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Enhancement of chitinolytic activity of by co-expression of endochitinase and chitobiosidase genes

Endochitinase와 Chitobiosidase 유전자의 동시발현에 의한 키틴분해 활성의 증가

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Abstract

Chitinolytic activity was enhanced by coexpression of endo-chitinase gene (*chiA*) and chitobiosidase gene (*chiB*) from *Serratia marcescens* KFRI314 using constitutive expression vector, pHCEIA, in *E. coli*. Coexpression vector was constructed by inserting ribosome binding site (RBS) into junction between two chitinase genes. SDS-PAGE analyses showed that two chitinase were constitutively expressed while *E. coli* clones expressing two chitinases simultaneously increased halo size on colloidal chitin plate. Furthermore, the chitinolytic activities were much enhanced in coexpressed clones when degradation patterns of substrate analogues such as 4-MU-(NAG), 4-MU-$(NAG)_2$,4-MU-$(NAG)_3$ were used. Consequently, the combined use of endochitinase and chitobiosidase greatly increased overall chitinolytic activities on recombinant *E. coli* clones

키워드 : *Serratia marcescens, chitinase, 키틴분해, 상승효과*
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1. INTRODUCTION

Serratia marcescens is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. Two chitinase genes, *chiA* and *chiB*, have been cloned from five different strains of *S. marcescens*, QMB1466, BJL200, KCTC2172, KCTC2216, and 2170, and third chitinase gene, *chiC*, was identified in the strain 2170, KCTC2172, KCTC2216 recently[1]~[5][13]. Among the three chitinases, ChiA has the highest hydrolyzing activity against insoluble chitinous substrates, while ChiC has the highest activity against soluble substrates[10]. In addition, the enzymatic properties from *S. marcescens* have been studied and synergism of the two chitinases have been demonstrated [3][14]. Recently, the results show that a combination of three chitinases of strain 2170 greatly enhanced the hydrolytic activity with respect to powdered chitin. When powder chitin was treated with ChiA plus ChiB or ChiA plus ChiC1, synergistic effects on chitin degradation were observed. But the combination ChiB and ChiC1 did not show any synergistic effect. In addition, similar results were also observed with chitinase of *B. circulans* WL-12 by T. Mizutani and T. Watanabe[15]. These observations suggest that multiple chitinases with different properties are necessary for an efficient

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synergistic hydrolysis of chitinous substrates [12]. As such, we tried to apply the co-expression method to produce the other two enzymes simultaneously[7][9]~[11][16].

In previous study, we have cloned and sequenced the chitinase genes involved in the chitin degradation using PCR techniques from *S. marcescens* KFRI314 strain. Furthermore, they were overexpressed in *E. coli* (JM109) and purified in homogeneity by Phenyl-Toyopearl column chromatography and studied on enzymatic properties. In this study, synergistic effects on the chitin degradation using the chitinases with other enzymatic properties were investigated. We tried to co-express by inserting the two chitinase genes (*chiA* and *chiB*) in the one plasmid. Successfully, we could demonstrate the co-expression of two different chitinases in one plasmid.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

Serratia marcescens KFRI314 was obtained from Korea Food Research institute and used in this study. It was grown at 30℃ in a nutrient broth medium containing 0.04% colloidal chitin for chitinase production. *E. coli* (JM109) was used as the host strain for the plasmid preparation and grown at 37℃ with shaking at 180 rpm in LB broth containing 50 ㎍/㎖ ampicillin or LB ampicillin agar plate. Plasmid pGEM-T easy Vector (Promega, USA) and pHCEIA (Bioleaders Corp.) were used for the cloning and expression of recombinant chitinases.

2.2 PCR amplification and gene cloning

Three chitinases genes (*chiA, chiB, chiC*) from *Serratia marcescens* KFRI314 were amplified by PCR using a GenAmp PCR System 2700 (Applied Biosystems, USA). the oligonucleotide primers were synthesized on the basis of previously known chitinase sequences of *S. marcescens* KCTC2172 and *S. marcescens* 2170. The primers used in this study are listed in Table 1. The reaction conditions were as follows: DNA template was denatured by one cycle for 1 min at 94℃and followed 28 cycles of 15 sec at 94℃, 30 sec at 60℃, 2 min at 72℃ and last extended by one cycle for 7 min at 72℃. PCR products were purified by Qiagen PCR gel extraction kit. The purified DNA was cloned into pGEM T-easy vector.

Table 1. PCR primers used in chitinase gene amplification

Primer	Sequence (5′-3′)
ChiAF(NdeI)	GGAATTCCATATGCGCAAATTTAATAAACC
ChiAR(BamHI)	CCGGATCCCTTATTGAACGCCGGCGCTGT
ChiBF(NdeI)	GGAATTCGCATATGTCCACACGCAAATCCGT
ChiBR(HindIII)	CCCAAGCTTACGGGGTTTTTTTACGGCTTA

2.3 Chitinase subcloning and transformation

Three recombinant pGEM T-easy plasmids harboring chitinase genes were digested with restriction enzymes. *chiA* gene (1692 bp) was digested with NdeI and BamHI and *chiB* gene (1500 bp) was digested with NdeI and HindIII and *chiC* gene (1443 bp) was digested with BamHI and XbaI. Each of gene fragments was gel eluted, and ligated into pHCEIA plasmid previously digested with the same restriction enzymes. The ligation products were used to transform into *E. coli* (JM109) host cells. Transformaton was performed by calcium chloride method by the procedure of Cohen method. Positive chitinase colonies were detected by the appearance of a clearing zone around colonies on LB agar plates containing 0.4% colloidal chitin and ampicillin (50 ㎍/㎖).

2.4 Construction of the recombinant plasmids for co-expression

A pHCEIA/*chiB* was digested with *Nde*I and *Hind*III and then the fragment of 1.5 kb was eluted and subcloned into pET22b(+) digested with the same restriction enzymes to use the ribosome binding site of pET22b(+) vector. The recombinant pET22b/*chiB* was digested with *Xba*I and *Hind*III and then *chiB* fragment containing ribosome bindingsite was eluted. Subsequently, *chiB* fragment was ligated into pHCEIA/*chiA* previously digested with *Xba*I and *Hind*III. The constructed plasmid was shown in Fig.1.

2.5 Enzyme assay and protein measurement

Chitinase activity was assayed by the modified method of Yanai *et al.*[17]. The reaction mixture containing 400 ㎕ of 0.8% colloidal chitin, 400 ㎕ of 10 mM sodium phosphate buffer (pH 6.8) and 200 ㎕ of enzyme solution was incubated for 2 hr at 37℃. Subsequently, the reaction mixture was boiled for 10 min. After centrifugation, 500 ㎕ from the supernatant fluid was mixed with 100 ㎕ of 0.8 M potassium tetraborate and the solution was heated for 3 min in boiling water. After the mixture was cooled in ice bath, 3 ml of p-dimethyl aminobenzaldehyde (DMAB) solution (1 g of DMAB dissolved in 100 ml of glacial acetic acid containing 1% (v/v) hydrochloric acid) was added and the mixture was incubated for 20 min at 37℃. After 20 min reaction, the reaction mixtures are cooled in ice bath and measured a absorbance at 585 nm using an UV Mini-1240 UV-VIS spectrophotometer (Shimadzu, Japan). One unit of chitinase activity was defined as the amount of enzyme which produced sugars equivalent to 1 ㎛㏖ of N-acetyl- glucosamine per min under the above condition. Protein concentration was determined according to Bradford method using a protein assay kit (Bio-Rad, USA) with bovine serum albumin as a standard.

2.6 SDS-polyacrylamide gel electrophoresis and activity staining

SDS-PAGE was done with a polyacrylamide gel containing 0.1% SDS (sodium dodecyl sulfate) by the method of Laemmli[8]. The molecular weight of purified protein was separated on 10% SDS-PAGE gel. Activity staining was performed in 10% SDS-PAGE gel containing 0.05% CM-chitin. The protein samples were added to SDS-sample buffer and heated at 80℃ for 5 min and run at 50 mA for 40min using a Bio-Rad Mini-Protein III Gel Kit. After electrophoresis, the gel was washed with distilled water and incubated for 2 hr at room temp with shaking in a 50 mM sodium phosphate buffer (pH 6.8) containing 1% (v/v) Triton X-100 to remove SDS. SDS-PAGE gel was stained with coomassie blue R-250 and activity gel was stained with a 0.1% congo red solution for 15 min and destained with a 1 M NaCl solution.

3. RESULTS AND DISCUSSION

3.1 Synergistic effects of ChiA, ChiB, and ChiC on the degradation of collidal chitin

To identify whether the three chitinases degrade synergistically on colloidal chitin, the reaction mixtures were measured by incubating the combination of ChiA, ChiB and ChiC as described in the enzyme assay conditions. Each NAG released from the reaction mixture was determined. The synergistic effect was observed from the combination of ChiA and ChiB or ChiA and ChiC. On the other hand, the combination of ChiB and ChiC did not show any enhanced effect. (data not shown). The enhanced values of ChiA plus ChiB and ChiA plus ChiC were measured as 16% and 13%, respectively (Table 2). Furthermore, a little synergistic effect was observed on TLC using the concentrated products of digested analogues (data not shown).

Table 2. Synergistic effects of ChiA, ChiB, and ChiC on the chitin degradation

Enzyme	Reducing sugar (㎛㏖)
ChiA(5㎍)	89.5
ChiA(10㎍)	157.5
ChiB(5㎍)	66.2
ChiB(10㎍)	113.7
ChiC(5㎍)	57.5
ChiC(10㎍)	100.4
ChiA(5㎍)+ChiB(5㎍)	180.8
ChiA(5㎍)+ChiC(5㎍)	166.6

3.2 Co-expression of endo-chitinase (ChiA) and chitobiase (ChiB)

To enhance the chitinase activity, two different classes of chitinases (endochitinase and chitobiosidase) were tried to co-express simultaneously in *E. coli* (JM109) using one plasmid. The overall scheme for construction of the recombinant plasmid for co-expression wasdescribed in Fig.1. *E. coli* (JM109) was transformed by recombinant plasmid, and then

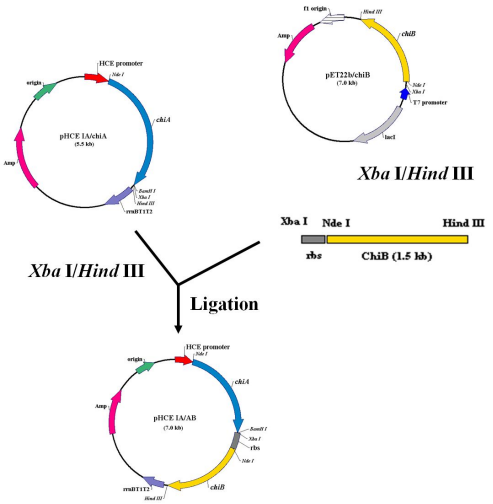

Fig. 1. Construction of the expression vector for chitinase co-expression

transformants were screened by comparing the halo size with control (ChiA) on LB plate containing ampicillin (50㎍/㎖) and colloidal chitin (0.4%). The recombinant plasmid was analyzed by restriction enzyme, further confirmed by PCR amplification. The recombinant plasmid was named as pHCEIA/*AB*.

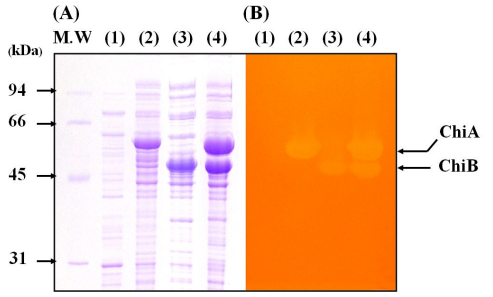

Fig. 2. Detection of the recombinant chitinase co-expressed in *E. coli* (JM109) by 10% SDS-PAGE and activity staining. (A) Coomassie blue staining, (B) Activity staining with 0.05% (w/v) CM-chitin; Lane 1: pHECIA (negative control), lane 2: pHECIA/*chiA*, lane 3: pHCEIA/*chiB*, lane 4: pHCEIA/*AB*

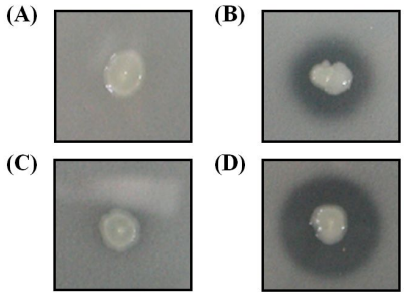

Fig. 3. Comparison of chitinolytic activities of the co-expressed recombinant *E. coli* clone on LB amp containing colloidal chitin. (A) *E. coli* clone harboring pHCEIA (negative control), (B) *E. coli* clone harboring pHCEIA/*chiA*, (C) *E. coli* clone harboring pHCEIA/*chiB*, (D) *E. coli* clone harboring pHCEIA/*AB* (co-expressed plasmid)

To identify whether *E. coli* clone harboring pHCEIA/*AB* can produce two chitinases simultaneously, the clone harboring the pHCEIA/*AB* was overexpressed and observed in 10% SDS-PAGE (Fig.2A). The protein bands of the co-expressed chitinases were identified to approximately 60 kDa (ChiA) and 50 kDa (ChiB). Furthermore, chitinase activities were confirmed by activity staining with native PAGE containing 0.05% CM-chitin (Fig. 2B). Enhanced chitinolytic activity was further tested by picking using a toothpick on colloidal chitin plate containing ampicillin, and then identified by comparing with the clearing zone size of substrate degraded by *E. coli* clones harboring pHCEIA/*chiA*, pHCEIA/*chiB* , and pHCEIA/*AB* (Fig.3). In order to test whether two chitinases are produced simultaneously or separately and to identify the amounts of the expression, *E. coli* clones harboring pHCEIA/*AB* are incubated by time-dependent incubation, and then confirmed to the protein expression patterns in 10% SDS-PAGE. The results revealed that two chitinases were expressed simultaneously and also appeared to the similar amounts of the expression (data not shown). Furthermore, three substrates, 4-MU-(NAG), 4-MU-$(NAG)_2$ and 4-MU-$(NAG)_3$, were used to see degradation

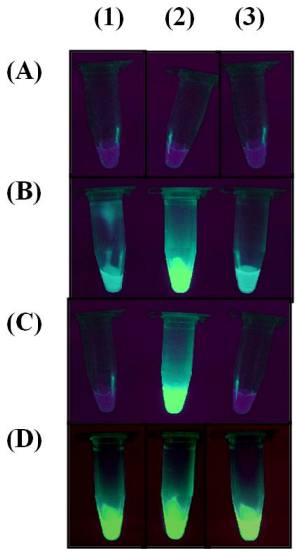

Fig.4. Flurorometric analyses of chitinolytic end-products by the chitinase with UV illuminator. (A) ChiA, (B) ChiB, (c) ChiC; Lane 1: 4-MU-NAG, lane 2: 4-MU-$(NAG)_2$, lane 3: 4-MU-$(NAG)_3$.

pattern of the co-expressed chitinase using a pattern of fluorometric assay. Interestingly, the results showed that the tube was fluoresced brightly in NAG and $(NAG)_3$ by the co-expressed crude chitinase than the crude ChiA (Fig. 4). These facts suggest that chitinolytic activity was enhanced by the co-expression of endochitinase and chitobiosidase. Consequently, combined use of two chitinases resulted in synergistic effect on chitin degradation.

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