International Journal of Biological Macromolecules 87 (2016) 488–497

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Immobilization of a novel cold active esterase onto Fe_3O_4~cellulose nano-composite enhances catalytic properties

Mohammad Asadur Rahman[a,b,1], Umma Culsum[a,b,1], Ashok Kumar[b], Haofeng Gao[a], Nan Hu[a,*]

[a] College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211800, China
[b] State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 8 March 2016
Accepted 9 March 2016
Available online 11 March 2016

Keywords:
Cold active
Immobilization
Nano-composites
Salt tolerance

ABSTRACT

A novel esterase, EstH was cloned, purified and characterized from the marine bacterium *Zunongwangia* sp. The purified EstH showed optimum activity at 30 °C and pH 8.5 with ~50% of original activity at 0 °C. EstH was stable in high salt conditions (0–4.5 M NaCl). To improve the characteristics and explore the possibilities for application, a new immobilization matrix, Fe_3O_4~cellulose nano-composite, was prepared and was characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM). Interestingly the optimal temperature of immobilized EstH elevated to 35 °C. Compared to its free form, immobilized EstH showed better temperature stability (48.5% compared to 22.40% at 50 °C after 30 min), prolonged half-life (32 h compared to 18 h), higher storage stability (~71% activity compared to ~40% after 50 days of storage), improved pH tolerance (~73% activity at pH 4 and 10), and, more importantly, reusability (~50% activity after 8 repetitive cycles of usage). Enzyme kinetics showed an increase in the V_{max} (from 35.76 to 51.14 μM/min) and K_{cat} (from 365 s^{-1} to 520 s^{-1}) after immobilization. The superior catalytic properties of immobilized EstH suggest its great potential in biotechnology and industrial processes.

1. Introduction

Esterases (EC 3.1.1.1) of microbial origin with good stability and activity have potential applications in food and dairy industries, detergents, pharmaceuticals, synthesis of optically pure compounds, bioremediation, perfume production and even in biomedical device production [1–4]. Esterases with some unusual characteristics such as cold active are energy saving and easy to be inactivated in synthetic reactions is useful in the production of heat sensitive materials [5–7]. On the other hand, esterases which are stable in high pH and salt conditions, can catalyze reactions in both nonaqueous and aqueous/organic media and can be utilized to degrade organic matters in saline water [8–10]. Moreover, structural modification and immobilization could contribute to the thermostability of cold adapted enzymes as compared to the free enzymes [11–14].

Immobilized enzymes can maintain the conformational change and the physiochemical properties, prevent the enzyme from being aggregated in organic media and continuous reaction processes, reduce the possibility of denaturation in the conditions of high temperature, pH and organic solvents, and also facilitate storage and maintenance compared with the free ones [15–20]. Furthermore, immobilization is very crucial for the production of fine chemicals by removing the enzyme from reaction system and control over product formation [21].

The chemical structure of immobilization support and large surface area are the important factors to achieve sufficient enzyme loading and catalytic efficiency in aqueous or organic media [22]. Organic and inorganic nanoparticles with a large surface area are proved to be excellent materials for immobilization of enzymes. Furthermore, selection of a suitable immobilization method is another important factor for the successful immobilization of an enzyme. Magnetic nanoparticles like Fe_3O_4 have been used efficiently in immobilization because of their superparamagnetism, high surface area, easy separation from the reaction mixture by applying magnetic fields as well as controlling mechanism of the orientation on the enzymes attached to the support [23–26]. Surface modification or coating of Fe_3O_4 nanoparticles with organic materials can enhance the binding efficiency of an enzyme with

* Corresponding author: College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211800, China.
E-mail addresses: nayongeb@gmail.com (M.A. Rahman), eshageb@gmail.com (U. Culsum), ashok.nadda09@gmail.com (A. Kumar), zane_gao1986@163.com (H. Gao), hunan@njtech.edu.cn (N. Hu).
[1] Authors have equal contribution to the work.

http://dx.doi.org/10.1016/j.ijbiomac.2016.03.016
0141-8130/

the support materials either by hydrogen bonding or van der Waals interaction or electrostatic interactions. In recent studies, nanocellulose has been synthesized from various natural sources which proved to be a good template for the formation of nano-composites [27]. Nanocellulose shows a unique property to self-assemble with improved thermal stability as well as solvent stability [28]. Thus, cellulose-based nano-composites with inorganic materials like Fe_3O_4 nanoparticles can sustain relatively high temperatures, pH and extreme physiochemical conditions and provide well-defined mesostructure with metal oxide scaffolds to protect any biomolecules like protein/enzyme from denaturation [29,30]. In recent years, cellulose of different origins has been used as a supporting matrix for the formation of iron oxide and as filler for the homogeneous distribution of pre-synthesized crystalline nanostructures [29,31,32].

In the present study, a novel family VII cold active and salt tolerant esterase from *Zunongwangia* sp. was purified and immobilized onto a Fe_3O_4~cellulose nano-composite. Both Fe_3O_4 nanoparticles and nanocellulose were synthesized separately and assorted by sol-gel method to prepare a hybrid nano-composite. The purified esterase was immobilized onto this nano-composite and the improved bio-catalytic properties were studied in comparison with those of the free esterase.

2. Material and methods

2.1. Strains, vectors and chemicals

The marine bacterium *Zunongwangia* sp. and its genome have been already reported [33]. It was grown in high-salt Luria-Bertani medium (HLB) (peptone 1%, yeast extract 0.5%, NaCl 2%) at 28 °C. *Escherichia coli* strains DH5α (Takara, Japan) and BL21 (DE3) (Novagen, USA) were used as the bacterial hosts for plasmid (pGEX-6P-1, GE Healthcare, USA) amplification and heterologous expression, respectively. The enzymes used such as restriction endonucleases, DNA polymerase, and T4 DNA ligase were purchased from Takara (Kyoto, Japan) and the substrates, *p*-nitrophenyl esters: *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl hexanoite (C6), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl laurate (C12), and *p*-nitrophenyl palmitate (C16) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Microcrystalline cellulose (MCC) was procured from Merck Schuchardt, Germany. All the other chemicals and buffers used were of high purity and analytical grade.

2.2. Gene cloning and recombinant plasmid construction

The putative esterase containing gene EstH encodes a protein (GenBank No. ADF54626.1) which was amplified using the genomic DNA of *Zunongwangia* sp. as template with the following primers EstH F: 5′ CGCGGATCCATGAAAAAAATCATACTGTTATTTGCA -3′ and EstH R: 5′- CCGCTCGAGTTATTGTTCGGTGTACTTTTTATCTAA -3′ with restriction enzyme sites of *Bam*HI and *Xho*I, (underlined) respectively. PCR was performed in a thermal cycler programmed with 95 °C for 30 s, 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1.40 min and a final elongation of 72 °C for 10 min. Then the PCR products were purified using 1% agarose gel using gel mini purification kit (AXYGEN, USA). Next, the vector and purified PCR products were digested with *Bam*HI and *Xho*I, and purified by gel. The digested and purified gene was cloned into the digested and purified pGEX-6P-1 vector using T4 DNA ligase and transformed into the competent *E. coli* DH5α cells, followed by incubation in solid LB medium (1% NaCl, 1% peptone, 0.5% yeast extract, 1.5% agar) supplemented with 100 μg/ml ampicillin at 37 °C overnight, and then the recombinant plasmids were extracted. Finally, the correct insert of the plasmid was confirmed by sequencing, and the recombinant plasmid of pGEX-6P-1-EstH was used for further study.

2.3. Expression and purification of EstH

The recombinant plasmid pGEX-6p-1-EstH was transformed into *E. coli* BL21 (DE3) competent cells for expression. *E. coli* BL21 (DE3) cells containing the recombinant plasmid pGEX-6P-1-EstH were grown in the liquid LB medium containing 100 μg/ml ampicillin at 37 °C overnight, followed by inoculation at 1:100 dilution into fresh LB liquid medium containing 100 μg/ml ampicillin and incubation at 37 °C till OD_{600} reached 0.6. Then 1 mM (final concentration) IPTG was added and cultured for 16 h at 18 °C and 180 rpm. Next, the cells were collected and washed twice with PBS buffer (0.8% NaCl, 0.02% KCl, 0.142% Na_2HPO_4, 0.027% KH_2PO_4; pH 7.4) by centrifugation at 8000 rpm for 10 min and resuspended in PBS buffer and then the cells were disrupted by a French press and the crude enzyme was obtained as supernatant by centrifugation at 12000 rpm for 40 min at 4 °C. Finally, glutathione-S transferase (GST)-tagged fusion esterase GST-EstH was purified according to manufacturer's instructions using Glutathione Sepharose 4B columns (GE Healthcare). 3C protease solution (10 U/μl PreScission; Pharmacia) was used to remove the GST tag and the purified protein was eluted with a moderate amount of PBS buffer (pH 7.4). The protein was quantified with Bradford reagent (Sigma, USA) using bovine serum albumin (BSA) as a standard [34] and the proteins were analyzed by 12% SDS-PAGE.

2.4. Sequence analysis

The sequence similarity was examined by Basic Local Alignment Search Tool (BLAST) program from the server at National Centre of Biotechnology, USA (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment was done by the Clustal W Method of BioEdit Sequence Alignment Editor Program. Phylogeny analysis was performed using the sequences of previously determined I-VIII family esterases/lipases [35] with MEGA 6.0 program [36].

2.5. Enzyme activity assay

One unit of esterase activity was determined by the production of 1 μmol of *p*-nitrophenol from *p*-nitrophenyl butyrate (*p*NPB, C4) in 1 min using *p*-Nitrophenol as standard. The volume of each standard reaction mixture was 200 μl consisting of 3 μl of 20 mM substrate, 2 μl of pure enzyme (2 μl of immobilized enzyme suspension) and 195 μl Tris–HCl buffer (50 mM, pH 8.5), and the reaction mixture without the addition of any enzyme was considered as standard. The reaction continued for 7 min at 30 °C for the free enzyme and 35 °C for the immobilized one with continuous shaking, followed by the separation of immobilized biocatalyst by applying magnetic field, and the absorbance of the released *p*-nitrophenyl was recorded at 405 nm using 96-well plate with Thermo Scientific Multiscan Spectrum.

2.6. Synthesis of Fe_3O_4~cellulose nano-composite

The spherical cellulose nanogel was prepared by controlled acidic hydrolysis as previously described [37] with minor modifications [38]. Nanogel was prepared by mixing 5 g of MCC in 50 ml distilled water and H_2SO_4 was as added drop by drop to a final concentration of 63.4 wt% under shaking condition. Later on, the solution was transferred into 10-fold volume of cold water, separated by centrifugation and neutralized with dd H_2O and Na_2CO_3 (2% v/v). Then, the cellulose nanogel was washed, diluted (5 wt%)