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Functional GNA expressed in *Escherichia coli* with high efficiency and its effect on *Ceratovacuna lanigera* Zehntner

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Abstract The mannose-specific GNA (*Galanthus nivalis* agglutinin, snowdrop lectin) are the resistant proteins with many bioactivities. Snowdrop lectin is different with plant organs and development periods in lectin species, content, and bioactivities. It is an effective and cheap way to obtain much active GNA through overexpression of GNA gene in *Escherichia coli*. Constructs encoding mature GNA fused with an N-terminal *pelB* signal sequence protein (PelB) were expressed in *E. coli* with high efficiency. Recombinant protein productivity was higher than values published before. The insecticidal activity of purified recombinant proteins was assayed on feeding sugarcane wooly aphid (*Ceratovacuna lanigera* Zehntner), as well as spraying on sugarcane plants infected by aphids. The insecticidal activity was found to be comparable to native GNA. Oral delivery has obvious positive implications for crop protection against insect pests since peptides can be present in, or sprayed on, plant tissues susceptible to damage. A highly efficient expression of functional recombinant GNA would decrease the cost of GNA and promote its wide use, especially to give crop protection in the field.

Introduction

The mannose-binding lectin from snowdrop bulb [*Galanthus nivalis* agglutinin (GNA)] exhibits several unique properties. It characterized as a tetrameric protein of 50 kDa, composed of four identical subunits, and is specific for terminal α-1-3-linked mannose (Shibuya et al. 1988). A detailed study of the properties of plant lectins showed that GNA had uses in preventing or interfering with colonization by pathogenic microorganisms (Pusztai et al. 1993), pest control agents, glycoprotein purification, etc. (Lu et al. 2002). A number of biological properties of GNA has been confirmed, such as (1) it has been found to readily agglutinate rabbit erythrocytes but does not affect human red blood cells (Van Damme et al. 1987, 1988); (2) it has shown inhibitory activity against retroviruses (Hammar et al. 1995); (3) it has been used for the selective isolation of the major glycoprotein of human immunodeficiency virus (Gillijam 1993); (4) it has been shown to significantly reduce pathogen numbers in the lower part of the small bowel and the large intestine of rats infected with *Salmonella typhimurium* S986 and significantly improved rat growth (Naughton et al. 2000); (5) it has shown toxic effects on nematodes and a number of important insect pests, including Homoptera such as aphids, the rice plant hopper, whitefly, and froghopper, and Coleoptera such as bruchid beetles, etc. (Couty et al. 2001b; Luo and Zhangsun 2001); and (6) it has been shown to act as a carrier protein to deliver an insect neuropeptide as novel insect contol agents (Fitches et al. 2002, 2004).

Natural GNA isolated from bulbs exists as a high-complex mixture of isoforms, and the extraction processes are interfered with rich polysaccharides in snowdrop bulbs (Van Damme et al. 1998). The difficulty of separating multiple lectin isoforms by conventional techniques has led to functional properties and biological activities being defined for protein mixtures, and while many of the naturally occurring lectin variants probably differ little in functional properties, in other cases, the differences in sequence between isoforms causes significant effects on the biological activity of the molecule. So native snowdrop is expensive and limits its use severely. Expression of GNA in heterologous systems offers a means of producing proteins of a defined amino acid sequence and allows sequence–function relationships in lectins to be explored. It is wise to express a GNA protein in *Escherichia coli* efficiently and feed pests, which have significance as a new insecticide (Longstaff et al. 1998; Adar et al. 1997; Allsopp and McGhie 1996; Young et al. 1995; Powell et al. 1995; Pan

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et al. 2002). The development of resistance in aphid populations highlights the importance of biological control as a pest management tactic. Recombinant GNA has the potential to be a biocontrol aphicide. Sugarcane wooly aphid (*Ceratovacuna lanigera* Zehntner) is one main pest of sugarcane plantations, which usually decreases sugar production by 20–30%. The effect of GNA on *C. lanigera* was not described before, so it is important to investigate functional GNA expressed in *E. coli* with high efficiency and its effect on *C. lanigera*. The result will be significant for sugarcane aphid control.

In this paper, we described that constructs encoding mature GNA, fused with an N-terminal pelB signal sequence protein, were expressed in *E. coli* with high efficiency, followed by a demonstration of its insecticidal activity in insect-feeding and foliar-spraying studies using *C. lanigera*. Evidence is presented that the purified recombinant snowdrop lectin fused with an N-terminal pelB signal sequence possesses activity corresponding to the natural mixture of isoforms.

Materials and methods

Materials and GNA expression constructs

Standard native GNA was purchased from Sigma, and polyclonal rabbit anti-GNA serum were provided by Prof. Kexuan Tang (one of the authors), Fudan University, China. Expression vector pET22b and expression host BL21 (DE3) cells were from Novagen. A cDNA sequence encoding for a GNA precursor protein (Accession M55556.1, GenBank, 1993) in pCAMBIA1300 (provided by Prof. Kexuan Tang) was used as a template for polymerase chain reaction (PCR) amplification of the mature GNA peptide to yield the constructs described before (Luo et al. 2002). Four PCR primers corresponded to the GNA mature coding sequence (MGNA). A *Bam*HI, *Sal*I, *Nde*I, and *Nco*I site was included at the 5′ end of each primer, which were synthesized by SABC, China. The primer sequences were:

- P1 5′GNA/B*am*HI 5′ GCGGATCCATGGACAATATT TTGTACTCC 3′(29 bp);
- P2 3′GNA/SalI 5′ TGGTCGACTCATCCAGTAGCC CAACGATC 3′ (29 bp);
- P3 5′GNA/*Nde*I 5′ GCCATATGGACAATATTTTGTA CTCC 3′ (26 bp); and
- P4 5′GNA/*Nco*I 5′ GCCCATGGACAATATTTTGTA CTCC 3′ (25 bp).

Polymerase chain ractions were carried out using Taq DNA polymerase (SABC) as described by the enzyme supplier. After amplification, PCR products, with three primer pairs of P1-P2 (*Bam*HI-*Sal*I, MGNA1), P3-P2 (*Nde*I-*Sal*I, MGNA2), and P4-P2 (*Nco*I-*Sal*I, MGNA3) were cloned into pGEM-T easy vector (Promega) and sequenced to separately check for the absence of PCR errors. Restriction endonucleases and T4 DNA ligase were bought from TaKaRa Biotechnology (Dalian) Co., Ltd. Plasmid template DNAs were sequenced on an ABI3100A DNA sequencer (Applied Biosystems). All final constructs described were sequenced to verify that no misincorporation occurred during PCR amplification.

MGNA1, MGNA2, and MGNA3 of mature GNA gene were inserted downstream of T7 promoter at three different sites of *E. coli* expression vector pET22b (Novagen), resulting in the construction of three prokaryotic expression vectors: pG1 (*Bam*HI-*Sal*I site), pG2 (*Nde*I-*Sal*I site), and pG3 (*Nco*I-*Sal*I site). The construct pG1 for the expression of GNA fused the pelB leader signal peptide sequence (66 bp, 22aa) and seven extra amino acids. The construct

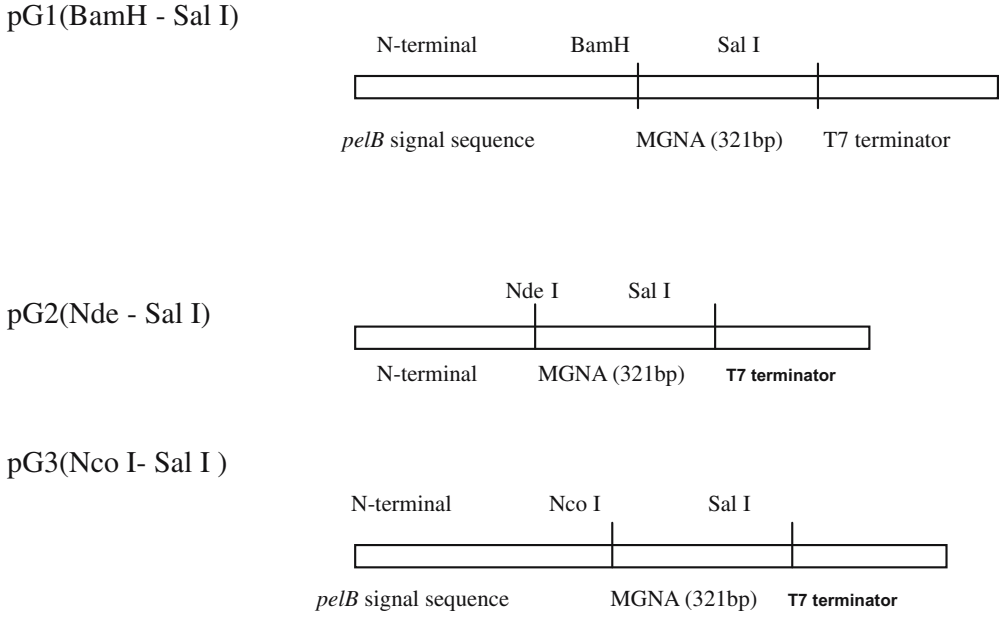

Fig. 1 Diagrammatic representation of three GNA constructs pG1 (*Bam*HI-*Sal*I), pG2 (*Nde*I-*Sal*I), and pG3 (*Nco*I-*Sal*I) showing restriction sites introduced by PCR. The position of N-terminal pelB signal sequence and C-terminal T7 terminator is shown

pG2 did not contain the pelB leader. The construct pG3 contains the pelB leader with a consensus ATG initiation codon without C-terminal His•Tag (Fig. 1). The sequence and size of pelB leader were:

– DNA sequence: ATGAAATACCTGCTGCCGACCG CTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAG CCGGCGATGGCC (66 bp); and
– Peptide sequence: MetLysTyrLeuLeuProThrAlaAlaA laGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAla (22aa).

Expression and purification of GNA construct (pG3)

The resulting plasmid, pG3 (GNApET22b), was transformed into expression *E. coli* BL21 (DE3) cells. For protein overproduction, 2-l cultures of *E. coli* containing pG3 were grown in a Luria broth (LB) containing 100 mg/l ampicillin, with shaking at 37°C. Cultures were induced to express the inserted genes with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) when an optic density (OD) of 0.6–0.8 had been attained. Cultivation at 37°C was continued for 6 h postinduction. Cells were harvested by centrifugation (20 min at 8,000×*g*) and resuspended in 100 ml buffer A (50 mmol/l Tris–HCl (pH 7.9), 0.5 mmol/l ethylenediaminetetraacetic acid (EDTA), and 50 mmol/l NaCl). Cells were lysed by sonication and centrifuge to collect supernatant of soluble cytoplasmic fraction and the insoluble fraction pellet of inclusion bodies separately. The inclusion bodies were washed in buffer A and dissolved in buffer A containing 6 M urea. The soluble protein samples were diluted to 0.05 mg/ml in 20 mM Tris, 4 M urea, and pH 7.8, and dialysed sequentially against 20 mM Tris, pH 7.8 and dH2O, then concentrated to the appropriate volume. After dialysis, half of renatured proteins were lyophilized directly for spraying on insecticidal assay (RG3); the other half were filtered (0.45 μm) and purified by binding to a mannose–agarose (Sigma) affinity column (10 mL) (RG3A) as described before (Raemaekers et al. 1999). Fractions containing RG3A were pooled, frozen in liquid nitrogen, and freeze-dried.

Sodium dodecyl sulfate/poly-acrylamide gel electrophoresis and Western blotting

All the samples of total cell proteins (TCP), soluble cytoplasmic fraction, inclusion bodies, and renatured proteins were analyzed using denaturing sodium dodecyl sulfate (SDS)/poly-acrylamide gel electrophoresis (PAGE) with 15% gels run under reducing conditions. The bands were visualized by Coomassie Brilliant Blue R250 staining. Samples were treated with 2× sample buffer and heated in boiling water for 3 min before loading onto gel. Low molecular mass markers [LMW-SDS Marker Kit (A.P.)] were used to calibrate the gels. Recombinant GNA was analyzed by Western blotting for concentration and reactivity with polyclonal rabbit anti-GNA serum (1:5,000 dilution) antibodies. Electrophoretic transfer of proteins from gels to nitrocellulose membranes (Amersham) was carried out by the semi-dry blotting technique (BioRad) and was followed by immunodetection. The membrane was incubated in Tris-buffered saline (TBS, pH 8.0) containing 1% (w/v) bovine serum albumin (BSA) and 1% Tween 20 for 1 h at room temperature with gentle shaking. Membranes were incubated in a primary antibody (anti-GNA) solution at room temperature for 1 h with gentle shaking. These were then washed in antibody dilution buffer for three times for 5–10 min at room temperature. Secondary antibodies were goat antirabbit IgG-AP conjugates (SABC) diluted 1:500 (as above), and incubation was for 1 h at room temperature, followed by three 5-min washes in antibody dilution buffer and a rinse in distilled water. A nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) staining kit was used to detect specifically bound secondary antibodies as instructed by the manufacturer (SABC), and bands were visualized by color.

Hemagglutination assay

Hemagglutination assays were carried out in round-bottomed microtiter plates as described (Mao et al. 1999). Recombinant protein (RG3A) concentrations were estimated by a microtiter-based Bradford assay (Biorad) using native GNA as the standard protein. A total volume of 100 μl was used in each well: 50-μl aliquots of serial twofold dilutions of the lectin in NaCl/P_i and 50 μl of 2% rabbit erythrocyte suspension in NaCl/P_i. The microtiter plate with the 100-μl erythrocyte suspension containing the serial double dilutions of the sample lectin was incubated for 1 h at room temperature. The lowest concentration required to completely agglutinate the red blood cells was determined visually.

Insect maintenance and artificial diet preparation

Stocks of *C. lanigera* were continuously reared and maintained on sugarcane (*Saccharum officinarum* L.) plants of the susceptible varieties (New Taitang No. 1 and No. 10) in controlled growth chambers (26–28°C, 16:8 light–dark regime). Native GNA obtained from Sigma was used as control. Artificial diet was prepared according to Li et al. (1997), and its components were improved by the authors (Table 1).

Feeding and spraying-on trials

Affinity-purified GNA (RG3A) was tested for biological activity by an artificial diet feeding trial. Twenty instar female nymphs, of second- or third-stadium larvae of *C. lanigera*, were placed in a plastic feeding chamber for trials.

Feeding trials Recombinant and native snowdrop lectins were incorporated into a diet at the concentration of 0.2 g/l

Table 1 Concentration of artificial diet components for *C. lanigera* Zehntner. (mg/100 ml)

L-amino acids	*C. lanigera*	Components	*C. lanigera*
Glycin	100	Vitamin B1	2.5
Alanine	100	Vitamin B2	2.0
Lysine	150	Vitamin B5	12
Methionine	80	Vitamin B6	2.5
Glutamic acid	200	Vitamin B3	5.0
Glutamine	300	Folacin	0.5
Valine	160	Inositol	60
Proline	100	Choline chloride	40
Threonine	150	Vitamin C	50
Leucine	120	Biotin	0.2
Arginine	360	$Ca(C_3H_5O_3)2{\cdot}5H_2O$	2.0
Tyrosine	30	KH_2PO_4	500
Tryptophan	80	$Ca(H_2PO_4)_2$	0.7
Serine	60	$FeC_6H_5O_7{\cdot}5H_2O$	0.2
Cystine	10	$CuCl_2{\cdot}2H_2O$	0.3
Histidine	200	$ZnCl_2$	1.3
Isoleucine	150	$MgCl_2{\cdot}6H_2O$	200
Phenylalanine	60	NaCl	0.3
Cysteine	50	$MnCl_2$	0.5
Aspartic acid	100	Sucrose	30,000

or so. Neonate female aphids of second- or third-stadium larvae, collected from sugarcane plants, were divided into four groups: (1) RG3 aphids, which were subsequently fed an artificial diet containing RG3 0.2% (w/v); (2) native GNA aphids, fed an artificial diet containing native GNA 0.2% (w/v); (3) negative-control aphids, fed an artificial diet only without GNA; and (4) positive-control aphids, fed on no diet. Ten diet chambers containing GNA 0.2% and ten containing a control diet were established. Twenty neonate female aphids of second- or third-stadium larvae were placed in each chamber and reared for 12 days. The number of surviving aphids was recorded daily. Mortality was recorded at 24-h intervals. *C. lanigera* survival rates of four treatments were compared by survival analysis, with differences being assessed for significance using a statistics test (Table 2).

Spraying-on trials in the field Insecticidal activity of RG3 without affinity purification was tested by spraying on sugarcane leaves infected by *C. lanigera* in the field. GNA insecticide (RG3N) was prepared by mixing RG3 and additive surfactant nonylphenol polyoxyethylene (10) (NP10) with a volume ratio of 3:2. Application efficacy with RG3N was assayed using *C. lanigera* abundance on shoots and leaves when mean aphid densities exceeded 30 aphids per leaf. Aphid numbers were assessed on sugarcane seedlings treated with various concentrations of RG3 at the range of 0.1–0.5% recombinant GNA (Table 3). The survival percentage was recorded at 72-h intervals for 12 days after foliar application under field conditions. Control sugarcane with aphid infection was sprayed by water containing only the same NP10 rate surfactant. Ten replicates were set up as described above for each treatment.

Statistical analyses

All data were analyzed statistically. Treatment differences were tested using Wald (χ^2) statistics. Confidence intervals (CI) of 95% were used to compare means when there were more than two treatments; means with nonoverlapping CIs can be considered significantly different at the 5% level.

Results

Expression and purification of recombinant GNA (RG3)

Start codon (ATG) and stop codon (TGA) were added on 5′ and 3′ end of DNA fragment encoding mature GNA using PCR primers, respectively. Constructs encoding mature GNA, including methionine (106 amino acids), fused with an N-terminal pelB signal sequence protein (22 amino acids), were prepared and cloned into the expression vector pET22b (*Nco*I/*Sal*I). The predicted protein products do not carry a histidine tag, which also contain an N-terminal extension of 23 amino acids. Protein expressions were monitored in the TCP, the soluble protein fraction, and the inclusion bodies produced by *E. coli* G3, following induction with 0.1 mM isopropylthiogalactoside (IPTG), by an analysis of each fraction on SDS/PAGE and immunoblots using antisera prepared against native GNA. A Western analysis of purified GNA confirmed that RG3 reacted

Table 2 Survival rates for nymphs of *C. lanigera* aphid in artificial diet bioassays

Survival percentage (%)						Differences
Feed time (days)		3	6	9	12	
C. lanigera	CK^+	40	0	0	0	C
	CK^-	90	70	55	20	A
	RG3	58	25	0	0	B
	Native GNA	53	22	0	0	B

CK^+ No diet (positive control), CK^- diet without GNA, *RG3* recombinant GNA from *E.coli* and G3, *Differences* different significance (P=0.01)

Table 3 Mean survival percentage of *C. lanigera* by spraying 0.1–0.5% RG3 solution (RG3N)

% RG3	% Survival at different times after spraying				
	3 days	6 days	9 days	12 days	significance (P,0.01)
Control	120	150	190	225	A
0.1	92	86	90	100	B
0.2	85	50	43	5	C
0.3	83	46	36	3	C
0.4	70	32	5	0	D
0.5	65	30	0	0	D

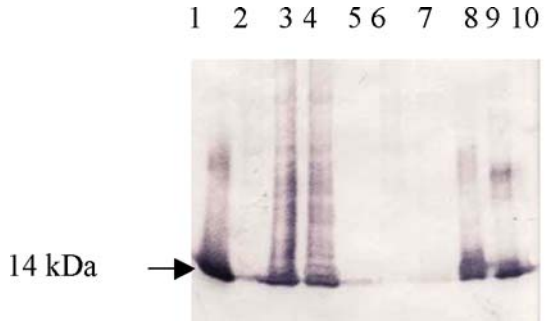

Fig. 2 Western analysis of recombinant G1, G2, G3, and native GNA. *Lane 1*, native GNA (Sigma); *Lane 2*, G1 TCP after IPTG inducing; *Lane 3*, G2 TCP after IPTG inducing; *Lane 4*, G3 TCP after IPTG inducing; *Lane 5*, G1 TCP before IPTG inducing; *Lane 6*, G2 TCP before IPTG inducing; *Lane 7*, G3 TCP before IPTG inducing; *Lane 8*, G3 inclusion bodies after dissolving; *Lane 9*, G2 inclusion bodies after dissolving; and *Lane 10*, LMW-SDS Marker Kit (A.P.) without prestained

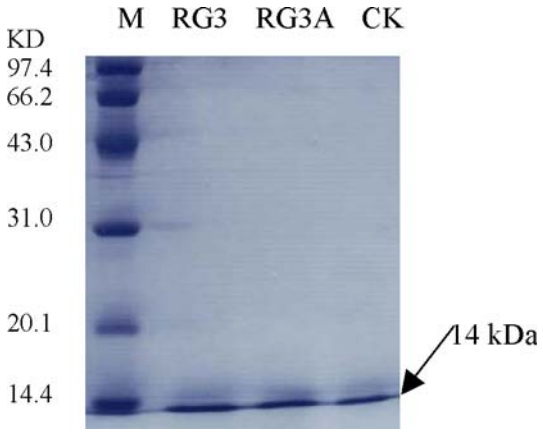

Fig. 4 Purified recombinant protein of GNA construct (pG3). *M*, LMW-SDS Marker Kit (A.P.); *RG3*, recombinant GNA without affinity purification; *RG3A*, recombinant GNA without affinity purification; and *CK*, native GNA (Sigma)

positively with antiGNA antibodies (Fig. 2). In this way, it was ascertained that RG3 was produced after IPTG induction. RG3 productivity was not affected by the IPTG concentration at the range of 0.1–1.0 mM (Fig. 3). Approximately 14 kDa RG3 were accumulated at a 42 mg/l culture from clones containing expression vectors in *E. coli* strain BL21 (DE3). RG3 accumulate in insoluble inclusion bodies and were purified to greater than 95% homogeneity by inclusion bodies isolation, solubilization, refolded, and dialysis. RG3 were renatured by dialysis. RG3A purity can be greater than 99% by an affinity purification step after renatured protein dialysis. This is consistent with predicted increases in molecular mass due to the presence of a pelB leader peptide (Fig. 4).

Recombinant GNA (RG3A) are functional proteins

Hemagglutination assays were carried out using RG3A and native agglutinin in a serial dilution assay. RG3A agglutinated rabbit erythrocytes at concentrations similar to those of native lectins assayed under the same conditions. The lowest concentration of purified RG3A required to completely agglutinate untreated rabbit erythrocytes was 0.25 ug/ml (variable over one serial dilution in different samples). Under identical conditions, samples of native protein gave a minimum concentration for complete agglutination of 0.2 ug/ml. χ^2 statistics showed that RG3A agglutination values were similar to GNA purified from snowdrop, which indicated that the N−terminal residues encoding the pelB leader peptide did not interfere with GNA functionality.

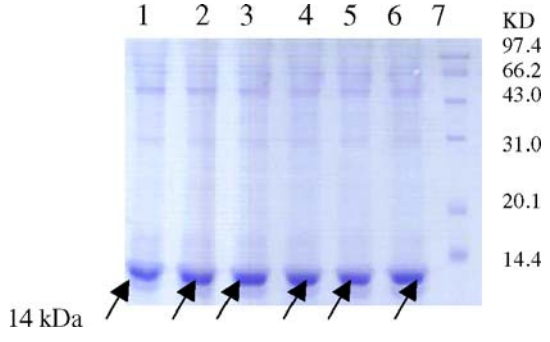

Fig. 3 RG3 productivity was not affected by the IPTG concentration at the range of 0.1–1.0 mM. *Lane 1*, 0.1 mM IPTG; *Lane 2*, 0.2 mM IPTG; *Lane 3*, 0.4 mM IPTG; *Lane 4*, 0.6 mM mM IPTG; *Lane 5*, 0.8 mM IPTG; *Lane 6*, 1.0 mM IPTG; and *Lane 7*, LMW-SDS Marker Kit (A.P.)

Insect bioassay by artificial diet feeding

Survival rates for nymphs of *C. lanigera* fed a control diet, a diet containing native GNA, and a diet containing RG3A are shown in Table 2. The survival rates for insects on a control diet and “no diet” act as negative and positive controls for this assay. After 3 days, 90% of negative-control aphids survived, while only 40% of positive-control (no diet) aphids survived; survival rates of RG3A and native GNA aphids were 58 and 53%, respectively, which were in the middle of control aphids. After 6 days, all the aphids with no diet died, 75% of RG3A and 78% of native GNA aphids were also dead, but most of the aphids on a diet without GNA (negative control) also survived. After 9 days, more than half of the negative-control aphids survived; all of the RG3A and native GNA aphids died. The results showed that survival rates for diets containing native snowdrop lectin (Sigma) and RG3A were similar, and gave levels of survival intermediate between the “no diet” control (all insects dead by day 6) and the diet without additions (70% survival by day 6). Comparison analysis of the survival rates showed that survival on all the diets was significantly different from the no-diet control (P, 0.01), and that survival on the control diet was significantly dif-

ferent from both the GNA diets (P, 0.01). However, survival on the diets containing native snowdrop lectin and RG3 did not differ significantly (P, 0.05); survival rates were similar over the assay period (Table 2).

Insecticidal bioassay by spraying on *C. lanigera* in the field

Insecticidal activity of RG3N by spraying on *C. lanigera* in the field was shown in Table 3. Control *C. lanigera* kept a high growth speed with an 87.5% increase rate. Control's survival rate (225% by day 12) was significantly higher than all the treatment of 0.1–0.5% RG3 spraying. Significantly, more mortality was observed in the 0.2–0.5% RG3 group than in the 0.1% RG3 group. The differences were not significant at the 1% level for both between 0.2 and 0.3% RG3, and between 0.4 and 0.5% RG3. The survival percentage of the 0.1% RG3 group changed slightly—aphids propagated slowly without population increase. There were less aphids that survived (3–5%) at 12 days after 0.2–0.3% RG3 spraying, as well as at 9 days after 0.4–0.5% RG3 spraying. All the aphids of the 0.4% group were dead in 12 days, and all of the aphids of the 0.5% group were dead in 9 days. The results of these studies showed that greater than 0.2% RG3N can be used to control *C. lanigera* by spraying on sugarcane plants in the field; the insecticidal effects increased and the action time decreased with increasing RG3 concentration. However, none of these differences were significant at the range of 0.2–0.5% RG3 to control *C. lanigera*. Therefore, a more than 20-mg/l yield of the recombinant GNA would be feasible for field protection. Recombint GNA of RG3 without affinity purification is likely to be effective as components of insecticidal sprays to control *C. lanigera*.

Disscussion

To our knowledge, this is the first description of construct (pG3) encoding mature GNA (MGNA) fused with an N-terminal pelB signal sequence protein that were expressed in *E. coli* with high efficiency, and this is also the first in evaluating its insecticidal activity against sugarcane wooly aphid (*C. lanigera*). At the same time, MGNA gene was inserted in *Bam*HI-*Sal*I site (pG1) and *Nde*I-*Sal*I site (pG2) of *E. coli* expression vector pET22b (Novagen), respectively (Luo et al. 2002). All the pG1, pG2, and pG3 constructs were transformed into *E. coli* BL21 (DE3) cells and induced by IPTG to produce recombinant with the same methods as pG3. The result showed that recombinant GNA were produced in *E. coli* carrying pG2 and pG3 constructs separately after induction with IPTG; however, an *E. coli* carrying pG1 construct cannot produce recombinant snowdrop lectin at all (Fig. 3). It indicated that the fusion protein pelB/MGNA produced by an *E. coli* carrying pG3 construct with a consensus ATG initiation codon might be processed correctly inside the cells, while an *E. coli* carrying pG1 construct failed to produce the recombinant GNA with a few extra amino acids in its N-terminal. pG2 constructs were similar with N-terminal of GNA11 and different with C-terminal of pGNA11, which had the extra amino acids introduced for metal affinity purification (Longstaff et al. 1998), both of which had a similar specificity for producing recombinant GNA. The majority recombinant GNA produced by *E. coli* carrying pG2 and pG3 constructs was inclusion bodies, which cannot be detected in soluble cytoplasmic fractions by SDS-PAGE and Western blotting in this experiment. Our results demonstrated that the recombinant GNA yield and solubility of pG3 were higher and better than that of pG2. The recombinant GNA yield of *E. coli* carrying pG3 construct (42 mg/l culture) in this research is much higher than that of GNA11 (5 mg/200 ml culture) in the report of Longstaff et al. (1998). The expression difference between *E. coli* carrying pG2 and pG3 constructs remain under further investigation. The recombinant GNA can be renatured to a fully active form by dilution and dialysis, which has similar functions of native snowdrop lectin such as mannose-binding, hemagglutination, and insecticidal activities. Further research will be necessary to optimize the expression conditions of RG3 such as growth temperature, initiate inducing OD 600 of culture by IPTG and inducing time, etc., as well as simpler renature methods of active GNA from *E. coli*.

The biological activity of recombinant GNA (RG3) was determined by its toxicity toward *C. lanigera* Zehntner collected from sugarcane, which was not observed in previous studies (Luo et al. 2003). Survival rates for insects on artificial diets containing RG3 or lectin purified from snowdrops were similar; the corrected mortality shown by snowdrop lectin at day 6 was 75–78% (Table 2), which was higher than the value determined previously against other aphids (*Myzus persicae*, *Macrosiphum euphorbiae*, etc.) for a similar concentration of GNA (Couty et al. 2001a). It was likely that GNA had a higher toxicity against *C. lanigera* than against other aphid species, which was confirmed by RG3N insecticide spray in the field. A significantly higher mortality was observed with foliarly application of RG3N containing 0.2–0.5% RG3 to sugarcane leaves over the testing period of 12 days. NP10 surfactant can increase insecticidal efficiency by improving the wetness, penetration, and dispersing of RG3N spray (Chen et al. 1999). The production cost of RG3N insecticide is low because it contains renatured recombinant protein from inclusion bodies directly without expensive affinity purification. So it is likely that RG3N can be effective as components of aphicide by foliar spray to control *C. lanigera*. Contrary to previous reports, our results demonstrated that recombinant GNA has a direct negative effect on the reproductive physiology of *C. lanigera*. Negative effects can mostly be attributed to the antifeedant activity of this compound and the protracted time to death. The results of this study contribute to a better understanding of the most suitable techniques for assessing aphid mortality after exposure to these new protein insecticides and provide a baseline susceptibility to recombinant GNA for sugarcane wooly aphid.

Snowdrop lectin preprotein gene was transferred into many kinds of crops such as tobacco, potato, wheat, rice, tomato, etc. GNA expressed in transgenic tobacco and potato plants has effects on homopteran and lepidopteran insect pests in plants. Transgenic lines of potato and tobacco showed significant reductions in the fecundity of the peach-potato aphid, *M. persicae* (Gatehouse et al. 1996; Hilder et al. 1995), and transgenic potato lines exhibited enhanced resistance to the tomato moth, *Lacanobia oleracea*, in terms of a reduction in larval biomass concomitant with a reduction in the extent of leaf damage and larval survival (Fitches et al. 1997). Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown planthopper (BPH; *Nilaparvata lugens*) and green leafhopper (GLH; *Nephotettix virescens*) (Foissac et al. 2000; Rao et al. 1998; Sun et al. 2001). Transgenic wheat plants from lines expressing GNA at levels greater than about 0.04% of total soluble protein decrease the fecundity, but not the survival, of grain aphids (Stoger et al. 1999; Xu et al. 2001). Therefore, many scientists have attached importance to transfer GNA isoform gene into plants as protectants. However, recombinant GNA used as a biocontrol agent by foliar spray has been ignored until now. The results of this study can improve and extend GNA usage in aphicide development. The studies of Fitches et al. (2002, 2004) have demonstrated that GNA can be utilized as a delivery system to transport the linked peptide to the hemolymph of larvae of the lepidopteran, *L. oleracea*, which would expand the control insects' species with GNA insecticide by spray-on, or be present in plant tissues susceptible to damage. For example, GNA linked C-terminally to an insect neuropeptide allatostatin (Manse-AS) was expressed in *E. coli*, and GNA linked to an insecticidal spider venom neurotoxin (*Segestria florentina* toxin 1; SFI1) was expressed in *Pichia pastoris*. The insecticidal activity of fusion proteins to tomato moth (*L. oleracea*) was found to be comparable to values for linked neuropeptide or neurotoxin by oral administration.

A number of factors that result in plant lectins occur as heterogenous mixtures of isoforms in plants. In some cases, imprecise trimming of the C-termini of mature polypeptides can give rise to heterogeneity in lectin sequences (Young et al. 1995). Most lectins are encoded by multigene families, members of which can be expressed in the same tissue, or differentially expressed in different tissues and/or developmental stages (Shi 2001). A detailed study of the properties of many plant lectins has been hindered by their heterogenous mixtures. Our successful results showed that attempts to express plant lectins fusing prokaryotic signal peptide in bacteria would be the wise selection; it provided the basis for studying GNA and using it as a new insecticide and reagent.

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