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Expression and characterization of the chitinases from *Serratia marcescens* GEI strain for the control of *Varroa destructor*, a honey bee parasite

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ABSTRACT

Serratia marcescens GEI strain was isolated from the gut of the workers of Chinese honey bee *Apis cerana* and evaluated in the laboratory for the control of *Varroa destructor*, a parasite of western honey bee *A. mellifera*. The supernatant and the collected proteins by ammonium sulfate from the bacterial cultures showed a strong miticidal effect on the female mites, with 100% mite mortality in 5 days. Heat (100 °C for 10 min) and proteinase K treatment of the collected proteins destroyed the miticidal activity. The improved miticial activity of this bacterial strain on chitin medium indicated the involvement of chitinases. The expressed chitinases ChiA, ChiB and ChiC1 from *S. marcescens* GEI by recombinant *Escherichia coli* showed pathogenicity against the mites in the laboratory. These chitinases were active in a broad pH range (5–9) and the optimum temperatures were between 60 and 75 °C. Synergistic effects of ChiA and ChiB on the miticidal activity against *V. destructor* were observed. The workers of both honey bee species were not sensitive to the spraying and feeding chitinases. These results provided alternative control strategies for *Varroa* mites, by formulating chitinase agents and by constructing transgenetic honey bees.

1. Introduction

The honey bee *Apis mellifera* is a beneficial insect, both for honey production and for crop pollination. From an economic standpoint, the value of crops pollinated by the honey bee is 100 times higher than that by honey production (Morse and Calderone, 2000). However, both pollination and honey production are being adversely affected by pathogens and parasites.

The external parasitic mite *Varroa destructor* (Anderson and Trueman, 2000) is currently one of the most destructive enemies of the honey bee world wide (De Jong et al., 1982). This haemolymph-feeding mite not only weakens adult and immature bees but also serves as a vector and inducer of viral infections, causing severe damage to bee populations world wide (Ball and Allen, 1988; Ball, 1994). Bee mortality due to *Varroa* mite infestations approaches 100% in untreated colonies (Sammataro et al., 1998). Sanford (1996) reported that the winter kill of managed honey bee colonies by *Varroa* mite was estimated at 13 million colonies worldwide, which was equivalent to a quarter of the global commercial population. Furthermore, the *Varroa* mite has been attributed, in part, to the recent widespread Colony Collapse Disorder (CCD) as a disease vector (van Englesdorp et al., 2007; Anderson et al., 2008).

The biology of this parasite is unique. Except for the mated female mite, all developmental stages live in the brood cells and feed on the haemolymph of the developing honey bees. Female mites feed on both adult and immature bees and reproduce only inside capped brood cells. It is because the mites spend much of their life cycle inside the capped brood cell that control is difficult.

Several methods have been developed or are being developed to control the mites, including physical, genetic, and chemical controls. Biotechnical control measures, such as natural products, smoke, thermal treatments, and cell size modifications, mite trapping devices, provide various degrees of success, but are labor-intensive (Fries and Hansen, 1993; Schmidt-Bailey et al., 1996; Sammataro et al., 2000). Although a longer-term solution is the development of genetically-resistant honey bee populations to limit the build-up of mite populations or reduce the effect of the secondary pathogens associated with the mite infection, more research and application on this genetic method are needed (Wilkinson et al., 2001). Currently, chemical treatments are essential to control *V. destructor* in the world honey bee management (De Guzman et al., 1996; Rinderer et al., 1997). The most effective and widely used acaricides to control *Varroa* are with the pyrethroid class of insecticide tau-fluvaliniate and with the organophosphate coumaphos. However, coumaphos and fluvalinate have been detected in wax and honey (Cabras et al., 1994; Wallner, 1995; Jimenez et al., 2005), which is a threat to the food chain for humans and compromising food and

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cosmetic sources. Other chemical acaricides such as flumethrin, amitraz, cymiazole, and bromopropylate are also associated with toxic residues (Gamber, 1990; Wallner, 1995). Control of *Varroa* mites with organic acids (formic, lactic, and oxalic acids) often require multiple applications to achieve a high degree of control, and pose a safety threat to beekeepers, and most are temperature-dependent (Mutinelli et al., 1997; Ven et al., 1998). Essential oils and their components usually lack consistency in their efficacy (Calderone et al., 1997). With the development of resistance in *Varroa* mite populations to miticides (Elzen et al., 1998; Elzen and Westervelt, 2002; Maggi et al., 2008), it is critical to develop new alternative control measures that are cost effective, environmentally friendly, and without mammalian toxicity.

One alternative approach is biocontrol. Entomopathogenic fungi, such as *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, and *Hirsutella thompsonii* have been considered a promising alternative to chemical miticides (Chandler et al., 2001; Shaw et al., 2002; James et al., 2006; Kanga et al., 2002, 2006; Meikle et al., 2007, 2008). However, unlike chemical miticides, fungal pathogens often take several days to kill their hosts, and may not be adapted to the brood nest environment (Kraus et al., 1998; Chandler et al., 2000). New biological control agents specific to *V. destructor* would be of great interest.

Serratia marcescens is an efficient biological degrader of chitin (Chernin et al., 1995; Tews et al., 1996), as well as potential biological agents for fungi and other organisms (Stevenson, 1959; Feng et al., 2002; Gómez-Ramírez et al., 2004; Tao et al., 2006). It secretes a number of extracellular proteins, including several chitinases, which were considered to be mainly active components (Xu et al., 2004).

On the basis of sequence similarities, chitinases are classified into two different families, families 18 and 19, that differ in structure and mechanism of catalysis (Henrissat and Bairoch, 1993). Three family 18 chitinase genes, *chiA*, *chiB*, and *chiC*, have been cloned from different strains of *S. marcescens* and expressed in *E. coli* for the chitin degradation (Suzuki et al., 2002). However, no reports were cited on using the chitinases from *S. marcescens* for the control of *V. destructor* mites.

The objective of this study was to evaluate the efficacy of the chitinases from a newly isolated *S. marcescens* GEI strain against *V. destructor* mites, in order to develop environmentally friendly agent for the control of this serious pest of honey bees.

2. Materials and methods

2.1. Insects

Workers and pupae of *A. mellifera* from a single honey bee queen in an apiary at Conghua, Guangdong Province, were collected for bioassay in the laboratory. Chinese honey bee *Apis cerana* was reared also in an apiary at Conghua, Guangdong Province.

2.2. Mite collection

Mites were collected from an infested brood of *A. mellifera* colonies during April, July and October. Drone and worker brood cells were opened and female mites were collected from larvae and pupae, using a camel hair brush. The mites were placed in sterile Petri dishes (diameter = 9 cm; 20 mites per dish) and were used for the bioassays within an hour after they were collected. White-eyed bee pupae were used in the present study as a food source for the mites, at a ratio of four adult female mites per pupa (Kanga et al., 2002).

The possible presence of honey bee viruses in the mites was checked by RT-PCR method (Chen et al., 2004; Yan et al., 2009). All the mites used in this study did not contain the following viruses, BQCV (black queen cell virus), CBPV (chronic bee paralysis virus), DWV (deformed wing virus), KBV (kashmir bee virus) or SBV (sacbrood virus).

2.3. Bacterial isolates

S. marcescens GEI was isolated from the gut of Chinese honey bee *A. cerana* workers, which were surface sterilized with 75% alcohol for 10 s, then with 0.1% mercuric chloride for 2 min, washed three times with sterile distilled water. The bacteria grew in Luria–Bertani medium (LB) or LB containing 0.2% (w/v) colloidal chitin, when appropriate, in a shaker (200 rpm, at 28 °C) for bioassay and for chromosomal DNA extraction. The cultures of *S. marcescens* GEI for laboratory bioassays contained approximately 10^8 cells ml^{-1} and with an OD_{620} = 2.

2.4. Laboratory bioassays

Twenty mites were transferred to a sterile Petri dish containing a sterile filter paper treated with the bacterial culture, bacterial supernatant or collected proteins (see Section 2.5) of *S. marcescens* GEI, or expressed proteins for 10 s. Mites treated with sterile LB medium and sterile distilled water served as controls. Five white-eyed honey bee pupae were introduced into each dish as food source for mites. The mites were incubated in the dark at 32 °C, 80% RH in the SANYO Growth Cabinet (auto-regulated relative humidity and lighting). Mite mortality was recorded every day, until all the mites were dead (no movement or response to stimulus). Three replicates for each treatment were established.

2.5. Characterization of toxic proteins from S. marcescens GEI

The cultures of the strain GEI in LB were centrifuged at 12,000 rpm, 4 °C for 20 min, then the supernatants were passed through a 0.22 μm syringe filter (Millipore, Bedford, MA). These culture filtrates were added slowly with ammonium sulfate (80% saturation) and kept overnight at 4 °C. After being centrifuged at 12,000 rpm for 30 min at 4 °C, the pellet collected was dissolved in PBS buffer (25 mmol/L, pH = 7.0), followed by exhaustive dialysis (6000–8000 Da cutoff) against the same buffer. The collected proteins in the closed tubes were treated by heat (100 °C, 10 min) and proteinase K (20 mg/ml, Sigma product). The bacterial culture, supernatant, collected proteins and the treated proteins were bioassayed against the mites in the laboratory as described above, to determine whether the proteins from *S. marcescens* GEI were involved in the toxic effect of the mites.

To investigate the possible involvement of the chitinases in the toxic effect of the mites, the collected proteins by ammonium sulfate from the LB medium with and without 0.2% (w/v) colloidal chitin were used to challenge the mites as described above.

2.6. Enzyme and protein assays

Chitinase activity was measured by a modification of Barboza-Corona's procedure (Brurberg et al., 1996), with colloidal chitin as the standard assay substrate. One unit of chitinase activity was defined as the amount of enzyme that produces 1 μmole of reducing sugar per min at 37 °C.

The protein content was determined by the Bio-Rad protein microassay kit using bovine serum albumin as standard.

2.7. Cloning and analysis of the chitinases from S. marcescens GEI

Chromosomal DNA from *S. marcescens* GEI was isolated according to the method described by Saito and Miura (1963) with some small

modifications. The *chiA*, *chiB* and *chiC1* genes were amplified from the chromosomal DNA by PCR with Easy-A™ high-fidelity PCR cloning enzyme (Stratagene, Germany), using the following oligonucleotide primers (ChiA-For: 5′-ATGGATCCATGCGCAAATTTAATA-3′, ChiA-Rev: 5′-GCGGCCGCTTATTGAACGCCGGCGC-3′; ChiB-For: 5′-ATGAATTCATGTCCACACGTAAAGCCGT-3′, ChiB-Rev: 5′-GAGCTCTTACGCTACGCGGCCCACCT-3′; ChiC-For: 5′-GGATCCATGAGCACAAATAACA-3′, ChiC1-Rev: 5′-GAGCTCTTAGGCGATGAGCTGCCACAGGGTG-3′), based on the nucleotide sequences from the GenBank (ChiA: accession number Z36294.1; ChiB: accession number Z36295.1; ChiC1: accession number AB019238.1). Underlined nucleotides represent the restriction sites *EcoR* I, *Not* I, *Sac* I, *BamH* I, and *Sac* I, respectively. The amplification consisted of 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The resulting PCR products were cloned into pMD18-T cloning vector (Takara) and sequenced by Shanghai Invitrogen Biotech Co. Ltd., Shanghai. Programs available in NCBI website (www.ncbi.nlm.nih.gov) were used to analyze the obtained DNA sequences of the chitinases.

2.8. Expression, purification and bioassay of the chitinases from S. marcescens GEI

To check the possible toxic effects of the *chiA*, *chiB* and *chiC1* gene products on the *Varroa* mites, the chitinases from GEI strain were expressed and purified. The amplified sequences were cloned into pMD™18-T Simple (Takara) to yield pMD18-T-chiA, pMD18-T-chiB, pMD18-T-chiC1, respectively. The constructs were verified by DNA sequence determination. The insert DNA was ligated into pET-32a(+) vector (Novagen) carrying an N-terminal His.Tag®/thrombin/S.Tag™ enterokinase configuration plus an optional C-terminal His.Tag sequence. The plasmid was transformed into *E. coli* strain BL21(DE3) to create BL21(DE3)/pET-32a(+)-chiA, BL21(DE3)/pET-32a(+)-chiB, and BL21(DE3)/pET-32a(+)-chiC1, respectively. The vector without the insert was transformed into the same *E. coli* strain as a control. A single bacterial colony was inoculated into 50 ml of LB broth containing carbenicillin and grown at 37 °C. When the culture reached an absorbance (OD_{600}) of 0.6, gene expression was induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) (Amresco, USA) for 4 h at 37 °C. The occurrence and accumulation of ChiA, ChiB or ChiC1 proteins during culturing were examined with 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The expressed proteins in the inclusion forms from *E. coli* BL21(DE3) were respectively extracted with BugBuster reagent, Benzonases Nuclease and rLysozyme™ solution (Novagen) according to pET system manual (11th edition, Novagen). The extracted recombinant protein in the form of inclusion bodies was solubilized under mildly denaturing conditions with the Protein Refolding Kit (Novagen), according to the instruction. Briefly, the inclusion bodies were isolated from the crude cell lysate. Following clarification, the solubilized fractions were dialyzed against a neutral pH buffer containing a reducing agent to encourage correct disulfide bond formation. A second dialysis step then removed excess reducing agent and transferred the protein into the buffer of choice. Protein concentrations were determined by the Bradford method as described above.

Confirming presence of the protein by SDS–PAGE and the chitinase activity as described above, the refolded proteins were respectively used for miticidal bioassay against *V. destructor* mites as above. The IB solubilization buffer and dialysis buffer (supplied in the Protein Refolding Kit) were used as the control.

To check the possible synergistic effects of the three expressed chitinases on the mite mortalities, mixed refolded proteins (1:1 or 1:1:1; v/v) of ChiA, ChiB and ChiC1 at 15.9, 14.5 and 14.7 U/ml, were used against *Varroa* mites. The IB solubilization buffer and dialysis buffer (supplied in the Protein Refolding Kit) were used as the control. Three replicates were established for each treatment. The whole experiment was done twice.

2.9. Effects of temperature and pH on the activities and stability of the expressed chitinases

The pH and temperature effects on recombinant enzymes were determined by incubating the reaction mixtures in Petri dishes at a pH range of 3–12 and a temperature range of 10–90 °C for 20 min. The chitinases at various pH values were adjusted by using 0.1 M sodium citrate (pH 3.5–6.0), sodium phosphate (pH 6.0–7.5), Tris–HCl (pH 7.5–9.0), glycine–NaOH (pH 9.0–10.5), or NaH_2PO_4–NaOH (pH 11.0–12.0) buffers according to the method (Suzuki et al., 2002).

The stability of the chitinases at different temperatures (4, 10, 30, 50 °C) was also evaluated every 24 h during 15 days, as described above.

2.10. The effects of the expressed chitinases on the workers of A. mellifera and A. cerana in laboratory

Groups of 30, 1 week-old adult worker bees were removed from their colony, briefly anesthetized with ether and then transferred to spray cages (20 × 20 × 26 cm). The bees were allowed to recover from anesthetization at room temperature (<1 h) and were then sprayed with 2 ml (34.8 U/ml) of the refolded proteins of ChiA plus ChiB (1:1, v/v) using a hand-held plant mister, or fed with the refolded proteins (5 ml) in an aqueous sugar solution (65% sugar in refolded protein solution, w/v) (Kanga et al., 2002). The bees were maintained in the dark at 32 °C, 80% RH as previously described. The numbers of living and dead honey bees in each cage were counted daily for 7 days. Dead bees (no movement or response to stimulus) were removed. Three replicates were established for each treatment.

2.11. Statistical data analyses

Data were analyzed with repeated measures analysis of variance (RMANOVA) with Bonferroni adjusted post hoc pair test, using SPSS statistical software. If Mauchly's test of sphericity showed no significant difference in the repeated measures data ($P > 0.05$), normal one-way analysis of variance was used and the significance between treatments in each experiment was evaluated by Duncan's multiple range test. The values are expressed as means ± SD. $P < 0.05$ was defined as statistically significant.

All data were analyzed using SPSS software. Differences of survival between the treatment and the control were analyzed by Paired-Sample *T*-Test. Differences of survival among different sample days and among different treatment of the same day were subjected to analysis of variance (ANOVA) and significant differences among treatments were determined using Tukey's repeated measure test at $P < 0.05$.

3. Results

3.1. Characterization of toxic proteins from S. marcecens GEI

As shown in Fig. 1, the bacterial culture, supernatant and the collected proteins by ammonium sulfate from the bacterial culture showed a strong miticidal effect on the mites, with 100% mortality of mites in 5 days. The treatment of the collected proteins by proteinase K and heat at 100 °C destroyed the miticidal activity, with only 10% mite mortality after 5 days, which was not significantly different from the control. These result indicated the involvement

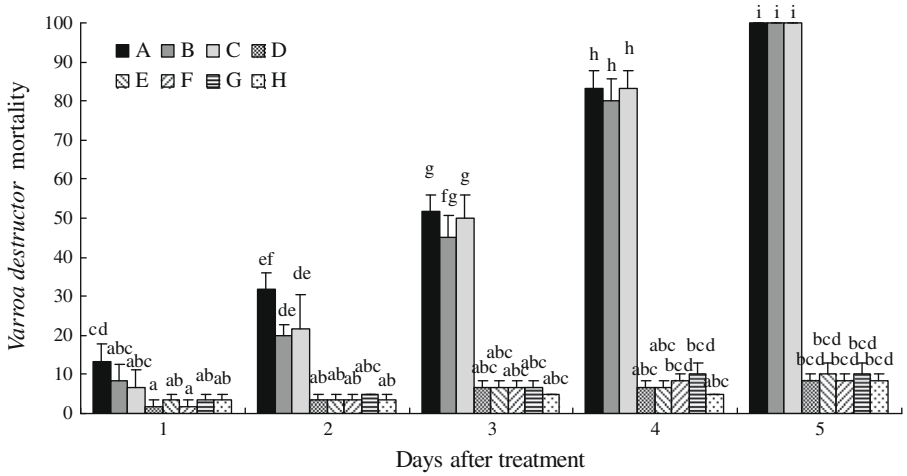

Fig. 1. Mortality of *Varroa destructor* mites treated with different components of *Serratia marcecens* GEI in 5 days: (A) the culture of *S. marcecens* GEI; (B) supernatant of the bacterial culture; (C) proteins collected by 80% ammonium sulfate from the bacterial culture; (D) the collected proteins heated at 100 °C for 10 min; (E) the collected proteins treated by proteinase K; (F) sterile LB medium; (G) PBS; and (H) sterile distilled water. Error bars standard deviation (SD). Bars with different letters indicate significant difference at $P < 0.05$ ($F_{(39,80)} = 98.9$).

of at least one of the proteins in the miticidal activity against *V. destructor* mites.

When chitin was added into LB medium, the miticial activities of the collected proteins increased significantly (Fig. 2). The chitinases activities of the collected proteins from the cultures with or without colloidal chitin were 58.5 U/ml and 38.3 U/ml (pH 7.0), respectively. Chitinases from *S. marcecens* GEI appeared to be the toxic factors against *V. destructor* mites.

3.2. Expression and purification of the chitinases ChiA, ChiB, ChiC1

Genes of *chiA, chiB and chiC1* were amplified from *S. marcecens* GEI. The open reading frames (ORF) of *chiA*, *chiB* and *chiC1* genes contained 1692, 1500 and 1443 bp nucleotides, encoding proteins of 561, 499 and 481 amino acids, respectively (Fig. 3). The similarities of *chiA*, *chiB* and *chiC1* genes were 96% to *S. marcescens* chiA gene (AF454462.1), 96% to *S. marcescens* chiB (Z36295.1), and 95% to *S. marcescens* chiC1 (AB019238.1), respectively. The sequences of *chiA, chiB* and *chiC1* were deposited in the GenBank with the accession numbers of GQ855217, GQ855218 and GQ855219, respectively.

ChiA, ChiB and ChiC1 proteins were expressed mainly in the inclusion forms, by *E. coli* carrying the corresponding cloned genes (Fig. 4). Their estimated molecular masses were 81.49, 75.93 and 72.3 kDa, respectively. A His.Tag protein from the expression vector was added to the C-terminal amino acid residue of each chitinase. These three expressed proteins were refolded with the Protein Refolding Kit. The refolded ChiA, ChiB and ChiC1 proteins showed chitinanse activity at 15.9, 14.5, and 14.7 U/ml, respectively. So these proteins were used for the bioassay of miticidal activity.

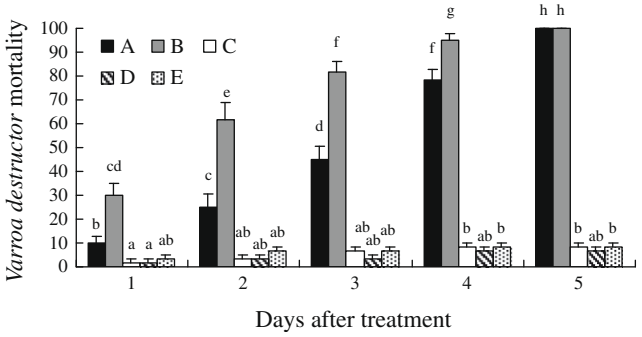

Fig. 2. Mortality of *Varroa destructor* mites treated with proteins collected by 80% ammonium sulfate from the bacterial culture of *S. marcecens* GEI with and without 0.2% (w/v) colloidal chitin in 5 days: (A) the proteins from LB medium; (B) the proteins from LB medium with colloidal chitin; (C) PBS with colloidal chitin; and (D) sterilized water. Error bars standard deviation (SD). Bars with different letters indicate significant difference at $P < 0.05$ ($F_{(24,50)} = 72.3$).

3.3. Effects of temperature and pH on the activities and stability of three chitinases

Effects of temperature and pH on the activities of ChiA, ChiB, and ChiC1 were determined using colloidal chitin as the assay substrate. Maximum activities of ChiA and ChiB at pH 7.0 were observed at 60 °C, but the optimum temperature for ChiC1 was about 75 °C, which was a little higher than for the other two chitinases. The activities of all three chitinases decreased sharply at temperature over 80 °C. The activities of these chitinases at 37 °C were approximately 50% of their corresponding maximum activities.

All three chitinases maintained significant activities in a broad pH range from pH 5 to 9 as shown in Fig. 5 ChiC1 had relatively lower activity at pH 10 than other two chitinases.

As shown in Fig. 6, all three chitinases activities decreased sharply from 1 to 7 days at 10 °C, 30 °C, and 50 °C. After 7 days, ChiA, ChiB, and ChiC1 almost had no chitinase activities. The chitinases activities of ChiA, ChiB, and ChiC1 decreased slowly at 4 °C, as they were still active until the 15th day.

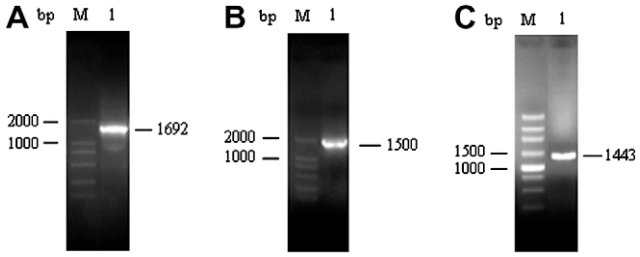

Fig. 3. *chiA*, *chiB* and *chiC1* genes amplified from *Serratia marcescens* GEI: (A) lane 1, *chiA* PCR product; (B) lane 1, *chiB* PCR product; and (C) lane 1, *chiC1* PCR product; Lane M. DNA marker.

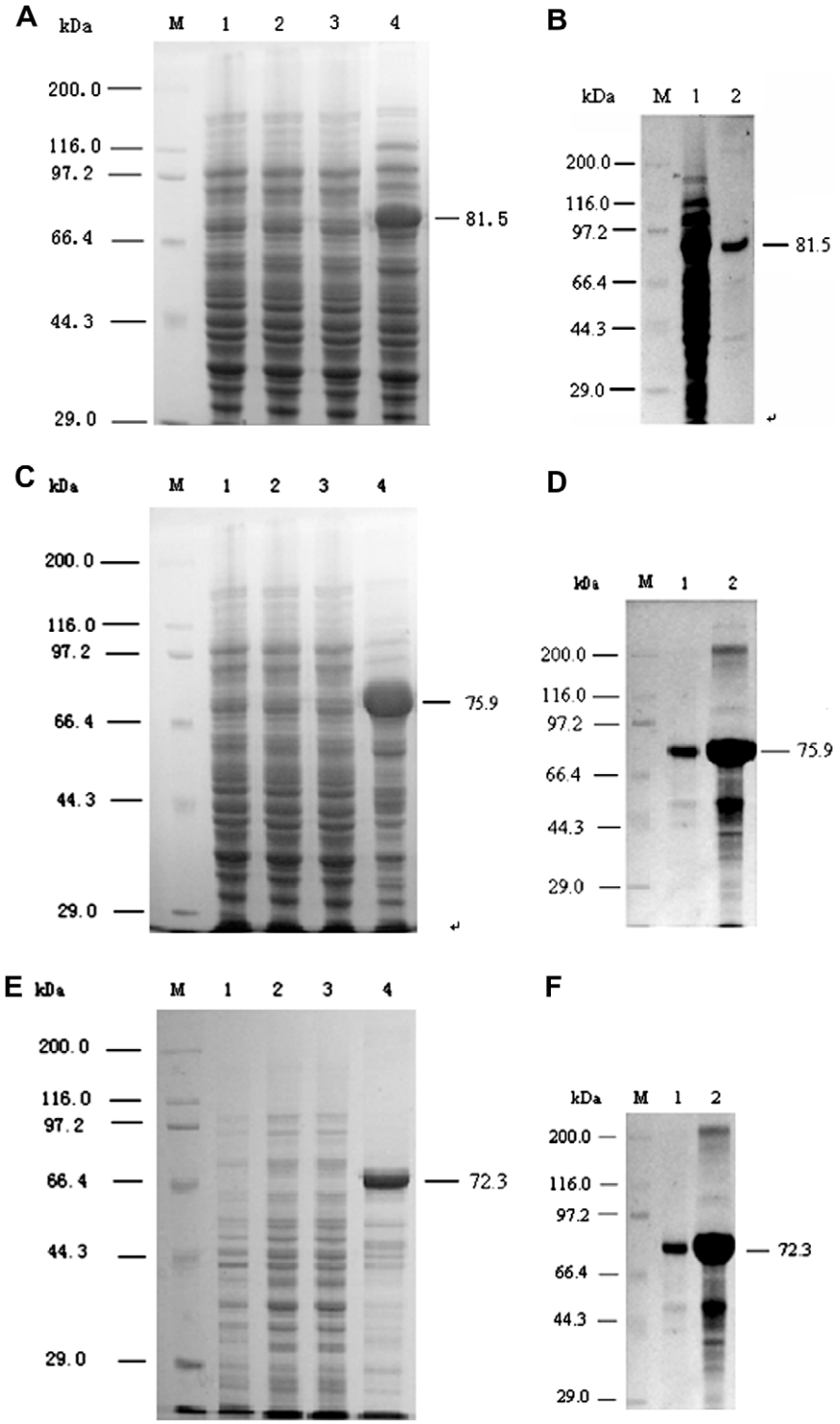

Fig. 4. Expression and purification of the recombinant ChiA, ChiB and ChiC1 from *S. marcescens* GEI: (A) expression of the recombinant ChiA: lane 1. BL21(DE3)/pET-32a(+) uninduced with IPTG; lane 2. BL21(DE3)/pET-32a(+) induced with IPTG; lane 3. BL21(DE3)/pET-32a(+)-chiA uninduced with IPTG; lane 4. BL21(DE3)/pET-32a(+)-chiA induced with IPTG; lane M. Protein marker. ChiB shows in (C), and ChiC1 shows in (E); (B) refolded of the recombinant ChiA. lane 1. BL21(DE3)/pET-32a(+)-chiA induced with IPTG; lane 2. Refolded of the recombinant ChiA; lane M. Protein marker. ChiB shows in (D), and ChiC1 shows in (F).

3.4. Synergistic effects of ChiA, ChiB, and ChiC1 on the miticidal activity against V. destructor

When the ratios of ChiA to ChiB, ChiA to ChiC1, ChiB to ChiC1, ChiA to ChiB and ChiC1 were 1:1 or 1:1:1, the chitinase activities were 34.8, 15.8, 16.2, and 27.5 U/ml, respectively. Combination of ChiA and ChiB significantly increased the chitinase activity. However, the addition of ChiC1 to ChiA or/and ChiB did not increased the activity.

The synergistic effects of ChiA and ChiB on the miticidal activity against *V. destructor* was in accordance with the chitinase activities (Figs. 7A and 7B). Markedly synergistic effects on the mite mortal-

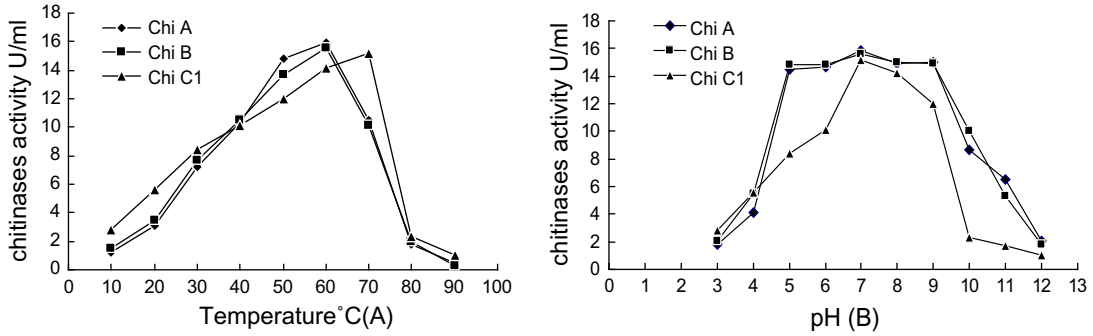

Fig. 5. Effects of temperature (A) and pH (B) on the activities of ChiA, ChiB, and ChiC1.

ity were observed in 5 days with ChiA plus ChiB. When *Varroa* mites were treated with the combination of ChiA plus ChiB, an approximately twofold increase in the mite mortality was recorded in comparison with ChiA or ChiB only. On the other hand, the combination of ChiA plus ChiC1, ChiB plus ChiC1 did not show any synergistic effects.

3.5. The susceptibility of the workers of A. mellifera and A. cerana to the chitinases

The maximum average mortalities of the workers of *A. mellifera* and *A. cerana* challenged with ChiA plus ChiB were 13.3% by spraying and 14.4% by feeding assay after 7 days. No significant differences in worker mortalities were found between the treatment with ChiA plus ChiB and the control, or between the assay methods (spraying or feeding assays). It seemed that the workers of two honey bee species were not sensitive to the test chitinases (ChiA plus ChiB at 34.8 U/ml).

4. Discussion

S. marcescens GEI isolated from the gut of *A. cerana* was found to be virulent to the honey bee mite *V. destructor*. Furthermore, the expressed chitinases ChiA, ChiB and ChiC1 from *S. marcescens* GEI showed toxicity against the mites in the laboratory. The chitinases acted as a miticidal agent possibly directly through the exoskeleton and did not have to be consumed. Although the bacteria themselves may pose a risk to humans or the honey bee products when considered for mite control in a hive environment, the chitinases can be inactivated in a short period and then potentially used for mite control. These results provided alternative control strategies

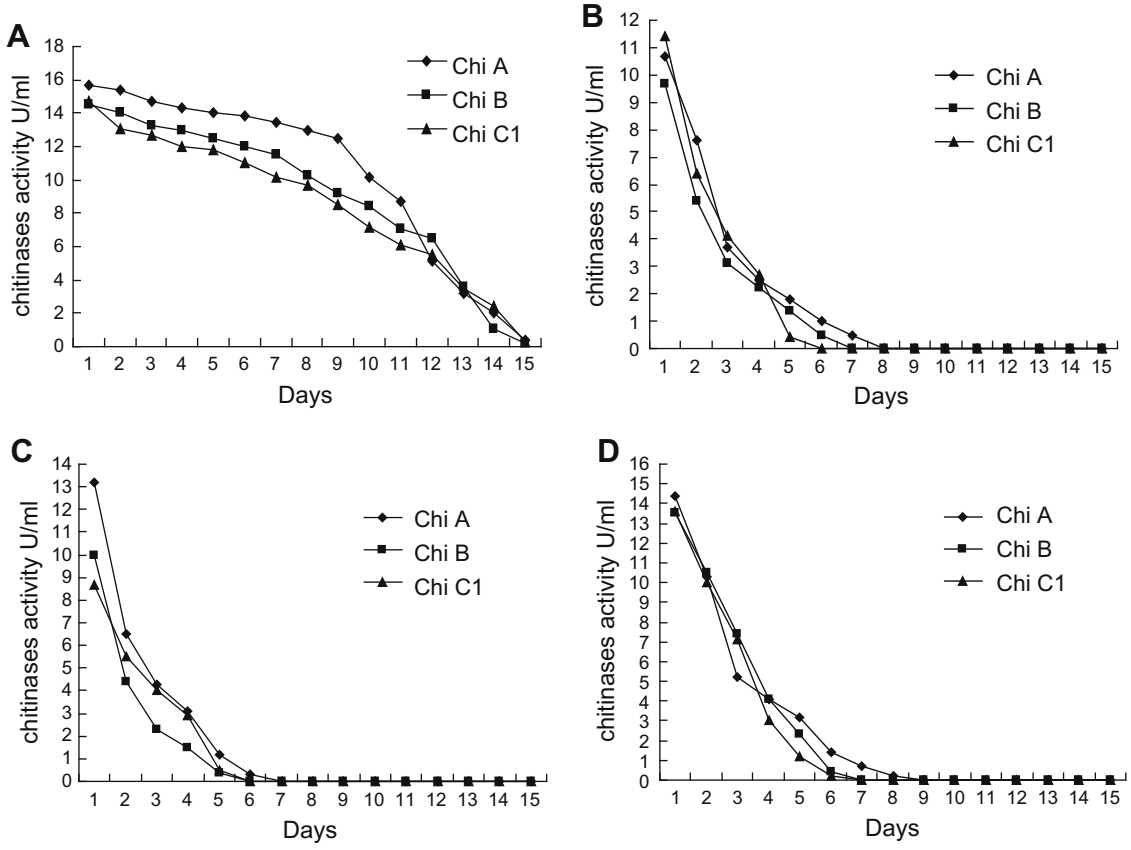

Fig. 6. The stability of the chitinase activity of ChiA, ChiB, and ChiC1 proteins under different temperatures: (A) 4 °C; (B) 10 °C; (C) 30 °C; and (D) 50 °C.

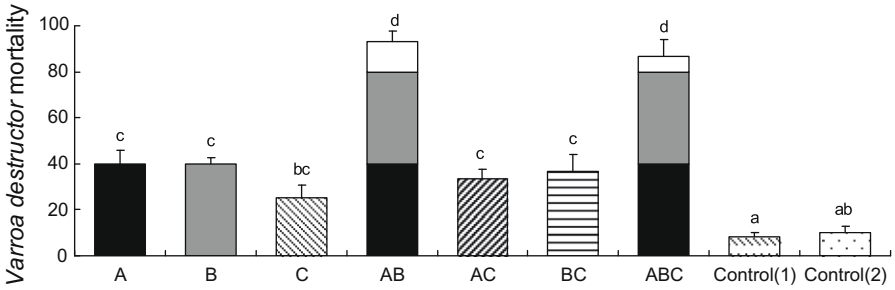

Fig. 7A. Synergistic effects of ChiA, ChiB, and ChiC1 on the miticial activity against *Varroa destructor*: (A) ChiA; (B) ChiB; (C) ChiC1; (AB) ChiA plus ChiB; (AC) ChiA plus ChiC1; (BC) ChiB plus ChiC1; (ABC) ChiA plus ChiB plus ChiC1. Control (1). solubilization buffer; Control (2). Dialysis buffer. Open boxes indicate the amount of mortality increased by the synergistic effect of the chitinases. Error bars standard deviation (SD). Bars with different letters indicate significant difference at $P < 0.05$ ($F_{(8,18)} = 24.3$).

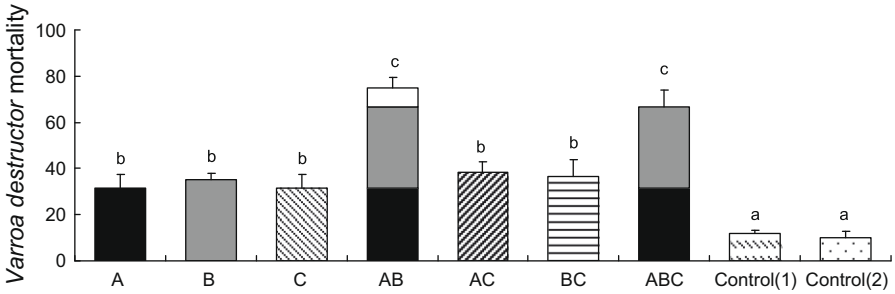

Fig. 7B. Synergistic effects of ChiA, ChiB, and ChiC1 on the miticial activity against *Varroa destructor*: (A) ChiA; (B) ChiB; (C) ChiC1; (AB) ChiA plus ChiB; (AC) ChiA plus ChiC1; (BC) ChiB plus ChiC1; (ABC) ChiA plus ChiB plus ChiC1. Control (1). Solubilization buffer; Control (2). Dialysis buffer. Open boxes indicate the amount of mortality increased by the synergistic effect of the chitinases. Error bars standard deviation (SD). Bars with different letters indicate significant difference at $P < 0.05$ ($F_{(8,18)} = 26.8$).

for the formulation of chitinase agents and the construction of transgenetic honey bees for the mite control.

S. marcescens GEI was isolated from the gut of *A. cerana*, however, it is unlikely that the resistance of *A. cerana* to mite infestation is due to the presence of *S. marcescens* in the gut, as the chitinase activity was undetectable in the gut of *A. cerana*.

Strains of *S. marcescens* have already been reported to be pathogens of insects (Boucias and Pendland, 1998). The insecticidal toxins from *S. marcescens* are the chitinases (Smirnoff, 1974; Shapiro, 1987). Chitinases are widely distributed and are found in viruses, bacteria, fungi, plants, and animals. They are the enzymes that hydrolyze β-1,4-linkages in chitin, one of the most abundant biopolymers. The *chiA*, *chiB* and *chic1* encode family 18 chitinases, chitinases A (ChiA), B (ChiB), and C1 (ChiC1) (Suzuki et al., 2002). The sequences of ChiA, ChiB, and ChiC1 from *S. marcescens* GEI shared at most 96%, 96% and 95% amino acid identities to the other strains of *S. marcescens*. As the other strains of *S. marcescens* also showed miticidal activity against this mite (data not shown), it seemed that the enzymatic properties of these chitinases with high sequence similarity were also very similar. These three chitinases were active in a broad pH range (5–9) and the optimum temperatures were between 60 and 75 °C. Although the brood nest temperature (about 35 °C) of the honey bee colonies is lower than the optimum temperature for the enzyme activity, these chitinases are expected to be resistant to high temperatures during formulation and transportation.

Synergistic effects of chitinases on colloidal chitin degradation were described (Brurberg et al., 1996; Suzuki et al., 2002). In this study, combination of ChiA and ChiB significantly increased the miticidal activity against *V. destructor*. However, the addition of ChiC1 to ChiA or/and ChiB did not increased this activity. The synergistic effects of ChiA, ChiB, and ChiC1 on the miticidal activity was in accordance with the chitinase activities. The reason why ChiC1 did not exhibit the synergistic effect was not known. ChiC1 was reported to be structurally unique from the other two chitinases (Suzuki et al., 2002; Horn et al., 2006; Synstad et al., 2008), which may be attributable to the different activity.

Although the activities of the expressed chitinases decreased sharply in the liquid formulation under the temperatures over 4 °C, it is possible to increase the shelf life of these enzymes by the improved formulation. The activity of the chitinases can be improved also by fusing a specific chitin-binding domain to form engineered chitinases, which may have higher chitinolytic ability and, consequently, increase their toxicity toward mites.

The combination of the expressed ChiA and ChiB caused 93.3% mite mortality in 5 days in the laboratory. The miticidal mechanisms of the chitinases are still unknown. As chitin is an important component of arthropod cuticle, the enzymes may kill the mites by destroying their cuticles. Although they may influence the insects, the chitinases used in this study did not show insecticidal activity toward honey bee workers which were sprayed with or fed on these enzymes. However, in the colonies of honey bees, it is necessary to develop proper application technology for optimum use of these chitinases in the control of the mites. In the colonies, mites are probably protected from exposure to the chitinases when they are sealed inside brood cells. The mites could become challenged only after they emerged from the brood cells and when sufficient quantities of chitinases are present in the hive.

The effect of chitinases on the third instar larvae of *A. mellifera* and *A. cerana* was determined. The chitinases showed toxicity against the larvae of the two honey bee species to some extent. The mortalities of larvae challenged with ChiA plus ChiB were 30–50% at the concentrations of 10–30 U/ml by spraying after 4 days (unpublished data). To protect the uncapped larvae from the possible damage in the brood cells, in practice, the chitinases are best applied when young larvae are not present in the brood by preventing the oviposition of the queens. When the mites do not reproduce, most of the mites reside on adult bees, thus they

are more accessible to chitinase treatments (Koeniger and Fuchs, 1989; Calderone et al., 1997; Le Conte et al., 2001).

No results have been reported on the use of chitinases as control agents of *Varroa* mites. To develop these chitinases as commercial agents, additional tests are in progress on the stability and persistence of these chitinases in the hives, nontarget effects, proper formulations for hive environment, any deleterious effects on queen fecundity, and application methodology.

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