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Detection of chitinase ChiA produced by *Serratia marcescens* PRC-5, using anti-PrGV-chitinase

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ABSTRACT

In this study, a bacterium *Serratia marcescens* PRC-5 that displayed strong chitinolytic activity on 0.5% colloidal chitin-containing agar medium was isolated from soil. The chitinase activity increased rapidly with a maximum level (6.14 U/mL) on 4 days of incubation with swollen chitin (pH 5.0). Three active bands of chitinase isozymes were observed (53, 44, and 34 kDa) on SDS-PAGE gel and there p*I* values ranged from p*I* 5.4 to 5.8 on 2D gels. The chitinase from the PRC-5 strain was also able to produce GlcNAc monomers on TLC plates. The chitinase of PRC-5 inhibited the mycelial growth of *Rhizoctonia solani* KACC40111, which indicates that it could be used as a biocontrol agent for phytophathogens. The chitinase isozyme N1, which had a molecular weight of 62 kDa, was transferred from a native and SDS-PAGE gel onto an immunoblot and was probed using an anti-PrGV-chitinase.

1. Introduction

Chitinase has the ability to degrade chitin, a major component of most fungal cell walls and the exoskeleton of anthropods (Jollès & Muzzarelli, 1999). Chitinolytic enzymes obtained from bacterium are the key enzymes in biological control strategies (Jung et al., 2003). Chitinases belong to families of glycosyl hydolases (GH) 18 and 19 (Henrissat & Davies, 1997). The family GH 18 enzymes are derived from a variety of prokaryotic and eukaryotic organisms, whereas the family GH 19 enzymes have only been found in higher plants and bacteria. Recently, these enzymes were simply classified with both chitinase (EC 3.2.1.14), and *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52).

Chitinase-producing *Serratia marcescens* is a very important bacterium for the degradation of chitin substrates in culture medium (Monreal & Reese, 1969). *S. marcescens* produces various extracelluar enzymes in different culture media that includes chitinases (Hines, Saurugger, Ihler, & Benedik, 1988). Also, this bacterium produces both chitinolytic enzymes and chitinbinding proteins in medium containing chitin as a substrate (Fuchs, McPherson, & Drahos, 1986). These enzymes were shown to exist in several isomer forms on SDS-PAGE gels. In addition, the precise molecular weight of these enzymes cannot be easily calculated based on biochemical experiments. *S. marcescens* produces five extracelluar enzymes, including chitinases (ChiA, ChiB, and ChiC), a chitobiase and a putative chitin-binding protein (CBP21) (Brurberg, Eijsink, Haandrikman, Venema, & Nes, 1995; Fuchs et al., 1986). These five proteins were well characterized and shown to display chitinolytic activity. Recently, the crystal structures of these enzymes were reported on chitinase (ChiA and ChiB) and chitobiase (Perrakis et al., 1994; Tews et al., 1996; Van Aalten et al., 2000).

The potential use of antibodies to assess chitinase produced from *S. marcescens* has not been extensively studied. In this work, a chitinase (ChiA) antibody was used to identify different expression pattern of *S. marcescens* between native PAGE and SDS-PAGE gel. The objective of this study was to investigate the properties of chitinase produced from *S. marcescens* PRC-5 on TLC plates and 2D electrophoresis gels.

2. Experimental

2.1. Isolation and identification of chitinolytic bacterium

The waste soils samples were collected in Gwangju, Korea. The samples were serially diluted in sterile water and appropriate soil dilutions (10^{-3}) were inoculated on 0.5% swollen chitin agar medium at 30 °C for 3 days. One bacterium displaying strong chitinolytic activity was selected for further characterization.

To identify the bacterium, polymerase chain reaction (PCR), (GeneAmp 9700, Applied Biosystems, USA) was performed to amplify a part of the 16S rRNA gene of the bacterium. The forward primer was 9F (5′-GAG TTT GAT CCT GGC TCA G-3′) and the reverse primer was 1412R (5′-ACG GCT ACC TTG TTA CGA CTT-3′). The nucleotide sequence of the 16S rRNA gene of the PRC-5

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was determined using an ABI PRISM BigDye™ Terminator Cycle Sequencing Kits (Applied Biosystems, USA) and ABI PRISM 3730xl Analyzer (Applied Biosystems) in Geno Tech Co. (Daejeon, Korea). The nucleotide sequence of the 16S rRNA gene of the PRC-5 was compared with published 16S rRNA sequences using a blast search at NCBI (Jung et al., 2002).

2.2. Mycelial growth inhibition

To investigate the inhibition of mycelial growth with strain PRC-5, *Rhizoctonia solani* KACC 40111 was grown in potato dextrose agar (PDA). The plate was incubated at 26 °C for 3 days, and the distances between the edges of the PRC-5 and fungal mycelium were measured (Jung, Kuk, Kim, Kim, & Park, 2005).

2.3. Determination of chitinase activity

The chitinase assay mixture consisted of 50 μL of sample, 500 μL of 0.5% swollen chitin and 450 μL of 50 mM sodium acetate buffer (pH 5.0) (Monreal & Reese, 1969). Following incubation at 37 °C for 1 h, 200 μL of 1 N NaOH was added. The sample was briefly centrifuged (10,000 × *g*, 5 min), after which 500 μL of the supernatant was mixed with 1 mL of Schales' reagent and then heated in boiling water for 15 min. The absorbance was immediately measured at 420 nm using a spectrophotometer (Mecasys, Optizen 3220UV, Korea). The amount of reducing sugar was calculated based on a comparison with a standard curve generated from known concentrations of GlcNAc (0–100 μg). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour. The protein concentration was determined using the method described by Bradford (1976).

2.4. Assay of the reaction products by thin layer chromatography

The reaction products were then spotted on silica gel thin-layer plates. After developing the plates in n-propanol:water:ethylacetate:ammonia solution (6:3:3:1, v/v), *N*-acetyl-chitooligosaccharides were visualized using the aniline–diphenylamine reaction (Tanaka et al., 1999).

2.5. Gel electrophoresis and activity staining of chitinase

SDS-PAGE was performed, according to the method described by Laemmli (1970). Electrophoresis was performed using a Bio-Rad Mini-PROTEAN (80 mm × 73 mm × 1.5 mm). The 12% SDS-PAGE gel was stained with 0.12% Coomassie brilliant blue R-250 and silver staining (Blum, Beier, & Gross, 1987). To evaluate the active staining of chitinase, 12% SDS-PAGE containing 0.01% glycol chitin was conducted according to the method described by Trudel and Asselin (1989). The gel was incubated in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and 1% skim milk at 37 °C for 2 h with reciprocal shaking. A subsequent incubation step was then conducted overnight under the same conditions, but without skim milk in the buffer solution. The gel was then immersed in a 500 mM Tris–HCl (pH 8.9) solution containing 0.01% calcofluor white M2R (Sigma F3397). The lysed zones were visualized and photographed using a UV transilluminator (Daihan Sci. Co., WGD-30, Korea). 8% or 10% Native-PAGE (w/v) was conducted according to the method described by Ornstein (1964). Two dimension native/native PAGE and 2D native/SDS PAGE analyses were performed using the method described by Wittig, Braun, and Schägger (2006) and the blue-native PAGE technique, with slight modifications.

2.6. Two dimensional polyacrylamide gel electrophoresis

The 2DE protocol in the Mini-PROTEAN Tube Cell Manual (Bio-Rad, USA) was used. Tube gels were used in the Mini-PROTEAN tube cell (Bio-Rad, USA) for isoelectric focusing (IEF). Following the manual, the tube gels were casted using the provided tube-cell module and a reservoir was created using a stopper that was inserted into the tube adaptor. An equal volume of the first-dimension sample buffer (8.0 mol/L urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte (Bio-Rad, USA), 0.4% Bio-Lyte 3/10 ampholyte) was then added to the sample and incubated at room temperature for 10 min. The mixture was added into the reservoir at the top of the gel and overlaid with 20 μL sample overlay buffer (4.0 mol/L urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte, bromophenol blue). Electrophoresis was conducted at 200 V for 15 min, 300 V for 15 min, 400 V for 30 min, and then the voltage was increased to 750 V for an additional 6 h. After the first round of electrophoresis, the capillary gel was pushed out from the tube with an ejector. To equilibrate the capillary gel, it was submerged in SDS sample equilibration buffer (0.0625 mol/L Tris–HCl, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, bromophenol blue) for 10 min. Equilibrated gels were subsequently overlaid on a 12% SDS-PAGE gel. A Mini-PROTEAN 3 Cell was used for the second electrophoresis on a 12% SDS-PAGE gel (Chen et al., 2008).

2.7. Immunoblotting

For immunoblotting, proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA) using an electric transfer kit (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad) according to the manufacturer's manual. Membrane-bound enzymes were detected using polyclonal anti-PrGV-chitinase (rat IgG, primary antibody, provided from Prof. Y.S. Han, Chonnam National University). The membrane was blocked for 1 h with 5% skim milk powder in TBS with 0.2% Tween 20, followed by incubation for 3 h with primary antibody (dilution 1:1000). Membranes were then washed in 2.5% skim milk, 0.01% Tween-20 in TBS (pH 7.2), and reacted with the goat anti-rat antibody conjugated with alkaline phosphatase (dilution 1:5000, Santa Cruz) and visualized using a NBT/BCIP solution (B5655, Sigma–Aldrich Co., USA) (Oh, 2012).

2.8. Purification of chitinase

For chitin affinity column chromatography, regenerated chitin from chitosan (2 g) was obtained according to a modified version of the method described by Molano, Duran, and Cabib (1977). The regenerated chitin was equilibrated in 20 mM sodium carbonate buffer (pH 8.4), after which the crude enzyme of the PRC-5 was loaded onto a regenerated chitin column (4 cm × 10 cm) at a flow rate of 0.5 mL/min. The first washing step was conducted using 600 mL of 20 mM sodium carbonate buffer (pH 8.4) until the eluate was free of proteins based on the absorbance at 280 nm. The second washing step was conducted with 100 mL of 20 mM sodium acetate buffer (pH 5.5). The proteins were finally released from the chitin matrix with 20 mM acetic acid (pH 3.2) and then 3 mL were collected in individual tubes to give a total of 122 fractions (Park, Lee, & Park, 1991).

3. Results and discussion

3.1. Identification of chitinolytic bacterium

The isolate PRC-5 displayed strong chitinolytic ability on agar medium containing 0.5% (w/v) swollen chitin and clear zone around the colonies were observed on 3 days of incubation at 30 °C (Fig. 1A).

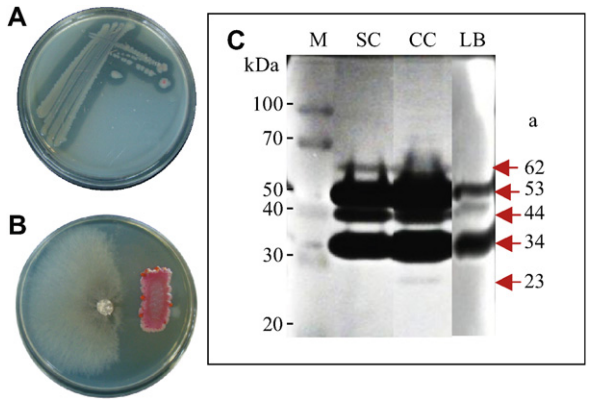

Fig. 1. *Serratia marcescens* PRC-5 showed halo formation around their colonies on 0.5% colloidal chitin agar at 30 °C after 3 days of incubation (A). Antifungal activity of PRC-5 against *Rhizoctonia solani* KACC 40111 (B). Chitinase activity, which was stained with calcofluor white M2R, using glycol chitin as a substrate (C). Chitinase activity on SDS-PAGE gels from a crude enzyme of PRC-5 after 5 days incubation with swollen chitin (SC), colloidal chitin (CC) and LB medium (LB). Protein marks (M).

The chitinase of PRC-5 inhibited the mycelial growth of *R. solani* KACC40111, which implies that PRC-5 could be used as a biocontrol agent for phytophathogens (Fig. 1B). *S. marcescens* B2 has been used as effective agents for biological of soilborne diseases, such as *R. solani* AG-4 and *Fusarium oxysporum* f. sp. *cyclaminis* IHF-1 (Someya et al., 2000). This bacterium was a Gram-negative species that was rod-shaped. On the basis of the nucleotide sequence of a conserved segment of 16S rRNA gene, the bacterium was identified as *S. marcescens* and named *S. marcescens* PRC-5. Within a 1418 bp sequence, there was only one base difference between PRC-5 (accession no. JN816402) and *S. marcescens* 16S rRNA sequences (accession no. HQ260324).

The chitinase activity of *S. marcescens* PRC-5 was detected on a modified SDS-PAGE gel containing 0.01% (w/v) glycol chitin as a substrate. Two major bands (53 and 34 kDa) and minor bands (62 and 44 kDa) from a crude enzyme obtained from PRC-5 incubated on swollen chitin were positively stained for chitinase activity on the SDS-PAGE gel (Fig. 1C). In addition, two major bands (53 and 34 kDa) and minor bands (62, 44 and 23 kDa) from the crude enzyme of PRC-5 that had been incubated on colloidal chitin stained positive for chitinase activity. In LB medium, one minor band (44 kDa) was observed. Chitinases from *S. marcescens* were reported to consist of four isomers (ChiA, ChiB, ChiC1, and ChiC2) and a chitin binding protein (CBP21) on SDS-PAGE gels (Brurberg et al., 2001; Synstad et al., 2008). Chitinase isozymes of *Paenibacillus chitinolyticus* MP-306 in LB medium showed as two major bands and two minor bands on SDS-PAGE gel (Song, Seo, Kim, Park, & Jung, 2012). Based on these results we found that the PRC-5 strain produced chitinase on LB medium containing yeast extract which is composed of some chitin.

3.2. Characterization of chitinase isozyme

S. marcescens PRC-5 was incubated in 0.5% swollen chitin medium for 6 days. Cell growth of PRC-5 gradually increases over 4 days and rapidly increased on 5 days when cultured on 0.5% swollen chitin liquid medium (Fig. 2A). Chitinase activity increased rapidly with a maximum level (6.14 U/mL) on 5 days of incubation with swollen chitin (pH 5.0). The expression pattern of chitinase was examined on SDS-PAGE gels (Fig. 2C) and degradation products from the swollen chitin were measured using TLC plates (Fig. 2D). The same amount of culture medium was loaded and strong bands were observed on the SDS-PAGE gel on 4 days of incubation

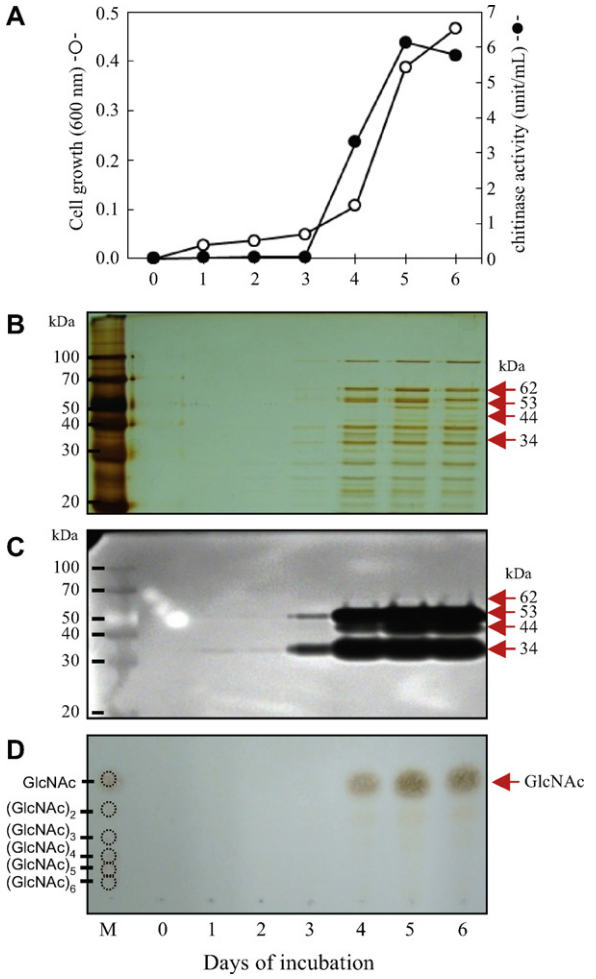

Fig. 2. Chitinase activity of the crude enzyme of *Serratia marcescens* PRC-5 after 0, 1, 2, 3, 4, 5, and 6 days incubation in 0.5% swollen chitin medium. Time course of cell growth (-○-) in 0.5% swollen chitin medium and chitinase activity (-●-) of PRC-5 (A). Silver staining of the crude enzyme in culture medium (B), chitinase activity by M2R staining (C), TLC chromatogram of reaction products obtained after 0, 1, 2, 3, 4, 5, and 6 days incubation in 0.5% swollen chitin medium. GlcNAc (*N*-acetyl-glucosamine). Marks (M).

(Fig. 2B). The major chitinase isozyme bands (53 and 34 kDa) were shown to display activity on the SDS-PAGE gels on 3 days of incubation and minor chitinase isozymes (62 and 44 kDa) were found to be active on 4 days of incubation (Fig. 2C). Gal et al. (1998) reported that two chitinase isomers, ChiC1 and ChiC2 (52 and 35 kDa), of *S. marcescens* KCTC 2172 were encoded from same chitinase gene (*chiC*). These results indicated that the same chitinase gene likely expressed both the 53 and 34 kDa enzymes during incubation with swollen chitin. We found that the 53 and 34 kDa chitinase isozymes of *S. marcescens* PRC-5 were expressed first regardless of the chitin substrate and the level of expression of others chitinase isozymes was dependent on the chitin substrates. Therefore, *S. marcescens* PRC-5 could possess three different genes related with chitinase expression. Degradation products of swollen chitin by the crude enzyme produced from *S. marcescens* PRC-5 were mainly found to be monomers of *N*-acetyl-glucosamine by TLC analysis on 4 days of incubation (Fig. 2D). Brurberg, Nes, and Eijsink (1996) reported that ChiA and ChiB enzymes of *S. marcescens* converts $(GlcNAc)_4$ exclusively to $(GlcNAc)_2$. In addition, these enzymes were also shown to convert the $(GlcNAc)_4$ analogue 4-methyumbelliferyl-$(GlcNAc)_3$ almost exclusively to 4-methyumbelliferyl, $(GlcNAc)_2$, and GlcNAc. ChiA and ChiB of *S. marcescens* indicated a strong activity as processive exo-enzymes on chitin, whereas ChiC1 was reported

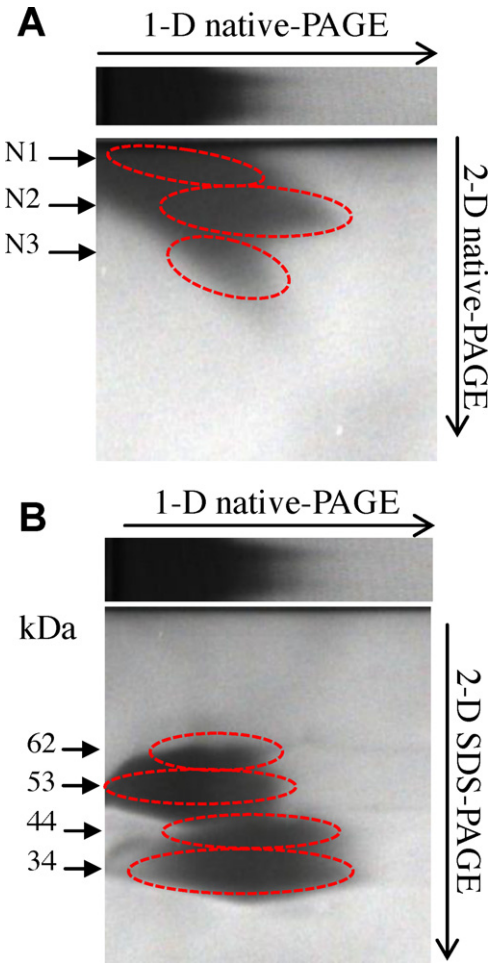

Fig. 3. Chitinase activity on 2D native/native PAGE gel (A) and 2D native/SDS PAGE gel (B) from the crude enzyme of *S. marcescens* PRC-5.

to be a non-processive endo-enzyme (Hult, Katouno, Uchiyama, Watanabe, & Sugiyama, 2005).

Three bands corresponding to active isozymes of *S. marcescens* PRC-5 (N1, N2 and N3) were observed on the 2D native/native PAGE gel (Fig. 3A) while four active isozymes (62, 54, 44, and 34 kDa) were observed on the 2D native/SDS PAGE gel (Fig. 3B). It was previously demonstrated that chitinase isozymes of *S. marcescens* QMB1466 contained two activity bands on native-PAGE gel, while the isozymes of this species showed five activity bands at 57, 52, 48, 36, and 21 kDa on SDS-PAGE-PAGE gel (Fuchs, McPherson, & Drahos, 1986).

For immunodetection of chitinase (ChiA), chitinase isozyme N1 was subjected to native-PAGE analysis and then transferred to an immunoblot (Fig. 4A) as was the 62 kDa chitinase isozyme band on the SDS-PAGE gel (Fig. 4B). The membranes were then probed using an anti-PrGV-chitinase (rat IgG). In the peptide sequence analysis, PrGV-chitinase 232–236 (–LQKPQKGVEAYNEPYKGNFGQLMAIKRHNPDL–) (Oh, 2012) was shown to have approximately 70% homology to *S. marcescens* chitinase ChiA (–LQKAQKGVTAWDDPYKGNFGQLMALKQAHPDL–) (Synstad et al., 2008). However, Brurberg et al. (2001) reported that chitinase ChiA had a molecular weight between 57 and 58 kDa on SDS-PAGE gel. Also, Watanabe et al. (1997) demonstrated that the five chitinase isomers produced from *S. marcescens* 2170 were 57, 52, 48, 36 and 21 kDa, while the four chitinase isomers in mutant of *S. marcescens* 2170 were 52, 48, 36 and 21 kDa except 57 kDa. Based on these results, chitinase isozymes of *S. marcescens* PRC-5,

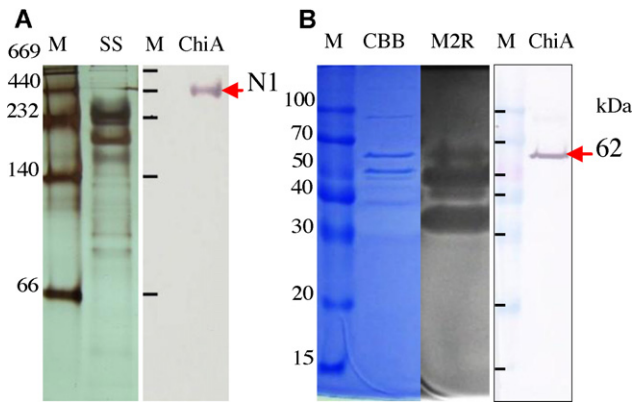

Fig. 4. Immunoblot of chitinase ChiA stained with anti-PrGV-chitinase (ChiA) on native-PAGE (A) and SDS-PAGE (B) gel. Silver staining (SS), coomassie brilliant blue R-250 staining (CBB), M2R staining (M2R) on crude enzyme of *S. marcescens* PRC-5. Protein marks (M).

S. marcescens QMB1466 and *S. marcescens* 2170 showed similar expression patterns.

From the 2D-PAGE analysis, the p*I* values of chitinase isomers produced from *S. marcescens* PRC-5 were shown to range from 5.3 to 5.8 (Fig. 5B). Thus, the different *S. marcescens* PRC-5 chitinase isomers, which had the same molecular weight, were shown to have different p*I* values. *S. marcescens* Nima Chi-180.5 had a wide focusing zone and contained two other small dots with a lower p*I*, which may be due to the different degrees of post-translation modifications (Ruiz-Sanchez, Cruz-Camarillo, Salcedo-Hernandez, Barboza-Corona, 2005).

Chitinase activity was measured on each fraction after chitin affinity purification (Fig. 6A). A new isomer produced from *S. marcescens* PRC-5, which had a molecular weight of 23 kDa on SDS-PAGE gels, was identified after chitin affinity purification (Fig. 6B). Based on these results, the low molecular isozymes of PRC-5 might be generated from sequential cleavage of chitin binding domain (CBD) at the C-terminus of high molecular isozyme.

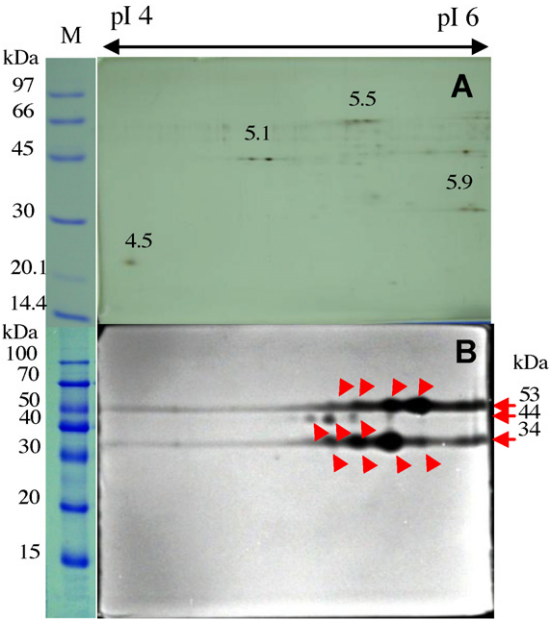

Fig. 5. Two-dimensional electrophoresis of the crude enzyme obtained from *S. marcescens* PRC-5 after 5 days of incubation in 0.5% swollen chitin medium. p*I* marks (A) and chitinase activity by M2R staining (B). Protein marks (M).

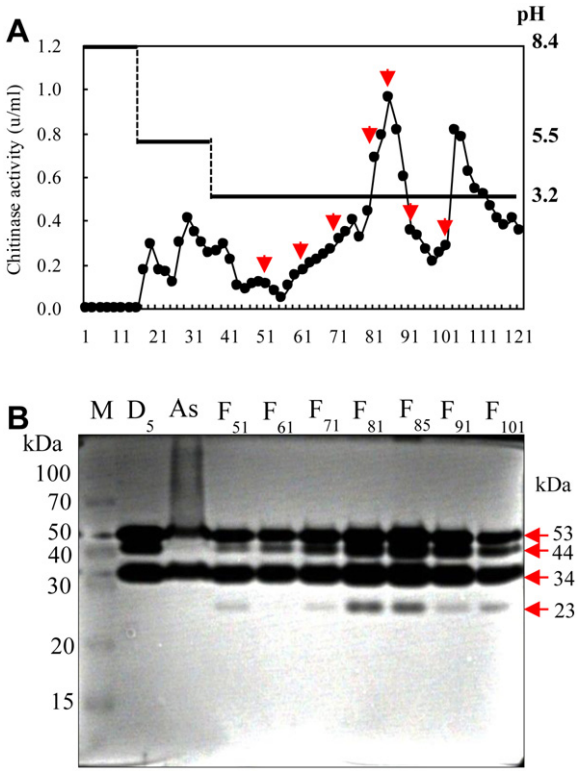

Fig. 6. Chitinase activity of *S. marcescens* PRC-5 after chitin-affinity chromatography (A) and chitinase activity staining of the fraction solution on SDS-PAGE gel (B).

Four chitinases (66, 60, 47, and 32 kDa) have the same N-terminal amino acid sequence (Thamthiankul, Suan-Ngay, Tantimavanich, & Panbangred, 2001). They suggested that two chitinases (60 and 47 kDa) was generated from sequential cleavage of CBD at the C-terminus of the 66 kDa protein. Chitinase Ch1 and Ch2 were previously shown to be expressed from crude enzymes of rape seeds, while chitinase Ch1, Ch2, and Ch3 were expressed after partial purification using chitin affinity column (Song, Seo, & Jung, 2010), which are similar to the results observed in this study. Also, they demonstrated that chitinase activity displayed different patterns at different buffer pH values, while chitinase from *S. marcescens* PRC-5 showed the same pattern of activity (Fig. 6B).

4. Conclusions

We investigated the chitinase ChiA (62 kDa) and the 23 kDa enzyme from *S. marcescens* PRC-5 were expressed in the presence of swollen or colloidal chitin substrate, while the chitinases were not expressed on LB medium in the absence of chitin substrates. For degradation of the swollen chitin substrate, the crude enzyme obtained from *S. marcescens* PRC-5 produced mainly monomers on 4 days of incubation, which was determined by TLC analysis. Chitinase N1 on native-PAGE gel was confirmed to be chitinase ChiA, which had a molecular weight of 62 kDa on SDS-PAGE gel, using immunodetection of anti-PrGV-chitinase. In conclusion, the expression pattern of chitinase ChiA was identified using an anti-PrGV-chitinase antibody. We found that the several p*I* values of chitinase isozymes were derived from post-translation on 2D gel. In addition, chitinase 23 kDa, which had a similar molecular weight as the 21 kDa chitin binding protein and did not display chitinase activity (Watanabe et al., 1997), was strong activity after partial purification using regenerated chitin column chromatography.

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