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Preparation of Monoclonal Antibody against Celangulin V and Immunolocalization of Receptor in the Oriental Armyworm, *Mythimna separata* Walker (Lepidoptera: Noctuidae)

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The botanical insecticide celangulin V (CA-V) is an insect digestive poison acting on midgut tissue of the target insect larvae. With the aim of localizing the receptor enacted by CA-V, monoclonal antibodies (MAbs) specific to the compound were developed. A hapten was synthesized by introducing a succinoyl into the CA-V structure and conjugated with three carrier proteins. From mice immunized with one conjugate, three MAbs were obtained with a potential capacity of detecting protein-bound residue forms of CA-V in the biological tissues. The oriental armyworm larvae ingested CA-V were examined by the technique of immuno-electron-microscopy (IEM) using the anti-CA-V MAb as the primary antibody and goat anti-mouse/IgG labeled with colloidal gold as the secondary antibody. Electron micrographs of the armyworm midgut tissues showed that the CA-V was associated with the midgut epithelia of the insects. These results demonstrated the existence of a receptor enacted by CA-V on the midgut cells of the oriental armyworm larvae.

KEYWORDS: Celangulin V; botanical insecticide; monoclonal antibody; hapten; ELISA; receptor; immunolocalization; *Mythimna separata* Walker

INTRODUCTION

Chinese bittersweet, *Celastrus angulatus* Max., a widely distributed plant and a traditional herb medicine in China (*1*), was studied intensively in recent years because of its cytotoxic (*2*), antitumor (*3*), and insecticidal (*3*−*5*) activities. Since the 1980s, Wu et al. isolated a series of sesquiterpene polyol esters with β-dihydroagarofuran skeleton from the bark of the plant (*6*−*10*). These compounds showed obvious anti-insect activities (including antifeedant, narcotic, and mortality) against important agricultural pests (*11*−*13*). Celangulin V (CA-V), one of the insecticidal components, was studied systematically due to its effectiveness against insects. Although significant progresses have been made on its chemical identity, its mechanism of toxicology still remains unclear. Recent symptomatological and anatomical studies suggest that CA-V acts on the midgut of the insects, resulting in a series of symptoms: excitement, paralysis, and body fluid loss. Further investigation on the ultramicrostructure of the midguts of the intoxicant oriental armyworm larvae revealed that the microvilli were disordered, mitochondria swelled irregularly, and the cisternae of rough endoplasmic reticulum were distended (*14*). These results suggested that there might be a putative receptor targeted by CA-V in the midguts of affected insects.

Many natural products are complex and difficult to synthesized in the laboratory. Therefore, the labeling with radioactivity seems to be problematic. For over a decade, scientists have realized that the immune system is a rich source of intriguing and highly efficient catalysts for common organic compounds. Many antibodies, including polyclonal antibodies (PAbs) and monoclonal antibodies (Mabs), are used for testing and marking the compounds (*15*). MAbs, owing to their high homogeneity and specificity, are used extensively in basic biomedical research and have led to many medical advances (*16*). The problems of MAbs production against small molecules had been well-documented and discussed (*17*−*19*). Traditionally, small molecules considered to be lacking immunogenicity should be rationally designed and conjugated with a larger immunogenic protein carrier to elicit antibodies (*20*). Electron microscopy in combination with immunohistochemistry techniques, namely immuno-electron-microscopy, has been widely used in clinical laboratories, as a fast, sensitive, and cost-effective tool for trace analysis. In particular, such techniques are useful in ultrastructural localization and receptor identification (*21*−*25*).

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However, the toxicological mechanism of the CA-V in lepidopteran insects is still not well understood. The objectives of the present study were to develop specific MAbs against CA-V and to identify CA-V receptors in the midgut cells of *Mythimna separata* using the method of IEM.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. All reagents were of analytical grade unless specified otherwise. CA-V (99.3% purity) was prepared by the Institute of Pesticides Science, Northwest Agriculture and Forestry University (NWAFU, Yangling, China). *N*-Hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), Freund's adjuvant, polyethylene glycol 1500 (PEG 1500), goat anti-mouse IgG-horseradish peroxidase (IgG-HRP), goat anti-mouse IgG labeled with colloidal gold nanoparticle (15 nm), tetramethylbenzidine (TMB), Epon812, dodecenyl succinic anhydride (DDSA), methyl nadic anhydride (MNA), 2,4,6-tridimethylamino-methylphenol (DMP-30), paraformaldehyde, glutaraldehyde, and osmium tetroxide (OsO_4) were purchased from Sigma-Aldrich Inc. (Shanghai, China). RPMI 1640 supplements, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and fetal bovine serum (FBS, inactivated at 56 °C for 30 min prior to use) were obtained from Gibco, Co. (Xi'an, China). Normal goat serum (NGS), penicillin G sodium, streptomycin sulfate, and gelatin were purchased from DingGuo Biotechnology (Beijing, China).

The 96-, 24-, 12-, and 6-well polystyrene microplates, flasks, and cryovials for ELISA, cell culture, and freezing were purchased from Nunc. ^{1}H NMR and ^{13}C NMR spectra were recorded on a model AM-400 spectrometer (Bruker) with $CDCl_3$ as the solvent and tetramthylsilane (TMS) as the internal standard. Chemical shifts were recorded in δ (ppm) values. The data of high-resolution mass spectroscopy (HRMS) were recorded under electrospray ionization (ESI) conditions using an APEX II 49e (ESI) instrument (Bruker). Optical rotation was measured by a Perkin-Elmer 241 MC. The ultraviolet absorbance spectra of the protein carriers and hapten−proteins were measured with a Perkin-Elmer Lambda 25 UV/vis spectrometer in the range of 200−400 nm. Infrared spectra of the protein carriers and hapten−proteins were taken on a Nicolet FT-IR-20SX instrument (Thermo Nicolet) using a potassium bromide disk, scanning from 400 to 4000 cm^{-1}. The absorbance of the ELISA was recorded on a Sunrise microplate reader (TECAN). The insect tissue sections were examined and photographed with a JEM-2000EX (JEOL) transmission electron microscope (TEM) at 80 kV.

Media and Buffers. Phosphate-buffered saline (PBS) consisted of 8.0 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4{\cdot}12H_2O$, and 0.2 g of KH_2PO4 in 1000 mL of distilled water, pH 7.4. Carbonate−bicarbonate buffer contained 0.159 g of Na_2CO_3 and 0.293 g of $NaHCO_3$, in 100 mL of distilled water, pH 9.6. Citrate−phosphate buffer was prepared with 24.3 mL of 0.1 M citrate and 25.7 mL of 0.2 M Na_2HPO_4, pH 5.0. Phosphate buffer (PB) was prepared with 19 mL of 20 mM NaH_2PO_4 and 81 mL of 0.2 mM Na_2HPO_4, pH 7.4. RPMI 1640 medium contained 10.4 g of RPMI-1640, 2 g of $NaHCO_3$, 2.38 g of HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine in 1000 mL of pure water (Mini-Q grade). Epon-embedding reagent was a mixture of 9.5 mL of Epon 812, 4.8 mL of DDSA, 5.6 mL of MNA, and 0.3 mL of DMP-30 (air removed with a pump).

Animals and Cell Line. Inbred BALB/c mice, 8-weeks-old, were obtained from the Experimental Animal Center of Fourth Military Medical University (FMMU, Xi'an, China), while Murine myeloma cell line Sp2/0 was provided by the Department of Microbiology of FMMU (Xi'an, China). Larvae of *M. separata* were provided by the Institute of Pesticide Science, NWAFU (Yangling, China). The colony of *M. separata* had been maintained in the laboratory for 15 years under 28 °C, 70% relative humidity, and a photoperiod of 16 h light: 8 h dark with periodic introduction of the field-collected insects.

Hapten Synthesis. CA-V hapten used in this work was prepared by introduction of carboxyl group into the CA-Vstructure. The hapten used for immunization and antigen coating was synthesized by the following procedure shown in **Figure 1**.

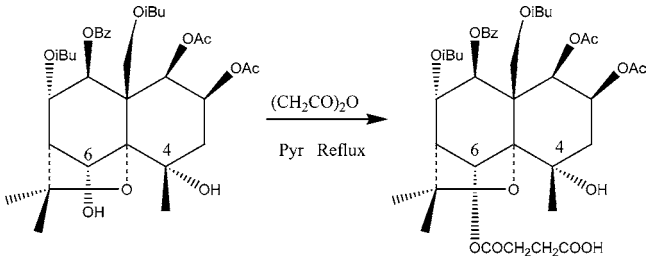

Figure 1. Synthetic route of hapten.

1β,2β-Diacetyloxy-8α,13-diisobutanoyloxy-6α-succinoyl-9-benzoyloxy-4α-hydroxy-β-dihydroagarofuran. Succinic anhydride (50 mg, 0.5 mM) and CA-V (100 mg, 0.15 mM) were added in 10 mL of anhydrous pyridine (dried by boiled with KOH and then distilled) and refluxing at 120 °C under nitrogen atmosphere for 8 h. The reaction was monitored by TLC developed in a solvent system of toluene/ethyl acetate/dioxane (50:30:20). The mixture was transferred into saturated NH_4Cl and then extracted with ethyl acetate. Subsequently, the extraction was washed sequentially by 10% $CuSO_4$, saturated NaCl, and anhydrous sodium sulfate and then separated on a silica gel column with a gradient of petroleum ether (60−90 °C)/ethyl acetate. After evaporation in a vent hood, the hapten was obtained as a white solid (72 mg). The structure was characterized by NMR, IR, optical rotation, and HRMS analysis. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 5.48 (1H, d, J = 3.6 Hz), 5.39 (1H, dd, J = 3.6 Hz, J = 2.8 Hz), 2.08 (1H, dd, J = 4.0 Hz, J = 15.2 Hz), 1.93 (1H, dd, J = 2.8 Hz, J = 15.2 Hz), 6.49 (1H, s), 4.88, 4.65 (2H, Abq, J = 13.6 Hz), 1.47 (3H, s), 1.55 (3H, s), 1.77 (3H, s), 2.09 (3H, s), 1.54 (3H, s), 7.85 (2H, d, J = 7.2 Hz), 7.56 (1H, t, J = 7.2 Hz), 7.41 (2H, t, J = 8.0 Hz), 2.92 (1H, m), 2.39 (2H, m), 0.91 (6H, m), 1.39 (6H, m), 2.76 (2H, m, OCOC*H*$_2$CH$_2$-COOH), 2.67 (2H, m, OCOCH$_2$C*H*$_2$COOH). ^{13}C NMR (DEPT, $CDCl_3$, 100 MHz) δ (ppm): 75.2, 67.7, 41.9, 69.8, 92.0, 76.1, 52.3, 73.5, 75.5, 51.5, 84.3, 61.8, 24.7, 29.7, 25.8, 169.5, 169.4, 21.1, 20.5, 165.5, 133.3, 129.4, 129.2, 128.6, 176.9C, 176.2, 34.3, 34.1, 19.3, 18.9, 18.6, 18.4, 175.7 C (O*C*OCH$_2$CH$_2$COOH), 170.7 (OCOCH$_2$CH$_2$*C*OOH), 29.5 CH$_2$ (OC*H*$_2$CH$_2$COOH), 28.5 CH$_2$ (CH$_2$*CH*$_2$COOH). Optical rotation: $[\alpha]^{20}_D$ −5°, (c = 10.8 mg/mL, $CHCl_3$). IR, KBr: 3420 (vs, *OH*), 1740 (vs, O*C*=O). HRMS, *m/z*: 780.3437 (M + NH_4), calculated for $C_{38}H_{50}O_{16}$, found, 780.3434 (M+ NH_4), error 0.4 ppm.

Preparation of Hapten−Protein Conjugates. Once the hapten has been designed and prepared, it was immunized as a conjugate with a carrier protein to provide sufficient immunogenicity to elicit an immune response. Hapten was covalently attached to KLH to be used as immunogen and was attached to BSA and OVA to be used as the coating antigens for serum and hybridoma supernatants screening assay. The method of conjugation used was the active ester method (*26*). The procedures for coupling haptens to the carrier proteins were as follows. Hapten (114 mg, 0.15 mM) was dissolved in 2.4 mL of *N*,*N*-dimethylformamide (DMF), to which NSH (17 mg, 0.15 mM) and DCC (31 mg, 0.15 mM) were added. The mixture was stirred for 2 h at room temperature, filtered, and added dropwise to a vigorously stirred solution of BSA (20 mg, 0.0003 mM) in 5 mL of PBS (previously cooled to 4 °C) containing 0.5 mL of dioxane. After a 2-h ice bath with gentle stirring, the reaction mixture was allowed to slowly warm to room temperature overnight. Simultaneously, another two conjugates (hapten−OVA and hapten−KLH) were prepared using the same method as described above. After dialyzing exhaustively against PBS and lyophilization to remove the unbounded hapten and residual solvent, the artificial antigens were obtained as a white powder and stored at −20 °C. The hapten−protein conjugates were confirmed by the methods of UV (200−400 nm) and IR (400−4000 cm^{-1}).

Production of MAbs to CA-V. The procedures for generating the immune response in mice were similar to those described by Shelver et al. (*27*). Four female Balb/c mice, 8-weeks-old (Experimental Animal Center, FMMU, Xi'an, China), were bled from tails for unimmunized serum and initially immunized interapertoneally (ip) with 50 μg per mouse of hapten−KLH dissolved in 50 μL of PBS and emulsified in Freund's complete adjuvant (1:1 v/v). After four twice-weekly intraperitoneal booster immunizations, serum samples were taken. SP2/0

myeloma cells were fused with the spleencytes from the immunized mice at a ratio of 2:1 in the presence of 50% PEG 1500 and were then cultured in HAT medium (100 μM sodium hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine in RPMI 1640 medium supplemented with 15% FCS) until all nonfused SP2/0 cells were eliminated. The hybridoma cells were selected by indirect ELISA as outlined below and the highly positive holes were continued with a determination of specificity for CA-V using carrier proteins (OVA and KLH) as the coating antigens. Hybridomas chosen for further studies were cloned two times by the method of limited dilution (*28*), and the final hybridoma cells, which develop monoclonal antibody against CA-V, were expanded and transferred into liquid nitrogen for long-time storage. The production of MAbs was carried out in accordance with the method of Jackson et al. (*29*). Balb/c mice (8-weeks-old) were inoculated ip with hybridoma cells (about 10^6 cells per capita), the ascites were collected 12 days later, and the titer of anti-CA-V antibodies was measured by indirect ELISA. Unless otherwise specified, all subsequent experiments were done with MAb C_6-E_9-C_5 ascites.

Indirect ELISA. Each hybridoma supernatant was titrated against the coating antigen to measure the reactivity of antibodies. Briefly, a modification of the method of Hoffman et al. (*30*) was used for the ELISA. Microtiter wells were coated with 100 μL/well hapten−OVA or hapten−BSA (10 μg/mL in carbonate−bicarbonate buffer) overnight at 4 °C. The following day, the plates were blocked by incubation with 1% gelatin in PBS (200 μL/well) for 2 h. Upon removal of the blocking buffer, the plates were incubated with hybridoma supernatants and goat anti-mouse IgG-HRP (diluted in PBS-2% NGS, 1:5000) sequentially. All incubations were carried out at 37 °C, and the plates were washed three times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) after each incubation. In a final step, the plates were developed with 100 μL/well of substrate solution (0.1 mL of 10 mg/mL TMB-DMSO diluted with 10 mL of citrate−phosphate buffer, supplemented with 2 μL of 30% hydrogen peroxide before use) for 20 min at room temperature. The reaction was stopped by adding 2 M sulfuric acid (50 μL/well), and optical density (OD value) was recorded at 450 nm. Samples and standards were generally analyzed in four replicate wells (*31*). A test sample was considered to be positive if the mean OD value was at least equal to twice that of the negative control (Sp2/0 supernatants) (*32*).

Determination of Specificity. A determination of specificity was carried out with OVA, KLH, and hapten−OVA as the coating proteins. The subsequent procedure was the same as the procedures of indirect ELISA.

Receptor Localization. *Insect Tissue Preparation.* The armyworms were treated using the leaf disc bioassay method (*33*). One day prior to the assay, 20 newly molted sixth instar larvae were placed individually in Petri dishes (6 cm diameter). After a starvation of 24 h, a 5 mm × 5 mm excised wheat leaf disc, which had been overlaid with 1 μL of 5% (w/v) CA-V diluted in acetone (using leaf disc dropped with pure acetone as control), was supplied to each animal. The individuals who had consumed whole leaf discs in 10 min were picked out for advanced observation. Approximately 2 h later, the intoxicant insects were dissected and the midgut was truncated from the intestinal tract, cut into 2 mm × 2 mm segments with a blade, and then transferred into 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate at pH 7.2, 4 °C for 4 h. This was followed by a post-fixed performance in 1% (v/v) OsO_4 4 °C for 4 h, washing five times in PBS, and dehydrating in a graded series of acetone/water solutions (in 30%, 50%, 70%, 80%, 90%, and 95% concentrations for 30 min each, in 100% acetone twice for 20 min each). Subsequently, tissues were infiltrated with increasing ratios of Epon-embedding reagent to acetone at room temperature (1:3 for 2 h, 1:1 for 4 h, 3:1 for 12 h) and embedded with pure Epon-embedding reagent (100% Epon-embedding reagent twice for 48 h each) (*34*). After polymerizing at 30 and 60 °C for 48 h, respectively, the specimens were sectioned, and the sections (200 nm) were prepared on carbon-coated nickel grids for electron microscopy observation.

Colloidal Gold Immunostaining and Observation. The nickel grids were floated on a drop of water for 5−10 min and incubated with 1% BSA in PB for 5 min. Then, the grids were incubated with MAb in PBS−BSA (150 mM NaCl, pH 7.4, 0.5% BSA, and 0.1% gelatin in PB) for 1 h and washed with PBS−BSA (3 × 1 min) afterward. This was followed by incubation in a solution (goat-anti-mouse IgG labeled with 15 nm colloidal gold diluted 1:100 in PBS−BSA) for 30 min. After another washing step, the grids with the stained samples were rinsed in distilled water twice, air-dried, and examined and photographed with TEM (*35*).

RESULTS AND DISCUSSION

Synthesis of Artificial Antigen. Synthesis of Hapten. In the current study presented, we designed a hapten by introducing a carboxyl group (−$OCOCH_2CH_2COOH$) into the CA-V structure. Successful synthesis was demonstrated by the presence of a new spot on TLC with an R_f value (0.55) that is different from that of CA-V (0.73) and succinic anhydride (0.44). All results of NHR, HRMS, and IR analysis confirmed that the target product was accurate. Besides the structure of CA-V being modified, the hapten retained its basic characteristic features of the structure, such as the groups of isobutyrate, benzoate, acetate, and hydroxyl. The design for hapten used the specific features of the succinyl arm that enabled the CA-V to conjugate with proteins by the carboxyl (CA-V−O−$COCH_2CH_2OCNHPr$) and thus be more likely to be recognized by the immune system.

Confirmation of Hapten−Protein Conjugates. The UV spectrum absorbance of the hapten, carriers alone, and hapten−protein conjugates were recorded from 200 to 400 nm (**Figure 2**). The maximum absorbance of the hapten−proteins (hapten−BSA was at 277.18 nm, hapten−KLH at 234.50 nm, and hapten−OVA at 276.46 nm) had a shift away from those of the carriers (i.e., BSA at 278.04 nm, KLH at 278.55 nm, and OVA at 279.65 nm). All absorbance values of antigens between 230 and 280 nm, which were contributed by the hapten, were higher than those of the carriers. IR spectra of the carriers and protein conjugates showed that all hapten−protein conjugates had a hydroxyl (3420, vs, unique to hapten) and amide carbonyl (1650, vs, unique to protein) absorber. Both UV and IR spectrum demonstrated that the hapten and carrier protein were coupled successfully.

Since Landsteiner (*36*) first introduced the concept of hapten−protein conjugation to induce immune responses against nonimmunogenic compounds in 1917, conjugation of nonimmunogenic or poorly immunogenic antigens with highly immunogenic foreign protein carriers has been applied to induce immune responses against a variety of antigens, including widespread use of compounds with relatively small molecular weight. In our study, the carefully designed hapten was coupled to three different carrier molecules: BSA, KLH, and OVA. Because of its capacity to couple more haptens and therefore have a strong immunogenicity, KLH was chosen for immunizing carrier to maximize the chance of stimulating a strong immune response.

Production of MAbs to CA-V. To ensure the development of MAbs with relatively high affinity for CA-V, mice were immunized with hapten−KLH conjugate, and antibody titrations (mice sera and hybridoma supernatants) were estimated by the method of indirect ELISA using two homologous hapten conjugates (hapten−OVA and hapten−BSA). Following a twice-weekly selective culture with HAT medium, hybridoma supernatants were screened for reactivity against CA-V. Eight hybridoma supernatants showed different positivity against the screening compounds when compared with the negative control (Sp2/0 cell supernatants from nonimmune mouse) for the experiment. The specificities of those positive clones were determined subsequently using hapten−OVA, OVA, and KLH as coating proteins, and the results are represented in **Table 1**. The hybridoma colony designated C_6 was selected as the

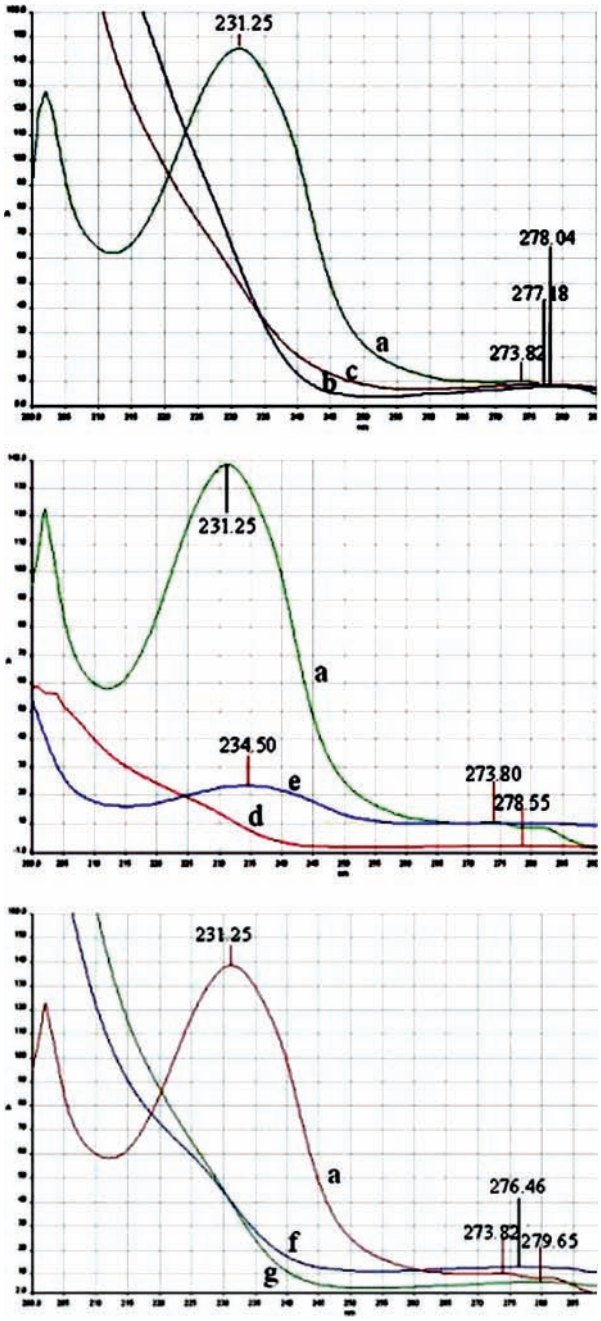

Figure 2. Verification of the linkages of hapten and carrier proteins with UV spectra scaning at 200–400 nm: (**a**) hapten, (**b**) BSA, (**c**) hapten–BSA, (**d**) KLH, (**e**) hapten–KLH, (**f**) OVA, and (**g**) hapten–OVA, with a concentration of 100 μg/mL.

material for advanced screening of monoclonal cells based on its characterization of a higher titer against hapten−OVA and a lower reactivity with coating-carrier (OVA) and immunocarrier (KLH). After two rounds of limited dilution of candidate clones, we obtained three hybridoma cell clones designated C_6-E_9-B_{11}, C_6-E_9-C_5, and C_6-E_9-H_3, which present stable and constant antibody production against CA-V.

Characterization of the MAbs. MAbs produced by each of the three selected hybridomas were small-scale purified from ascites and were subsequently characterized for their titering and specificity to CA-V.

The hybridoma cell clones, C_6-E_9-B_{11}, C_6-E_9-C_5, and C_6-E_9-H_3, were proliferated and injected into the peritoneal cavities of mice, and then the ascites were tapped 12 days later. The titrate of MAbs in ascites was determined to be 7.5×10^4 (data shown in **Table 2**).

Table 1. Antibody Specificities of Eight Hybridoma Cell Clones[a] to **CA-V**

coating protein	antibody specificities								antiserum (1:2000)	Sp2/0 supernatants
	A_7	A_9	C_6	D_2	D_7	E_6	E_8	H_5		
hapten–OVA	0.425	0.786	1.932	0.521	0.463	0.441	1.642	0.460	0.631	0.097
OVA	0.155	0.166	0.169	0.241	0.195	0.217	0.182	0.193	0.195	0.065
KLH	0.382	0.491	0.255	0.277	0.375	0.327	1.260	0.262	0.675	0.076

[a] Using 10 μg/mL OVA-hapten as coating antigen and the OD values were recorded at 450 nm.

Table 2. Titrates of MAbs against **CA-V** in Diluted Ascites[a]

cell clones	1:10 000[b]	1:25 000[b]	1:50 000[b]	1:75 000[b]	1:100 000[b]
C_6-E_9-B_{11}	0.446/+[c]	0.398/+	0.228/+	0.192/+	0.116/–[d]
C_6-E_9-C_5	0.426/+	0.330/+	0.255/+	0.204/+	0.106/–
C_6-E_9-H_3	0.408/+	0.306/+	0.226/+	0.164/+	0.119/–
antiserum	0.213/+	0.097/–	–	–	–
Sp2/0 supernatants	0.077				

[a] Using 10 μg/mL OVA-hapten as coating antigen, and the OD values were recorded at 450 nm. [b] Ascites were diluted with PBST containing 0.1% BSA. [c] + denotes that the OD values of ascites dilutions were more than two times that of Sp2/0 supernatants and considered to be positive. [d] – denotes that the OD values of ascites dilutions were less than two times that of Sp2/0 supernatants and considered to be negative.

To evaluate the specificity of the MAbs to CA-V, the primary eight hybridomas cell clones and the final three MAbs were detected by the method of antibody titering using hapten−OVA, OVA, and KLH as coating proteins. This selection performance was propositional for a rapid screening of MAbs with its advantage of saving time and labor. Otherwise, there would be a deficiency that some antibodies might not be selected or may even be lost, if the positive holes containing more than two hybridoma cell lines could also recognize CA-V or KLH.

Immunolocalization of Receptor by CA-V. The previous report indicated that CA-V led to serious damage of the midgut cells of the target insect, a confirmation also was given by the ultramicroscopic structure examination (*14*). To investigate the receptor localization of CA-V in the midgut tissues of *M. separata*, IEM was performed. As expected, the CA-V-treated midgut exhibited broken off microvilli, mitochondrial ridge deletion, and dilation and vacuolization of the cisternae lumen of endoplasmic reticulum (ER), as shown in **Figure 3**, parts **B**, **D**, and **F**. Immunohistochemical staining of the midgut sections revealed that while there was no specific CA-V staining detected in the tissue sections of the control insects (**Figure 3A,C,E**); the most colloidal gold staining was located on the treated midgut epithelia of the insects (**Figure 3B,D,F**). The results were in support of the previous finding and proved that there are receptors of CA-V in the midgut tissue of lepidopteran insects. **Figure 3** also indicated that the gold nanoparticles distributed both inside and outside the midgut cells, which should be owing to the high liposolubility of CA-V to a great extent and thus it has a tendency to cross the plasmalemma. The observation that the drug did not efficiently exhibit specificity for organelles suggested that the receptor was nonspecifically binding to the midgut epithelia, and its exact mechanism of action remains to be determined.

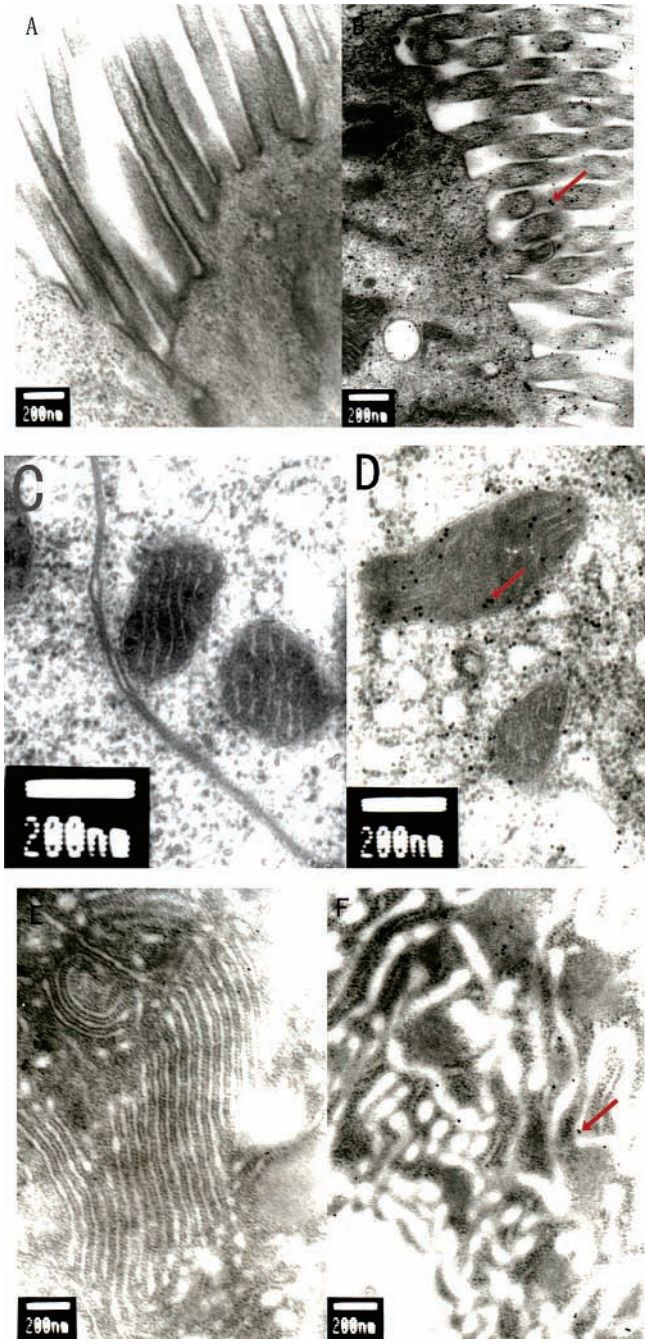

Figure 3. The receptor localization of CA-V by immunostaining with colloidal gold nanoparticle (15 nm). Paraformaldehyde–glutaraldehyde fixed and OsO_4 postfixed insect midgut slices (200 μm thick) were stained with anti-CA-V McAb (dilution 1:100), and the immunostaining was detected by transmission electron microscopy (80 kV). The black dots are immunogold particles. Panels **A**–**F** show microvillus, mitochondria, and endoplasmic reticulum of untreated (**A**, **C**, and **E**) and treated (**B**, **D**, and **F**) insect tissue, respectively.

Conclusions. Following the general guidelines about hapten design, 1β,2β-diacetyloxy-8α,13-diisobutanoyloxy-6α-succinoyl-9-benzoyloxy-4α-hydroxy-β-dihydroagarofuran was synthesized and the structure was characterized. An immunogen (hapten−KLH) and two types of coating-antigen (hapten−BSA and hapten−OVA) were prepared for the production of highly sensitive MAbs against CA-V.

After application of the hybridoma technology, three MAbs, named C_6-E_9-B_{11}, C_6-E_9-C_5, and C_6-E_9-H_3, were selected and evaluated for their antibody secreting ability and specificity to CA-V by the method of indirect ELISA. To our knowledge, the MAbs for CA-V herein presented are the first reported for this botanical insecticide.

The goal to localize the receptor acted on by CA-V in the armyworm midgut tissue was achieved from insects administrated with CA-V, and the MAbs had the ability to recognize the residues of this chemical, as verified by IEM. The observation of the insect midgut sections immnostaining indicates that there are receptors acted on by CA-V in the midgut epithelia of the target insect. Work is in progress to isolate the receptor and investigate the insecticidal mechanism of CA-V.

ABBREVIATION USED

CA-V, celangulin V; MAbs, monoclonal antibodies; PAbs, polyclonal antibodies; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; IEM, immunoelectron-microscopy; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; NHS, *N*-hydroxysuccinimide; DCC, *N*,*N*-dicyclohexylcarbodiimide; PEG, polyethylene glycol; IgG-HRP, IgG-horseradish peroxidase; TMB, tetramethylbenzidine; DDSA, dodecenyl succinic anhydride; MNA, methyl nadic anhydride; DMP-30, 2,4,6-tridimethylaminomethylphenol; OsO_4, osmium tetroxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FBS, fetal bovine serum; NGS, normal goat serum; PBS, phosphate-buffered saline; PB, phosphate buffer; HRMS, high-resolution mass spectroscopy; ESI, electrospray ionization; TMS, tetramthylsilane; TEM, transmission electron microscope; HAT, sodium hypoxanthine, aminopterin, and thymidine; ELISA, enzyme-linked immunosorbent assay; OD, optical density.

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