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Mannose-Binding Lectin from Yam (*Dioscorea batatas*) Tubers with Insecticidal Properties against *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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The amino acid sequence of mannose-binding lectin, named DB1, from the yam (*Dioscorea batatas*, synonym *Dioscorea polystachya*) tubers was determined. The lectin was composed of two isoforms DB1(Cys86) and DB1(Leu86) consisting of 108 amino acid residues with 90% sequence homology between them. DB1 showed a high sequence similarity to snowdrop (*Galanthus nivalis*) bulb lectin, GNA; especially, the carbohydrate-binding sites of GNA were highly conserved in DB1. DB1 interacted with D-mannose residues of oligosaccharides, and the oligosaccharides carrying two mannose-α-1,3-D-mannose units showed high binding affinity. DB1 was examined for insecticidal activity against *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae at different stages of development. The rate of adults successfully emerging from pupae fed on DB1 was 33%, when incorporated into an artificial diet at a level of 0.01% (w/w). Although DB1 had no or marginal inhibitory effects on gut proteolytic and glycolic enzymes, the lectin strongly bound to larval brush border and peritrophic membrane detected by immunostaining. The results show that DB1 may fulfill a defense role against insect pests.

KEYWORDS: ***Dioscorea batatas*; *Helicoverpa armigera*; insecticidal activity; mannose-binding lectin, yam**

INTRODUCTION

Lectins are carbohydrate-binding proteins that are widespread in the biosphere and occur in almost every living organism. The defensive role of plant lectins, such as anti-insect, antifungal, antimicrobial, as well as being toxic to birds and mammals, has been proposed on the basis of indirect and direct evidence (*1–8*). The interaction between lectins and the intestinal tract is considered to be a prerequisite for insecticidal action (*8–10*). During the past decades, most trials to assess the insecticidal action of lectins as a potential biological pesticide have focused on D-mannose (Man)-binding lectin, named GNA, from snowdrop (*Galanthus nivalis*) bulb (*3, 11, 12*). GNA has been shown to be insecticidal to a range of economically important pests. Initial experiments, in which various insects were fed with artificial diets supplemented with GNA, were followed by transgenic plants containing GNA gene. Such an approach gave promising results on increased plant resistance evaluated against aphids, plant hoppers, and moths (*12–16*).

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a very destructive polyphagous pest occurring on cotton, tomato, chickpea, pigeon-pea, chilli, maize, sorghum, and many other crops, inflicting substantial crop losses every year (*17*). The ability of insect species to thrive on diverse host plants is an adaptive advantage for their better survival in the ecosystem. *H. armigera* is also characterized by its high mobility and fecundity. Exogenous chemical means to counteract an *H. armigera* attack have become less feasible, mainly due to the development of pesticide resistance and inherent possible environmental hazards (*18*).

Yam is an important staple crop, which constitutes about 500 species of the genus *Dioscorea* of the Dioscoreaceae family. Yam has also been considered as herbal medicines in East Asia.

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For example, dioscorin, the storage protein of yam tuber, has been shown to have various beneficial effects on health, such as antioxidative activity (*19*) and angiotensin converting enzyme inhibitory activity (*20*). We isolated and characterized four major proteins designated DB1, DB2, DB3, and DB4 from the yam tuber *Dioscorea batatas* (*21*). The ratios of their yields were 20:50:20:10. DB1 is a mannose-binding lectin (20 kDa) consisting of 10-kDa subunits. DB2 and DB3 are maltose-binding lectins, which are composed of two kinds of dioscorin-like storage proteins (31 kDa) with 72% sequence identity, and they could not be classified into any of the known plant lectin families. DB4 (28 kDa) is a Chitinase.

In this study, we analyzed the amino acid sequence and sugar binding specificity of DB1 and its insecticidal activity toward *Helicoverpa* species. DB1 is composed of two isoforms DB1(Cys86) and DB1(Leu86), consisting of 108 amino acid residues with 90% sequence homology between them. DB1 was homologous to GNA in terms of the amino acid sequence and sugar binding specificity, and it showed insecticidal activity on Lepidoptera pests.

MATERIALS AND METHODS

Materials. The tubers of *Dioscorea batatas* were harvested in Aomori prefecture, Japan, and stored at 4 °C until use. Endoproteinase Lys-C was purchased from Wako Chemical (Osaka, Japan). Soybean lectin (SBA) was isolated from soybean (*Glycine max* var. Miyagishirome) flour by affinity chromatography using cross-linked guar gum as described (*22*). Fluorescein isothiocyanate I (FITC) was purchased from Sigma (St. Louis, MO). Pyridylaminated oligosaccharides were obtained from Takara Bio (Kyoto, Japan) and Seikagaku Kogyo (Tokyo, Japan), and their structures were numbered as described (*23*).

FITC labeled casein was prepared as follows: casein (Nacalai Tesque, Kyoto, Japan) (10 mg) and FITC (4 mg) were dissolved in 2 mL of 0.1 M sodium carbonate buffer (pH 9.0) containing 8 M urea and left for 3 h at 20 °C. FITC labeled casein was separated by gel chromatography on a Sephadex G-25 column (10 mL) equilibrated with 10 mM phosphate buffered-saline (pH 7.5) (PBS). The visible FITC-casein fraction was pooled, desalted by dialyzing against distilled water, and lyophilized.

Preparation of DBs and anti-DBs Antisera. DBs were isolated from the tubers of *Dioscorea batatas* by hydrophobic chromatography and anion-exchange chromatography successively as described (*21*). Anti-DB1 and anti-DB3 antisera were raised in rabbits by repeated injection of the lectins emulsified with Freund's complete adjuvant. The titer and specificity of antibodies were estimated by double immunodiffusion analysis (Ouchterlony test) on agar gels (*24*) and enzyme-linked immunosorbent assay. After three booster injections, antisera were collected and stored at −20 °C.

Amino Acid Sequencing of DB1. DB1 was reduced with 10 mM dithiothreitol in 0.25 M Tris-HCl buffer (pH 8.6) containing 10 mM EDTA and 6 M guanidine hydrochloride at 37 °C for 2 h, and it was reacted with 20 mM iodoacetamide for 20 min at room temperature in the dark. After removing excess reagent by dialysis against distilled water, the solution was lyophilized. Reduced and carboxamidomethylated (CAM-) DB1 was digested with endoproteinase Lys-C (S/E = 100:1) and endoproteinase Arg-C (Takara Bio) (S/E = 100:1). Each digest was separated by reversed-phase HPLC on a 250 mm × 4.6 mm i.d. TSKgel ODS 120T column (Tosoh, Tokyo, Japan) using a linear gradient increase of acetonitrile in 0.1% trifluoroacetic acid (TFA). The amino acid sequences of isolated peptide fragments were determined by the combined use of a PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan) and a matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) Voyager-DE STR mass spectrometer (Applied Biosystems) as previously described (*25*). Homologous sequences were searched by the BLAST program.

cDNA Sequencing of DB1. Oligonucleotide primers (DB1F/DB1R) specific to DB1 were designed on the basis of the amino acid sequence of DB1 to amplify cDNA fragments of the lectins by means of RT-PCR as follows (F and R indicate sense and antisense primers, respectively): DB1F, 5′-TAYGAYAAYGGNAARGCNATHTGGGC-3′; DB1R, 5′-GCNGCNCCRTADATNACNACRTT-3′. Total RNA was extracted using Concert Plant RNA reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Poly $(A)^+$RNA was purified with a Micro-Fast Track mRNA Isolation kit (Invitrogen) and reverse transcribed with oligo dT primer using an Access Quick RT-PCR system (Promega, Madison, WI). PCR was done with a combination of DB1F and DB1R primers and first strand cDNA as template on a PCR express. DNA was denatured at 95 °C for 2 min, followed by three step cycles (35 cycles)—95 °C for 0.5 min, 45 °C for 0.5 min, 72 °C for 1 min—further extension at 72 °C for 5 min. The amplified DNA fragment (0.6 kbp) generated by PCR with DB1F/DB1R specific primers was subcloned into the pCR-Blunt II TOPO vector (Invitrogen). The flanking regions of cDNA encoding DB1 were obtained by the 5′- and 3′-RACE method using the Marathon cDNA Amplification kit (Clonetech, Palo Alto, CA, USA). Further, to obtain the full sequence of cDNAs encoding DB1 and related proteins, PCR was carried out by using primer set, DB1-5S (5′-CTCCTCTTTGCTGCATGGCC-3′) and DB1-3AS (5′-GAAAGGCATAGGATGCATTCACA-3′) and KOD plus polymerase (Toyobo Co., Tokyo, Japan). DNA was sequenced on an ABI DNA sequencer (Model 377) by cycle sequencing using T7, SP6, and M13 forward (−20) primers and the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech). The nucleotide sequence data reported in this article are available from DDBJ, EMBL, and Genbank databases with accession numbers of AB178474 and AB178475 for DB1(Cys68) (DB1-1) and DB1(Leu68) (DB1-4), respectively.

Sugar Binding Specificity. DB1 was coupled to NHS-activated Sepharose 4FF (Amersham Biosciences) according to the manufacturer's instructions. The DB1-Sepharose was packed into a capsule-type 10 mm × 2 mm i.d., 31 μL, miniature column. Frontal affinity chromatography (FAC) was performed using an automated system (FAC-1) as described previously (*23*). The DB1 column was equilibrated with running buffer consisting of 10 mM Tris-HCl (pH 7.4) and 0.8% NaCl. The flow rate and column temperature were kept at 0.125 mL/min and 25 °C, respectively. Pyridylaminated oligosaccharides (2.5 nM) dissolved in the running buffer were successively injected into the column by an autosampling system. Elution of pyridylaminated oligosaccharides was monitored by measuring fluorescence (E_x/E_m = 310/380 nm). Relative binding affinity was compared by the values of $V - V_0$, where V and V_0 were the elution volumes of analyte and a control substance, respectively.

Insect Feeding Trials. Larval cultures of *Helicoverpa armigera* and *H. assulta* were obtained from Zennoh (Hiratsuka, Kanagawa, Japan) and JA (Oyama. Tochigi, Japan), respectively, and reared on an artificial diet, Insecta LFS (Nosan, Yokohama, Japan), at 24 ± 1 °C under a L16:D8 light regime. Third instar larvae of *H. armigera* and *H. assulta* were reared on artificial diets containing lectins (DB1, DB2/DB3/DB4 (5:2:1), or SBA) at concentrations of 0.01%. Fifteen larvae were used per treatment. The control diet was supplemented with an equivalent weight of casein instead of lectins. Insect survival was estimated daily, and the weights of pupae were measured.

Gut Enzyme Assays. Midguts were dissected from the fifth instar larvae and stored at −80 °C until use. The gut tissue was mixed with 3 volumes of 0.1 M glycine−NaOH buffer (pH 10.0) and kept for 15 min on ice to extract proteases. The gut luminal contents were recovered by centrifugation at 10,000*g* for 10 min at 4 °C (*26*). The resulting supernatant was preincubated with 15−150 μM DB1 for 15 min at 37 °C. The protease solution (15 μL) was added to 40 μL of FITC−casein (1 μg/mL) in 0.1 M glycine−NaOH buffer (pH 10.0) and incubated for 1 h at 37 °C. The reaction was stopped by adding 5 μL of 60% trichloroacetic acid. The solution was mixed with 0.2 mL of 0.2 M Tris-HCl buffer (pH 9.0) containing 0.5% sodium dodecylsulfate (SDS) and 0.02% NaN_3. The fluorescence polarization of the solutions was measured for protease activity with a Beacon 2000 (Takara Bio) with E_x = 490 nm and E_m = 520 nm (*27*).

Midguts dissected from the fifth instar larvae were homogenized in cold distilled water (1 mL/gut) and centrifuged at 10,000*g* for 5 min at 4 °C. The supernatant was collected and used for an amylase assay by using a glucose B test kit (Wako Chemical) with soluble potato starch

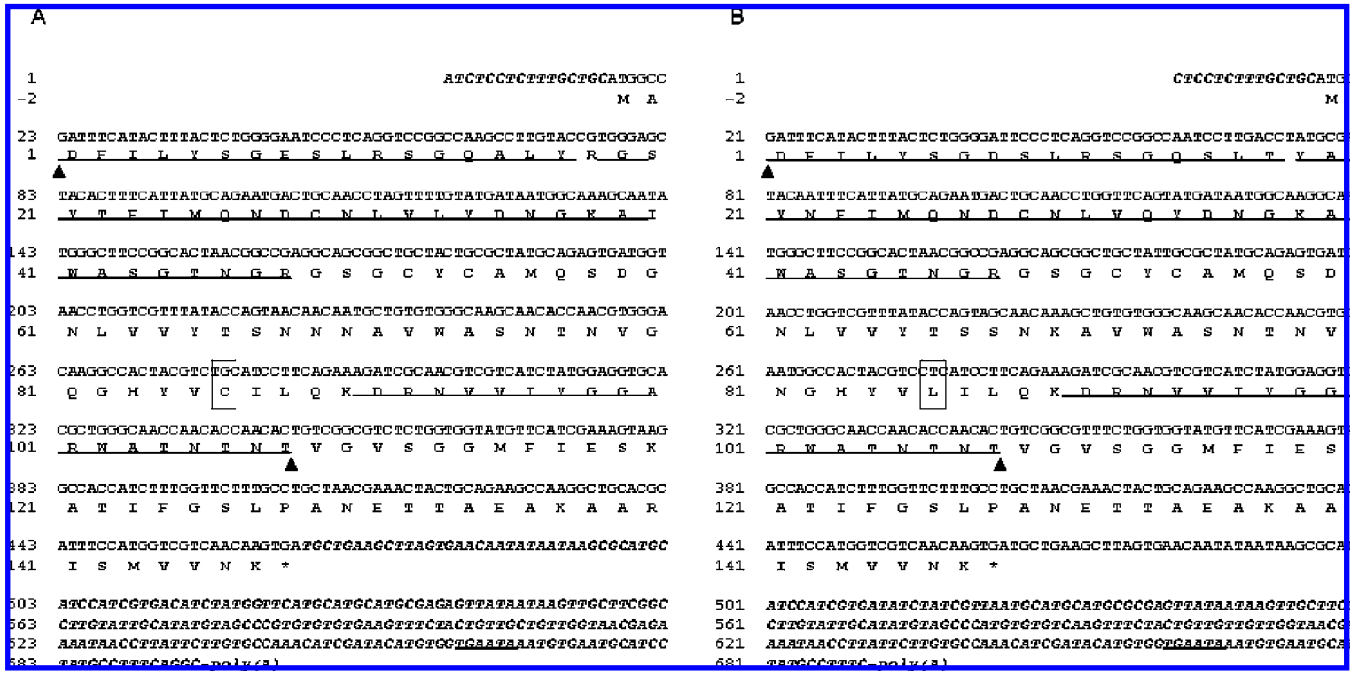

Figure 1. Nucleotide and amino acid sequences of DB1(Cys86) and DB1(Leu86). (**A**) DB1(Cys86). (**B**) DB1(Leu86). Nucleotides and amino acid residues are numbered on the side. Dotted underlines represent amino acid sequences determined by analysis of the peptide fragments derived from enzymatic digestions. The putative processing sites for the signal sequence and the C-terminal extension are indicated by arrowheads.

(Wako Chemical) as substrate in 0.1 M sodium phosphate buffer (pH 5.8) containing 20 mM NaCl and 0.1 mM $CaCl_2$. The midgut extracts (10 μL) were preincubated with 0−150 μM DB1 or DB3 at 37 °C for 15 min prior to the addition of 10 μL of 1% soluble starch solution. After 1-h incubation, the reaction was stopped by the addition of 0.3 mL of coloring reagent followed by developing color by placing the reaction tubes at 37 °C for 20 min. The absorbance was measured at 510 nm.

Digestibility of Lectins in Guts. Newly enclosed fifth instar larvae were exposed to the control diet or an artificial diet containing DB1 or DB3 at a concentration of 0.5% (2.5 mg of DB1 or DB3 per 0.5 g dry weight of diet) for 48 h at 24 ± 1 °C. The control diet was supplemented with an equivalent weight of casein. Gut tissues from larvae flushed free of gut contents prior to homogenization and feces were extracted with 2% SDS and 20% glycerol in 0.2 M Tris-HCl buffer (pH 6.8) for 2 h at room temperature. The supernatant obtained by centrifugation (12,000*g* for 5 min) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (*28*). Following electrophoresis, proteins in the gels were transferred onto polyvinylidene difluoride (PVDF) membranes by semidry electroblotting (*29*). The membrane was incubated in blocking buffer (50 mM Tris-HCl buffer, pH 7.5, containing 5% skim milk and 0.15 M NaCl) for 1 h at 37 °C. The membrane was incubated with anti-DBs antisera (1:10,000 dilution) in blocking buffer for 1.5 h at 37 °C and washed three times with 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20 (TBS-T). It was subsequently incubated with peroxidase-conjugated antirabbit IgG goat antibody (Jackson Immunoresearch Laboratories, West Baltimore, PA) (1:1,000 dilution) for 1.5 h at 37 °C and washed three times with TBS-T. Blots were developed with 0.025% 3,3′-diaminobenzidine (DAB) and 0.01% H_2O_2 in 50 mM Tris-HCl buffer (pH 7.5).

The quantitative analysis of DB1 in midgut was carried out by dot-blotting. Aliquots of gut proteins (~30 μg) were dot-blotted onto PVDF membranes using a dot-blot apparatus (Bio-Rad). The membrane was incubated in blocking buffer for 1 h at 37 °C. The membrane was then incubated with anti-DB1 antiserum (1:10,000 dilution) in blocking buffer for 1.5 h at 37 °C, washed three times with TBS-T, subsequently incubated with the secondary antibody conjugate (1:1,000 dilution) for 1.5 h at 37 °C, and washed three times with TBS-T. Bound secondary antibody was detected using the BM chemiluminesence blotting substrate (POD) kit (Boehringer Mannheim, Germany). A series of DB1 standards were used to calibrate the dot-blot, and quantitative densitometry on LAS 1000 (Fujifilm, Tokyo, Japan) was performed to integrate the densities of individual spots for both standards and samples.

Localization of Lectin in Guts. Newly enclosed fifth instar larvae were exposed to control diet or artificial diet containing DB1 or DB3 at a concentration of 0.5% for 48 h at 24 ± 1 °C. Insects were fixed by 4% paraformaldehyde in PBS for 48 h at room temperature. The specimens were dehydrated through a graded ethanol, cleared through toluene, and embedded in paraffin.

The dewaxed and rehydrated 4-μm sections were treated with 10% hydrogen peroxide for 10 min for blocking endogenous peroxidases. They were rinsed three times in PBS and incubated with 3% normal goat serum for 20 min and then with anti-DB1 or anti-DB3 antisera (1:4000 dilution) for 30 min at room temperature in a humidity chamber. After washing with PBS, the sections were incubated with biotinylated antirabbit goat IgG solution (1:200 dilution) for 30 min. They were washed with PBS and incubated with avidin−biotinylated peroxidase complex (ABC) reagent (VECTASTAIN Elite ABC Kit) (Vector Laboratories, CA) for 30 min. The tissue-bound peroxidase was visualized with 0.025% DAB and 0.01% H_2O_2 in PBS. The sections were washed in running tap water for 5 min and then counterstained with Mayer's hematoxylin. To test the specificity of immunostaining, negative controls were run without the primary antiserum and with antibodies preabsorbed with DB1 or DB3.

RESULTS

Amino Acid Sequence of DB1. The amino acid sequence of DB1 was determined by both Edman degradation and cDNA sequencing as summarized in **Figure 1**. DB1 was found to be composed of two isoforms, DB1(Cys86) and DB1(Leu86), consisting of 108 amino acid residues with molecular masses calculated to be 11,807 Da and 11,779 Da, which were in good agreement with the values (11,813 Da and 11,785 Da) obtained from MALDI-TOF mass spectrometry, respectively. Two isoforms had 90% sequence identity. DB1(Cys86) and DB1(Leu86) contained four and three half-cystine residues, respectively. The cDNA of DB1(Cys86) included 696 nucleotides with an open reading frame of 447 nucleotides encoding for a protein of 149

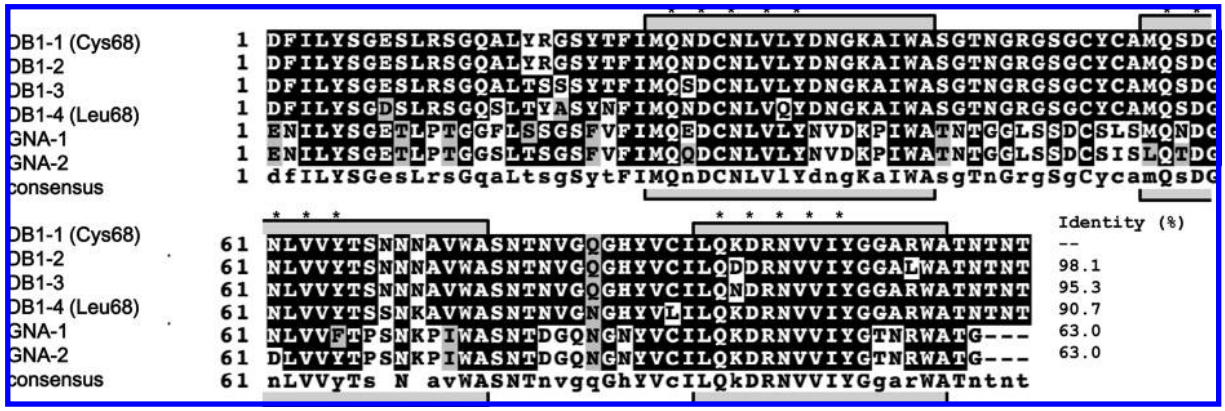

Figure 2. Aligned amino acid sequences of DB1s and snow drop lectins (GNA-1 and GNA-2). Carbohydrate recognition domains (CRD) are in the boxes, and the conserved amino acid residues in CRD are indicated by asterisks.

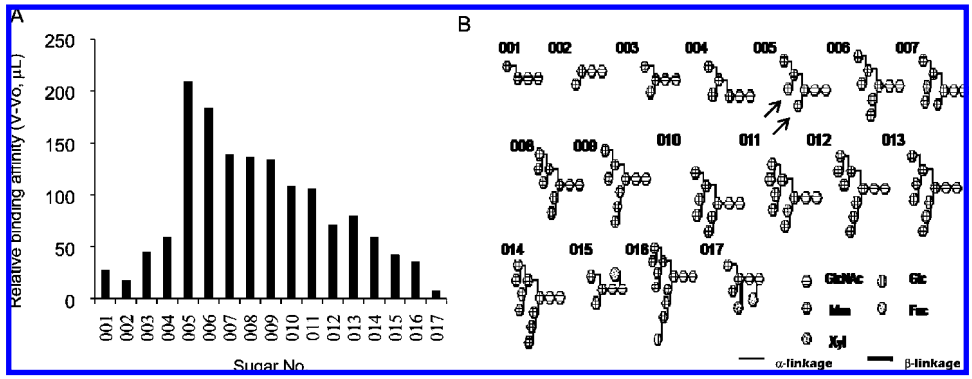

Figure 3. Sugar-binding specificity of DB1 analyzed by frontal affinity chromatography. (**A**) Bar graphs indicate relative binding affinity, which is compared by the values of $V - V_0$, where V and V_0 are the elution front volumes of analyte and a control sugar, respectively. The structures of pyridylaminated oligosaccharides assigned by Arabic figures are shown in part **B**. (**B**) Schematic representation of pyridylaminated oligosaccharides. The reducing terminals are pyridylaminated. Symbols represent pyranose rings of monosaccharides. Thin and bold lines indicate α- and β-linkages, respectively. The positions of Man(α1-3)Man units are indicated by arrows in sugar No. 005 as an example.

amino acid residues without a signal peptide sequence. The stop codon at nucleotide position 448 was followed by a polyadenylation signal AATAAA, starting at position 664 (**Figure 1A**). The cDNA of DB1(Leu86) included 689 nucleotides with an open reading frame of 447 nucleotides encoding for a protein of 149 amino acid residues. The stop codon at nucleotide position 462 was followed by a polyadenylation signal AATAAA, starting at position 662 (**Figure 1B**). It should be noted that the C-terminal amino acid sequence, Val-Gly-Val-Ser-Gly-Gly-Met-Phe-Ile-Glu-Ser-Lys-Ala-Thr-Ile-Phe-Gly-Ser-Leu-Pro-Ala-Asn-Glu-Thr-Thr-Ala-Glu-Ala-Lys-Ala-Ala-Arg-Ile-Ser-Met-Val-Val-Asn-Lys, which was deduced from the cDNA sequence, could not be detected in any digest prepared with various proteases, indicating that the C-terminal region of DB1 had been excised by processing proteases to produce mature proteins. Furthermore, cDNA clones encoding the polymorphic variant of DB1, DB1-1 to DB1-4, were obtained by PCR with high-fidelity KOD polymerase and a specific primer set for DB1, DB1-5′S, and DB1-3′AS. DB1-1 (corresponding to DB1(Cys86)), DB1-2, and DB1-3 were Cys86-type isolectins, while DB1-4 (corresponding to DB1(Leu86)) was Leu86-type (**Figure 2**). In DB1-2, only two amino acid residues, Lys (AAA) at position 90 and Arg(CGC) at position 101, of DB1-1 were replaced by Asp (GAT) and Leu (CTC), respectively, while Tyr17-Arg18-Gly19 (TAC CGT GGG), Asn27 (AAT), and Lys90 were replaced by Thr-Ser-Ser (ACC TCT TCG), Ser (TCT), and Asn (AAC), respectively, in DB1-3 (**Figure 2**).

Sugar Binding Specificity. The binding specificity of DB1 against oligosaccharides was examined by frontal affinity chromatography using 100 oligosaccharides. DB1 showed specific binding affinity for high-mannose-type *N*-glycans (**Figure 3**) but no affinity for agalacto-type, galactosyl-type, sialylated-type, and glycolipid-type glycans (data not shown). In particular, the lectin interacted strongly with the oligosaccharides carrying two Man(α1-3)Man units (sugar Nos. 005 and 006), whereas the binding affinity decreased by incorporating two Man(α1-2)Man units (sugar Nos. 008−014).

Effect of Lectins on Larval Development and Survival. Third instar larvae were reared on an artificial diet containing lectins (DB1, DB2/DB3/DB4, SBA) at a concentration of 0.01%. In a bioassay of the *H. armigera* from third instar to adult, the rates of adults successfully emerging from pupae fed on DB1 or DB2/DB3/DB4 were 33% and 47%, respectively (**Figure 4A**). These rates were much lower than those of control insects (67%). Final instar larvae and pupae, fed on DB1 or DB2/DB3/DB4, also showed lower surviving rates than control insects. On the other hand, SBA had no effect on the rates.

For *H. assulta* from third instar to adult, SBA had an insecticidal activity as well as DB1. The rates of pupae emerging from larvae and adult were 40% and 33%, respectively (**Figure 4B**). The control insects showed higher emerging rates (80% and 87%). The rates of pupation and hatch of the insects fed on DB1 were 73% and 40%, respectively. The number of adults significantly decreased. *H. assulta* usually advances from the egg to pupation in 30 days, with 5 larval stages. The insects fed on SBA emerged seventh instar larvae (13%). Days to reach pupation and pupae period, and pupae weight were not significantly different among each treatment on both insects (**Table 1**).

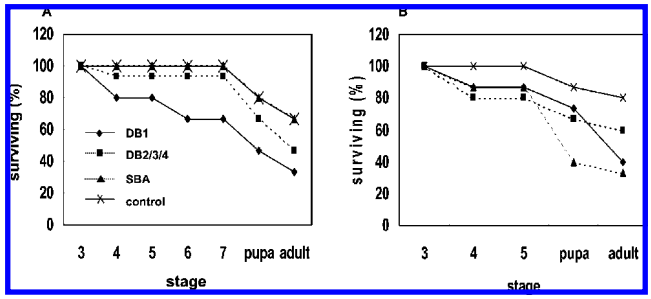

Figure 4. Effect of yam lectins on survival and development of *H. armigera* (**A**) and *H. assulta* (**B**) when incorporated into an artificial diet at 0.01%. Insects were newly emerged third instar larvae at the start of the assay.

Table 1. Effect of Lectins on Larval Development of *H. armigera* and *H. assulta*[a]

	days to reach pupation	pupae period (days)	pupae weight (mg)
	(**A**) *H. armigera*		
DB1	21.9 ± 1.1	13.4 ± 0.8	286.8 ± 5.4
DB2/3/4	207 ± 1 5	139 ± 0 7	2831 ± 6 4
SBA	19.5 ± 0.5	13.5 ± 0.5	314.4 ± 25.4
control	20.0 ± 0.6	13.7 ± 0.3	314.4 ± 25.4
	(**B**) *H. assulta*		
DB1	213 ± 2 0	139 ± 0 3	220.7 ± 9.1
DB2/3/4	20.4 ± 2.2	14.8 ± 0.5	239.3 ± 11.6
SBA	18.2 ± 1.5	13.8 ± 0.7	224.4 ± 18.1
control	16.7 ± 0.5	12.9 ± 0.5	217.2 ± 15.7

[a] Values are means ± standard deviation, $n = 15$.

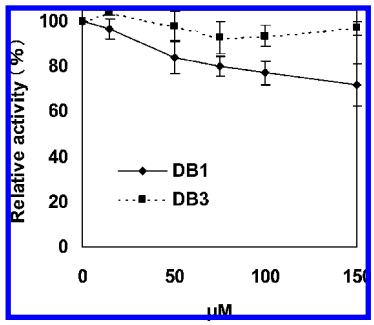

Figure 5. Effect of yam lectins on the amylase activity of midgut extracts from *H. armigera*. Values are means ± standard deviations, $n = 5$.

Effect of Lectins on Proteolytic and Amylase Activity of Insect Midgut. The proteolytic activity of the midgut extracts from fifth instar *H. armigera* was not affected by DB1 at a concentration of 150 μM (data not shown). On the other hand, DB1 showed a weak inhibitory activity (28% at 150 μM) against *H. armigera* amylase (**Figure 5**). DB3 showed no inhibitory activity.

Digestibility of Lectins in Guts. The extracts of midgut from larvae exposed to lectins for 48 h were analyzed by Western blotting after SDS-PAGE. DB1 was detected with the midgut extract (**Figure 6A**), whereas DB3 could not be found (**Figure 6B**), indicating that DB1 bound to midgut wall and/or peritrophic matrix in an intact form. The amount of DB1 bound to midgut was estimated to be 1.03 ± 0.29 μg/midgut by dot-blotting analysis. Both DB1 and DB3 were found in feces with accompanying degraded fragments.

Localization of Lectins in Guts. Immunohistochemical localization of DB1 and DB3 was carried out in the guts of fifth instar larvae fed an artificial diet containing DB1 or DB3 (2% of total dietary protein) for 48 h. DB1 was detected predominantly in the brush border region and the peritrophic

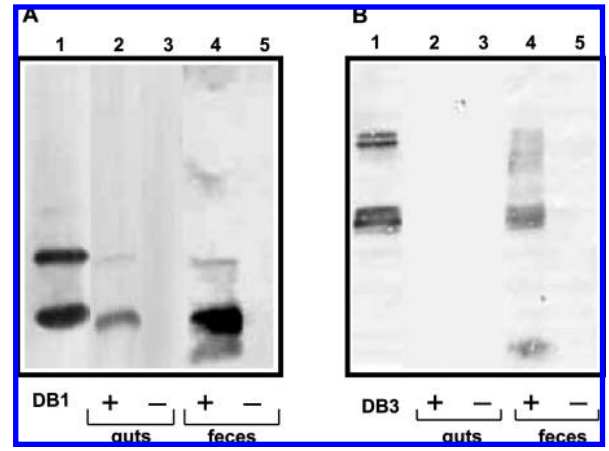

Figure 6. Western-blot analysis of gut and feces of insects fed on artificial diets containing DB1 or DB3. Gut and feces samples were subjected to SDS-PAGE and semidry blotting onto PVDF membranes. The membranes were immunostained with anti-DB1 (**A**) or anti-DB3 (**B**). Lane 1, DB1 or DB3 (100 ng); lane 2, gut extracts from insects fed on a DB1 or DB3 containing diet; lane 3, gut extracs from insects fed on an artificial diet without lectin; lane 4, feces extract from insects fed on a DB1 or DB3 containing diet; lane 5, feces extract from insects fed on an artificial diet without lectin.

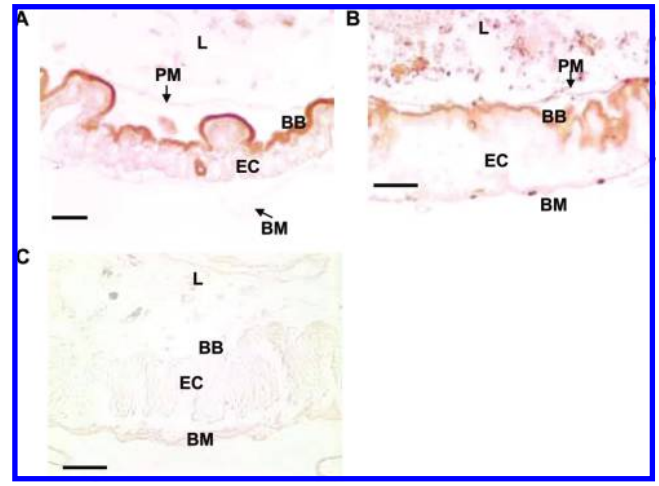

Figure 7. Localization of yam lectins in midgut sections from *H. armigera* fed on an artificial diet containing DB1 or DB3. (**A**) Midgut section from *H. armigera* larvae fed on a DB1 containing diet. (**B**) Midgut section from *H. armigera* larvae fed on a DB3 containing diet. (**C**) Midgut section from *H. armigera* larvae fed on a control diet. BB, brush border; EC, epithelial cells; PM, peritrophic membrane; BM, basement membrane; L, lumen. Scale bars are 50 μm.

membrane of larval midgut tissues (**Figure 7A**). The basement membrane and the epithelial region were slightly stained. DB3 was also detected predominantly in the brush border region of larval midgut (**Figure 7B**). In contrast, the control section showed no staining with anti-DB1 or anti-DB3 antibodies (**Figure 7C**).

DISCUSSION

DB1 accounted for 20% of the total tuber protein in yam (Dios*corea batatas*, synonym *Dioscorea polystachya*) (*21*). DB1 was composed of two isoforms, DB1(Cys86) and DB1(Leu86), both of which consisted of 108 amino acid residues, though the cDNA sequencing predicted a protein of 147 amino acid residues. This fact indicates that post-translational processing

resulted in the removal of a C-terminal segment of 39 amino acid residues (3984 Da). A similar post-translational processing has been reported for other plant lectins including GNA (*30*). The two isoforms, both of which formed dimers with or without a disulfide bond, could not be separated by conventional chromatography under nondenaturing conditions; thus, DB1 was used for sugar-binding specificity and insecticidal assay without separating the isoforms.

DB1 was classified into the monocot mannose-binding (GNA-related) lectin family and had a high sequence homology to GNA. X-ray crystal structure analysis of GNA in complex with D-mannose-containing sugars revealed the molecular basis for the specific mannose-binding abilities of the GNA-related lectin family (*31*, *32*). GNA monomer, which consists of a single polypeptide chain of 109 amino acid residues, forms a β-prism fold including the three bundles composed by four-stranded β-sheets and connecting loops. Each bundle comprises a subdomain (I, II, and III), corresponding to the carbohydrate-binding site. The mannose-binding sites of GNA consist of five conserved residues (Gln26, Asp28, Asn30, Val32, and Tyr34 in subdomain I, Gln57, Asp59, Asn61, Val63, and Tyr65 in subdomain II, and Gln89, Asp91, Asn93, Val95, and Tyr97 in subdomain III), which connect to mannose by a network of hydrogen bonds and hydrophobic interactions. In the case of DB1, these key amino acid residues were also conserved in all three subdomains (**Figure 2**).

GNA contains three half-cystine residues at positions 29, 52, and 86, with one intrachain disulfide bond formed between Cys29 and Cys52 (*30*). On the other hand, DB1(Cys86) contained four half-cystine residues at the positions of 29, 52, 54, and 86, while DB1(Leu86) contained three half-cystine residues (**Figure 2**). Based on the structural similarity between GNA and DB1, Cys29 and Cys52 of DB1 may form an intrachain disulfide bond. Since the free SH group could not be detected in DB1, Cys54 and Cys86 may form an intra- or interchain disulfide bond. In fact, DB1 containing an interchain disulfide bond can be observed as a dimer on SDS-PAGE under nonreducing conditions (**Figure 6A**). The dimer seems to be more susceptible to protease digestion, since it disappeared quickly in guts.

Frontal affinity chromatography analysis for binding specificity of DB1 showed the binding affinity of DB1 for high-mannose-type *N*-glycans, whereas the binding affinity decreased by incorporating two Man(α1−2)Man units carrying two Man(α1−3)Man units (**Figure 3**). DB1 preferred Man(α1−3)Man compared to Man(α1−2)Man, and its affinity was increased for sugars having more than one Man(α1−3)Man. Recently, the relationship between the structure and the carbohydrate-binding specificity of two-domain GNA-related lectins including *Arum maculatum* agglutinin (AMA) and *Tulipa* hybrid lectin-I (TxLC-I) was studied in detail using glycan arrays and frontal affinity chromatography (*33*). AMA, of which five key residues corresponding to Gln26, Asp28, Asn30, Val32, and Tyr34 in subdomains I and II were substituted, showed specificity toward both high-mannose-type and complex-type *N*-glycans, while TxLC-I recognized a broad range of complex-type *N*-glycans. Compared with AMA, DB1 showed slightly but distinct different profiles against high-mannose-type *N*-glycans. DB1 and AMA showed different affinities for glycans 007, 010, 011, 013, and 014, although both lectins exhibited a much higher affinity for glycans 005, 006, 008, and 009. These results suggest that DB1 can widely recognize the high-mannose-type *N*-glycans containing α1-2Man residues at the nonreducing end of Man(α1-3)Man(α1-6)Man, while AMA did not interact. X-ray crystal structure analysis of GNA in complex with Man(α1-3)Man revealed that two charged amino acid residues, Asp37 and Lys38, contributed the interaction with mannose residues besides the five conserved residues (*32*). In DB1, however, Asp37 was substituted with Gly, while the Lys38 residue was conserved (**Figure 2**).

GNA has been extensively studied in terms of its insecticidal properties to a range of economically important pests (*12*–*16*). In the present study, DB1 showed insecticidal activity when incorporated in an artificial diet at 0.01%, resulting in 33% survival rate of larva and pupae, whereas the rate of the control was 67%. The precise mechanism how the lectin exerts the activity has not been fully elucidated. The DB1 seems to be susceptible to gut protease, as shown in **Figure 4A**. DB1 did not affect the protease activity of insect guts, whereas the amylase activity decreased by 28% when exposed to a high concentration of lectin (150 μM). Binding of lectins to the midgut epithelium or the peritrophic membrane has been shown to be required for expressing insecticidal activity, though the binding may not always be attributed to direct damage of gut epithelia (*9*, *34*–*37*). The insecticidal activity of DB1 may be attributed to the disruption of membrane function, such as transport and/or digestion of nutrients.

The yam tubers of *D. batatas* are stored for a year after harvesting, despite its high water content, and are generally consumed without cooking in Japan. The good preservability may be correlated with the presence of defense-related proteins such as DB1.

ABBREVIATIONS USED

DB1, *Dioscorea batatas* tuber lectin 1; FITC, fluorescein isothiocyanate I; GNA, snowdrop (*Galanthus nivalis*) bulb lectin; CAM-, carboxamidomethylated; PBS, 10 mM phosphate buffered-saline (pH 7.5); PVDF, polyvinylidene difluoride; SBA, soybean lectin; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBS-T, 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20; TFA, trifluoroacetic acid.

Supporting Information Available: Schematic representation of oligosaccharides used in this study. The reducing terminals are pyridylaminated. Symbols used to represent pyranose rings of monosaccharides are shown in the box at the bottom of the figure. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Peumans, W. J.; Van Damme, E. J. M. Lectins as plant defense proteins. *Plant Physiol.* **1995**, *109*, 347–352.
(2) Rudiger, H.; Gabius, H. J. Plant lectins: Occurrence, biochemistry, functions and applications. *Glycoconjugate J.* **2001**, *18*, 589–613.
(3) Carlini, C. R.; Grossi-de-Sá, M. F. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon* **2002**, *20*, 1515–1539.
(4) Vasconcelos, I. M.; Oliveira, J. T. A. Antinutritional properties of plant lectins. *Toxicon* **2004**, *44*, 1737–1747.
(5) Macedo, M. L. R.; Damico, D. C. S.; Freire, M. G. M.; Toyama, M. H.; Marangoni, S.; Novello, J. C. Purification and characterization of an N-acetylglucosamine-binding lectin from Koelreuteria paniculata seeds and its effect on the larval development of Callosobruchus maculatus (Coleoptera: Bruchidae) and Anagasta kuehniella (Lepidoptera: Pyralidae). *J. Agric. Food Chem.* **2003**, *51*, 2980–2986.
(6) Boleti, A. P. A.; Freire, M. G. M.; Coelho, M. B.; Silva, W.; Baldasso, P. A.; Gomes, V. M.; Marangoni, S.; Novello, J. C.; Macedo, M. L. R. Insecticidal and antifungal activity of a protein

from Pouteria torta seeds with lectin-like properties. J. Agric. Food Chem. **2007**, *55*, 2653–2658.
(7) Moura, F. T.; Oliveira, A. S.; Macedo, L. L. P.; Vianna, A. L. B. R.; Andrade, L. B. S.; Martins-Miranda, A. S.; Oliveira, J. T. A.; Santos, E. A.; Sales, M. P. Effects of a chitin-binding vicillin from Enterolobium contortisiliquum seeds on bean bruchid pests (Callosobruchus maculatus and Zabrotes subfasciatus) and phyropathogenic fungi (Fusarium solani and Colletrichum lindemuntianum). J. Agric. Food Chem. **2007**, *55*, 260–266.
(8) Majumder, P.; Mondal, H. A.; Das, S. Insecticidal activity of Arum maculatum tuber lectin and its binding to the glycosylated insect gut receptors. J. Agric. Food Chem. **2005**, *53*, 6725–6729.
(9) Habibi, J.; Backus, E. A.; Huesing, J. E. Efffect of phytohemagglutinin (PHA) on the structure of the midgut epithelial cells and localization of its binding sites in western tarnished plant bug, *Lygus Hesperus* Knight. J. Insect Physiol. **2000**, *46*, 611–619.
(10) Fitches, E.; Woodhouse, S. D.; Edwards, J. P.; Gatehouse, J. A. In vitro and in vivo binding of snowdrop (Galanthus nivalis agglutinin; GNA) and jackbean (Canavalia ensiformis; Con A) lectins within tomato moth (Lacanobia oleracea) larvae; mechanisms of insecticidal action. J. Insect Physiol. **2001**, *47*, 777–787.
(11) Van Damme, E. J. M.; Allen, A. K.; Peumans, W. J. Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (Galanthus nivalis) bulbs. FEBS Lett. **1987**, *215*, 140–144.
(12) Hilder, V. A.; Powell, K. S.; Gatehouse, A. M. R.; Gatehouse, J. A.; Gatehouse, L. N.; Shi, Y.; Hamilton, W. D. O.; Merryweather, A.; Newell, C. A.; Timans, J. C.; Peumans, W. J.; Van Damme, E.; Boulter, D. Expression of snowdrop lectin in transgenic tobacco results in added protection against aphids. Transgenic Res. **1995**, *4*, 18–25.
(13) Down, R. E.; Gatehouse, A. M. R.; Hamilton, W. D. O.; Gatehouse, J. A. Snow drop lectin inhibits development and decreases fecundity of the glasshouse potato aphids (Aulacorthum solani) when administered in vitro and via transgenic plants both in laboratory and glasshouse trials. J. Insect Physiol. **1996**, *42*, 1035–1045.
(14) Bell, H. A.; Fitches, E. C.; Down, R. E.; Marris, G. C.; Edwards, J. P.; Gatehouse, J. A.; Gatehouse, A. M. R. The effect of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on Eulophus pennicornis (Hymenoptera: Eulophidae) a parasitoid of the tomato moth Lacanobia oleracea (Lepidoptera: Noctuidae). J. Insect Physiol. **1999**, *45*, 983–991.
(15) Rao, K. V.; Rathore, K. S.; Hodges, T. K.; Fu, X. D.; Stoger, E.; Sudhakar, D.; Williams, S.; Christou, P.; Bown, D. P.; Powell, K. S.; Spence, J.; Bharathi, M.; Gatehouse, A. M. R.; Gatehouse, J. A. Expression of snowdrop lectin (GNA) in the phloem of transgenic rice plants confers resistance to rice brown planthopper. *Plant J.* **1998**, *14*, 529–539.
(16) Maqbool, S. B.; Riazuddin, S.; Loc, N. T.; Gatehause, A. M. R.; Gatehause, J. A.; Christou, P. Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. Mol. Breeding **2001**, *7*, 85–93.
(17) Armes, N. J.; Jadhav, D. R.; DeSouza, K. R. A survey of insecticide resistance in Helicoverpa armigera in the Indian subcontinent. Bull. Entomol. Res. **1996**, *86*, 499–514.
(18) Ramasubramaniam., T.; Regupathy, A. Pattern of cross resistance in pyrethroid selected populations of Helicoverpa armigera from India. J. Appl. Entomol. **2004**, *128*, 583–587.
(19) Hou, W. C.; Lee, M. H.; Chen, H. J.; Liang, W. L.; Han, C. H.; Liu, Y. W.; Lin, Y. H. Antioxidant activities of dioscori, the storage protein of yam (*Dioscorea batatas* Decne) tuber. J. Agric. Food Chem. **2001**, *49*, 4956–4960.
(20) Hsu, F. L.; Lin, Y. H.; Lee, M. H.; Lin, C. L.; Hou, W. C. Both dioscorin, the tuber storage protein of yam (*Dioscorea alata* cv. Tainong No. 1), and its peptic hydrolysates exhibited angiotensin converting enzyme inhibitory activities. J. Agric. Food Chem. **2002**, *50*, 6109–6113.
(21) Gaidamashvili, M.; Ohizumi, Y.; Iijima, S.; Takayama, T.; Ogawa, T.; Muramoto, K. Characterization of the yam tuber storage proteins from Dioscorea batatas exhibiting unique lectin activities. J. Biol. Chem. **2004**, *279*, 26028–26035.
(22) Ohno, Y.; Naganuma, T.; Ogawa, T.; Muramoto, K. Effect of lectins on the transport of food factors in Caco-2 cell monolayers. J. Agric. Food Chem. **2006**, *54*, 548–553.
(23) Nakamura, S.; Yagi, F.; Totani, K.; Ito, Y.; Hirabayashi, J. Comparative analysis of carbohydrate-binding properties of two tandem-type Jacalin-related lectins, *Castanea crenata* agglutinin and Cycas revolute leaf lectin. FEBS J. **2005**, *272*, 2784–2799.
(24) Munoz, J. Double diffusion in plates. Methods Immunol. Immunochem. **1971**, *3*, 146–160.
(25) Tateno, H.; Saneyoshi, A.; Ogawa, T.; Muramoto, K.; Kamiya, H.; Saneyoshi, M. Isolation and characterization of rhamnose-binding lectins from eggs of steelhead trout (Oncorhynchus mykiss) homologous to low density lipoprotein receptor superfamily. J. Biol. Chem. **1998**, *273*, 19190–19197.
(26) Harsulkar, A. M.; Giri, A. P.; Patankar, A. P.; Gupta, V. S.; Sainani, M. N.; Ranjekar, P. K.; Deshpande, V. V. Successive use of non-host plant proteinase inhibitors required for effective inhibition of Helicoverpa armigera gut proteinases and larval growth. Plant Physiol. **1999**, *121*, 497–506.
(27) Maeda, H. Assay of proteolytic enzymes by the fluorescence polarization technique. Anal. Biochem. **1979**, *92*, 222–227.
(28) Laemmli, U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **1970**, *227*, 680–685.
(29) Lauriere, M. A semidry electroblotting system efficiently transfers both high- and low-molecular weight proteins separated by SDS-PAGE. Anal. Biochem. **1993**, *212*, 206–211.
(30) Van Damme, E. J. M.; Kaku, H.; Perini, F.; Goldstein, I. J.; Peeters, B.; Yagi, F.; Decock, B.; Peumans, W. J. Biosynthesis, primary structure and molecular cloning of snowdrop (*Galanthus nivalis* L.) lectin. Eur. J. Biochem. **1991**, *200*, 23–30.
(31) Hester, G.; Kaku, H.; Goldstein, I. J.; Wright, C. S. Structure of mannose-specific snowdrop (*Galanthus nivalis*) lectin is representative of a new plant lectin family. Nat. Struct. Biol. **1995**, *2*, 472–479.
(32) Hester, G.; Wright, C. S. The mannose-specific bulb lectin from *Galanthus nivalis* (snowdrop) binds mono- and dimannosides at distinct sites. Structure analysis of refined complexes at 2.3 Å and 3.0 Å resolution. J. Mol. Biol. **1996**, *262*, 516–531.
(33) Van Damme, E. J. M.; Nakamura-Tsuruta, S.; Smith, D. F.; Ongenaert, M.; Winter, H. C.; Rouge, P.; Goldstein, I. J.; Mo, H.; Kominami, J.; Culerrier, R.; Barre, A.; Hirabayashi, J.; Peumans, W. J. Phylogenetic and specificity studies on two-domain GNA-related lectins: generation of multispecificity through domain duplication and divergent evolution. Biochem. J. **2007**, *404*, 51–61.
(34) Sauvion, N.; Rahbe, Y.; Peumans, W. J.; Van Damme, E. J.; Gatehouse, J. A.; Gatehouse, A. M. R. Effect of GNA and other binding lectins on development and fecundity of the peach-potato aphid Myzus persicae. Entomol. Exp. Appl. **1996**, *79*, 285–293.
(35) Powell, K. S.; Spence, J.; Bharathi, M.; Gatehouse, J. A.; Gatehouse, A. M. R. Immunohistochemical and development studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal). J. Insect Physiol. **1998**, *44*, 529–539.
(36) Harper, S. M.; Hopkins, T. L.; Czapla, T. H. Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrina nubilalis*) larvae. Tissue Cell **1998**, *30*, 166–176.
(37) Zhu-Salzman, K.; Shade, R. E.; Koiwa, H.; Salzman, R. A.; Narasimhan, M.; Bressan, R. A.; Hasegawa, P. M.; Murdock, L. L. Carbohydrate binding and resistance to proteolysis control insecticidal activity of *Griffonia simplicifolia* lectin II. Proc. Natl. Acad. Sci. U.S.A. **1998**, *95*, 15123–15128.

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