International Journal of Biological Macromolecules 96 (2017) 171–176

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Improvement of chitinase Pachi with nematicidal activities by random mutagenesis

Junpeng Chen, Yangdongfang An, Ashok Kumar, Ziduo Liu *

College of Life Science and Technology, State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430 070, China

ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form
23 November 2016
Accepted 24 November 2016
Available online 15 December 2016

Keywords:
Pseudomonas aeruginosa
Chitinase
Nematicidal
Caenorhabditis elegans
Random mutant library

ABSTRACT

Chitinase, an enzyme that can degrade the main compositions of insect intestine and cuticle, has been used in the bio-control field. Our previous work has reported the chitinase Pachi with nematicidal activity (*Caenorhabditis elegans*). In the present study, to improve the chitinolytic and nematicidal activities of Pachi, a random mutant library was constructed by error-prone PCR and screened by bacteriophage T7-based high-throughput screening system. One mutant, $Pachi_{N35D}$ was obtained from about 10,000 clones. The kinetics analysis revealed that $Pachi_{N35D}$ exhibited a 63% decrease in K_m value against chitosan, a 2.1-fold enhancement in k_{cat}/K_m value and a 1.2-fold increase in specific activity over the wild-type Pachi. Moreover, the mortality analysis against *Caenorhabditis elegans* showed that the 50% lethal concentration (LC_{50}) of $Pachi_{N35D}$ is 309.6 ± 1.1 μg/ml and a 20% increase in nematicidal activity over the wild-type Pachi (with a LC_{50} value of 387.3 ± 31.7 μg/ml). The structure modeling and superimposition indicated that the substitution N35D reduced the distance between substrate and substrate-binding site Asp141, finally resulting in an increase in substrate affinity, catalytic efficiency and specific activity. These results provide useful information for the study of structure-function relationship of Pachi and lay a foundation for its potential applications in agro-biotechnology.

1. Introduction

Nematodes can cause serious damages to crops by parasitizing the roots, stems and leaves of plants. Plant-parasitic nematodes give rise to an annual global agricultural loss up to $157 billion [1]. The extensive use of various chemicals to control nematodes results in problems like environmental pollution and human health. In recent years, much research has focused on biopesticides. It is known that microorganisms can attack and kill nematodes by a variety of methods like capturing, parasitizing, and producing toxins and enzymes [2]. The crystal proteins produced by *Bacillus thuringiensis* are one of the most widely used natural insecticides in agriculture [3]. These insecticidal crystal proteins are not only toxic to nematodes, but also have some inhibitory effects on insects like *Lepidoptera, Dipteran, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophage* [4]. Extracellular hydrolytic enzymes involved in infection against nematodes are serine proteases, chitinases, collagenases and other hydrolytic enzymes [5]. It was reported that extracellular protease could be employed in infection against nematodes by the *Brevibacillus laterosporus* G4 strain [6]. Endochitinase (CHI43) and the serine protease P32 from *Verticillium suchlasporium* caused obvious damage to eggs of the nematode *Globodera pallida* [7]. Chitinases from *Lecanicillium antillanum B-3* and *Pochonia chlamydosporia* were responsible for degradation of eggshell structures of *Meloidogyne incognita* [8,9].

With the introduction of genetic engineering into biocontrol research, scientists have paid more attentions to the improvement of biological insecticides. Ahman, J.et al firstly reported that genetic engineering can be used to improve the pathogenicity of a nematode-trapping fungus *Arthrobotrys oligospora* by transforming an additional copy of a subtilisin-like extracellular serine protease gene *PII* [10]. Luo, X., et al. found a novel nematicidal virulence factor Bmp1 protein in *B. thuringiensis* which is toxic to nematodes and also improves the pathogenicity of *Cry5Ba* against nematodes because of its ability to damage the intestinal tissues of nematodes [11]. In our previous work, nematicidal potential of the chitinase Pachi from *Pseudomonas aeruginosa* was enhanced significantly when association with *Cry21Aa* like mixed proteins or fusion proteins [12]. Interestingly, Ni, H., et al. have improved the chitinolytic, synergistic Lepidopteran-larvicidal and nematicidal activities of a *B. thuringiensis* chitinase by molecular docking and site-directed mutagenesis [13].

However, few research attempts have been made concerning how to rationally improve the nematicidal activity of hydrolytic

* Corresponding author.
E-mail address: lzd@mail.hzau.edu.cn (Z. Liu).

http://dx.doi.org/10.1016/j.ijbiomac.2016.11.093
0141-8130/

enzymes which are toxic to nematodes. In this study, we enhanced the chitinolytic and nematicidal activity of the chitinase Pachi from *Pseudomonas aeruginosa* by random mutagenesis and directed screening. We used the model organism *Caenorhabditis elegances* (*C. elegans*) as the object for bio-control of nematodiasis due to its genetic manipulability and short life cycle [14,15].

2. Materials and methods

2.1. Chemicals, strains, vectors and culture conditions

Substrate chitosan was purchased from Sigma (USA). Taq DNA polymerase, T4 DNA ligase and restriction enzymes were purchased from TAKARA (Japan). The DNA purification kit and GST Bind Resin were purchased from Axygen (USA) and Novagen Co. (Germany). Oligonucleotide primers and sequencing service were offered by Beijing Tsingke Biotech Co. (Beijing, China). All the other chemicals used in this study were of analytical grade and commercially available. The host strains *Escherichia(E.) coli* DH5α (TaKaRa, Japan) for random mutant library construction and E.coli BL21 (DE3) (Novagen, USA) for protein expression were grown at 37 °C in Luria-Bertani medium (LB) [16] with ampicillin (100 μg/ml), and the bacteriophage T7 was cultured as described previously [17]. Plasmid pGEX-6P-1 was used as the vector for gene cloning and protein expression. The *C. elegans* N2 wild-type (WT) strain was offered by the *Caenorhabditis Genetics Center (CGC)* and cultured on nematode growth medium (NGM) agar plates with *E. coli* OP50 as its food at 20 °C and stored at−80 °C [15].

2.2. Construction and screening of random mutant library

The random mutant library was constructed by error-prone PCR described by Cadwell, R.C. and G.F. Joyce [18]. Universal primer of pGEX-6P-1 was used and $MnCl_2$ was added to increase the error-rate in the PCR reaction. PCR products were purified, digested with *EcoR* I and *Xho* I, cloned into pGEX-6P-1, and transformed into *E. coli* DH5α to create the random mutant library.

In order to get mutants with higher chitinolytic activity under low temperature,the bacteriophage T7 based high-throughput screening was performed [17]. Mutants of the library were cultured in the 96-deeep well plates including 0.6 mL LB medium and the corresponding copies were cultivated on LB agar plates with ampicillin (100 μg/ml) overnight at 37 °C. After induction by IPTG (0.2 mM) and simultaneous lysis by bacteriophage T7 at 28 °C for 6 h [19], 100 μL of cellular supernatant was transferred into another 96-deep well plate containing 100 μL 10 mg/ml chitosan. After reaction at 20 °C for 20 min, 200 μL DNS was added into each well and boiled at 100 °C for 10 min. The absorbance of 540 nm was measured by the Multiskan Spectrum spectrophotometer (Thermo Scientific, Finland), and the mutants with higher absorbance than the wild-type were selected for further studies [20].

2.3. Enzyme expression and purification

The expression host strain *E. coli* BL21 (DE3) containing recombinant plasmid pGEX-6P-$pachi_{N35D}$ was cultured in LB medium with ampicillin (100 μg/ml) at 37 °C until the exponential phase, and IPTG (0.1 mM) was added to induce the expression of enzyme at 18 °C for 16 h. Cells were collected by centrifugation, re-suspended in phosphate buffer and crushed with the high pressure homogenizer (NS100IL 2K, Niro Soavi, Germany). Subsequently, the insoluble part was removed by centrifugation and the crude enzyme was purified by glutathione-S-transferase(GST) affinity chromatography. The GST tag was removed by using 3C protease as described by L, C., et al. [21], the purity of pure enzyme was analyzed by 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and its concentration was determined by Bradford assay [22].

2.4. Activity assay for chitinase

10 mg/mL chitosan was prepared as the substrate as described by Ilyina, A.V., et al. [23]. 3 uL chitinase was mixed with 147 uL 10 mg/ml chitosan, after reaction at 37 °C for 20 min, 150 uL DNS was added and the reaction system was boiled at 100 °C for 10 min. The OD_{540} value of supernatant was measured by the Multiskan Spectrum spectrophotometer after centrifuged [20]. One unit of chitinase was defined as the amount of enzyme that liberated 1 mg of D-glucosamine per minute under standard conditions.

2.5. Characterization of WT and the mutant

The optimal temperature of WT and the mutant was tested within the 20–80 °C range. The pH effect on the activities was determined in different buffers at pH 4.0–9.0[0.2 M Na_2HPO_4-0.1 M citric acid buffer (pH 4.0–7.5), 0.2 M boric acid-sodium borate buffers (pH 8.0–9.0)]. The thermo-stability of the chitinase was studied under the optimal pH by heat treatment from 30 to 65 °C for 1 h and the residual activity was measured as described above. To study the pH stability, the remaining activity was measured after incubation in buffers (pH 4.0–9.0) at 4 °C for 72 h. The effects of different metal ions on the enzymes activity were tested under standard assay conditions.

2.6. Enzyme kinetic parameters

The kinetic parameters of WT and the mutant were determined using different concentrations of chitosan under standard assay conditions according to Lineweaver-Burk plot. The K_m and V_{max} values were calculated from the Michaelis-Menten equation.

2.7. Structure modeling

The homology models of WT Pachi and the mutant were extracted from SWISS-MODEL (http://www.swissmodel.expasy.org/) after submitting the amino acid sequence. The 3D structures with some further modification of WT and the mutant were generated using the MOE software.

2.8. Bioassay of nematicidal activity

To determine the nematicidal activity against *C. elegans*, the pure chitinase $Pachi_{N35D}$ was used for bioassays including mortality tests, brood size assays, and quantitative growth analyses. The mortality tests of $Pachi_{N35D}$ were performed against L4 stage of *C. elegans* after synchronization. 30–40 nematodes were added to the 96 wells plate containing 100 uL purified Pachi and the plate was incubated at 20 °C for 24 h. When we checked the results under the inverted microscope, a nematode was considered dead when showing no response to several touches by a needle. The brood size assays were performed by determining the number of progeny. The quantitative growth analyses of the nematode started at L1 stage. The sizes of nematodes were measured and compared by taking pictures and calculating the area of nematodes using the software ImageJ. All experiments were performed in triplicates and were repeated at least three times. The quantitative analyses of the 50% lethal/inhibition/growth concentration (LC_{50}/IC_{50}/GC_{50}) were undertaken according to the method of Bischof et al. [24].