CURRENT MICROBIOLOGY Vol. 50 (2005), pp. 196–201
DOI: 10.1007/s00284-004-4423-3

Current Microbiology
An International Journal

Cloning and Characterization of a Carboxylesterase from *Bacillus coagulans* 81-11

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Received: 15 July 2004 / Accepted: 18 October 2004

Abstract. A genomic library of *Bacillus coagulans* strain 81-11 was screened in *Escherichia coli* JM83 for lipolytic activity by using tributyrin agar plates. A 2.4 kb DNA fragment was subcloned from a lipolytic-positive clone and completely sequenced. Nucleotide sequence analysis predicted a 723 bp open reading frame (ORF), designated *estC1*, encoding a protein of 240 amino acids with an estimated molecular mass of 27 528 Da and a pI of 9.15. The deduced amino acid sequence of the *estC1* gene exhibited significant amino acid sequence identity with carboxylesterases from thermophilic *Geobacillus* spp. and sequence analysis showed that the protein contains the signature G-X-S-X-G included in most esterases and lipases. Enzyme assays using *p*-nitrophenyl (*p*-NP) esters with different acyl chain lengths as the substrate confirmed the esterase activity. EstC1 exhibited a marked preference for esters of short-chain fatty acids, yielding the highest activity with *p*-NP butyrate. Maximum activity was found at pH 8 and 50°C, although the enzyme displayed stability at temperatures up to 60°C.

Hydrolases (EC 3) are the most predominant class of enzymes employed in biocatalysis research and in the production of fine chemicals by biocatalytic resolution [7]. Most important in this class of enzymes are hydrolytic enzymes cleaving carboxylic ester bonds, such as lipases (EC 3.1.1.3) and carboxylesterases (EC 3.1.1.1), as they accept a broad range of natural and non-natural substrates, are usually stable in organic solvents, and exhibit good to excellent stereoselectivity [7, 10]. The hydrolytic mechanism of most esterases and lipases resembles that of serine proteases and they all contain a similar catalytic triad, generally consisting of a nucleophilic serine residue that acts in conjunction with a histidine and an acidic residue (aspartic acid or glutamic acid) [20].

Unlike lipases, esterases show a preference toward short-chain acetyl esters and can be found in many organisms including animals, plants, and microorganisms [19]. The physiological functions of bacterial esterases are, however, not clear; although some may be involved in plant pathogenicity, carbon source provision, and biocide detoxification [11]. Nevertheless, esterase enzymes play an important role as catalysts for biotechnological application. In addition to ester hydrolysis, esterases are capable of catalyzing interesterification, aminolysis, and peracid formation [3, 7]. These biochemical reactions are applicable in the pharmaceutical and food industries [7].

Despite their important commercial value, esterases are used less frequently in industrial processes than lipases, mainly due to their lack of availability [3, 7]. Accordingly, we have attempted to explore esterases from different bacterial sources. By extensively screening our strain collection, strain 81-11, identified as *Bacillus coagulans* by 16S rDNA sequence analysis, was found to display the highest lipolytic activity and was selected for further studies. Here, we report the biochemical characterization of a new *B. coagulans* esterase, as well as the cloning and characterization of the *estC1* gene encoding the esterase.

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Materials and Methods

Bacterial strains, plasmids, and growth conditions. *B. coagulans* strain 81-11, used as the source of chromosomal DNA, and *Escherichia coli* JM83, used as the host in the cloning procedures, were routinely cultured at 44 and 37°C, respectively, in Luria–Bertani broth (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7.4). For selection and maintenance of plasmid DNA in *E. coli* JM83, the medium was supplemented with 20 μg/mL of chloramphenicol or 100 μg/mL of ampicillin. Plasmids pBS19, an *E. coli*/*B. subtilis* shuttle vector, and pBC(SK) were used as cloning vectors.

Library construction and screening. Genomic DNA from *B. coagulans* strain 81-11 was isolated according to the method of Lovett and Keggins [14] and partially digested with *Sau*3AI. DNA fragments (3–5 kb) were ligated with *Bam*HI-digested, dephosphorylated pBS19 to generate a genomic library. Competent *E. coli* JM83 cells were prepared and transformed by electroporation, as described by Tung and Chow [22]. Transformants were screened for lipolytic activity by replica-plating onto tributyrin agar plates [18] and investigation of halo-formation after incubation at 37°C for up to 72 h.

DNA sequence analysis. The nucleotide sequence of both strands of the cloned esterase-active DNA fragment was determined by automated sequencing with an ABI PRISM BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems), in a Model 377 automated DNA sequencer. A combination of subclones constructed in pBC(SK) for sequencing with the universal M13/pUC forward and reverse primers were used. Homology was analyzed by using BLAST at NCBI [2], sequence alignments were performed with ClustalW (1.74) Multialign Software, and the program SIGNALP was used for identification of potential signal peptides. The physicochemical parameters of the deduced amino acid sequence and presence of defined protein patterns was determined by using the ProSite database at ExPASY (http://www.expasy.org).

Enzyme activity assays. Enzyme activity in crude cell extracts was determined spectrophotometrically by measuring the liberation of *p*-nitrophenol from *p*-NP butyrate in 50 mM potassium phosphate buffer (pH 8) at 50°C, as previously described [5]. One unit of activity was defined as the amount of enzyme that released 1 μmol of *p*-NP per min under the assay conditions. The protein concentrations were determined according to Bradford [4] with serum albumin as standard. Substrate specificity towards various *p*-nitrophenyl esters (Sigma-Aldrich) was determined using *p*-NP propionate (C_3), *p*-NP butyrate (C_4), *p*-NP caproate (C_6), *p*-NP laurate (C_{12}), *p*-NP myristate (C_{14}), *p*-NP palmitate (C_{16}), and *p*-NP stearate (C_{18}) as substrates. The pH and temperature profiles were established using *p*-NP butyrate as the substrate. The pH profile was established at 50°C and the temperature profile at pH 8. Thermostability was investigated by measuring the residual enzyme activities after incubation of the enzyme for 3 h at different temperatures between 30 and 65°C in 50 mM potassium phosphate buffer (pH 8).

Results

Cloning of a gene from *B. coagulans* strain 81-11 encoding lipolytic activity. *B. coagulans* strain 81-11 displayed significant lipolytic activity on tributyrin agar plates. In order to isolate the gene(s) responsible for the observed activity, a genomic library of this strain was prepared and screened in *E. coli* JM83, which exhibits no lipolytic activity. From approximately 9000 chloramphenicol-resistant transformants screened on tributyrin agar plates, only one, designated *E. coli*/pSEI, showed hydrolysis of tributyrin and was selected for further characterization. Subsequent digestion of the recovered plasmid DNA revealed that it contained a DNA insert of 3.4 kb. The plasmid DNA of *E. coli*/pSEI was digested with various restriction enzymes, and fragments were subcloned into pBS19. The transformants resulting from electroporation of *E. coli* JM83 cells were examined for lipolytic activity on tributyrin agar plates. In this way, a 2.4 kb DNA fragment that encoded the lipolytic activity was identified. The corresponding plasmid was designated pSEI-PH. Since plasmid pBS19 lacks a promoter, the results also indicated that the gene was transcribed from its own promoter and probably contained the full-length enzyme-encoding gene(s).

Sequence analysis. The nucleotide sequence of the 2.4 kb DNA insert from pSEI-PH was determined and submitted to GenBank under the accession number AY634688. An open reading frame (ORF) of 723 nucleotides that encoded for a protein of 240 amino acids with similarity to known esterases was found. The predicted molecular weight and pI of the protein were 27 529 Da and 9.15, respectively. The ORF, representing a gene designated *estC1*, was preceded by a potential Shine–Dalgarno sequence (AAGAGG). No signal peptide was found, suggesting that the cloned enzyme was a cell-bound esterase. The deduced amino acid sequence of EstC1 contains the sequence G-L-S_{94}-L-G, which agrees with the consensus sequence surrounding the active-site serine, G-X_1−S-X_2−G, commonly found within the sequences of lipases and esterases [3, 10].

The deduced amino acid sequence of EstC1 showed 72% and 70% identity, respectively, with the esterases from *Geobacillus stearothermophilus* [12] and *G. thermoleovorans* (accession no. AAG53982), while lower identity (66%) was found with the BsubE (YvaK) esterase from *B. subtilis* [13]. EstC1 also displayed significant homology (61–67% identity) to putative esterases from *B. halodurans* C-125 (accession no. NP_244421), *B. anthracis* A2012 (accession no. NP_653-555), and *B. cereus* ATCC 14579 (accession no. NP_834798). Although the similarities were lower than those toward enzymes from the *Bacillus* spp., the EstC1 esterase also showed notable homology (48–51% identity) with putative carboxylesterases from *Listeria monocytogenes* strain EGD-e (accession no. AB1381), *L. innocua* strain Clip 11262 (accession no. AC1750),

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Est_Gs      -MMKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPE    59
Est_Gt      -MMKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPITKG-MVPPE    58
Est_Bs      MSMKVVTPKPFTFKGGDKAVLLLHGFTGNTADVRMLGRYLNERGYTCHAPQYEGHGVPPE    60
EstC1_Bc    -MMRIVPPKPFLFEAGKRAVLLLHGFTGSSADVRMLGR---KKGYTCHAPQYKGHGVPPE    56
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Est_Gs      ELVHTGPDDWWQDVMNGYEFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPM    119
Est_Gt      ELVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPM    118
Est_Bs      ELVHTGPEDWWKNVMDGYEYLKSEGYESIAACGLSLGGVFSLKLGYTVPIKGIVPMCAPM    120
EstC1_Bc    NLLHTGPSDWWQDVMNGYQLLKEKGYHEIAVAGLSLGGVFSLKLGYTVPVKGIITMCAPM    116
            :*:****.***::**:**: **.:**..**..*****************::**:.*****

Est_Gs      YIKSEETMYEGVLEYAREYKKREGKSEEQIEQEMEKFKQTPMKTLKALQELIADVRDHLD    179
Est_Gt      YIKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLD    178
Est_Bs      HIKSEEVMYQGVLSYARNYKKFEGKSPEQIEEEMKEFEKTPMNTLKALQDLIADVRNNVD    180
EstC1_Bc    YIKSEQTMYEGVVAYARKYKQFEGKSEAQIEQEMREFKKTPMHTLQSLQELIQDVRNHVD    176
            :****:.**:**: ***:**: ****  ***:**..*::***:**::**:** *** ::*

Est_Gs      LIYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIY    239
Est_Gt      LVYARTFVVQARHDKMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIY    238
Est_Bs      MIYSPTFVVQARHDHMINTESANIIYNEVETDDKQLKWYEESGHVITLDKERDLVHQDVY    240
EstC1_Bc    HIYAPVFVVQARHDEMIRPESANIIYSEVETDHKQLKWYENSG---MLLHSRRKDHFTKM    233
             :*: .********.**..:******.*:*:  **:****:**    * :.:   *

Est_Gs      AFLESLDW  247
Est_Gt      AFLESLDW  246
Est_Bs      EFLEKLDW  248
EstC1_Bc    LFRSGVT-  240
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Fig. 1. Alignment of the amino acid sequence of *B. coagulans* strain 81-11 EstC1 esterase with the amino acid sequences of other bacterial esterases. Alignment was maximized by introducing gaps, which are indicated by dashes. Identical (*), highly similar (:) and similar (.) amino acids are indicated. The consensus active site sequence of serine esterases is indicated in bold. Abbreviations: Est_Gs, esterase (BstE) from *G. stearothermophilus* (Q06174); Est_Gt, carboxylesterase from *G. thermoleovorans* (AAG53982); Est_Bs, esterase (YvaK, BsubE) from *Bacillus subtilis* subsp. *subtilis* 168 (CAB15367); and EstC1_Bc, EstC1 from *B. coagulans* strain 81-11 (this study).

Staphylococcus epidermidis ATCC 12228 (accession no. NP_764119), and *S. aureus* subsp. *aureus* N315 (accession no. BAB41967). An alignment of the EstC1 deduced amino acid sequence with the most homologous proteins is shown in Fig. 1.

Localization of esterase activity. When crude extracts from *B. coagulans* strain 81-11 and *E. coli*/pSEI-PH were prepared by sonication, the highest activity was observed in the intracellular fractions, whilst low activity was recovered in the cell-free culture supernatants. By contrast, a control nonrecombinant *E. coli* JM83 culture did not show lipolytic activity in identically prepared fractions. Therefore, EstC1 seems to be a cell-bound esterase, a result that agrees well with the in silico-predicted absence of a signal peptide. Similar to the EstC1 homologues from *B. subtilis* and *G. stearothermophilus* [8], zymographic staining on a renatured SDS-PAGE gel using 1-naphthylacetate/Fast Red was unsuccessful.

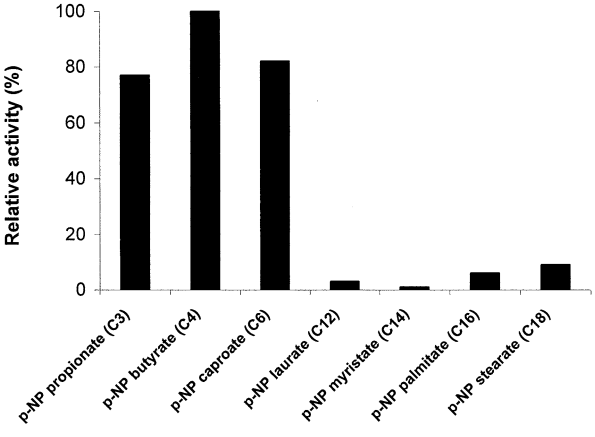

Fig. 2. Esterase-specific activity of EstC1 of *B. coagulans* strain 81–11, assessed using *p*-NP esters with different carbon chain-lengths as substrates. Values are the means of results of duplicate experiments.

Substrate specificity. Cell extracts from *E. coli*/pSEI-PH were tested for lipolytic activity using *p*-nitrophenyl

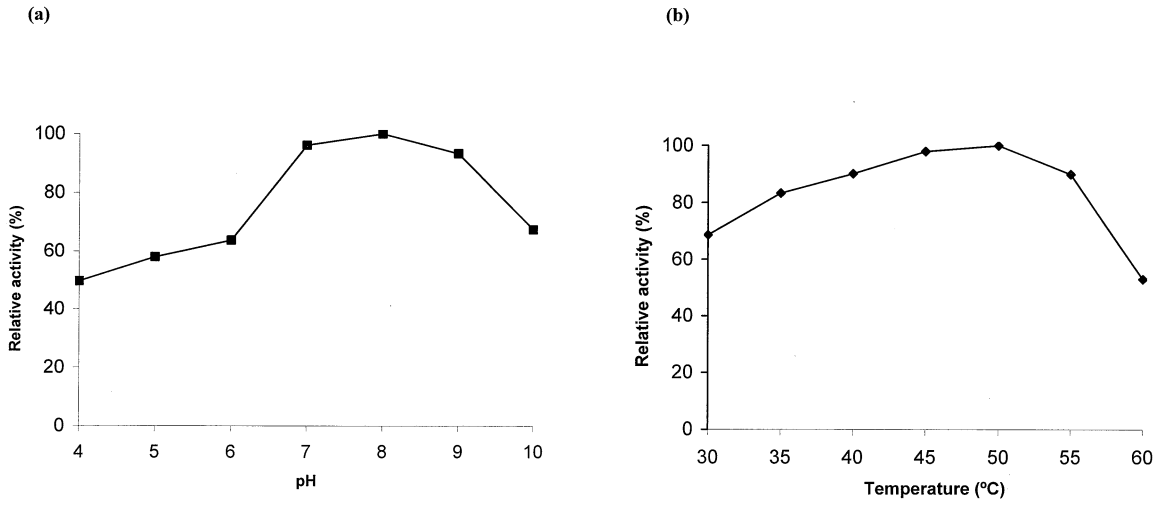

Fig. 3. Influence of (a) pH and (b) temperature on the esterase activity of EstC1 of *B. coagulans* strain 81-11. For the pH profile, activity was measured in buffers of different pH values. For the temperature profile, activity was measured in 50 mM potassium phosphate (pH 8) at different temperatures. Values are the means of results of duplicate experiments.

esters with chain lengths ranging from C_3 to C_{18} as substrate. As shown in Fig. 2, the enzyme exhibited a marked preference for short-chain fatty acids, yielding the highest activity against *p*-NP butyrate (C_4). High hydrolytic activities were also obtained with *p*-NP propionate (C_3) and *p*-NP caproate (C_6), which exhibited activities of 77% and 82%, respectively. The hydrolytic activity of the protein dropped abruptly toward *p*-NP esters with chain lengths ranging from C_{12} to C_{18} and less than 10% activity was detected against these long-chain fatty acids. Lipases are, by definition, carboxylesterases that have the ability to hydrolyze long-chain acylglycerols ($\geq C_{10}$), whereas esterases hydrolyze ester substrates with short-chain fatty acids ($\leq C_{10}$) [23]. The decrease in activity as the length of the fatty acid chains increases is behavior typical for true esterases. These results therefore served to confirm that the enzyme produced by *E. coli*/pSEI-PH is indeed an esterase.

Effect of pH and temperature on enzyme activity. The effects of pH and temperature on the activity of EstC1 were determined using cell extracts prepared from *E. coli*/pSEI-PH and *p*-NP butyrate as substrate. The activity of the esterase at various pH values was measured following adjustment of the reaction pHs from 4 to 10 with various buffers (Fig. 3a). The enzyme showed maximal activity at pH 8, but activity levels were above 80% in the range between pH 9 and pH 7. The optimum temperature of the enzyme was determined by varying the reaction temperature at pH 8 (Fig. 3b). The enzyme had maximal activity at 50°C, but exhibited about 90% of the maximum activity at 40 and 55°C, respectively. The thermal stability of the enzyme was determined by incubating the enzyme extract at pH 8 for 3 h at different temperatures and then measuring the residual esterase activity (Fig. 4). Heat treatment at 30°C and 50°C did not result in significant loss of esterase activity, but incubation at higher temperatures induced activity loss. Approximately 35% of the initial activity was lost after incubation at 60°C and almost all activity was lost after incubation at 65°C.

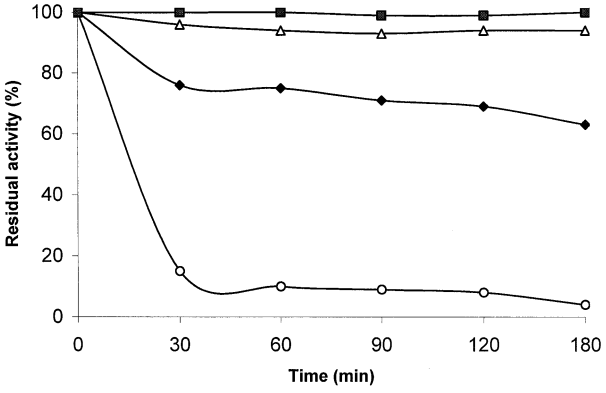

Fig. 4. Thermostability of EstC1 of *B. coagulans* strain 81-11. The residual enzyme activity was measured after incubation of the enzyme extract for the indicated times at temperatures of 30°C (filled squares), 50°C (open triangles), 60°C (filled diamonds), and 65°C (open circles). Values are the means of results of duplicate experiments.

Discussion

B. coagulans is known to have esterase activity and two intracellular carboxylesterases, carboxylesterases A and

B, have been purified and partially characterized [17]. The purified monomeric carboxylesterases A and B have apparent molecular weights of 70 and 60 kDa, respectively, on SDS-PAGE. Whereas esterase A hydrolyzes short-chain fatty acid esters (C_6) optimally at pH 9 and 50°C, esterase B is unable to hydrolyze esters with an acyl portion of 4 or more C atoms. The newly identified EstC1 enzyme differs from the previously reported esterase A and B enzymes by having a predicted molecular mass of 28 kDa, preference for *p*-NP butyrate (C_4), and displaying maximum activity at pH 8 and 50°C. The esterase EstC1 reported in this study therefore appears to be novel.

The production of different esterases in the same bacterial species has been reported for other microorganisms as well. In addition to *B. coagulans*, both *B. subtilis* [9] and *B. acidocaldarius* [15, 16] produce two intracellular esterases with different properties. Furthermore, at least four kinds of esterases have been identified in *P. fluorescens* strains, which differ in substrate specificity and cellular location [6, 11]. Since esterases from bacteria and fungi are not essential for growth, their physiological functions are not clear [19]. Nevertheless, it is tempting to speculate that the presence of different esterases in a single bacterium may function to hydrolyze ester compounds (short-chain), carboxylic acids and/or alcohols that are to be assimilated by the cells.

Sequence analysis indicated that the *B. coagulans* EstC1 protein displayed the highest identity with esterases from thermophilic *G. stearothermophilus* and *G. thermoleovorans*, followed by a slightly lower identity to the BsubE esterase isolated from the mesophile *B. subtilis*. Notably, less than 12% of all amino acids are completely different between the esterases of *B. coagulans*, *G. stearothermophilus*, and *G. thermoleovorans*. These small changes may, however, have a strong influence on the properties of each esterase. This is in accordance with directed-evolution studies, in which it could be demonstrated that only seven amino acid changes were necessary to increase the thermostability of a *p*-nitrobenzyl esterase from *B. subtilis* by 17°C [21].

Whereas the esterase enzymes from the thermophilic *Geobacillus* spp. are active at temperatures in excess of 65°C [8], the *B. coagulans* esterase showed activity in a range typical for mesophilic enzymes. Enzymes from thermophiles are often considered to be advantageous for use in industrial-level biotransformations, since they exhibit high thermostability that can be correlated with enhanced resistance to denaturation in organic solvents [1]. However, thermophilic enzymes have a significant operational disadvantage. One of the major advantages of enzymes over industrial catalysis is their potential for high activity at low temperatures, often a critical factor in protecting labile substrates from deleterious reactions. The activity of thermophilic enzymes at room temperature is often relatively low, and the apparent high temperature requirements for thermophilic enzymes would appear to impart some limitation on their applications as industrial biocatalysts. Ideal enzyme characteristics might thus include high molecular stability and high mesophilic activity. The temperature activity and stability of the *B. coagulans* strain 81-11 EstC1 esterase may therefore provide a good basis for enzyme utilization at moderate temperatures such as 30–50°C. In this range, the enzyme is very active (>70% of the maximal activity) and highly thermostable, as less than 5% of the activity was lost after 3 h of incubation at these temperatures.

Although the biological function of EstC1 and its natural substrate remain unknown, the enzyme exhibits properties that suggest it could be useful for biotechnological applications. Indeed, EstC1 exhibits considerable similarity to the biochemically characterized BstE esterase from *G. stearothermophilus* and *B. subtilis* BsubE (YvaK) esterase, both of which catalyze the hydrolysis of menthyl acetate to (–)-menthol, a key substance in the fragrance industry [8]. Further experiments are in progress to overproduce the described carboxylesterase in a *Bacillus* secretory host in order to evaluate the biotechnological applications of EstC1.

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