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Cloning of *Serratia marcescens* KFRI314 chitinase genes and its role on chitin degradation

Serratia marcescens KFRI314 chitinase 유전자의 클로닝과 키틴분해에 관한 효소의 역할

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Abstract

Three chitinase genes (*chiA, chiB*, and *chiC*) were cloned into *E. coli* by PCR amplification from *Serratia marcescens* KFRI314. The sizes of cloned chitinase genes were 1692 bp, 1500 bp, and 1443 bp which correspond to 563, 499, and 480 amino acids, respectively. Recombinant chitinases were overexpressed using pHCEIA expression vector and purified to homogenity. The molecular weights of chitinases were about 60kDa, 50 kDa, 52 kDa, respectively. Optimum pHs were around pH 5~6 and optimum temperatures were 45~50℃ while 90% of enzyme activities were stable up to 50℃. The specific activities of ChiA, ChiB, and ChiC were 233.1, 278.8, 111.3 μmol $(min)^{-1}mg^{-1}$ against colloidal chitin. From experiments using TLC and fluorescent substrate analogues, it was demonstrated that ChiA was endo-chitinase while ChiB and ChiC were chitobiosidase.

키워드 : *Serratia marcescens, Chitinase, 유전자, 키틴분해*
Keywords : *Serratia marcescens, Chitinase, Gene, Chitin degradation*

1. INTRODUCTION

Chitin degrading enzymes are ubiquitous and are found in prokaryotic and in eukaryotic organism as well as in archae. *Serratia marcescens* has been used as a model system for the utilization of chitin as a carbon source. In the presence of chitin, *S. marcescens* express chitinase A, chitinase B, chitinase C and chitobiase that degrade chitin to mono N-acetylglucosamine (NAG). Chitinase A, B, and C were assigned to glycosyl hydrolase protein family 18, while chitobiase belongs to protein family 20. The structures of *S. marcescens* chitinolytic enzymes, chitinase A and chitinase B were solved. The cleft in ChiA has a more groove-like character, typical for endo-enzymes, whereas the substrate-binding cleft fo ChiB has a more tunnel-like character, which is often observed in exo-enzymes.

The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries. Chitin degradation is initiated by the action of endochitinases, probably chitinase A and chitinase C. Chitinase A also act as an exochitinase, cleaving di-NAG dimmers from the reduced end. Chitinase B act as an exochtinase cleaving tri-NAG and di-NAG from the

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non-reducing end of the chitin oligo NAG chains generated by the action of chitinase A and chitinase C.

Chitinases are also great biotechnological interest because these enzymes may be used to convert abundant chitin-containing biomass in earth into useful depolymerized components. As an approach to screen new chitinase gene. *Serratia marcescens* KFRI314 strain was employed to chitinase gene sources and the corresponding genes were cloned into *E. coli*. The enzymatic properties of recombinant chtinases were studied and its enzymatic roles in chitin degradation were described in this paper.

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 subcloning

2.2 PCR amplification and Gene Cloning

Three chitinase 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
ChiCF(BamHI)	CCGGATCCCATGAGCACAAATAACATTAT
ChiCR(XbaI)	GCTCTAGATTAGGCGATGAGCTGCCACA

2.3 Chitinase sublconing 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 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 Overexpression and purification of the recombinant chitinases

E. coli clones harboring the pHCEIA/*chiA*, pHCEIA/*chiB* and pHCEIA/*chiC* were cultivated overnight in 2 ml LB broth supplemented with 50㎍/㎖ ampicillin at 37℃. These overnight cultures were inoculated into 30 ml of the same medium and cultivated at 37℃ for 12 hr without an induction process by addition of IPTG. After cultivation, *E. coli* cells were harvested by centrifugation at 12,000 rpm for 5 min, washed with buffer A (10mM Na_2HPO_4-NaH_2PO_4, pH6.8, 1mM DTT, 1mM PMSF, 5mM EDTA) and further centrifuged at 12,000 rpm for 10 min to obtain cell pellets. The cell pellets were resuspended in 5 ml of the same buffer. The cell suspension was placed on ice and disintegration of the cells was performed using a

UP50H sonicator (Dr. Heilscher GmbH, Stahnsdorf, Germany) on ice bath. Then the sonicated cells were centrifuged at 12,000 rpm for 15 min, the supernatants obtained were used to purify the proteins. The sample was prepared by adjusting to 0.5 M ammonium sulfate in the supernatants. A KONTES FLEX-COLUMN^{TM} (1.5 x 5 ㎝) was used for this step. Purification procedure was performed as follows. Phenyl-Toyopearl 60S (TOSOH, Japan) was equilibrated with buffer A containing 0.5 M ammonium sulfate and packed in the column. The sample was then applied to the column. The column was washed several times with 20 ml of the same buffer. Subsequently, the protein was eluted with decreasing a linear gradient of 0.5 M to 0 M ammonium sulfate at a flow rate of 20 ml/h. The fractions exhibiting chitinase activity were monitored with an UV Mini-1240 UV-VIS spectrophotometer (Shimadzu, Japan) at 280 nm and analyzed on a SDS-PAGE gel and used for the enzymatic characterizations.

2.5 Enzyme assay and protein measurement

Chitinase activity was assayed by the modified method of Yanai *et al.* [14]. 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 Enzymatic characterizations of recombinant chitinases

To investigate the optimal pH of chitinase, the activity was measured by incubating the reaction mixture in various pH at 50℃ for 2 hr as described in the enzyme assay conditions. The used buffers were a 20 mM Citric acid-Na_2HPO_4 buffer (pH 4.0~6.0), 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH6.0~8.0) and 20 mM Glycine-NaOH buffer (pH 8.0~10.0). To investigate the pH stability of chitinase, the enzyme was measured from the residual activity after pre-incubation in various pHs at 4℃ for 12 hr. The optimal temperature of chitinase was measured by incubating the reaction mixture at various temperatures between 20℃ and 80℃ for 2 hr in a 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH6.8) with colloidal chitinase substrate. Thermostability was measured from residual activity after pre-incubation at various temperatures between 20℃ and 80℃ for 1 hr in a 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH6.8), as described in the enzyme assay conditions.

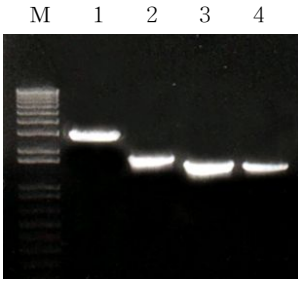
2.7 Thin-layer chromatography of sugar oligomers

The products of the chitinolytic enzyme was analyzed by thin-layer chromatography (TLC) using colloidal chitin and chitin oligosaccharides ($GlcNAc_{2-3}$, Sigma) as a substrate, further confirmed by fluorometric method using 4-MU-GlcNAc, 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$. A 5㎕ of a 10 mM colloidal chitin was incubated with 40 ㎕ of the purified chitinase and 5 ㎕ of a 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH6.8) at 37℃ for 1 ~24 hr and then hydrolysis products were analyzed by TLC. Enzyme reaction products were analyzed on silica gel plate (Kieselgal 60; Merck Co., Berlin, Germany) using isopropyl alcohol:ethanol:water (5:2:1). The plates were run and sugars were visualized by spraying with 10% sulfuric acid in ethanol, followed by heating at 110℃ for 6 hr to detect any dark spots[5][6].

3. RESULTS AND DISCUSSION

3.1 Cloning and nucleotide sequencing of the chitinase genes from *S. marcesens*

Four DNA fragments of 2.6 kb, 1.7 kb, 1.5 kb and 1.4 kb were obtained through PCR using the oligonucleotide primers synthesized on the base of previously published chitinase gene sequences of *S. marcescens* strains (Fig. 1). Each PCR products were initially analyzed by restriction enzymes based on previous published sequences to determine whether it was amplified correctly. Subsequently, each fragments were cloned into pGEM-T easy vector and then transformed into *E. coli* (JM109). The *E. coli* clones showing chitinase activities were screened on LB plate containing ampicillin (50 ㎍/㎖) and colloidal chitin (0.4%). Each recombinant plasmid was analyzed by DNA sequencing (data not shown). These analyses revealed the existence of open reading frames (ORFs) which represent *chiA-full* (containing promoter region, 2211 bp), *chiA* (1692 bp), *chiB* (1500 bp) and *chiC* (1443 bp), respectively. The 2.2 kb fragment of *chiA-full* revealed that it had been a short deletion region (29 bp) in upstream from the promoter region by comparing with other chitinases from *S. marcescens*. A putative ribosome binding site (AAGGAA) and promoter region (-10, -35) was identified at upstream of the start codon (ATG). An inverted repeated sequence(CCGGGGGATATCCTTTCGCCCCCGG) was located 17 bp downstream of the termination codon (TAA). The *chiA* (1.6 kb) fragment of KFRI314 was encoded for 563 amino acids with estimated molecular mass of 60 kDa. The computer graphic N-terminal analyses of ChiA using a signal sequence prediction program (Swiss-prot, Geneva, Switzerland) revealed the existence of a putative signal sequence cleaved between Ala-23 and Ala-24. A NCBI BLAST searches showed that the *chiA* nucleotide sequence of *S. marcescens* KFRI314 was 99.5% similarity to *chiA* of *S. marcescens* 2170 (AB015996), 98.8% similarity to *chiA* of *S. marcescens* KCTC2216 (AF454462), 97.8% similarity to *chiA* of *S. marcescens* ATCC27117 (L01455), 96.6% similarity to *chiA* of *S. marcescens* QMB1466 (X03657), 97.7% similarity to *chiA* of *S. marcescens* BJL200 (Z36294) published previously[1][5][10][13]. The *chiB* (1.5kb) fragment of KFRI314 was encoded for 499 amino acids with estimated molecular mass of 50 kDa. The putative signal sequence revealed that it cleaved between Ala-41 and Lys-42. A NCBI BLAST searches showed that the *chiB* nucleotide sequence of *S. marcescens* KFRI314 was 99.7% similarity to *chiB* of *S. marcescens* 2170 (AB015997), 99.4% similarity to *chiB* of *S. marcescens* KCTC2216 (AF454463), 99.6% similarity to *chiB* of *S. marcescens* QMB1466 (X15208), 96.1% similarity to *chiB* of *S. marcescens* BJL200 (Z36295) published previously[2][4][10][13]. The *chiC* (1.4 kb) fragment of KFRI314 was encoded for 480 amino acids with estimated molecular mass of 52 kDa. The computer graphic N-terminal analyses of ChiC using a signal sequence prediction program revealed that it had not a signal sequence. A NCBI BLAST searches showed that the *chiC* nucleotide sequence of *S. marcescens* KFRI314 was 99.4% similarity to *chiC* of *S. marcescens* 2170 (AB019238), 98.3% similarity to *chiC* of *S. marcescens* KCTC2216 (AF454464), 98.4% similarity to *chiC* of *S. marcescens* KCTC2172 (L41660)[3][9].



Lane M: Size marker
Lane 1: *chiA*-full (2659 bp)
Lane 2: *chiA* (1692 bp)
Lane 3: *chiB* (1500 bp)
Lane 4: *chiC* (1443 bp)

Fig. 1. Agarose gel electrohoresis of PCR products of chitinase from *Serratia marcescens* KFRI314

3.2 Overexpression and purification of the recombinant chitinases from *E. coli*

pHCEIA vectors were used for over-expression of chitinases. The advantage of this vector could be overexpressed without an induction process by addition of IPTG and with short expression time for about 16 hr. Therefore, they were subcloned into pHCEIA vector (e.g.

High Constant Expression vector) to overexpress each chitinase genes (*chiA*, *chiB*, *chiC*), and then introduced into *E. coli* (JM109). The cloned chitinases activity was confirmed by clear zone around cells on LB ampicillin containing colloidal chitin (Fig. 2). *E. coli* clones harboring the pHCEIA/*chiA*, pHCEIA/*chiB* and pHCEIA/*chiC* were overexpressed and recombinant chitinases were detected in 10% SDS-PAGE (data not shown). The overexpressed chitinase proteins were purified as described in methods. Three chitinases were efficiently attached to Phenyl-Toyopearl 60S at low concentrations of ammonium sulfate. Each chitinase proteins were purified conveniently by one-step purifications. The purified ChiA proteins were eluted between 30 and 150 mM ammonium sulfate as only symmetrical peak and each fractions (43~53 fractions) were stained with coomassie blue. The specific activity and recovery of purified ChiA were estimated to 233.1U/mg and 96.1%. The purified ChiB and ChiC proteins were eluted between 20 and 100 mM ammonium sulfate as only symmetrical peak and each fractions were stained with coomassie blue. The specific activity and recovery of purified chiB and ChiC were estimated to 278.8 U/mg, and 111.3 U/mg, respectively. The molecular weights of the purified chitinases (ChiA, ChiB, ChiC)

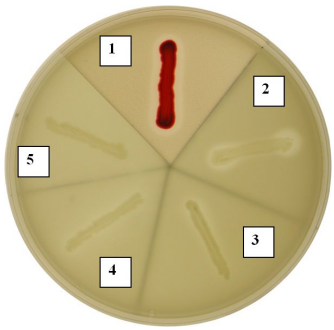


Fig. 2. Detection of chitinase activities of *S. marcescens* KFRI314 and recombinant *E. coli* clones on LB and LB ampicillin plate containing colloidal chitin. (1) *S. marcescens* KFRI314, (2) *E. coli* (JM109) containing pHCEIA/chiA, (3) *E. coli* (JM109) containing pHCEIA/chiB, (4) *E. coli* (JM109) containing pHCEIA/chiC, (5) *E. coli* (JM109) containing pHCEIA as a negative control.

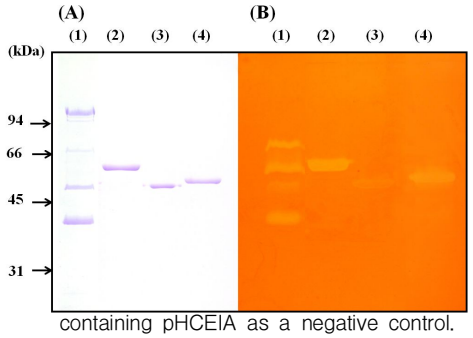


Fig. 3. Activity staining of the purified chitinases on 10% SDS-PAGE gel containing 0.05% CM-chitin. Lane 1: *Serratia marcescens* KFRI314, Lane 2: ChiA, Lane 3: ChiB, Lane 4: ChiC; (A) SDS-PAGE gel stained with coomassie brilliant blue R-250 (B) Activity band stained with 0.1% congo red.

were estimated to be approximately 60 kDa, 50 kDa and 52 kDa by SDS-PAGE, respectively (Fig. 3A). Each purified chitinases were confirmed by activity staining with SDS-PAGE containing 0.05% CM-chitin (Fig. 3B). The estimated molecular weight of cloned ChiA was similar to the chitinases from other *S. marcescens* strains. But, ChiB was estimated to about 50 kDa that it was decreased about 4 kDa by cleavage of the signal sequence of 41 amino acids. On the other hand, ChiC was estimated to about 52 kDa because ChiC did not have the signal sequence even if ChiB was longer than ChiC in nucleotide sequence.

3.3 Enzymatic properties of *Serratia marcescens* chitinase

Three chitinases activities were measured at various pHs ranging as described in methods. The optimum pH of ChiA and ChiC were estimated at pH 5.0, and ChiB was pH 6.0 . To investigate the effect of pH on the stability of three chitinases, the enzymes were preincubated at various pHs for 12 hr at 4℃, and then remaining activity was determined. More than 80% of ChiA, ChiB and ChiC were stable at pH 5.0 to 9.0, pH 6.0 to pH 8.0, pH 5.0 to pH 6.0, respectively. These results indicate that chitinase

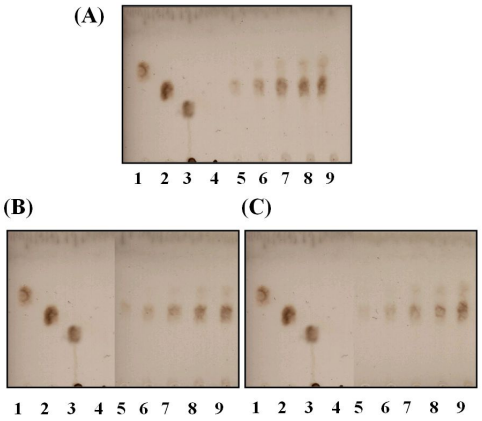


Fig. 4. TLC analyses of products from colloidal chitin for incubation times by the purified chitinases. (A) ChiA, (B) ChiB (C) ChiC; Lane 1: GlcNac, lane 2: $(GlcNAc)_2$, lane 3: $(GlcNAc)_3$, lane 4: colloidal chitin (control), lane 5: 1 hr, lane 6: 3hr, lane 7: 6 hr, lane 8: 12 hr, lane 9: 24 hr.

produced by *S. marcescens* KFRI314 are nearly similar to the previously reported *Serratia* chitinases[8][11][13][14].

To investigate the optimum temperature of ChiA, ChiB and ChiC, reaction mixtures were treated at between 30℃ and 80℃ for 2 hr using colloidal chitin as the assay substrate. The optimum temperature of ChiB and ChiC were observed at 50℃, while ChiA was a little lower than the other two chitinases. More than 80% of the activity of ChiA was found to be stable below 50℃. On the other hand, ChiB and ChiC were stabled below 55℃. These enzymatic properties are almost similar to the results of previously published chitinases from *Serratia* strains[7][11]~[13].

3.4 Analyses of chitinolytic activities by TLC and fluorescence

Enzyme reaction products obtained by time-dependent incubation were initially analyzed by TLC. Subsequently, the end products were confirmed by fluorometric analysis using 4-MU-(NAG), 4-MU-$(NAG)_2$ and 4-MU-$(NAG)_3$ as substrates. When colloidal chitin was used as a substrate, GlcNAc and chitobiose $(GlcNAc)_2$ was detected as the products in incubation times of 1~24 hr (Fig. 4). Colloidal chitin was gradually degraded to GlcNAc and $(GlcNAc)_2$ when the incubation time was prolonged. Finally, the end products were degraded to $(GlcNAc)_2$ as major product and GlcNAc as minor product. When using $(GlcNAc)_2$ as a substrate, as described in Fig.5A, ChiA degraded it to a monomer form of the faint spot, but ChiB and ChiC were not degraded and when using $(GlcNAc)_3$ as a substrate all chitinases degraded it to a monomer form and dimmer form. At the same time, when 4-MU-(NAG), 4-MU-$(NAG)_2$ and 4-MU-$(NAG)_3$ were used as a substrate, a 4-MU-$(NAG)_2$ was strongly fluoresced and 4-MU-(NAG) and 4-MU-$(NAG)_3$ were slightly fluoresced by ChiA, however ChiB and ChiC were detected by a only 4-MU-$(NAG)_2$ (data not shown). These results suggest that ChiA is an endo-type chitinase and the final products of the enzyme reaction are GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$, while ChiB and ChiC are a chitobiosidase and the final product of the enzymes reaction is $(GlcNAc)_2$. The proposed hydrolysis pattern of Chia, ChiB, and ChiC on chitin degradation was suggested (Fig. 6) In conclusion, we have cloned and sequenced the chitinase genes involved in the chitin degradation using PCR techniques from *S. marcescens* KFRI314 strain. Recombinant

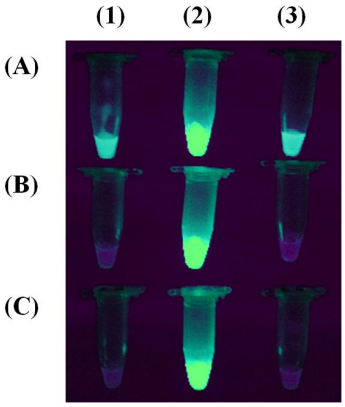


Fig. 5. Flurorometric analyses of chitinolytic end-products by the chitinase with UV illuminator. (A) ChiA, (B) ChiB, (c) ChiC; Lane 1: 4-MU-NAG, lane 2: 4-MU-$(NAG)_2$, lane 3: 4-MU-$(NAG)_3$.

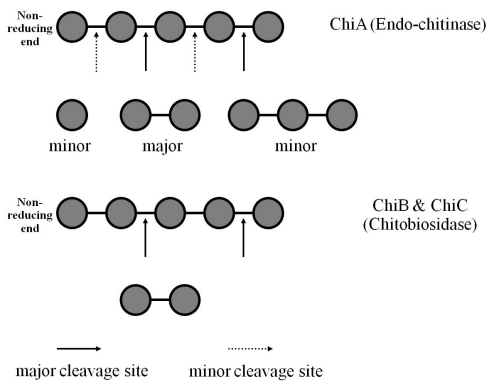


Fig. 6. Proposed hydrolysis pattern of ChiA, ChiB, and ChiC

chitinases were overexpressed in *E. coli* (JM109) and purified in homogeneity by Phenyl-toyopearl column chromatography. The enzymatic properties on stability were studied. Chitin degradation mechanism was also suggested from analyses using fluorescent substrate analogues.

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