Ann Microbiol (2013) 63:879–885
DOI 10.1007/s13213-012-0539-z

ORIGINAL ARTICLE

# Cloning, expression and characterization of a novel esterase from *Bacillus pumilus*

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Received: 27 February 2012 / Accepted: 27 August 2012 / Published online: 13 September 2012
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**Abstract** The gene encoding esterase (CE1) from *Bacillus pumilus* ARA with a calculated molecular weight of 28.4 kDa was cloned, sequenced and efficiently expressed in *Escherichia coli*. The open reading frame of 747 nucleotides encoded a protein, which was classified as a carboxylesterase with an identity of 87 % to esterase from *Bacillus subtilis* 168. Recombinant CE1 was purified in a single step to electrophoretic homogeneity by IMAC ($Ni^{2+}$). The enzyme displayed maximum activity toward *p*-nitrophenyl (*p*NP) acetate at 37–40 °C and pH 6.5–7.0. It was stable in the pH range from 6.5 to 8.0, and at temperature from 25 to 40 °C. Among four *p*-nitrophenyl esters tested, the best substrate was *p*NP acetate with $K_m$ and $k_{cat}$ values of 0.33 mM and 4.07 $s^{-1}$, respectively. Amounts of 2 mM $Ca^{2+}$ and $Co^{2+}$ significantly increased the esterase activity to 190 and 121 %, respectively. These results suggest that CE1 has very attractive applications of increasing feed digestibility in animal nutrition in this moderate temperature range.

**Keywords** Carboxylesterase · Gene expression · Purification · Characterization · *Bacillus pumilus*

## Introduction

Lipases and esterases have been recognized as very useful biocatalysts used in organic synthesis and industrial processes because of their high regio- and stereo-specificity, and efficient activity in organic solvents (Bornscheuer 2002; Jaeger and Eggert 2002). Lipolytic enzymes are hydrolases (E.C. 3.1.1.-) catalyzing the hydrolysis of esters into the corresponding carboxylic acids and alcohols. These enzymes' activities mainly rely on a catalytic triad formed by Ser, His and Asp residues, with the serine embedded in the consensus sequence Gly-Xaa-Ser-Xaa-Gly at the active site (Arpigny and Jaeger 1999). The enzymes display the common α/β hydrolase fold (Ollis et al. 1992), which is commonly found in other hydrolases.

The enzymes are classified into two major families: the carboxylesterases (EC 3.1.1.1) and the lipases (EC 3.1.1.3) (Arpigny and Jaeger 1999). A fundamental difference in kinetics between carboxylesterases and lipases has been demonstrated, based upon the properties of the substrates they hydrolyze. While similar in molecular structure and catalytic mechanisms, carboxylesterases are distinguishable from lipases since they show preferential activity toward acylglycerols with short chains (<10 carbon atoms). Lipases catalyze the hydrolysis of fats and oils at the water–lipid interface and reverse the reaction in non-aqueous media, due to a hydrophobic domain covering the active site of lipases (Jaeger et al. 1999). Every year, novel biotechnological applications are established using lipases and esterases for synthesis of biopolymers and biodiesel, for the production of pharmaceuticals, agrochemicals and flavor compounds, and for degradation of roughages in animal nutrition (Bornscheuer 2002). Therefore, identification and isolation of novel lipases and esterases genes are of special interest for the industry.

Previously isolated *Bacillus pumilus* ARA, which was grown at 50 °C in a minimal medium containing xylan, has been reported to produce a number of highly xylan degrading enzymes, e.g., xylanase, β-xylosidase, and α-L-arabinofuranosidase (Pei and Shao 2008; Qu and Shao 2011). Recently, we found that this organism produced the extracellular thermostable feruloyl esterase for the enzymatic degradation of agricultural by-products, for example

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wheat bran and maize bran (Xue et al. 2012). This paper describes the cloning, expression, and characterization of a novel esterase (named CE1) in order to evaluate its potential applicability in biotechnological processes.

## Materials and methods

### Strains, plasmids and culture conditions

*Bacillus pumilus* ARA (Pei and Shao 2008) was used as the source of chromosomal DNA for cloning the CE1 gene. *Escherichia coli* DH10B (Promega, Madison, WI, USA) was used as a host for gene cloning. *Escherichia coli* JM109 (DE3) (Promega) was used as a host for expression of the CE1, via the T7 RNA polymerase expression system with pET-28a plasmids (Novagen, USA). They were cultured at 37 °C in Luria-Bertani (LB) medium containing kanamycin (50 μg $ml^{-1}$).

### Cloning and sequence analysis

Genomic DNA was isolated as described by Sambrook et al. (1989). Based on the conserved sequence of CE1 gene of *Bacillus pumilus* SAFR-032 (accession number YP_001488246.1), Two primers, 5′-CATGCCATGGGC ATGAAAGTTGTTACACC-3′ (the underlining indicates the *Nco*I restriction site) and 5′-CCGCTCGAGAATT GACCAATCAAGTG-3′ (the underlining indicates the *Xho*I restriction site), were designed and synthesized (Sangon, China). PCR amplification with Ex-Taq polymerase (TaKaRa, China) was carried out. Each cycle consisted of denaturation at 94 °C for 40 s, annealing at 44 °C for 40 s, and extension at 72 °C for 50 s. The PCR products were purified (QIA quick PCR purification kit), digested with *Nco*I and *Xho*I (TaKaRa) restriction enzymes, and then ligated into the vector pET-28a with the 6-His tag, resulting in the plasmid pET-28a-*CE1*. The homology of the CE1 deduced amino acid sequence was analyzed by using Blast at NCBI (Altschul et al. 1997). The program SIGNALP was used for identification of potential signal peptides. Sequence alignments were performed with Clustal W (1.80) Software.

### Expression and purification

*Escherichia coli* JM109 (DE3) containing pET-28a-*CE1* was grown in 300 ml LB with kanamycin (50 μg $ml^{-1}$) at 37 °C to $OD_{600}$ of 0.8 and incubated further with 0.5 mM isopropylthio-β-galactoside (IPTG) for 8 h. The cells were harvested by centrifugation at 9,600 *g*, 4 °C, 30 min, and resuspended in 20 ml binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris/HCl, pH 7.9), and lysed by three cycles through a FRENCH press (Thermo Electron, USA). The crude extract was obtained by centrifugation at 9,600 *g*, 4 °C, 30 min, and loaded onto an immobilized metal affinity column (IMAC) filling Ni-chelating resin (Novagen). Unbound proteins were washed off by binding buffer and then by washing buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris/HCl, pH 7.9), the his-tagged CE1 was eluted by eluting buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris/HCl, pH 7.9), and collected in 10-ml fractions. The fraction was used as the purified enzyme for subsequent analysis by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 10 % polyacrylamide running gels with 4 % polyacrylamide stacking gels.

### Enzyme assays

Esterase activity was determined by measuring the amount of *p*-nitrophenol released during enzymatic hydrolysis of different *p*-nitrophenyl (*p*NP) esters (Kademi et al. 2000). Unless otherwise indicated, in a standard assay, CE1 activity was measured with 10 μl 1 mM *p*NP butyrate (*p*NP-C4) as a substrate in 100 mM citrate-phosphate buffer (pH 6.5) at 40 °C. Stock solutions of *p*NP esters were prepared by dissolving substrates in isopropanol. The reaction was started by adding 0.8 μg of purified enzyme to the reaction mix, which was pre-incubated at the respective temperature. After 10 min, the reaction was stopped by chilling on ice, the color was developed by the addition of 0.6 ml 1 M pH 8.0 Tris/HCl, and the $A_{405}$ was read. Measurements were corrected for background hydrolysis in the absence of enzyme. A standard curve was prepared by using *p*NP (Sigma). One unit of CE1 activity was defined as the amount of enzyme producing 1 μmol *p*NP per min. Protein concentrations were determined according to Bradford with bovine serum albumin (BSA) as a standard.

### Inhibition studies

The effect of metal ions on CE1 (0.8 μg purified enzyme ) activity was determined using different metal salts ($Cu^{2+}$, $Pb^{2+}$, $Zn^{2+}$, $Fe^{3+}$, $Mn^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $K^{+}$, $NH^{4+}$, $Co^{2+}$, $Ca^{2+}$, and $Na^{+}$) at final concentrations of 2 mM using the standard activity assay. In addition, the effect of some organic solvents and inhibitors [methanol, ethanol, isopropanol, ethyleneglycol, glycerol, n-hexane, dimethylsulfoxide (DMSO), Tween 80, Triton-X 100, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-*O, O*'-bis (2-aminoethyl)-*N, N, N', N*'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), and diethyl pyrocarbonate (DEPC)] on the enzyme were tested at 40 °C and pH 6.5. The activity of CE1 without inhibitor addition was defined as 100 %.

Kinetic parameters

For determinations of the kinetic parameters ($K_m$, $V_{max}$, $k_{cat}/K_m$), 0.8 μg purified enzyme was assayed at substrate concentrations for *p*NP acetate (0.1–2.0 mM), *p*NP butyrate (0.1–1.0 mM) and *p*NP caprylate (0.2–2.4 mM) at their optimal conditions. Kinetic parameters ($K_m$ and $V_{max}$) were determined by the Lineweaver-Burk plot. The specificity constant, $k_{cat}/K_m$, was calculated to determine the substrate specificity of CE1. Each experiment was done in duplicate. The standard error was recorded to be <2 %.

## Results

Cloning and sequence analysis of the esterase gene

The nucleotide sequence of the CE1 gene from *B. pumilus* ARA was determined and submitted to GenBank under the accession number HQ441253. Its open reading frame of 747 nucleotides encoded a protein of 249 amino acids. No signal sequence was found, suggesting that this enzyme was an intracellular esterase. An alignment of the CE1 deduced amino acid sequence with the most homologous proteins is shown in Fig. 1. BLAST-P analysis revealed the highest similarity with known esterases from the *Bacillus* spp., such as the esterase gene product from *B. pumilus* SAFR-032 (95 %, accession number YP_001488246.1), *Bacillus subtilis* 168 (87 %, CAB15367), esterase from *Bacillus cereus* ATCC 14579 (78 %, NP_834798), and esterase from *Bacillus halodurans* C125 (71 %, NP_244421). The CE1 also exhibited remarkable homology with other bacterial proteins, such as esterase gene products from *Geobacillus stearothermophilus* (77 %, Q06174), and *Geobacillus thermoleovorans* (75 %, AAG53982). It contains the highly conserved Gly-Lys-Ser-Lys-Gly, in which the $^{93}$Ser residue is a part of the lipase box pentapeptide Gly-Xaa-Ser-Xaa-Gly, found in lipases, esterases, and serine proteases. Moreover, the putative catalytic triad components ($^{192}$Asp and $^{222}$His) in the translated CE1 sequence were also well conserved (Kim et al. 2008).

Overexpression and purification

The pET-28a-*CE1* was transformed into *E. coli* JM109 (DE3), the resulting *E. coli* JM109 (DE3) containing pET-28a-*CE1* was grown in LB medium supplemented with kanamycin in 1-L bioreactor. The recombinant CE1 was overexpressed in *E. coli* JM109 (DE3) by IPTG induction, and made up about 38 % of the total proteins present in the crude extract fraction (Table 1). It was purified to gel electrophoretic homogeneity by immobilized Ni-affinity chromatography (Fig. 2, lane 2), and had apparent molecular masses of 29 kDa on SDS-PAGE, which was in accordance

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CE1_Bp    --MKVVTPKPFTFKGGEKAVLLLHGFTGNTADVRMLGRYLNERGYTCHAPQYKGHGVPPE 58
Est_Bs    MSMKVVTPKPFTFKGGDKAVLLLHGFTGNTADVRMLGRYLNERGYTCHAPQYEGHGVPPE 60
Est_Gt    -MMKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPITKGM-VPPE 58
Est_Gs    --MKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPE 58
Est_Bh    --MKLVAPKPFTFEGGTRAVLLLHGFTGTTADVRMLGRYLQEKGYTCHAPLYKGHGVPPE 58
            **:*.**** *:.* :**********.:********:*:.:*******  :*  ****

CE1_Bp    ELVHTGPTDWWKDVEEGYQFLKDEGYEEIAVCGLSLGGVFSLKLGYTVPVKGIVPMCAPM 118
Est_Bs    ELVHTGPEDWWKNVMDGYEYLKSEGYESIAACGLSLGGVFSLKLGYTVPIKGIVPMCAPM 120
Est_Gt    ELVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPM 118
Est_Gs    ELVHTGPDDWWQDVMNGYEFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPM 118
Est_Bh    ELIQTGPDDWWEDVEDGYQHLKEQGYEEIAVCGLSLGGVFSLKIGYTLPVKGIVPMCAPM 118
          **::*** ***::* :**:.**.:***.**..***********:***:*::***.*****

CE1_Bp    YIKSEEVMYQGVLDYARNYKKFEGKSEDQIEQEMEEFKKTPMGTLKALQELIADVRNNVD 178
Est_Bs    HIKSEEVMYQGVLSYARNYKKFEGKSPEQIEEEMKEFEKTPMNTLKALQDLIADVRNNVD 180
Est_Gt    YIKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLD 178
Est_Gs    YIKSEETMYEGVLEYAREYKKREGKSEEQIEQEMEKFKQTPMKTLKALQELIADVRDHLD 178
Est_Bh    RPKTDDAIYKGVLEYAEEYKRREKKSDEQIEEEMERFKSAPQTTLFGLKQLIEDVRDHLD 178
           *:::.:*:***.**.:**: * ** :***:**:.*:.:*  ** .*::** *** ::*

CE1_Bp    MIYSPTFVVQARHDHMINTDSANIIYNEVETDEKHLKWYEESGHVITLDKEREKVHQDVY 238
Est_Bs    MIYSPTFVVQARHDHMINTESANIIYNEVETDDKQLKWYEESGHVITLDKERDLVHQDVY 240
Est_Gt    LVYARTFVVQARHDKMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIY 238
Est_Gs    LIYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIY 238
Est_Bh    HIYAPVFVVQARHDEMIDVESANVIHDTVESDEKSLKWYEDSTHVITLDKEKEQLHEDVY 238
           :*: .********.**: :***:*:: :*:  * :****:* ******:*:: :*:*:*

CE1_Bp    AFLESLDWSI 248
Est_Bs    EFLEKLDW-- 248
Est_Gt    AFLESLDW-- 246
Est_Gs    AFLESLDW-- 246
Est_Bh    RFLEGLNWSE 248
           *** *:*
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**Fig. 1** Alignment of the amino acid sequence of *B. pumilus* ARA CE1 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 and the putative catalytic triad components ($^{93}$Ser, $^{192}$Asp and $^{222}$His) are indicated in *bold*. *CE1-Bp* esterase from *B. pumilus* ARA (this study), *Est-Bs* esterase from *B. subtilis* 168 (CAB15367), *Est-Gt* esterase from *G. thermoleovorans* (AAG53982), *Est-Gs* esterase from *G. stearothermophilus* IFO 12550 (Q06174), *Est-Bh* esterase from *B. halodurans* C-125 (NP_244421)

with the theoretically calculated molecular mass. The data on the purification are summarized in Table 1. The enzyme was purified 1.6-fold to a specific activity of 5.7 U $mg^{-1}$ proteins from the cell with a yield of 60 %.

## Substrate specificity and kinetics

The substrate specificity of purified CE1 was analyzed using various *p*NP esters with acyl chains of different lengths [*p*NP acetate (C2), *p*NP butyrate (C4), *p*NP caprylate (C8), *p*NP palmitate (C16)] as substrates at 40 °C (Table 2). A Lineweaver-Burk plot showed a linear response over the tested concentration range. The catalytic efficiency represented by the value of $k_{cat}/K_m$ (12.37 $s^{-1}\cdot mM^{-1}$) indicated that *p*NP acetate was the best substrate for CE1 among four *p*NP esters examined.

## Effects of temperature and pH on enzyme activity and stability

The effects of temperature and pH on CE1 activity were evaluated. The highest level of CE1 activity using *p*NP acetate as substrate was observed at 37–40 °C and pH 6.5–7.0 (Fig. 3a, b). The enzyme retained almost 100 % activity at 40 °C after incubation for 2 h at the optimum pH, and gradually decreased above 40 °C. Its half-life was about 30 min at 45 °C (Fig. 3c). The activity of CE1 was stable over a pH range of 6.5–8.0 (Fig. 3d).

## Effects of metals, solvents and inhibitors

Effect of various agents on CE1 activity is shown in Table 3. Amounts of 2 mM $Ca^{2+}$ and $Co^{2+}$ increased the esterase activity to 190 and 121 %, respectively. In contrast, 2 mM $Cu^{2+}$, $Pb^{2+}$, $Zn^{2+}$, and $Fe^{3+}$ reduced the esterase activity to 8, 40, 64, and 76 %, respectively. *B. pumilus* CE1 exhibited low tolerance to some organic solvents, but full inhibition by isopropanol was established. The effects of various inhibitors on esterase activity were examined using SDS, DTT, EDTA, EGTA, PMSF, DEPC, Tween 80, and Triton-X 100. The esterase activity was completely inhibited by 0.5 % (w/v) SDS, 2 mM PMSF, and 1 % (v/v) Tween 80, and strongly inhibited by DEPC, and slightly inhibited by EDTA and EGTA, but was increased to 124 % by 2 mM DTT.

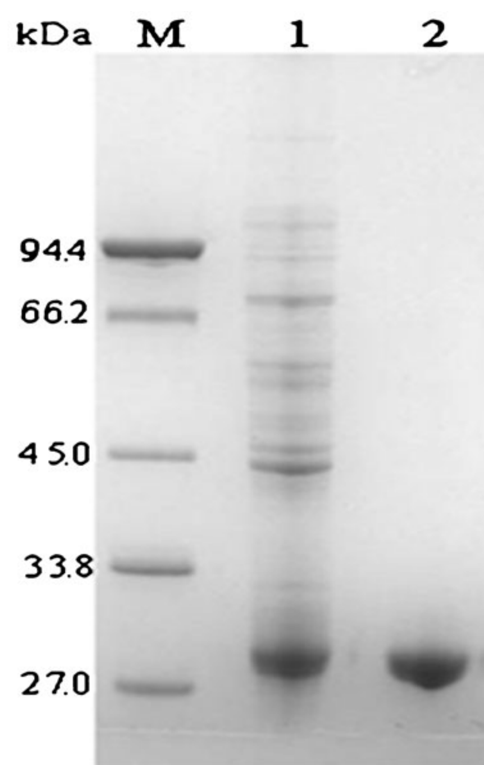


**Fig. 2** SDS-PAGE analysis of purified CE1. *Lane M* molecular mass marker; *lane 1* crude extract; *lane 2* purified CE1

## Discussion

A novel CE1 gene encoding esterase (CE1) from *Bacillus pumilus* ARA was cloned and sequenced. It encodes a protein of 249 amino acids that exhibits high homology to carboxylesterase from *Bacillus subtilis* 168, and this fact suggests that the coding genes of both carboxylesterases are evolutionarily conserved among close *Bacillus* strains. The alignment of *B. pumilus* CE1 showed high similarity to the esterases from thermophilic *G. stearothermophilus* and *G. thermoleovorans*. Recombinant CE1 was successfully overexpressed in *E. coli* and purified to homogeneity. The experimental results revealed that the CE1 displayed maximum activity toward *p*NP-acetate at 40 °C and pH 6.5–7.0. It has been previously reported that *G. stearothermophilus* esterase showed maximum activity at pH 7.0 and 60 °C using *p*-NP propionate as the substrate (Wood et al. 1995), while *G. thermoleovorans* esterase was most active at pH 9.5 using *p*NP palmitate as substrate and

**Table 1** Purification of the recombinant CE1 from *E.coli* JM109 (DE3) containing pET-28a-*CE1*

| Step | Total protein (mg) | Total activity (U) | Specific activity (U $mg^{-1}$) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 18.4 | 66.5 | 3.6 | 1 | 100 |
| Nickel affinity chromatography | 7.0 | 40.0 | 5.7 | 1.6 | 60 |

The enzyme activity was measured towards 1 mM *p*NP butyrate at 40 °C and pH 6.5.

**Table 2** Kinetic parameters for the recombinant CE1 activity on various *p*NP-esters

| *p*NP-esters | $K_m$ (mM) | $V_{max}$ (U mg$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$·mM$^{-1}$) |
|---|---|---|---|---|
| Acetate (C2) | 0.33±0.02 | 8.28±0.11 | 4.07±0.05 | 12.37±0.61 |
| Butyrate (C4) | 0.37±0.01 | 5.15±0.13 | 2.53±0.06 | 6.82±0.32 |
| Caprylate (C8) | 3.89±0.57 | 2.95±0.26 | 1.45±0.13 | 0.37±0.02 |

The kinetic parameters were determined at substrate concentrations ranging from 0.1 to 2.0 mM for *p*NP acetate, from 0.1 to 1.0 mM for *p*NP butyrate, and from 0.2 to 2.4 mM for *p*NP caprylate. Data are reported as means ± standard deviation of triplicates.

temperature optima and stabilities were 70 °C (Soliman et al. 2007). The alignment of the CE1 deduced amino acid sequence with those of the esterases from *G. stearothermophilus* and *G. thermoleovorans* showed that less than 10 % change in all amino acids was responsible for the difference in their temperature and pH optimum. This is in accordance with a directed-evolution study (Spiller et al. 1999).

Generally, enzymes exhibiting high thermostability are often considered to be advantageous for use in industrial-level biotransformations. However, thermophilic enzymes have an operational disadvantage that their activity at room temperature is often relatively low, and the high temperature requirements for thermophilic enzymes would be harmful to labile substrates (Kademi et al. 2000). Therefore, ideal enzyme characteristics might include high stability and high mesophilic activity (Mnisi et al. 2005). The CE1 from *B. pumilus* ARA showed high activity and stability at 37–40 °C and neutral pH, with an optimal pH of 6.5–7.0 (a range typical for mesophilic enzymes), suggesting that CE1 is very attractive for applications in this moderate temperature range, such as in food processes, livestock breeding, or fermentation using a recombinant strain.

In general, enzyme activity on fatty acids shorter than 10 carbon atoms or longer than 10 carbon atoms are referred to as

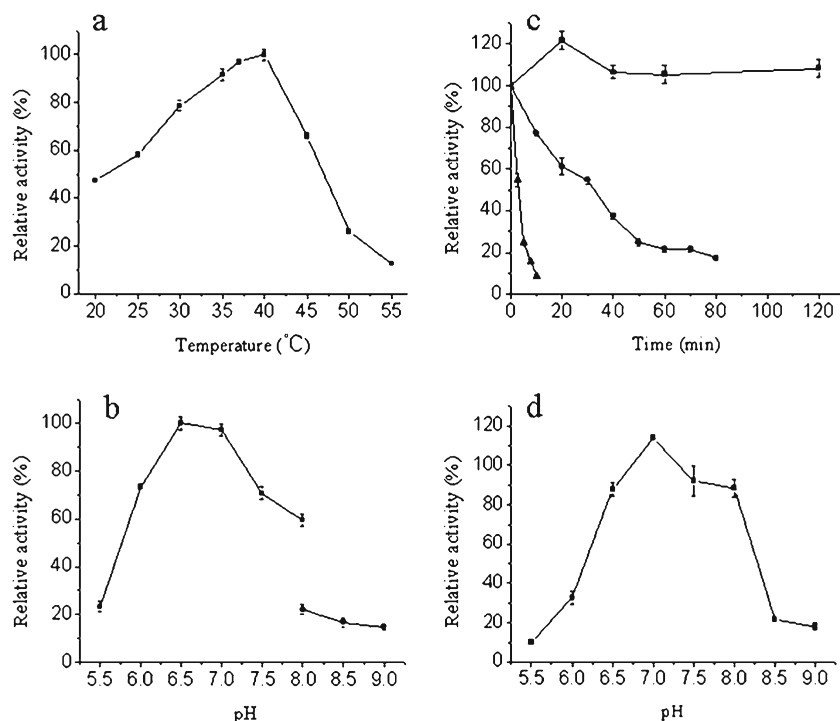


**Fig. 3** Effects of temperature and pH on the recombinant CE1 activity. **a** Effect of temperature (22–55 °C) on CE1 activity at pH 6.5. **b** Effect of pH (5.5–9.0) on CE1 activity at 40 °C. **c** Themostability: 0.8 μg purified enzyme in 100 μl mixed buffer of 100 mM citrate-phosphate (pH 6.5) was pre-incubated for various times at 40 °C (*black square*), 45 °C (*black circle*), and 50 °C (*black up-pointing triangle*) in the absence of substrate. The activity of the enzyme without pre-incubation was defined as 100 %. **d** Stability at different pH values: 0.8 μg purified enzyme in 100 μl mixed buffer of 100 mM citrate-phosphate (pH 5.5–8.0) and 100 mM barbitone buffer (pH 8.0–9.0) was pre-incubated for 1 h at 40 °C in the absence of substrate. Full activity was determined at each pH value. The initial activities were determined at pH 6.5 and defined as 100 %. The substrate was *p*NP butyrate (1 mM)

**Table 3** Effects of various agents on the recombinant CE1 activity; enzyme activities are the mean of three independent assays on *p*NP-butyrate

| Reagent | Concentration | Relative activity (%) |
|---|---|---|
| None | – | 100 |
| $Cu^{2+}$ | 2 mM | 8 |
| $Pb^{2+}$ | 2 mM | 40 |
| $Zn^{2+}$ | 2 mM | 64 |
| $Fe^{3+}$ | 2 mM | 76 |
| $Mn^{2+}$ | 2 mM | 93 |
| $Na^{+}$ | 2 mM | 95 |
| $Ni^{2+}$ | 2 mM | 96 |
| $Mg^{2+}$ | 2 mM | 102 |
| $K^{+}$ | 2 mM | 104 |
| $NH_4^{+}$ | 2 mM | 108 |
| $Co^{2+}$ | 2 mM | 121 |
| $Ca^{2+}$ | 2 mM | 190 |
| Methanol | 10 % (v/v) | 24 |
| Ethanol | 10 % (v/v) | 8 |
| Isopropanol | 10 % (v/v) | UD |
| Ethyleneglycol | 10 % (v/v) | 66 |
| Glycerol | 10 % (v/v) | 39 |
| n-Hexane | 10 % (v/v) | 15 |
| DMSO | 1 % (v/v) | 90 |
| DMSO | 10 % (v/v) | 25 |
| Tween 80 | 1 % (v/v) | 58 |
| Tween 80 | 10 % (v/v) | UD |
| Triton-X 100 | 0.5 % (w/v) | 18 |
| SDS | 0.5 % (w/v) | UD |
| DTT | 2 mM | 124 |
| EDTA | 2 mM | 90 |
| EGTA | 2 mM | 82 |
| PMSF | 2 mM | UD |
| DEPC | 2 mM | 35 |

*UD* undetectable

esterase activity and lipase activity, respectively (Jaeger et al. 1999). As expected, the CE1 showed high preference for substrates with short chain fatty acids rather than the long chain fatty acids. This confirms that CE1 is an esterase rather than a lipase. The same was found in the case of an esterase from an isolated moderate thermophilic bacterium *Bacillus circulans* MAS2: it showed the highest activity on *p*NP-C2 and no activity against *p*NP-C16 (Kademi et al. 2000). These esterases can be used for synthesis of biopolymers and biodiesel, and for the production of pharmaceuticals, agrochemicals, and flavor compounds (Bornscheuer 2002).

To gain more knowledge on the presence of essential catalytic or structural amino acids, CE1 activity was tested upon incubation with various chemicals. Inhibition by PMSF and DEPC suggested that the enzyme belongs to the serine hydrolase group, and serine and histidine residues play key roles in the catalytic mechanism. The CE1 activity is increased with DTT, and possible mechanisms that could contribute to this phenomenon include the possibility that the DTT reduces the formation of the disulfide bond in CE1, in which the putative disulfide components ($^{45}$Cys and $^{115}$Cys) in the translated CE1 sequence were well conserved (Fig. 1). EDTA and EGTA did not inhibit activity indicating that there is no requirement for divalent cations. Altogether, the inhibition pattern is similar to that described for the esterases from *Bacillus* sp. (Karpushova et al. 2005).

Some esterases have a $Ca^{2+}$-binding GXXGXD motif, which plays an important role in the enzyme activity and thermostability. Analysis of the effects of $Ca^{2+}$ and EGTA ($Ca^{2+}$ chelator) on the CE1 activity indicated that calcium ions obviously promote CE1 activity. However, any known $Ca^{2+}$-binding motif could not be found in the sequence of CE1, which are similar to that of the carboxylesterase (EST53) from *Thermotoga maritima* (Kakugawa et al. 2007). The CE1 exhibits biochemical properties that make it well suited for biotechnological applications, such as production of useful synthetic compounds (Bornscheuer 2002), and the enzymatic degradation of roughages for ruminant animals (Cao et al. 2012).

In conclusion, a novel CE1 gene from *B. pumilus* ARA was cloned, sequenced, and functionally expressed in *Escherichia coli* JM109 (DE3). It encodes a protein of 249 amino acids with a calculated molecular weight of 28.4 kDa. The specificity pattern showed a marked specificity for short chain length fatty acids (2–8 carbon atoms), which classified the enzyme as a carboxylesterase. Enzyme inhibition studies and sequence analysis of the CE1 revealed the typical catalytic mechanism of a serine hydrolase with a catalytic triad composed of serine, aspartic acid, and histidine. The enzyme exhibited high activity and stability at 37–40 °C and pH 6.5–7.0, suggesting that CE1 is very attractive for applications of increasing feed digestibility in animal nutrition in this moderate temperature range.

**Acknowledgments** We thank Prof. Weilan Shao and Dr. Jianjun Pei for providing *Bacillus pumilus* ARA. This work was supported by grants from NSF of Jiangsu Province of China (BK2010545).

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