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Characterization of a novel hormone-sensitive lipase family esterase from *Rhizomucor miehei* with tertiary alcohol hydrolysis activity

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ABSTRACT

A novel esterase gene (designated *RmEstB*) from the thermophilic fungus *Rhizomucor miehei* was cloned and functionally expressed in *Escherichia coli*. Sequence analysis revealed a 960-bp open reading frame encoding a protein of 319 amino acids. The deduced protein sequence contained an HGGG motif, suggesting that the enzyme is a hormone-sensitive lipase (HSL) family esterase. It showed highest identity of 52% with the esterase from *Pseudomonas mandelii*. The recombinant esterase was purified to homogeneity at 5.1-fold purification with a recovery yield of 85%. The molecular mass of RmEstB was estimated to be 37 kDa by SDS-PAGE. RmEstB was most active at pH 7.5 and 50 °C. The enzyme was highly stable in the presence of 30% ethanol, methanol, acetone, isopropanol, dimethyl sulfoxide and acetonitrile. RmEstB showed a broad range of substrate specificities toward various *p*-nitrophenol (*p*NP) esters (C_2–C_{10}) and triglycerides (C_2–C_6), with the highest specific activities obtained for *p*NP acetate (255 U/mg) and triacetin (1330 U/mg), respectively. In addition, RmEstB efficiently catalyzed the hydrolysis of sterically hindered esters of tertiary alcohols. This study presents a novel fungal HSL family esterase with potential for some industrial applications.

1. Introduction

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) belong to the general class of carboxylic ester hydrolases, catalyzing both the cleavage and formation of ester bonds of an extensive range of substrates [1]. Esterases exhibit maximum activity toward water-soluble or emulsified esters of short-chain (less than 10 carbon atoms) carboxylic acids, whereas lipases prefer long-chain (≥10 carbon atoms) esters [2]. Both types of enzymes share a definite order of α-helices and β-sheets, termed α/β hydrolase fold, in their three-dimensional structures [3], and most of them contain the consensus sequence Gly–X–Ser–X–Gly around the active site [4]. The most significant difference between esterases and lipases is their ability to act on surface displays: lipases exhibit interfacial activation whereas esterases do not [5].

Microbial esterases have attracted considerable attention in the last decades due to their broad array of potential applications in fields such as food, pharmaceuticals, cosmetics, organic chemicals, paper and pulp, detergents and the dairy industry, based on their widely varied enzymatic properties [2]. However, there are still some bottlenecks in the industrial usage of known esterases, such as low production yield, limited pH and thermal stability, and poor performance in organic solvents [6]. Several strategies have been used to overcome these limitations, such as molecular modification [7] and protein engineering [8,9]. Alternatively, it has been proposed that instead of modifying enzymes to enhance their properties, it is more desirable to isolate naturally evolved enzymes directly for industrial applications [10].

To date, a number of esterases have been purified and biochemically characterized from various microbes, such as *Pseudoalteromas* sp. strain 643A [11], *Rhodococcus* sp. [6], *Bacillus subtilis* strain DR8806 [12], *Psychrobacter celer* strain 3Pb1 [13] and *Sulfolobus solfataricus* [14]. Some esterase genes have been cloned and heterologously expressed, including those from *Oenococcus oeni* [15], *Mycobacterium tuberculosis* strain Rv0045c [16], *Thermus thermophilus* strain HB27 [17], *Pelagibacterium halotolerans* $B2^T$ [18], *Thermotoga maritima* [19] and *Bacillus* sp. strain BP-7 [20]. In addition, several esterase genes have been cloned, expressed and characterized from various metagenomic DNA libraries [21–24]. Despite growing interest in esterases, most of them are from bacteria, and only a few have been reported from fungi [25–29].

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In a previous study, we cloned and biochemically characterized an esterase (RmEstA) from the thermophilic fungus *Rhizomucor miehei* strain CAU432 [29]. This novel esterase showed high-efficiency flavor-ester synthesis. In a further experiment, another novel esterase (RmEstB) belonging to the hormone-sensitive lipase (HSL) family with relatively high molecular mass was found in the same strain. Hence, in this paper, we report on the cloning, expression and biochemical characterization of this second novel esterase. In addition, the potential application of RmEstB as a catalyst for the hydrolysis of tertiary alcohol esters was investigated.

2. Materials and methods

2.1. Materials

Linalyl acetate, linalool, *p*-nitrophenol (*p*NP), *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl caprylate (*p*NPC), *p*-nitrophenyl decanoate (*p*NPD), *p*-nitrophenyl laurate (*p*NPL), *p*-nitrophenyl myristate (*p*NPM), *p*-nitrophenyl palmitate (*p*NPP), 4-methylumbelliferyl butyrate (MUF-butyrate) and Fast Red TR Salt were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *p*-Nitrophenyl hexanoate (*p*NPH) was obtained from HEOWNS Company (Tianjin, China). Triacetin (C_2), tributyrin (C_4), tricaproin (C_6), tricaprylin (C_8) and tricaprin (C_{10}) were obtained from TCI Co. (Tokyo, Japan).

Escherichia coli strain DH5α (Biomed, Beijing, China) and the plasmid pMD18-T (TaKaRa, Tokyo, Japan) were used for DNA manipulations and amplification. *E. coli* strain BL21 (DE3) (Biomed) and the pET-30a(+) vector (Novagen, Madison, WI, USA) were used for protein expression. PrimeSTAR HS DNA polymerase and restriction endonucleases were purchased from TaKaRa. T4 DNA ligase was purchased from New England Biolabs (Ipswich, MA, USA). All other chemicals were of analytical grade unless otherwise stated.

2.2. Strain and cultivation

R. miehei strain CAU432 used in this study has been deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number 4967. For isolation of genomic DNA, *R. miehei* CAU432 was cultivated at 50 °C for 2 days in liquid medium containing (g/l): oat flour (30), soybean peptone (10), $MgSO_4{\cdot}7H_2O$ (0.3), KH_2PO_4 (5) and $CaCl_2$ (0.3), and the initial culture pH was adjusted to 7.0. The mycelia were collected and ground to a fine powder under liquid nitrogen.

2.3. Cloning of the esterase gene and sequence analysis

Recombinant DNA techniques described by Sambrook and Russell [30] were employed to perform DNA manipulations. Genomic DNA was isolated from *R. miehei* strain CAU432 mycelium using CTAB (hexadecyltrimethyl ammonium bromide) according to the method of Lodhi et al. [31]. Total RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, CA), and mRNAs were purified using the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany). Genomic DNA of *R. miehei* strain CAU432 was used as a template for subsequent PCR amplification. The degenerate primers EstDF and EstDR (Table 1) were designed based on two conserved motifs (LAVAGDSAGGN and DVLRDEGEAYA) of known esterases using the CODEHOP algorithm [32], and were used to amplify a partial esterase gene. PCR conditions were as follows: a hot start at 94 °C for 5 min, 10 cycles of 94 °C for 30 s, 60–55 °C for 30 s and 72 °C for 1 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The amplified PCR product was purified, ligated to vector pMD18-T and sequenced.

To obtain the full-length cDNA sequence of the esterase, 5′ and 3′ rapid amplification of cDNA ends (RACE) was performed using a BD SMARTTM RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). To amplify the 5′ end of the cDNA, the RACE product was amplified with primer Est5′GSP and an adapter primer (UPM), and subjected to nested PCR using nested gene-specific primer Est5′NGSP and adapter primer NUP (Table 1). The PCR conditions for RACE were: one cycle of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 68 °C and 1 min at 72 °C, and finally 10 min at 72 °C. For the 3′ RACE, the primary PCR was performed with primers Est3′GSP and UPM followed by a nested PCR using nested gene-specific primer Est3′NGSP and NUP. The obtained PCR product was thus purified, cloned and sequenced. To amplify the coding region from the genomic DNA, the same PCR conditions were used with specific primers EstDNAF and EstDNAR (Table 1). The PCR product was gel-purified, ligated to pMD18-T vector, transferred into *E. coli* strain DH5α for sequencing and finally subjected to BLAST analysis.

Sequence assembly was performed with DNAMAN software (LynnonBiosoft, Quebec, Canada). Database homology search of obtained nucleotide sequences was carried out using BLAST in GenBank at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment analysis was performed using Clustal W program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalW/). Signal peptide was analyzed by SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Search analysis of conserved domains was carried out using ScanProsite (http://prosite.expasy.org/scanprosite/). *N*-Glycosylation sites were predicted using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.4. Expression of the esterase gene in E. coli

To express the esterase gene in *E. coli*, the open reading frame (ORF) region of the cDNA gene was amplified by PCR with RmEstBF and RmEstBR (Table 1) as primers. *Nde*I and *Xho*I sites (underlined in Table 1) were added to the forward and reverse primers, respectively. The PCR program was as follows: a hot start at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension cycle at 72 °C for 10 min. The obtained cDNA product was purified, cloned into pMD18-T, and then transformed into *E. coli* strain DH5α. The recombinant plasmid containing the esterase gene was recovered and digested with the respective restriction enzymes *Nde*I and *Xho*I, subcloned into the corresponding sites of the pET-30a(+) vector, and transformed into *E. coli* strain BL21 competent cells for protein expression. The positive colonies were isolated from LB agar plates containing kanamycin (50 μg mL^{-1}). Transformants were identified by enzymatic digestion and PCR analysis, and further confirmed by DNA sequencing. Seed culture of *E. coli* strain BL21 harboring the esterase gene in vector pET-30a(+) was prepared by incubating cells at 37 °C in a rotary shaker with a rotation speed of 200 rpm for 4 h, and then inoculating on LB medium (1 l) containing 50 μg/ml kanamycin.

Table 1
Primers used in this study.

Primers	Primer sequence (5′→3′)
EstDF[a]	GCCGGCGACTCCgcnggnggnaa
EstDR[a]	CTCGCCCTCGTCTcknarnacrtc
Est5′GSP	TCGCCTTCCTCTCGGAGAACATC
Est5′NGSP	GGAGAACATCAGCTTCTGCGG
Est3′GSP	ACTCTGCAGGTGGTAACCTGTC
Est3′NGSP	TGGTAACCTGTCCGCGGTTGT
EstDNAF	ATGGCACCCACTGTGAAGC
EstDNAR	TTATTTCCAGGTCTTTTGCAACCAG
RmEstBF[b]	GGGTTTCATATGGCACCCACTGTGAAGC
RmEstBR[b]	ATTCCGCTCGAGTTTCCAGGTCTTTTGCAACCAG

[a] N = A/T/C/G, K = G/T, R = A/G.
[b] Restriction enzyme sites incorporated into primers are underlined.