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Expression of Genetically Engineered Chitinase Gene of *Pyrococcus furiosus*

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ABSTRACT: Wild-type *Pyrococcus furiosus* is most likely unable to grow on chitin in the natural biotope due to a nucleotide insertion which separates the chitinase gene into two ORFs, whereas a genetically engineered strain with the deleted nucleotide is able to grow on chitin. In the latest studies, the recombinant enzyme activity against the crystal chitins was examined. But there are still some conflictions. In our study, to shed a light on whether the construct composed of a catalytic domain and a chitin binding domain show any activity against crystalline chitin, the construct was created in the pET 28b (+) expression vector and expressed in *Escherichia coli*. The chitinase with an approximately 55 kDa molecular weight was determined. The activity of the enzyme was measured spectrophotometrically. Despite the presence of enzyme activity against the colloidal chitin, no significant activity against the crystal chitin has been measured.
Keywords:- Pyrococcus furiosus, chitinase, DNA polymerase, crystalline chitin, cloning.

I. INTRODUCTION

Chitin $(C_8N_{13}O_5N)_n$, β-1-4 glycosidic bonded N-acetylglucosamine subunits with various grades of acetylation, is the second most abundant biopolymer generated mostly by arthropoda and fungi on earth with an estimated 10^{10} to 10^{11} tons per year [1]. Although a large amount of its presence from deserts to the deep see, the industrial utilization of chitin on a large scale has not been achieved because of the deficiency of appropriate enzymes which can hydrolyze crystal chitin. If it will be possible, N-acetyl-glucosamine (GlcNAc) and its oligosaccharides derived from chitin like diacetylchitobiose, N-acetylglucosamine (NAG) monomers/oligomers, chitotrioses are expected to be useful as food additives and medicines [2].

Chitinase (EC 3.2.1.14), which are capable of hydrolyzing the β-1,4 bonds between N-acetyl glucosamines in chitins, are widely found in the eukaryal and bacterial domains. But in archaea domain, experimentally active chitinases are found just in three genera of *Halobacterium*, *Thermococcus* and *Pyrococcus*. *Thermococcus* chitinase was the first known chitinase in archaeal domain with two catalytic domains (A and B) and three chitinase binding domains. The N-terminal catalytic domain A functions as an exochitinase and liberates diacetyl-chitobiose. The C-terminal catalytic domain B acts as an endochitinase which produces N-acetyl-chitooligosaccharides of various lengths, which could be further hydrolyzed to diacetyl-chitobiose by the N-terminal catalytic domain A. Further degradation to N-acetylglucosamine is the result of a concerted action of diacetyl-chitobiose deacetylase and exo-β-D-glucosaminidase. By the bioinformatic tools, it was found that *Pyrococcus* encodes two chitinase open reading frames, ChiA (PF1234) and ChiB (PF1233), and the catalytic domain and the binding domain of ChiB are closely related to *T. kodakarensis* chitinase domain B and it may have endochitinase activity. By the annotations of the sequence comparisons, it was showed that *P. furiosus* enzyme doesn't have any signal sequence at the N-terminal region, so the enzyme is intracellular [3]. Oku and Ishikawa [2] explore that because of a one nucleotide insertion at the position 1006 in PF1234, the chitinase gene of the *P. furiosus* splits into two genes. They cloned and expressed catalytic domain and the binding domains alternately, and showed that some alterations have weakly active on alpha chitin and crystalline chitin. Kreuzer et al. [3] construct a mutant *P. furiosus* by homolog recombination with one chitinase coding genome showed that genetically engineered strain with deleted nucleotide is able to grow on colloidal chitin but not on crystalline chitin.

It has been reported that recombinant ChiA and ChiB enzymes exhibits chitinase activity towards crystalline chitin and the catalytic activity can be improved by adding the chitin binding domains (CBD) [2, 4]. But, there is a conflict in literature whether the recombinant domain constructs exhibit any activity toward crystalline chitin or not. In this study, it is aimed to construct an enzyme from the catalytic and chitin binding domain of chitinase from *P. furiosus* PF1234 genes and to show its expression in *E.coli*. It is expected to shade a light on the confliction between Oku and Ishikawa [2] and Kreuzer et al., [3].

II. MATERIALS AND METHODS

A. Strains and Growth Conditions

P. furiosus, a kindly provided from Dr. Winfried Hausner from Regensburg University, was grown on SME medium under anaerobic conditions at 90°C as described previously [5]. Luria-Bertoni (LB) Medium was used to cultivate *E. coli* DH5α with kanamycin (50 mg/mL) when it is necessary to select the transformants. Colloidal chitin is prepared from chitin from shrimp shells (Sigma) according to Kreuzer et al., [3].

B. DNA Manipulations and Plasmid Constructions

Genomic DNA isolation from *P. furiosus* was managed according to Waege et al., [6]. DNA concentration and integrity were determined by measuring the absorbances at 260 and 280 nm, as well as by 0.8% agarose gel analysis.

PCR amplification was managed with primers ChiForw 5'-tataGAATTCtatgactacccctgtcccagtct-3' and ChiRev 5'-ttctCTCGAGtcATGTTGGAACACTAGCTTcg-3' in the reaction mixture for Phire DNA Polymerase (Thermo) according to manufacturer's suggestions with optimization by using Thermal Cycler T100 (BioRad).

After purification, the PCR products were then double digested with two restriction enzymes XhoI and EcoRI and ligated to the similarly digested pET28b (+). The ligation products were transferred into *E.coli* by standard heat shock transformation. Plasmids from *E. coli* colonies appeared on LB agar + Km^{50} were screened on agarose gel according to their molecular weight and the selected plasmids with right size were sequenced in order to analyze the mutations.

Expression Screening of the Construct

Sequence proved pET28b:CD2AD2 construct was transferred into *E.coli* BL21(DE)pLysE cells by heat shock transformation. In order to screen the expression, the cells were grown on LB+Km^{50} at 37°C by vigorous shaking at 200 rpm until the cell concentration reach to OD_{600} 0.6 spectrophotometrically. The cooled cells were induced by various concentrations of IPTG. 3.5-4 h after inductions, cells were harvested by centrifugation at 6000 rpm for 10 min and the cells were homogenized in 10 mL of resuspension buffer (20 mM Tris–HCl pH 8.5, 0.5 M NaCl; 3,5 ml buffer per gram of cell pellet) containing 10 µL 100mM protease inhibitor cocktail, 10 µL 100 mM benzamine. The cells were disturbed by sonification at 4°C. The insolubilized cell debris was removed by centrifugation at 13000 rpm for 5 minutes. The expression level of the construct was showed by SDS-PAGE analysis.

Chitinase activity

The disturbed cell free supernatant was used as chitinase (CBD+CD) source to measure chitinase activity. Chitinase activity against crystalline and colloidal chitin was measured by Morgan- Elson's procedure according to Oku and Ishikawa [2].

III. RESULTS AND DISCUSSIONS

Chitinases are composed of two domains; i) catalytic domain (CD) ii) one or more chitin binding domain (CBD). CDs disturb the hydrogen bond in crystalline chitin and CBDs promote the hydrolysis of chitin. In *P. furiosus* there are two adjacent genes, PF1234 and PF1233, with two CBD and two CD in the combined gene from the sequence comparison with *T. kodakaraensis* TK1765 (ChiA) (Fig 1).

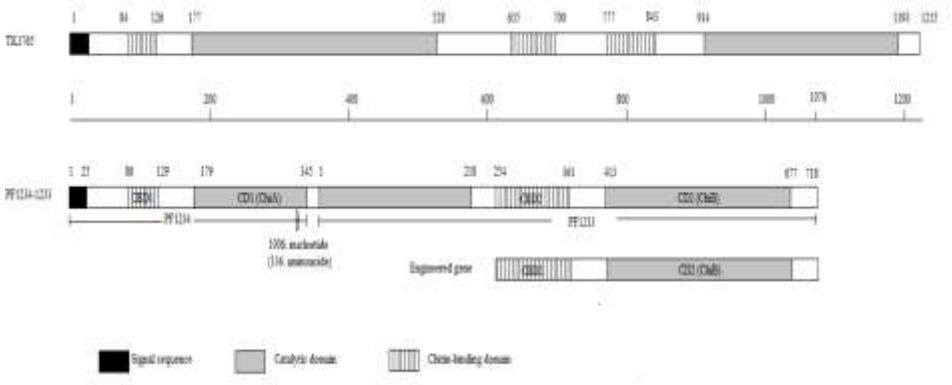

Fig. 1. Comparison of chitinase genes in *T. kodakaraensis* and *P. furiosus*

A. Cloning and Expression Screening

Mutation controlled pET28:CD2AD2 construct was transformed into *E.coli* BL21 (DE3)pLysE cells and induced with 0.2 M of IPTG. The level of the expression was showed by SDS-PAGE. It is known that *T. kodakaraensis* ChiA has 134 kDa, *S. tokodaii* has 77 kDa, *T. chitinophagus* has 50 kDa molecular weight chitinases. *P. furiosus* ChiA protein has 39 kDa and ChiB protein has 11 kDa molecular weight. But, the mutated and modified CD + CBD which is constructed in this study has 55 kDa molecular weight (Fig 2).

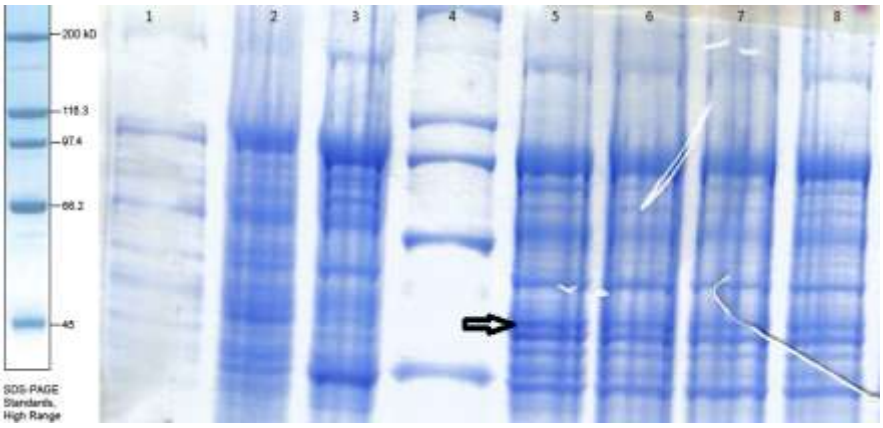

Fig. 2. Optimisation of IPTG concentration for chitinase expression. 30 ng protein was loaded into each well 1) Supernatant 2) Negative control (disturbed cells without IPTG) 3) 0.1 M IPTG 4) Marker 5) 0.2 M IPTG 6) 0.4 M IPTG 7) 0.5 M IPTG 8) 1M IPTG

Specific chitinase activity of the crude extract against colloidal chitin was recorded as 8.31±0.25 µmolGlcNAc/mg protein per minute. But it is not recorded any significant activity against crystalline chitin. Oku and Ishikawa [2] showed the increased activity against α-type crystalline chitin in mutated chitinase. But Kreuzer et al [3] couldn't reach the same activity in *P. furiosus*. Although we were able to show the expression of the protein, and the chitinase activity toward colloidal chitin, we couldn't show any activity against crystalline chitin.

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