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Effects of domains modification on the catalytic potential of chitinase from *Pseudomonas aeruginosa*

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ABSTRACT

Chitinase, an important enzyme in chitin-degrading, have extensive biophysiological functions and immense potential applications. Here, a chitinase gene *pachi* was cloned from *Pseudomonas aeruginosa* and overexpressed in *E. coli* (DE3). The structural analysis showed that chitinase pachi consists of catalytic domain (CHC), chitin binding domain (CBD) and both of these are linked by connective domain (FN_3). In this study, Pachi displayed optimal activity at temperature 65 °C and pH 6.5. To understand the structural and functional relationship of chitin-binding domain with catalytic domain, two mutants, CHA (without CBD) and CBD + FN_3-pachi with additional CBD have been constructed. Though the results showed that the two mutants have similar characteristics with Pachi, it is interesting to note that the deficiency of CBD caused an increase in expression level as well as solubility of the CHA. Moreover, the catalytic efficiency of CHA was increased 1.26-fold and substrate affinity in the absence of CBD was decreased 1.85-fold. Thus, the improved solubility and activity of CHA by domain deficiency is an interesting pathway to study the relationship of structure and function of chitinase and support its potential use in commercial applications.

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1. Introduction

Chitin is a natural homopolymer of *N*-acetylglucosamine (GlcNAc) act as a structural component in the cell walls of fungi, exoskeletons of insects, and in the cuticle or eggshells of nematodes. It acts as a barrier to protect hosts from microbial attack. Meanwhile, chitin is an important carbon source, metabolized by various bacteria and fungi [1–3]. The microbial enzymes like chitinases play key role in the digestion and degradation of chitin components of host cell. For the complete digestion of chitin both endo as well as exo-chitinases are required. These enzymes degrade the chitin into *N*-acetyglucosamine which can be easily metabolized by microbes to obtain energy for cell growth [4]. Chitinase belongs to glycosyle hydrolase superfamily, which included 18, 19 and 20 families [5]. In particular, chitinase of family 18 exists in prokaryotic organisms and eukaryotes commonly, while family 19 is exclusively in plants [6–9]. The pachi from *P. aeruginosa* belongs to family 18, which contain catalytic domain (CHC; 1–1026 bp; 37.68 kDa), connective domain (FN_3; 1027–1281 bp; 8.96 kDa), and chitin binding domain (CBD; 1282–1449 bp, 6.41 kDa). However, most chitinases from fungi only contained catalytic domain CHC, while others include *N*-terminal (signal peptide) region, catalytic domain, connective domain, chitin binding domain [10,11]. It has been reported that the chitinase were quite unstable and tended to lose their substrate-binding domain [12]. To understand the role of CBD and FN_3 domains on catalytic activity, stability and solubility a derivant which contained CHC merely had been constructed and characterized. Recently, though the research on cloning and application of chitinase gene increased progressively. The wild-type chitinase with moderate activity and characteristics cannot meet the demand of industry [11]. The activity and stability of an enzyme are major factors for commercial uses, moreover thermo-stability is an advantageous feature which decide the industrial potential of an enzyme. Thus finding the new chitinases with improved activity and thermo-stability will definitely be an asset to the biotechnology industry [13]. Directed evaluation and rational design have been widely used to obtain such enzymes with high activity and stability. As reported previously the catalytic activity of chitinase from *Beauveria bassiana* was increased considerably using DNA shuffling and throughput screening methods [14]. Another significant strategy to improve the properties of enzyme is by either addition or deletion of domains [15,16]. In order to improve substrate affinity an additional cellulose binding domain from cellobiohydrolase II was inserted to *N*-terminal of chitinase from *Trichoderma harzianum*

Abbreviations: CHC, catalytic domain; FN_3, connective domain; CBD, chitin binding domain; CBM, carbohydrate/cellulose binding domain.

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[17]. However the significant increase in the activity of chitinase has been reported in the present study by deletion of CBD and FN3 domains. The native CHA constructed by domain deletion displayed higher catalytic potential than wild chitinase.

It is well known that the activity of one enzyme related to its solubility probably. Especially in the crystal protein of insecticidal, the virulence is affected by its solubility. Thus using rational design and molecular biology tools researchers tried to increase the solubility and activity. Wang F et al. and Van Den Berg S et al. improved the solubility and toxicity and TEV protease using direct evolution strategy [18–20]. Chan et al. declared that chaperones altered the solubility property of the proteins [21]. The C-terminal region of enzymes was considered to contribute to solubility, stability, and catalysis by some researchers [22]. In the present study, an effort has been made to obtain the chitinase with high activity and more solubility by CBD and FN_3 domains deletion. The characteristics of domain deficient constructed protein were studied systematically. The study has provided a new and significant strategy to increase activity of enzymes.

2. Materials and methods

2.1. Materials

Substrate colloid chitin was prepared by the method of Jeniaux C [23] using 5 g of practical grad crab shell chitin (Sigma Chemical c-9213). The enzymes such as DNA polymerase, restriction endonuclease, and T4 DNA ligase were purchased from Takara and DNA purification kit from Zoman (Beijing, China). All the chemicals and buffers used were of analytical grade and commercially available.

2.2. Bacterial strains and vector

Pseudomonas aeruginosa was isolated from south lake, near Huazhong Agricultural University, Wuhan, China. The strain was grown in the Luria-Bertani medium [24] (peptone; 1%, yeast extract; 0.5%, NaCl; 1%) at 37 °C for 24 h. *Escherichia coli* DH5α (TaKaRa, Japan) and BL21 (DE3) (Novagen, USA) used as the bacterial hosts for cloning and heterologous expression, respectively were cultivated in LB medium containing ampicillin (100 μg/ml). The pGEX-6p-1 (GE Healthcare, USA) was used as the original plasmid for constructing expression vectors.

2.3. Construction of expression vectors

The DNA was extracted from *P. aeruginosa* and used as the template for amplifying the chitinase-encoding gene *pachi* ORF (Genbank: CP003149) by the polymerase reaction (PCR) using the primer pair pachi-F, pachi-R (Table 1). Subsequently, the gene pachi was cloned into the pGEX-6p-1 to construct the recombinant plasmid pGEX-6p-pachi. With the same method the recombinant plasmid pGEX-6p-CHA has been constructed.

The CBD and FN_3 were fused by overlapping PCR using the primer pairs CBD-F, CBD-R, FN_3-F, FN_3-R. The plasmid pGEX-6p-pachiB was constructed by adding an additional restriction site *Bgl* II using the rapid PCR-based site-directed mutagenesis. Finally, to construct the recombinant plasmid, CBD-FN_3 was inserted into the vector pGEX-6p-pachiB (Fig. 2A).

Table 1
Primers used in the study.

Primer	5′-3′	Purpose
CHA-F	CCGGAATTCATGATCAGGATCGACTTTTCCCAG-TTGCA	Cloning *CHA*
CHA-R	CCGCTCGAGTCAGCGCTGGTCGCCGCCC	Cloning *CHA*
Pachi-F	CCGGAATTCATGATCAGGATCGACTTTTCCCAG-TTGCA	Cloning *pachi*
Pachi-R	CCGCTCGAGTCAGCGCAGCGGCCGCC	Cloning *pachi*
PachiB-F	CCGAGATCTCCGGAATTCATGATCAGG	Adding *Bgl* II
PachiB-R	GCCTTCCGGAGATCTCGGGGATCCCAG	Adding *Bgl* II
CBD-F	GGAAGATCTATGAGCGGCGGGGCGGTGGATCCG	Cloning *CBD*
CBD-R	CTGCGGCGCCGCCGGGCGCAGCGGCCGCCA	Cloning *CBD*
FN_3-F	TGGCGGCCGCTGCGCCCGGCGGCGCCGCAG	Cloning *FN_3*
FN_3-R	CCGGAATTCGGTGCTGACCTCCAGGCCTTC	Cloning *FN_3*

F, forward; R, reverse. Restriction sites are underlined.

2.4. Heterologous expression and purification

All the recombinant plasmids were transformed into *E. coli* BL21 (DE3) to express the proteins. The transformants were cultured in LB medium containing ampicillin (100 μg/ml) at 37 °C until the OD_{600} of 0.6–0.8 reached. After 3 h IPTG (1 mM) was added to induce the protein synthesis and culture was grown for 20 h at 20 °C under shaking (200 rpm). The cells were harvested by centrifugation (8000 rpm; 10 min) and disrupted with High pressure Homogenizer (NS100IL 2 K, Niro Soavi, Germany). The collected cell hydrolysate was used to purify the protein using GST (Ge Healthcare, USA) affinity chromatography. The GST tag was removed by digestion with 3 C protease according to method described by Chanjuan et al. [25].

2.5. Enzyme and protein assay

The chitinase activity was determined by method described by Wirth et al. using colloid chitin as a substrate [26]. One unit of enzyme activity was defined as the amount of enzyme which released 1 mg of *N*-acetylglucosamine per min under standard assay conditions. To analyze the molecular mass of the purified proteins the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used and concentration was determined by Bradford assay using BSA as standard.

2.6. Physic-chemical properties of chitinase

The activity of enzymes was studied at temperature(s) range from 30 to 80 °C. The optimal pH was studied in different buffers at pH 4.0–10.0, namely 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), and 0.05 M and sodium borate-NaOH buffers (pH 9.5–10.0). The thermo-stability of the enzymes was determined at the optimum pH by incubating the enzymes at temperature(s) range from 30 to 70 °C for 1 h and the residual activity were measured using standard method. The enzyme activity without treatment was considered as 100%. The effect of pH on the enzymes stability was studied by measuring the residual activity after incubation in buffers (pH 4.0–10.0) at 4 °C for 72 h. The effects of the metal ions on the chitinase activity was analyzed by addition of different metal ions (NH_4^+, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^+, Zn^{2+}, K^+, Ca^{2+}, Sr^{2+} and Co^{2+}) in reaction mixture under standard assay conditions.

2.7. Analysis of kinetic parameters

The kinetic parameters were studied using Lineweaver-Burk plot method. The colloid chitin with different concentrations from 6 to 60 mg/ml was used to determine the *Km*, *kcat*, and *Vmax* values under the optimized temperature and pH.

2.8. Structure modeling

The homology models of all the three proteins were constructed automatically by SWISS-MODEL (http://swissmodel.expasy.org)

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