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Molecular Cloning and Expression of a cDNA Encoding Putative Chemosensory Protein from the Mole Cricket, *Gryllotalpa orientalis*

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We describe here the cloning, expression and characterization of a cDNA encoding a putative chemosensory protein (CSP) from the mole cricket, *Gryllotalpa orientalis*. The *G. orientalis* chemosensory protein cDNA sequences comprised of 384 bp with 128 amino acid residues. The *G. orientalis* chemosensory protein showed 75.4% protein sequence identity to the *Locusta migratoria* CSP. Northern blot analysis revealed that signal was stronger in head than leg and cuticle, indicating that the head part containing antennae is a main site for *G. orientalis* chemosensory protein synthesis. The cDNA encoding *G. orientalis* chemosensory protein was expressed as approximately 12 kDa polypeptide in baculovirus-infected insect cells.

Key words: Insect, Mole cricket, *Gryllotalpa orientalis*, Chemosensory protein, cDNA sequences, Phylogeny, Insect cells, Baculovirus

Introduction

Animals possess an olfactory sense organ that allows for the recognition and discrimination of chemosensory information in the environment. Insects perceive the chemical cues of their environment by chemosensory organs. Biochemical studies of insect antennae have identified two small soluble proteins: odorant-binding proteins (OBPs) and olfactory specific-D (OS-D) related proteins (Pelosi and Maida, 1995; Vogt *et al.*, 1999). OBPs are a multigene family of antennal-specific proteins including the pheromone-binding proteins (PBP), the general odorant-binding proteins and the antennal-binding proteins (ABP) X-related proteins (Robertson *et al.*, 1999; Vogt *et al.*, 1999). Perception of volatile semiochemicals in mosquitoes and other insects is mediated by specific olfactory sensilla, which are located on the antennae and maxillary palpi (Biessmann *et al.*, 2002). In *Mamestra brassicae*, OBPs specifically expressed in male and female antennae were previously characterized (Nagnan-Le Meillour *et al.*, 1996). The genes coding for three OBPs were also cloned (Maïbèche-Coisné *et al.*, 1998a, b). Furthermore, OBPs from other insect orders such as Hymenoptera (Danty *et al.*, 1998), Hemiptera (Dickens *et al.*, 1995), Coleoptera (Wojtasek *et al.*, 1998), Blattoidea (Picimbon and Leal, 1999) and Phasmida (Tuccini *et al.*, 1996; Mameli *et al.*, 1996) were characterized.

The mole cricket, *Gryllotalpa orientalis* (Burmeister), is a singly known species of the Family Gryllotalpidae in Korea, and distributed in Asia and many European and African countries. Information on this species, particularly on the genetic aspect, is extremely rare, not enough to cite scientifically (Nevo *et al.*, 2000). In order to obtain genetic information of the mole criket, therefore, we have previously constructed the cDNA library using *G. orientalis* whole bodies (Kim *et al.*, 2002). Sec61p γ subunit homologue gene has been identified through EST (Expressed sequence tag) sequencing from the mole criket (Kim *et al.*, 2002).

In this study, we report the cDNA sequence for putative chemosensory protein from *G. orientalis*. The cDNA for putative chemosensory protein from *G. orientalis* was expressed in baculovirus-infected insect cells.

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Materials and Methods

Animals

The mole cricket, *Gryllotalpa orientalis* (Burmeister), was collected in Kimhe city, Korea (Kim *et al.*, 2002).

cDNA library screening, nucleotide sequencing and data analysis

A cDNA library (Kim *et al.*, 2002) was constructed using whole bodies of *G. orientalis*. Sequencing of randomly selected clones harboring cDNA inserts was performed to generate the expressed sequence tags (ESTs). For DNA sequencing, plasmid DNA was extracted by Wizard mini-preparation kit (Promega). Sequence of each cDNA clone was determined using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (www.Ncbi.nlm.nih.gov). GenBank, EMBL and SwissProt databases were searched for sequence homology using a BLAST algorithm program (www.Ncbi.nlm. nih.gov/BLAST).

MacVector (ver. 6.5, Oxford Molecular Ltd.) was used to align the amino acid sequences of chemosensory proteins. Including the eleven GenBank-registered chemosensory proteins amino acid sequences, phylogenetic analysis was performed using PAUP* (Phylogenetic Analysis using Parsimony) version 4.0 (Swofford, 2000). The tree was obtained by bootstrap analysis with the option of heuristic search (1,000 replications). The accession numbers of the sequences in the GenBank are: *G. orientalis* (this study), *Locusta migratoria* CSP (AJ251077), *Manduca sexta* SAP3 (AF117585), *Periplaneta americana* P10 (AF030340), *M. sexta* SAP4 (AF117599), *Drosophila melanogaster* PebIII (AE003462), *Anopheles gambiae* SAP (AF437891), *Helicoverpa armigera* CSP (AF 368375), *M. sexta* SAP2 (AF117592), *M. sexta* SAP1 (AF117574), *D. melanogaster* A10 (AE003525), and *M. sexta* SAP5 (AF117594).

RNA isolation and Northern blot analysis

Total RNA was isolated from whole body, fat body, midgut, head, leg and cuticle of the *G. orientalis* using the Total RNA Extraction Kit (Promega, Madison, WI). Total RNA (10 μg/lane) from the *G. orientalis* was separated on glyoxalation gel (McMaster and Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a buffer containing 2 × PIPES, 50% formamide, 1% sodium dodecyl sulphate (SDS) and blocking agent (Boehringer Mannheim, Mannheim, Germany). The probe used to detect the chemosensory protein gene was 535 bp for *G. orientalis* chemosensory protein cDNA cloned in this study and labeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA). After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C, and finally exposed to X-ray film.

Construction of baculovirus transfer vector

The 535 bp *G. orientalis* chemosensory protein cDNA from pBlueScript-AgFer1 was subcloned between *Sac*I and *Kpn*I sites of pBacPAK9 (Clontech, Palo Alto, CA) to produce transfer vector pBacPAK9-*GoCSP*. In this transfer vector, chemosensory protein cDNA is under the control of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus.

Cell culture and virus

The *Spodoptera frugiperda* IPLB Sf21-AE (Vaughn *et al.*, 1977) clone 9 (Sf9) cells were maintained at 27°C in TC100 medium (GIBCO BRL LIFE Technologies), supplemented with 10% fetal bovine serum (FBS; GIBCO BRL LIFE Technologies) as described by standard methods (OReilly *et al.*, 1992). Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recom- binant AcNPV were propagated in Sf9 cells. The titer was expressed as plaque forming units (PFU) per ml (O'Reilly *et al.*, 1992).

Construction of recombinant virus

Thirty-five mm cell culture dishes were seeded with 1.0 – 1.5 × 10^6 cells and incubated at 27°C for 1 h to allow cell attachment. One microgram of BacPAK6 viral DNA (Clontech), five μg of pBacPAK9-*GoCSP* in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystyrene tube. Fifty microliters of 100 μg/ml Lipofectin™ (GIBCO BRL LIFE Technologies, Gaithersburg, MD) were gently mixed with the DNA solution and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC100 medium and fed with 1.5 ml serum-free TC100 medium. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubation at 27°C for 5 hr, TC100 medium containing antibiotics and 10% FBS was added to each dish and incubation at 27°C was continued. At 5 days of postinfection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C. Recombinant AcNPV was plaque purified on 6-well plates seeded with 1.5 × 10^6 Sf9 cells as described by O'Reilly *et al.* (1992). Cells were visualized under the inverted phase contrast microscope (Olympus, Tokyo, Japan).

SDS-polyacrylamide gel electrophoresis (PAGE)

Insect Sf9 cells were mock-infected or infected with the wild-type AcNPV and recombinant AcNPV in a 35-mm diameter dish (1×10^6 cells) at an multiplicity of infection (MOI) of 5 PFU per cell. After incubation at 27°C, cells were harvested at 1, 2, and 3 days p.i. For SDS-PAGE (Laemmli, 1970) of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with PBS and mixed with protein sample buffer and boiled. The total cellular lysates were subjected to 10% SDS-PAGE. After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250.

Results and Discussion

Cloning, sequencing and phylogenetic analysis of cDNA encoding putative chemosensory protein from *G. orientalis*

cDNA library was constructed using whole bodies of *G. orientalis*. Sequencing of randomly selected clones harboring cDNA inserts was performed to generate *G. orientalis* ESTs. Of these ESTs, one exhibited similarity to previously reported chemosensory proteins. The nucleotide and deduced amino acid sequences of *G. orientalis* chemosensory protein are presented in Fig. 1. The nucleotide and deduced amino acid sequences of cDNA encoding a putative member of the insect chemosensory protein revealed that the cDNA sequences comprised of 384 bp with. 128 amino acid residues. The *G. orientalis* chemosensory protein cDNA contained 535 nucleotides with a

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-32                                                    GGAAGAAGCCATCCGTCCGACCACAAGAAGCC
  1  ATGAACCGCTTTGTCTTAGTCTTCGTCGCCGTGGTGGTGGCCGTAGCCATGGCTGCACCA
  1   M  N  R  F  V  L  V  F  V  A  V  V  V  A  V  A  M  A  A  P

 61  GACGGCTACACCACCAAGTACGACAACGTGAACCTGGAGGAGATCCTCAGCAACGACAGG
 21   D  G  Y  T  T  K  Y  D  N  V  N  L  E  E  I  L  S  N  D  R

121  CTCAGGAACAAGTACGTGGAGTGCCTCACTAGCACTTCCGATGAGCACTGCACTCCTGAG
 41   L  R  N  K  Y  V  E  C  L  T  S  T  S  D  E  H  C  T  P  E

181  GGCAAGGAGCTCAAGAGCGTGGTGTCTGATGCTCTGACCACCGACTGCGCCAAATGTAAC
 61   G  K  E  L  K  S  V  V  S  D  A  L  T  T  D  C  A  K  C  N

241  GAGAAGCAGAAGAACGGCACGAAGTACGTGGTGGACACCCTCCTGGACAAGTACCCCGAC
 81   E  K  Q  K  N  G  T  K  Y  V  V  D  T  L  L  D  K  Y  P  D

301  GACTACGCCAAGCTGGAGAAGGGTGTACGACGCCGATGGCGCTTACCGCAAGAAGTACGAA
101   D  Y  A  K  L  E  K  V  Y  D  A  D  G  A  Y  R  K  K  Y  E

361  GTACTCAAGGCTGAAGGCAAGCTGTAAACAGCTGTTCCACGTCCATTTCACCGCACTATG
121   V  L  K  A  E  G  K  L  *

421  TAAATCATCTCTGTACACACATCTTACATTCACTACAATAGTTTCAATAAAAATCTCTTA
481  AGATATTAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide and deduced amino acid sequences of *G. orientalis* chemosensory protein. The start codon of ATG is boxed and the termination codon is shown by asterisk. The polyadenylation signal is underlined.

polyadenylation signal AATAAA occurred in the 3' untranslated region at positions 466 – 471. The hydropathy plot (data not shown) of *G. orientalis* chemosensory protein by the Kyte and Doolittle method (1982) showed that *G. orientalis* chemosensory protein is hydrophilic except for N-terminal and is similar to insect *L. migratoria* CSP (Picimbon *et al.*, 2000), *P. americana* P10 (Kitabayashi *et al.*, 1998) and *H. armigera* CSP (Deyts *et al.*, 2001), suggesting that cleavage of the putative signal peptide occurred between Pro^{20} and Asp^{21} in *G. orientalis* chemosensory protein. The mature *G. orientalis* chemosensory protein is predicted to be 108 amino acid residues.

A multiple sequence alignment of the deduced protein

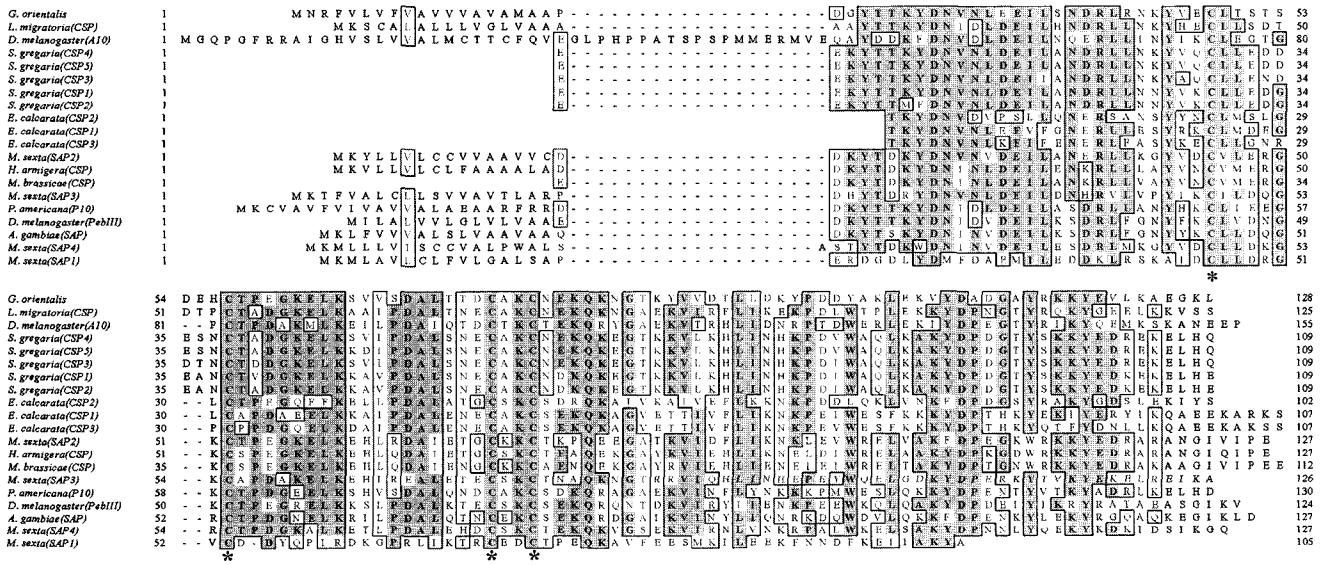

Fig. 2. Alignment of deduced amino acid sequences of *G. orientalis* chemosensory protein with nineteen insect chemosensory proteins. The identical residues are shown in solid boxes. The four conserved cysteine residues are marked by asterisk.

Table 1. Pairwise comparisons among amino acid sequences of the *G. orientalis* chemosensory gene and the known chemosensory genes

Species	GenBank No.	1	2	3	4	5	6	7	8	9	10	11	12
1. *G. orientalis*		-	0.246	0.327	0.304	0.312	0.304	0.304	0.315	0.323	0.392	0.408	0.750
2. *L. migratoria*(CSP)	AJ251077	64	-	0.292	0.265	0.281	0.265	0.265	0.288	0.300	0.385	0.373	0.719
3. *M. sexta*(SAP3)	AF117585	85	76	-	0.304	0.281	0.315	0.296	0.273	0.273	0.385	0.412	0.654
4. *P. americana*(P10)	AF030340	79	69	79	-	0.296	0.292	0.254	0.315	0.312	0.408	0.369	0.735
5. *M. sexta*(SAP4)	AF117599	81	73	73	77	-	0.281	0.265	0.281	0.288	0.365	0.392	0.719
6. *D. melanogaster*(PebIII)	AE003462	79	69	82	76	73	-	0.215	0.265	0.265	0.381	0.408	0.708
7. *A. gambiae*(SAP)	AF437891	79	69	77	66	69	56	-	0.269	0.269	0.385	0.377	0.704
8. *H. armigera*(CSP)	AF368375	82	75	71	82	73	69	70	-	0.123	0.396	0.408	0.696
9. *M. sexta*(SAP2)	AF117592	84	78	71	81	75	69	70	32	-	0.396	0.400	0.715
10. *M. sexta*(SAP1)	AF117574	102	100	100	106	95	99	100	103	103	-	0.500	0.808
11. *D. melanogaster*(A10)	AE003525	106	97	107	96	102	106	98	106	104	130	-	0.850
12. *M. sexta*(SAP5)	AF117594	195	187	170	191	187	184	183	181	186	210	221	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

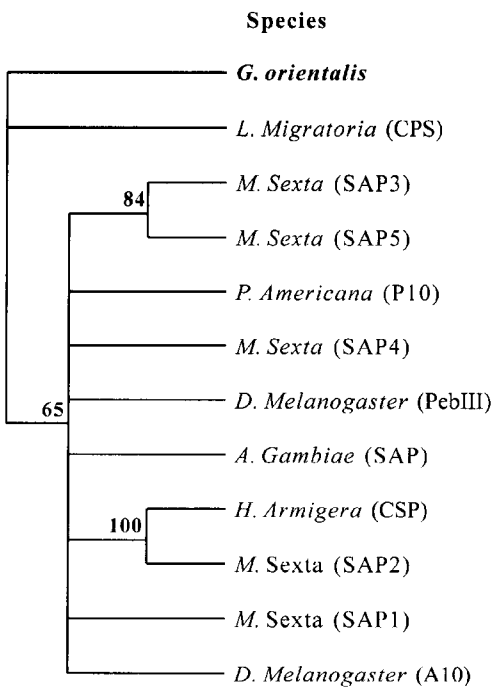

Fig. 3. Phylogenetic tree of aligned amino acid sequences of the *G. orientalis* chemosensory protein and the other known chemosensory proteins. The sequences were extracted from; *G. orientalis* (this study), *L. migratoria* CSP (AJ251077), *M. sexta* SAP3 (AF117585), *P. americana* P10 (AF030340), *M. sexta* SAP4 (AF117599), *D. melanogaster* PebIII (AE003462), *A. gambiae* SAP (AF437891), *H. armigera* CSP (AF368375), *M. sexta* SAP2 (AF117592), *M. sexta* SAP1 (AF117574), *D. melanogaster* A10 (AE003525), and *M. sexta* SAP5 (AF117594). The tree was obtained by bootstrap analysis with the option of heuristic search and the numbers on the branches represent bootstrap values for 1,000 replicates.

sequence of *G. orientalis* chemosensory protein cDNA with other chemosensory protein sequences is shown in Fig. 2. Alignment of the *G. orientalis* chemosensory protein sequences with those of chemosensory proteins from several other species indicates the extent of the identity that exists. As shown in Fig. 2, twenty of the insect chemosensory proteins have four conserved cysteine residues. The *G. orientalis* chemosensory protein showed 75.4% protein sequence identity to the *L. migratoria* CSP, while the lowest identity was found with *M. sexta* SAP5 (25.0% protein sequence identity) (Table 1).

A phylogenetic analysis using deduced amino acid sequences of known insect chemosensory protein genes revealed that the two insect species, *G. orientalis* and *L. migratoria*, which showed the highest homology, respectively formed its own subgroup exclusively (Fig. 3).

Expression at transcriptional level of the G. orientalis chemosensory protein cDNA

To confirm the expression of the *G. orientalis* chemosensory protein gene at transcriptional level, Northern blot analysis was performed using total RNA prepared from the whole body, fat body, midgut, head, leg and cuticle of *G. orientalis* (Fig. 4). Signals were detected for whole body, head, leg and cuticle. The whole body was used as positive control. Signal was stronger in head than leg and cuticle, indicating that the head part containing antennae is a main site for *G. orientalis* chemosensory protein synthesis. Although chemosensory protein mRNA is highly expressed in *A. gambiae* antennae, the chemosensory protein was detected in both the antennae and the body (Biessmann *et al.*, 2002). OBPs and PBPs are highly expressed in insect antennae, enabling isolation of their

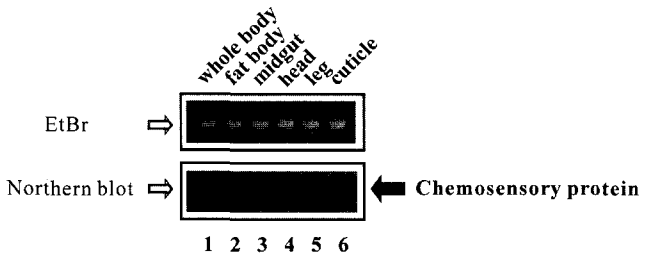

Fig. 4. Northern blot analysis of the *G. orientalis* chemosensory protein messages. Total RNA was isolated from a whole body (lane 1), fat body (lane 2), midgut (lane 3), head (lane 4), leg (lane 5) and cuticle (lane 6). The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred onto a nylon membrane, and hybridized with the appropriate radiolabelled probe (lower panel). *G. orientalis* chemosensory protein messages are indicated by arrow on the right side of the panel.

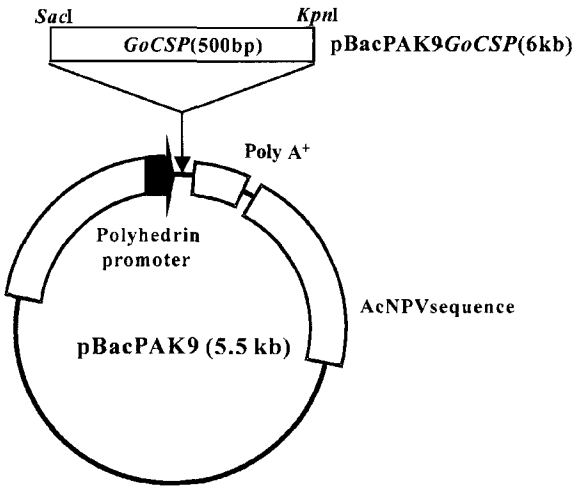

Fig. 5. Structure of the transfer vector used to generate the recombinant baculovirus. The transfer vector pBacPAK9-*GoCSP* was constructed by insertion of the *G. orientalis* chemosensory protein cDNA under the control of AcNPV polyhedrin promoter. The arrow shows the direction of transcription.

cDNAs from antennal libraries of lepidopteran insects (Breer *et al.*, 1990; Krieger *et al.*, 1993, 1996; Vogt *et al.*, 1991) and *D. melanogaster* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994) possible. In the cockroach, *P. americana*, chemosensory protein mRNAs have been detected in the antennae, leg and epidermis (Kitabayashi *et al.*, 1998). From these data, our present result is in good agreement with the previous findings in that the chemosensory protein gene is highly expressed in insect antennae.

Expression of cDNA encoding G. orientalis chemosensory protein in baculovirus-infected insect cells

The baculovirus transfer vector (pBacPAK9-*GoCSP*) was used to generate a recombinant virus expressing *G. orientalis* chemosensory protein (Fig. 5). Recombinant AcNPV named AcNPV-GoCSP was produced in insect Sf9 cells by cotransfection with wild-type AcNPV DNA and the transfer vector.

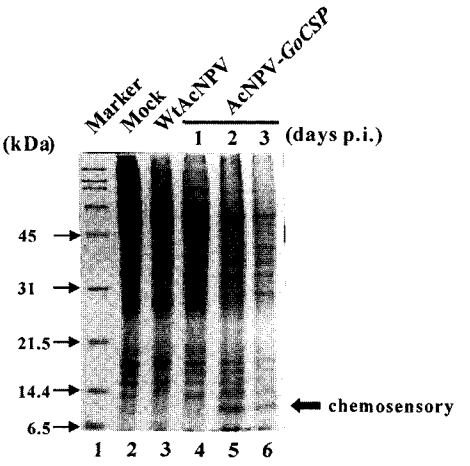

Fig. 6. SDS-PAGE analysis of *G. orientalis* chemosensory protein expression using recombinant baculovirus in Sf9 cells. Sf9 cells were mock-infected (lane 2) or infected with wild-type AcNPV (lane 3), recombinant AcNPV-GoCSP (lanes 4, 5 and 6) at an MOI of 5 PFU per cell. Cells were collected at 1 (lane 4), 2 (lanes 3 and 5) and 3 (lane 6) days p.i. Total cellular lysates were subjected to 10% SDS-PAGE. The solid arrows on the right of the panel indicate the *G. orientalis* chemosensory protein band of 12 kDa. Molecular weight standards were used as size marker (lane 1).

To determine whether the *G. orientalis* chemosensory protein gene can be expressed by baculovirus in insect cells, the protein synthesis in Sf9 cells infected with the recombinant virus was analyzed by SDS-PAGE (Fig. 6). In cells infected with AcNPV-GoCSP recombinant virus, a single band of ~12 kDa was observed. No such band was seen in the cells infected with wild-type AcNPV or mock-infected cells.

In conclusion, we have cloned and characterized a novel cDNA encoding putative chemosensory protein from the mole cricket *G. orientalis*. We hope that the molecular characterization of chemosensory protein in *G. orientalis* in this study will expand the understanding of insect chemosensory proteins.

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