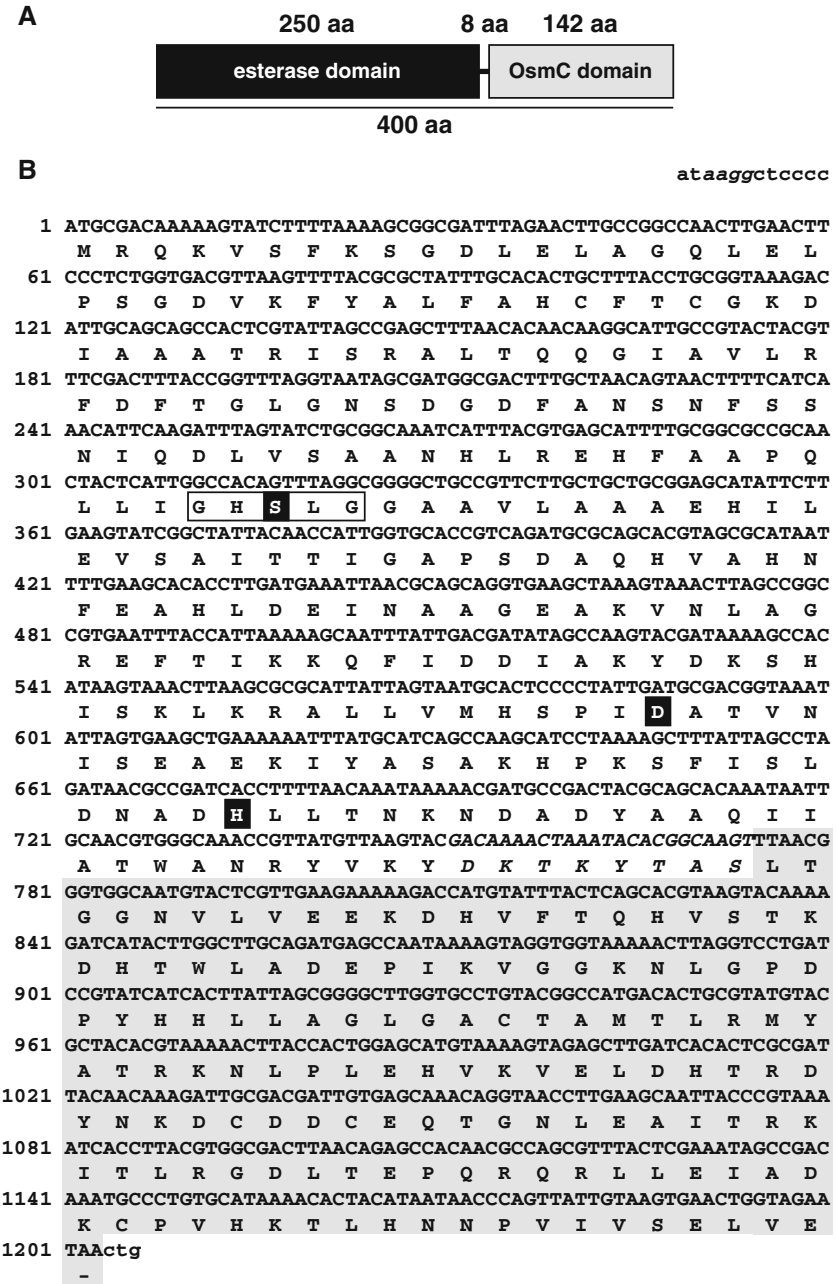

Fig. 1 Organization of EstO in *P. arctica*. **a** Schematic representation of the predicted 250 aa esterase domain separated by 8 aa from the 142 aa OsmC domain of EstO. **b** Nucleotide sequence of EstO with deduced peptide sequence. Flanking genomic regions are given in *lowercase letters* with a hypothetical Shine–Dalgarno-like sequence marked in *italics*. The highly conserved G × S × G motif typical for α/β hydrolases is *framed*. The catalytic triad consisting of Ser^{106}, Asp^{196}, and His^{225} is indicated in *white letters* and *boxed in black*. The predicted OsmC domain is shaded in *gray*

Purification of soluble recombinant esterase was carried out from *E. coli* Tuner™ (DE3) pLacI transformants, which were cultured in 1 L liquid LB medium at optimal growth conditions. Cells were harvested by centrifugation and 3 g wet weight was redissolved in 15 ml Tris–HCl buffer (25 mM, pH 7.5). French press was used to break bacterial cells open, and the supernatant was applied for purification via Ni–NTA column. Separation of eluted proteins by 12% SDS-PAGE revealed a coomassie stained band with deduced molecular weight of 45.2 kDa (Fig. 2), which is consistent with the joined molecular masses of the full-length protein (44.1 kDa) with the C-terminally fused His-tag peptide (1.1 kDa). After Ni–NTA superflow column chromatography the full-length recombinant esterase was purified 3.4-fold, with a specific activity of 1.7 U/mg, and yield of 96.8% (Table 1).

Substrate specificity of EstO

The catalytic activity of the *P. arctica* esterase EstO was studied toward a wide range of substrates. To accomplish this, the enzyme was incubated with 1 mM of different *p*-NP ester compounds in Tris–HCl buffer (25 mM, pH 7.0) at 20°C. In this approach, substrate specificity of EstO was

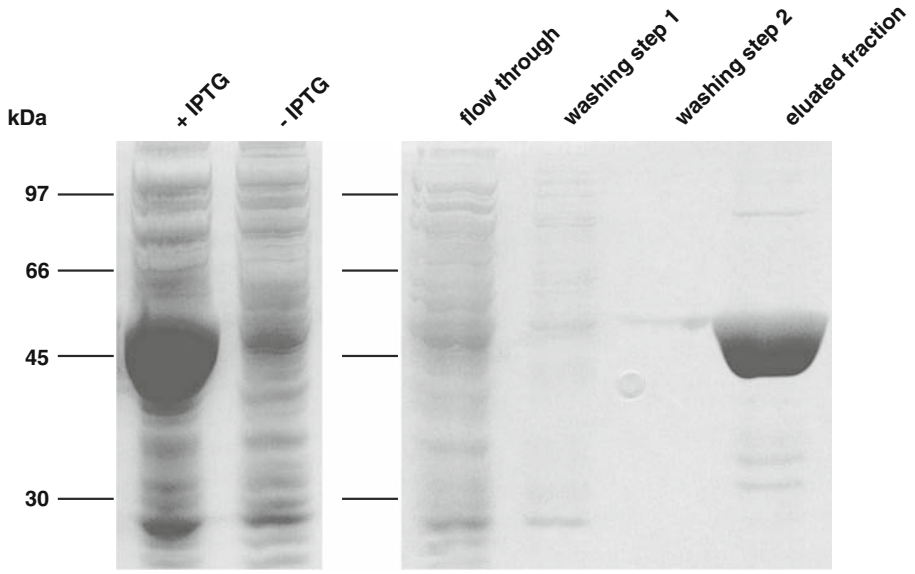

Fig. 2 Overexpression and purification of His-tagged version of the *P. arctica* EstO produced in *E. coli*. **a** SDS-PAGE (10%) of crude protein extracts from *E. coli* Tuner™ (DE3) pLacI transformed with pET24b-EstO. Cells were grown for 24 h at 30°C after induction with 2 mM IPTG (+IPTG) or without the addition of IPTG (−IPTG) and harvested by centrifugation. **b** After disruption of the cells using French press and centrifugation the supernatant was loaded on Ni–NTA superflow column followed by washing and elution steps as indicated in "Materials and methods". The flow through, proteins from washing steps 1 and 2, and the eluted fraction were tested on SDS-PAGE

Table 1 Purification of the recombinant esterase EstO after expression in *E. coli* Tuner™ (DE3) pLacI

Purification step	Fraction volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
EstO (crude extract)[a]	1	23.8	12.35	0.5	100	1
EstO (Ni–NTA)[b]	6	6.9	11.96	1.7	96.8	3.4

[a] Cells were disrupted by using the French Press, and cell debris was sedimented

[b] Purified protein fraction using Ni–NTA superflow column

analyzed toward the length of acyl chains of *p*-NP esters. The esterase hydrolyzes short chain esters (C_2–C_8), and is incapable to use medium and long chain length esters of carboxylic acids, such as *p*-NP laurate (C_{12}), *p*-NP myristate (C_{14}), *p*-NP palmitate (C_{16}) or *p*-NP-stearate (C_{18}), respectively, as substrate (Fig. 3a). The highest activity among the *p*-NP esters examined was measured by the utilization of the C_4 ester *p*-NP butyrate. Furthermore, several ester substrates were used to test positional specificity and ability to hydrolyze molecules with chiral carbons. The esterase is capable to hydrolyze ester bonds at the -1- position (*p*-NP 1-naphtoate) rather than at the -2- position (*p*-NP 2-naphtoate) (Fig. 3b). The lipolytic enzyme was also active using the plenary molecule *p*-NP benzoate as substrate, while EstO is not able to hydrolyze amide bonds in molecules such as 2-(4-isobutylphenyl)-*N*-(*p*-NP) propanamide. The enzyme displayed activity toward ester bonds in compounds, such as *p*-NP 2-phenylpropanoate, *p*-NP 3-phenylbutanoate, *p*-NP cyclohexanoate, *p*-NP 2-(6-methoxynaphthalen-2-yl) propanoate, *p*-NP 2-(3-benzoylphenyl) propanoate and *p*-NP 2-(4-isobutylphenyl) propanoate (Fig. 3b). The latter three compounds are the *p*-NP esters of the commercially available drugs naproxen, ketoprofen and ibuprofen.

In a further attempt, specificity of the enzyme toward the length of various acyl chains of ethyl, methyl, and vinyl esters was investigated (0.25 mg/ml) using an alternative assay (see assay B in "Materials and methods"). The esterase shows the highest activity in the presence of medium chain length (C_8–C_{12}) ethyl esters (Fig. 4). Ethyl nanoate (C_9) and ethyl laurate (C_{12}) were the most preferred substrates among the ethyl esters examined, but the esterase even showed higher catalytic activity toward the methyl ester methyl octanoate (C_8). However, a pronounced activity was also measurable using the short chain ester compound vinyl butyrate (C_4) as the sole substrate, while EstO did not hydrolyze vinyl acetate, which contains the shortest acyl chain of only two carbon atoms. Finally, no reactivity of EstO toward triacylglycerols such as olive oil, triolein ($C_{18:1}$) and tributyrin ($C_{4:0}$) was measured using assay B.

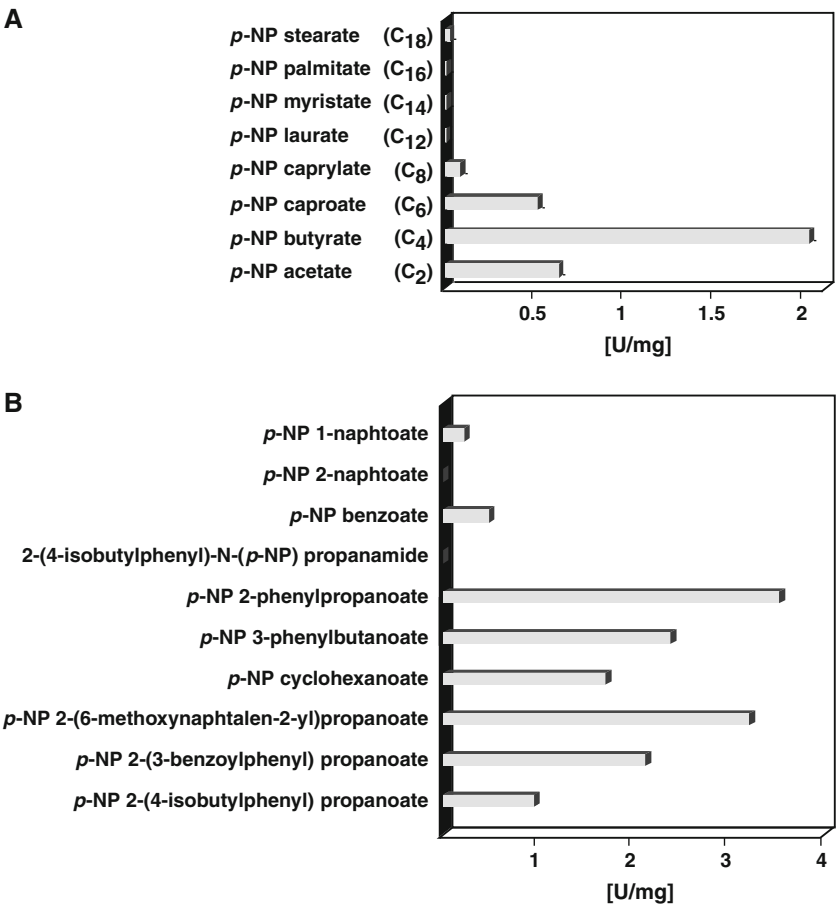

Fig. 3 Substrate specificity of the recombinant EstO from *P. arctica*. **a** EstO activity for selected *p*-NP ester substrates was tested using assay A as described in "Materials and methods". The enzyme assay was performed with 1 mM *p*-NP esters for 30 min at 20°C in Tris–HCl buffer (25 mM, pH 7.0). **b** EstO activity toward *p*-NP esters with chiral carbons and NSAIDs. The enzyme assay was performed using 0.25 mg/ml substrate concentration under the same conditions as described in **a**

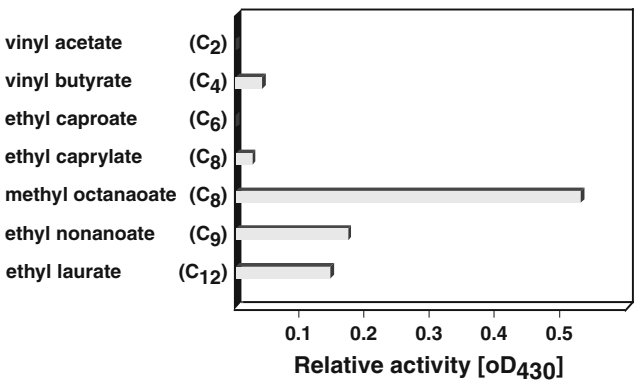

Fig. 4 Substrate specificity of EstO using non-*p*-NP esters as substrates. Activity was tested using assay B as indicated in "Materials and methods" with 0.25 mg/ml of substrate. Samples were incubated under constant shaking for 12 h in Tris–HCl buffer (25 mM, pH 7.0) at 20°C

Biochemical properties of recombinant esterase

The pH optimum of EstO from *P. arctica* heterologously produced in *E. coli* Tuner™ (DE3) pLacI cells was determined. The esterase retained about 40% activity over a pH range from 6.5 to 9.0 at 25°C. The optimal pH was indicated to be pH 7.5 (Fig. 5a). The influence of pH on the stability of EstO was examined by measuring the enzymatic activity after incubation at pH ranging from 5.0 up to 12.0. The enzyme is stable over a wide pH range (≥60% retained activity between pH 6.0 and 10.0), but completely instable at pH 5.0, and only about 20% of original activity was measurable at pH 12.0 (data not shown). The temperature optimum for catalytic activity was measured by the incubation of samples from 0 to 40°C. Up to 50% of activity is retained at 0°C, and the temperature optimum was calculated to be 25°C, while the activity dramatically decreases at temperatures above 25°C (Fig. 5b). In a further approach, thermostability of the recombinant esterase was examined by measuring the enzymatic activity after incubation at temperatures ranging from 4 up to 40°C. The enzyme is stable at 4 and 10°C. It lost about 35% of activity after 24 h of incubation at 20°C. The half-life at 30°C was determined to be 15 h and 5 h at 40°C.

The apparent kinetic parameters for Michaelis–Menten with different concentrations of *p*-NPB (0.05–2 mM) were determined at optimal pH 7.5 and 25°C. The K_m value for EstO is 0.511 (±0.17) mM and V_{max} is 0.166 (±0.021) U/min. Finally, the activation energy E_a for the EstO-catalyzed hydrolysis of *p*-NPB was calculated from Arrhenius

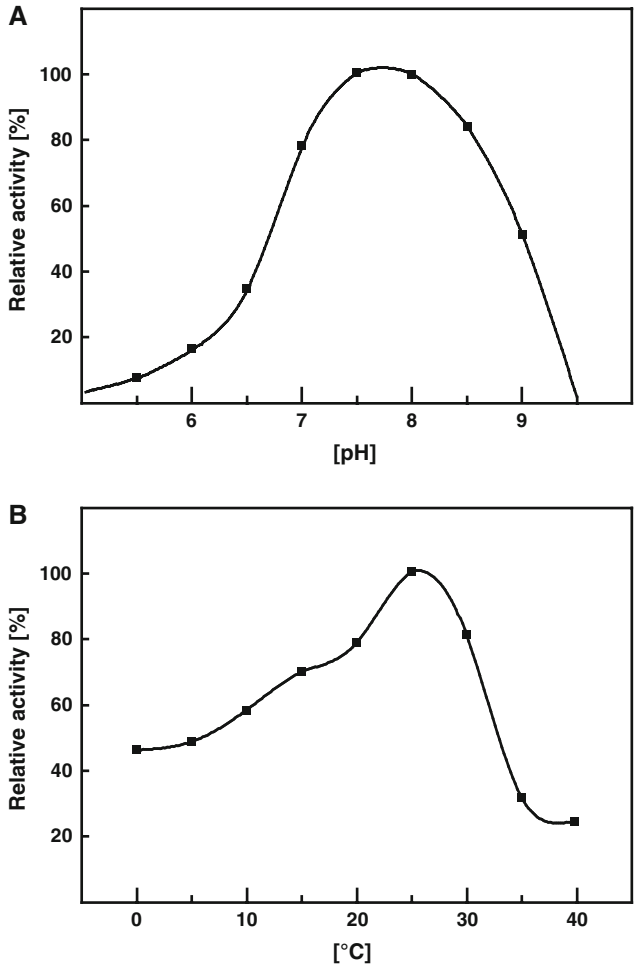

Fig. 5 Influence of pH and temperature on esterase activity from *P. arctica*. **a** For the determination of pH optimum, recombinant enzyme was incubated at 25°C in universal buffer (pH 5–9.5) for 30 min. *p*-NPB was used as substrate and the enzymatic reaction was carried out as described in "Materials and methods" (assay A). **b** The optimal temperature for the recombinant enzyme was investigated by incubation of 2 U/mg of EstO in Tris–HCl buffer at pH 7.5 for 30 min at a temperature range of 0–40°C

plot to be 5.1 kcal/mol in the temperature range from 0 to 25°C.

Effect of metal ions and other reagents on recombinant esterase activity

Inhibitory and activating effects of simple metal ions were investigated for the recombinant esterase from *P. arctica* (Fig. 6a). Divalent and monovalent cations affected the activity of the recombinant enzyme as follows: EstO was completely inhibited by the addition of 10 mM Al^{2+}, Cu^{2+}, Fe^{2+}, Cr^{2+}, and Co^{2+}, whereas Ca^{2+}, Mg^{2+}, Se^{2+} and Mn^{+2} had no or only minor effect. Interestingly, 10 mM of Na^{+} increased the activity by 30%.

Since esterases belong to a class of serine hydrolases with the catalytic triad Ser-Asp-His, classical serine inhibitors were used to study the structure and catalytic mechanism of the enzyme (Arpigny and Jaeger 1999). In order to confirm this experimentally, the effect of typical serine modifying reagents was tested, and total inhibition was observed using 10 mM PMSF or serineprotease-inhibitor Pefabloc. Since EstO amino acid sequence exhibits six cysteine residues, the specific effects of the disulfide bond disturbing reagents β-mercaptoethanol, PCMB, 2-iodoacetate and dithiothreitol (DTT) (10 mM each) were investigated (Fig. 6b). The latter reagents completely blocked the activity of the enzyme, whereas the mild disulfide bond reducing β-mercaptoethanol as well as PCMB decreases catalytic activity down to 30%. A concentration of 10% (v/v) of the detergents Tween 20 and SDS and 10 mM of the chelating agent EDTA totally abolished activity of the enzyme, while the chaotropic salt guanidine hydrochloride had only limited effects on catalytic activity (reduction down to 90%).

Generation and characterization of a truncated EstO variant

To investigate the influence of the predicted OsmC domain on the esterase activity of EstO from *P. arctica*, a truncated variant was expressed from plasmid pET24b-EstOΔOsmC in *E. coli* Tuner^{TM} (DE3) pLacI. The complete OsmC domain including the 8 amino acid linker, encoded by nucleotides 751–1,203, has been deleted in this construct. SDS-PAGE analysis of purified protein revealed a band of 28.2 kDa, which is consistent with the molecular weights of the N-terminal esterase domain (27.1 kDa) including the additional His-tag (1.1 kDa) (data not shown). In contrast to the full-length esterase, the truncated EstOΔOsmC could be purified 57-fold, with a specific activity of 11.4 U/mg and a yield of 96.8% (Table 2). No alteration on substrate specificity could be observed with regard to EstO. Interestingly, EstOΔOsmC also displayed a comparable pH and temperature optimum (pH 7.0; temperature optimum 25°C) to the wild type esterase, but a drastically change in its stability. While EstO is stable at alkaline rather than at acidic pH (≥60% retained activity between pH 6 and 10), the truncated isoform was found to be more stable at pH 4–9 (≥80% retained activity). Thermostability of EstOΔOsmC was measured between 4 and 90°C. Both proteins were stable at 4 and 10°C. In contrast to EstO, which lost about 50% of its activity after incubation for 5 h at 40°C, the mutated enzyme displayed a half life of 42 h at 60°C and was found to be stable even at 90°C (50% retained activity after 2.5 h).

In contrast to the wild type enzyme, activity of EstOΔOsmC was not completely blocked by the addition of the

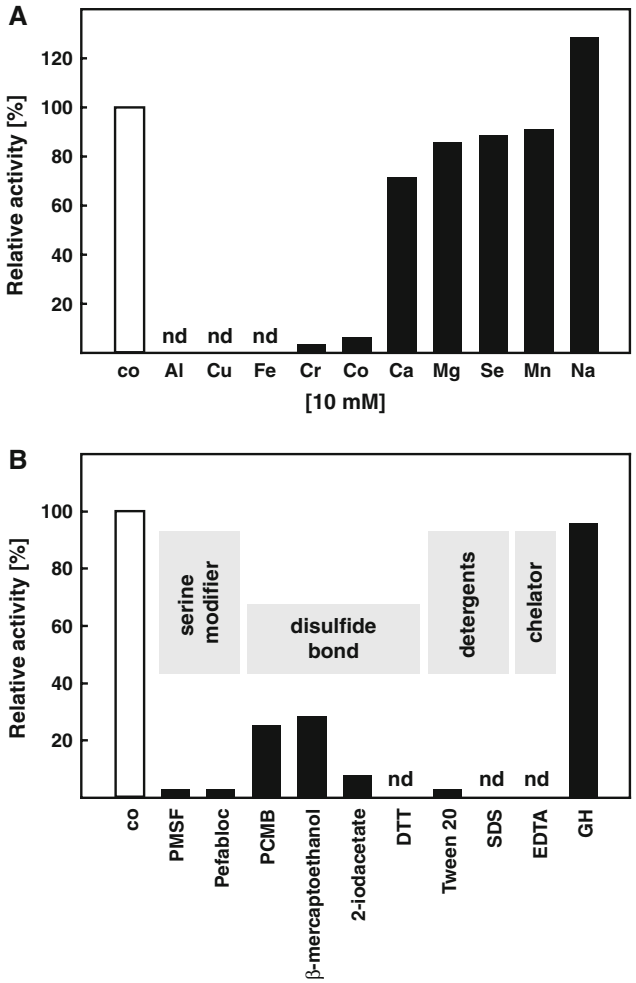

Fig. 6 Inhibitory effects on the catalytic activity of EstO by various compounds. **a** Simple metal ions were tested for inhibitory and activating effects on the esterase EstO from *P. arctica*. Esterase samples were incubated with 10 mM of various metal ions for 120 min at 20°C. For examination of the residual enzyme activity, 100 µl of preincubation mixture were mixed with 900 µl of substrate (*p*-NPB) in Tris–HCl buffer (25 mM, pH 7.0). The reaction was carried out for 20 min at 20°C. **b** Effect of putative esterase inhibitors with different characteristics (serine modifying reagents, disulfide bond disturbers, detergents, chelating agents, and chaotropic salts) was examined. Apart of the detergents, which were incubated with esterase at a concentration of 10% for 1 h at 20°C, incubation of esterase with other compounds (10 mM) took place for 1 h at 30°C. The assay was performed as described above; *co* control; *GH* guanidine hydrochloride; *nd* not determined

single metal ions Al^{2+}, Cu^{2+}, Fe^{2+}, Cr^{2+}, and Co^{2+}, respectively, but the mutated enzyme exhibited between 30 and 50% of residual catalytic activity. However, the increased activity of the EstO enzyme activity in presence of NaCl was not observed by the mutated isoform. In the presence of Na^{+}, activity of EstOΔOsmC was not influenced at all (data not shown). No changes between EstO and EstOΔOsmC were observed by the addition of serine or disulfide disturbing reagents, detergents or the chaotropic salt guanidine hydrochloride, whereas EDTA had only minor effects on the catalytic activity of EstOΔOsmC (ca. 60% residual activity in the presence of 10% EDTA), in contrast to the total inhibition of the *P. arctica* wild type esterase.

Discussion

In this study, we have identified a novel cold-adapted esterase from the gram-negative bacterium *P. arctica*. The α/β-hydrolase family of enzymes is one of the largest groups of structurally related proteins with diverse catalytic functions and is currently among the most important groups of biocatalysts in biotechnology. A potential catalytic triad consisting of one serine (Ser^{106}), one aspartic acid residue (Asp^{196}) and one histidine (His^{225}) was predicted, indicating that EstO belongs to the serine hydrolase family. Ser^{106} was found within the G × S × G (position 104–108) consensus sequence, known as the substrate-binding site from many esterases (Brenner 1988). Moreover, the prediction of secondary structure elements using the PSIPRED server revealed a typical distribution of the catalytic triad throughout the protein (Ser^{106} between β_5 and α_C, Asp^{196} between β_7 and α_E and His^{225} between β_8 and α_F), but an additional secondary structure element composed of a β-sheet and an α-helix was inserted after strand β_6 (data not shown) as described by Siew et al. (2005).

Homologs of EstO are well conserved among prokaryotes, but are mostly annotated as OsmC-like proteins, because they display homology to a group of osmotically inducible proteins (Atichartpongkul et al. 2001; Jenkins et al. 2007). Species have evolved a number of mechanisms to deal with environmental stress including that of heat, oxidative agents and osmotic shock. Although the function of these specific proteins remains unknown, the gene is highly conserved among prokaryotes (Rehse et al. 2004). The highest identity of EstO has been observed in a BLASTP analysis identifying an uncharacterized protein (90% identity in 400 aa overlap; YP_340440) from the closely related bacterium *Pseudoalteromonas haloplanktis* (Medigue et al. 2005). Computer analysis of the deduced amino acid sequence of the *P. arctica* esterase established that EstO does not belong to any of the eight described families of lipolytic enzymes (Arpigny and Jaeger 1999). Hitherto, a thermoalkalophilic hydrolase PhaZ7 from *Paucimonas lemoignei*, the carboxylesterase Est30 from *Geobacillus stearothermophilus* and the thermostable esterase EstD from *Thermotoga maritima* could also not be grouped into one of these families (Handrick et al. 2001; Liu et al. 2004; Levisson et al. 2007). While the conserved pentapeptides of PhaZ7 and Est30 do not show identity to

Table 2 Purification of the recombinant EstOΔOsmC after expression in *E. coli* Tuner™ (DE3) pLacI

Purification step	Fraction volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
EstOΔOsmC (crude extract)[a]	3	392.4	76.54	0.2	100	1
EstOΔOsmC (Ni–NTA)[b]	4.5	6.5	74.1	11.4	96.8	57

[a] Cells were disrupted by using the French Press, and cell debris was sedimented

[b] Purified protein fraction using Ni–NTA superflow column

the EstO sequence, Levisson et al. (2007) give the consensus sequence including a preceding leucine residue (LGHSLG), which is almost identical to IGHSLG of EstO. However, the lacking of the OsmC domain in EstD and related esterases and the fact that EstO and EstD merely display an identity of 20.5% in 127 aa overlap distinguishes our esterase from EstD. To the best of our knowledge, EstO is the first esterase with OsmC-domain that has been identified, purified, and characterized.

The *P. arctica* EstO exhibits high activity toward water-soluble substrates with short chains, such as *p*-NP esters with fatty acid chains shorter than C_8. A criterion has been proposed in which carboxylesterases are defined as enzymes that catalyze the hydrolysis of acylglycerols with short chains (<10 carbon atoms), while lipases are defined as enzymes that catalyze the hydrolysis of acylglycerols with long chains (≥10 carbon atoms), clearly indicating that EstO belongs to the group of esterases (Jaeger et al. 1999). Since triacylglycerols such as triolein were inert as substrates, the *P. arctica* EstO can be further classified as carboxylesterase (Bornscheuer 2002). Furthermore, EstO is capable of hydrolyzing esters from the medical relevant non-steroidal anti-inflammatory drugs (NSAID) naproxen, ketoprofen, and ibuprofen, which are extensively used to combat human diseases. The truncated EstO variant EstOΔOsmC displayed similar broad substrate specificities, indicating that the esterase domain hydrolyzes its substrates independent of the OsmC region.

Since the central serine of the pentapeptide is an active side residue of the catalytic triad, specific serine inhibitors, such as PMSF or Pefabloc can irreversibly abolish activity of esterases (Suzuki et al. 2002; Kulakova et al. 2004; Cieslinski et al. 2007). Furthermore, disulfide bond reducing reagents were investigated, because it is known that disulfide bonds are essential for the catalytic activity of some esterases (Cieslinski et al. 2007; Hess et al. 2008). DTT and 2-iodacetate completely inhibited activity of EstO and EstOΔOsmC, indicating that at least one of the two cysteine residues in the esterase domain (Cys^{34} and Cys^{37}) is important for maximal activity (Cys^{312}, Cys^{345}, Cys^{348} and Cys^{382} are located within the OsmC domain). EstO is completely inhibited by addition of Al^{2+}, Cu^{2+}, Fe^{2+}, Cr^{2+}, and Co^{2+}, in particular transition metals have been reported elsewhere to disturb esterase activity (Suzuki et al. 2002; Metin et al. 2006). The OsmC domain seems to help the esterase to retain its activity in the presence of metal ions. Additionally, the activity of EstO but not the OsmC-less variant is increased in the presence of NaCl. Since growth of *P. arctica* has been observed at 0–9% of NaCl, the Na^+ ions might be important for enzyme activation under physiological conditions (Al Khudary et al. 2008). Due to the fact that esterase activity is not influenced by some ions tested and activated by others, but completely inhibited in the presence of the chelating reagent EDTA might be possibly caused by a basal requirement of metal ions. Metin et al. (2006) propose that some ions are important for the structural conformation of esterases rather than for activity. Interestingly, the EstOΔOsmC variant is less sensitive toward EDTA, indicating that OsmC influences the hypothetical metal ion regulation of the esterase.

Biochemical parameters, in particular pH and temperature range, determine the conditions in which a specific industrial process must operate. Cold-adapted enzymes are mainly established in manifold applications, which offer potential economic benefits by saving energy (Feller and Gerday 2003). Furthermore, they also minimize undesirable chemical side-reactions that occur most probably at higher temperatures (Yang et al. 2008). EstO displays a temperature optimum in *p*-NBP hydrolysis of 25°C. Furthermore, the enzyme is active at temperatures below 0°C and drastically instable at temperatures above 25°C. In recent studies, similar cold-adapted esterases showing varying temperature optima between 30 and 45°C have been investigated from the bacterial species *Pseudoalteromonas* sp. strain 643A (35°C), *Psychrobacter* sp. strain Ant300 (35°C), *Acinetobacter* sp. strain 6 (45°C), *Pseudomonas* sp. strain B11-1 (45°C), from the basidiomycetous yeast *Rhodotorula mucilaginosa* (45°C) and from an Antarctic desert soil isolate (40°C) (Suzuki et al. 2002, 2003; Kulakova et al. 2004; Zimmer et al. 2006; Cieslinski et al. 2007; Heath et al. 2009). Moreover, a further psychrophilic esterase Lipo1 obtained from a metagenomic DNA library of activated sludge has been described to be highly active at 10°C (Roh and Villatte 2008). As to be expected for a psychrophilic enzyme, EstO is not stable at higher temperatures and can therefore easily be inactivated, but it is slightly more thermostable compared to the profile

of enzymes reported from other bacterial species such as *Psychrobacter* sp. strain Ant300, *Acinetobacter* sp. strain 6 or *Pseudoalteromonas* sp. strain 643A (Suzuki et al. 2002; Kulakova et al. 2004; Cieslinski et al. 2007). The truncated EstOΔOsmC variant displays an impressively increased amount of thermostability and retains 50% of its activity even after 2.5 h of incubation at 90°C, which is comparable to esterases from some hyperthermophiles (Levisson et al. 2009). This might indicate that the OsmC domain is required for higher flexibility of the esterase at low temperatures. The optimal pH of EstO and EstOΔOsmC to hydrolyze *p*-NPB is between pH 7.0 and 7.5, which matches with the optimum of the above-quoted enzymes, but pH stability of EstOΔOsmC is shifted toward a more acidic pH range.

The velocity of chemical reactions decreases exponentially at low temperatures according to the Arrhenius law, which is reflected by lower activation energy (E_a) of psychrophilic enzymes than their mesophilic counterparts (Marx et al. 2007). EstO displays a lower E_a (5.1 kcal/mol) than the values reported for the hydrolysis of *p*-NPB catalyzed by mesophilic and psychrophilic lipases/esterases from *Candida cylindracea* (21.0 kcal/mol), *Pseudoalteromonas* sp. strain B11-1 (LipP: 11.2 kcal/mol and PsEst1: 20.1 kcal/mol), *Acinetobacter* sp. strain 6 (9.0 kcal/mol), but was matched by $E_a = 4.6$ kcal/mol from cold-active esterase PsyEst from *Psychrobacter* sp. strain Ant300 (Choo et al. 1998; Suzuki et al. 2002, 2003; Kulakova et al. 2004).

Despite of being well characterized in vitro, the physiological function of esterases is mostly unknown and intensely discussed (Panda and Gowrishankar 2005). The function of the related OsmC proteins from other prokaryotes remains unclear as well.

This study provides a deeper insight into the catalytic functionality of cold-adapted esterases. *P. arctica* EstO is clearly one of the most active cold-adapted esterases known to date with impressively low activation energy. Furthermore, the characterization of the truncated variant revealed improved catalytic properties that are distinct to the wild type enzyme and should demonstrate the tremendous advantage to further engineer this esterase for biotechnological applications.

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