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Development and use of a monoclonal antibody to detect semi-digested proteins of the English grain aphid, *Sitobion avenae*, in the guts of ladybird beetle predators

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Abstract

A monoclonal antibody (McAb), EGA-4A9, was developed to detect the semi-digested proteins of the English grain aphid, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae), in predatory ladybird beetles (species of the genera *Adonia*, *Coccinella*, *Hippodamia*, and *Propylea*) using the gut homogenate of *Adonia variegata* (Goeze) (Coleoptera: Coccinellidae) adults which had fed on *S. avenae* as immunogen. The McAb was selected by screening hybridoma lines for antibodies that bound with the semi-digested aphid proteins in ladybirds. A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) using Clonotyping™ System/HRP showed that it belonged to the IgG2a isotype. It did not cross-react with any of the 21 arthropod species tested besides the ladybird beetles fed on *S. avenae* with an indirect ELISA. It could still detect the semi-digested proteins in the gut of a ladybird adult, kept at 25 °C, that had ingested one aphid 6 days before. The extended antigen detection period and the high specificity of the antibody indicated that EGA-4A9 could be used to study interactions between English grain aphids and their ladybird predators in the field. Between 28 and 72% of coccinellids collected from the field were positive for English grain aphid protein by ELISA. The percentage of McAb-positive predatory ladybird beetles was positively correlated with the density of *S. avenae* in wheat fields.

Introduction

Wheat is one of the most important food crops in the world. The English grain aphid, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae), is an important world-wide wheat pest. For a long time, application of chemical insecticides has been the predominant method of control for wheat aphids in many regions. However, chemical insecticides not only pollute the environment and potentially cause resistance of aphids to insecticides but also kill natural enemies (Chen et al., 2000). In most agricultural systems, arthropod predators fulfil an essential function in pest control (Symondson et al., 2002). Many species of arthropod predators such as ladybird beetles, hoverflies, green lacewings, carabid beetles, and spiders occur in wheat ecosystems (Sunderland et al., 1987; Gao et al., 2004). However, the importance of these predators in controlling wheat aphid populations is largely unknown.

The enzyme-linked immuno-sorbent assay (ELISA) has become the most frequently used method for studying arthropod predator–prey interactions in the field (Sheppard & Harwood, 2005). Sunderland et al. (1987) studied natural enemies of wheat aphids by ELISA, but the antiserum used was polyclonal, thus unsuitable for distinguishing between wheat aphid species. Symondson et al. (1999) developed a monoclonal antibody to *Metopolophium dirhodum* (Walker), but the antibody reacted not only to *M. dirhodum*, but also to *S. avenae* and *Rhopalosiphum padi* (L.). Recently, DNA-based molecular methods have been used to analyse gut contents of invertebrate predators

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(Agustí et al., 1999; Hoogendoorn & Heimpel, 2001; de León et al., 2006; Zhang et al., 2007; Schmidt et al., 2009). Chen et al. (2000) produced polymerase chain reaction (PCR) primers for gut content analysis of aphid predators, which could distinguish six species of cereal aphids including *S. avenae*. However, their detectability half-lives were short, 3.95 h in *Chrysoperla plorabunda* (Fitch) and 8.78 h in *Hippodamia convergens* Guerin.

Our objectives were to develop a species-specific monoclonal antibody against the semi-digested protein of *S. avenae* in ladybird predators having a longer detection period, and to use the antibody for evaluation of the relative importance of various ladybird species to control *S. avenae* in the wheat ecosystem.

Materials and methods

Monoclonal antibody production

The antigen was prepared by homogenizing the guts of ladybird beetles, *Adonia variegata* (Goeze) (Coleoptera: Coccinellidae), fed on *S. avenae* for 24 h in phosphate-buffered saline (PBS, pH = 7.2). After centrifuging at 8 000 ***g*** for 10 min at 4 °C, the supernatant was stored at −20 °C. Monoclonal antibody production was based on the general protocols in Liddell & Cryer (1991). When the cells covered 1/4 of the bottom area of the wells, the supernatants were screened by indirect ELISA to select the hybridomas which produced antibodies directed specifically against the above antigen but not against other insects (Table 1) or gut material of hungry *A. variegata*. The selected cells were then cloned by limiting dilution. The hybridoma cells were selected more than three times to obtain cell lines that produced the monoclonal antibodies specific to the above antigen. Highly specific hybrid cells were injected intra-peritoneally into a mouse to produce monoclonal antibodies. Isotyping of monoclonal antibodies was performed using Clonotyping™ System/HRP (SouthernBiotech, Birmingham, AL, USA). The monoclonal antibodies were purified with ammonium sulphate precipitation.

Hybridoma supernatant screening

Supernatant screenings of fused hybrid cells were performed using indirect ELISA (Hagler et al., 1993). The protocol was the same as that of Hagler et al. (1993) except goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA), HRP-IgG, instead of goat anti-mouse IgG/IgM alkaline phosphatase-labelled antibody. The absorbance of each well was measured with a Bio-Rad Microplate reader (Model 1680; Bio-Rad Corporate, Hercules, CA, USA) set at 490 nm. If the absorbance values exceeded the mean negative control values by three

Table 1 Arthropod species and stage(s) examined for cross-reactivity to the McAb to *Sitobion avenae*. N, nymph; A, adult

	Species	Order: family	Stages
Herbivores	*Sitobion avenae* (Fabricius)	Hemiptera: Aphididae	N, A
	Rhopalosiphum maidis (Fitch)	Hemiptera: Aphididae	N, A
	Aphis medicaginis Koch	Hemiptera: Aphididae	N, A
	Aphis glycines Matsumura	Hemiptera: Aphididae	N, A
	Aphis gossypii Glover	Hemiptera: Aphididae	N, A
	Aphis laburni Kaltenbach	Hemiptera: Aphididae	N, A
	Acyrthosiphon solani (Kaltenbach)	Hemiptera: Aphididae	N, A
	Schizaphis graminum (Rondani)	Hemiptera: Aphididae	N, A
	Laodelphax striatellus (Fallen)	Hemiptera: Delphacidae	N, A
	Cicadella viridis (L.)	Hemiptera: Cicadellidae	A
	Lygus lucorum Meyer-Dur	Hemiptera: Cicadellidae	A
	Trigonotylus ruficornis Geoffroy	Hemiptera: Miridae	A
Predators	*Propylea japonica* (Thunberg)	Coleoptera: Coccinellidae	A
	Adonia variegata (Goeze)	Coleoptera: Coccinellidae	A
	Hippodamia tredecimpunctata (L.)	Coleoptera: Coccinellidae	A
	Coccinella septempunctata L.	Coleoptera: Coccinellidae	A
	Coccinulea sinensis (Weise)	Coleoptera: Coccinellidae	A
	Adalia bipunctata (L.)	Coleoptera: Coccinellidae	A
	Chrysopa phyllochroma Wesmael	Neuroptera: Chrysopidae	A
	Syrphus corollae F.	Diptera: Syrphidae	A
	Misumenops tricuspidatus (F.)	Araneae: Thomisidae	A
	Orius minutus L.	Heteroptera: Anthocoridae	A
	Deraeocoris punctulatus Fallen	Heteroptera: Miridae	A

SDs, the hybridoma cell lines were scored as positive (Sutula et al., 1986).

Monoclonal antibody cross-reactivity tests

The McAb was tested by indirect ELISA for cross-reactions against material from a range of arthropod species presented in Table 1. Each sample (gut) was homogenized with PBS and the final concentration was 50 mg ml^{-1}. Each treatment was repeated 10 times. The indirect ELISA procedure was carried out exactly as described above. Individual wells of the plate were coated separately with a 100 µl aliquot of the arthropod homogenates diluted 1:1 000 in coating buffer. Optimum assay conditions were determined by checkerboard titrations. The stock solution, McAb and HRP-IgG were diluted 1:1 000, 1:20 000, and 1:5 000, respectively, in coating buffer.

Detection period within predators

Adonia variegata was chosen as a model predator because it is one of the most important predators in wheat fields in China (Gao et al., 2004). Beetles were collected from wheat fields at the experimental farm of Inner Mongolia Agricultural University. Beetles (n = 120) were fed and then starved for 7 days prior to the experiment. Ten beetles were frozen as negative controls (unfed controls). Remaining beetles were allowed to feed on one *S. avenae* adult each at 25 ± 1 °C. The beetles were then frozen at 0, 2, 4, 8, 12, 24, 48, 72, 120, 144, and 168 h, respectively, since the end of the period during which they had been feeding on aphids. Each beetle was dissected and the remaining gut was homogenized in 1 ml PBS and centrifuged at 8 000 ***g*** for 10 min. The supernatant was then removed and stored at −40 °C as stock solution. Each treatment (time after feeding) was repeated 10 times (10 beetles). Indirect ELISA was used to test these samples as described above.

Field experiment

Major predator species were collected from the experimental farm of Inner Mongolia Agricultural University every week during the wheat growth period. A sample of 10 net sweeps was randomly made at one of 10 sites from wheat fields (0.4 ha) and 10 samples were made each time. Meanwhile, the number of *S. avenae* on 200 wheat plants, 20 randomly selected plants from each site, was determined each time. After collection, each predator individual was placed in separate small tubes in an ice bag and transferred to the lab. The predators were killed immediately by freezing at −20 °C. Each individual was dissected and homogenized as described above, and stored at −40 °C until assayed. Indirect ELISA was performed on homogenized individual predators as described above.

Results

Monoclonal antibody production

A total of 206 positive wells were generated from 421 wells with hybridoma cells on five fusion plates. Twenty-seven percent of these positive wells generated antibodies that strongly reacted with the antigens. They were selected and monocloned by limiting dilution. Finally, cell line EGA-4A9 was selected to develop the monoclonal antibody against the semi-digested proteins of *S. avenae* aphids in ladybird beetles. This particular cell line was selected for its high specificity, its rapid growth rate, and its stability. Isotyping demonstrated the McAb to be the IgG2a isotype. EGA-4A9 was propagated in mice after screening.

Monoclonal antibody cross-reactivity tests

An indirect ELISA indicated that the McAb EGA-4A9 did not cross-react with *S. avenae* and other arthropods tested except with the ladybird beetles fed on *S. avenae* (Figure 1). Moreover, it did not react with the beetles fed on other aphid species tested (Figure 2). These samples all yielded optical densities similar to the negative controls.

Detection period

The absorbance values (Y) for detection of the epitope to which McAb EGA-4A9 binds first increased with time (X)

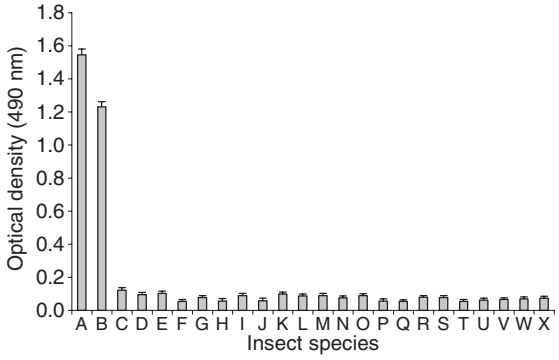

Figure 1 Reactivities of McAb EGA-4A9 to arthropod species: (A) *Adonia variegata* fed on *Sitobion avenae*, (B) *Propylea japonica* fed on *S. avenae*, (C) *S. avenae*, (D) *Rhopalosiphum maidis*, (E) *Aphis medicaginis*, (F) *Aphis glycines*, (G) *Acyrthosiphon solani*, (H) *Laodelphax striatellus*, (I) *Trigonotylus ruficornis*, (J) *Cicadella viridis*, (K) *A. variegata*, (L) *P. japonica*, (M) *Hippodamia tredecimpunctata*, (N) *Coccinella septempunctata*, (O) *Coccinulea sinensis*, (P) *Chrysopa phyllochroma*, (Q) *Syrphus corollae*, (R) *Misumenops tricuspidatus*, (S) *Orius agilis*, (T) *Deraeocoris punctulatus*, (U) *C. phyllochroma* fed on *S. avenae*, (V) *S. corollae* fed on *S. avenae*, (W) *D. punctulatus* fed on *S. avenae*, and (X) negative control. Bars represent mean + SE.

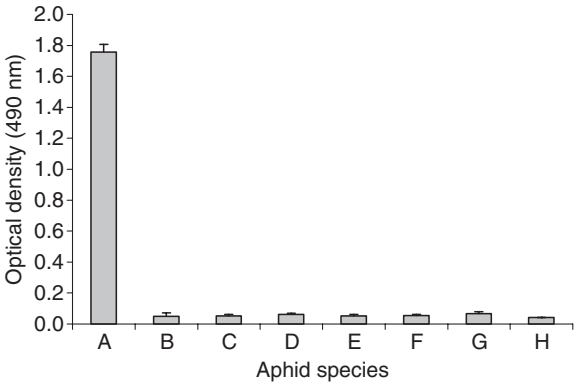

Figure 2 Reactivities of McAb EGA-4A9 to *Adonia variegata* fed on various aphid species: (A) *Sitobion avenae*, (B) *Aphis gossypii*, (C) *Myzus persicae*, (D) *Aphis medicaginis*, (E) *Rhopalosiphum maidis*, (F) *Aphis laburni*, (G) *Schizaphis graminum*, and (H) negative control. Bars represent mean + SE.

and then declined (Figure 3). This relationship is adequately described by the regression equation $Y = 1/(0.3076X^2 - 0.5906X + 0.7386)$ ($r = 0.9060$, $P = 0.0058$, non-linear regression). The absorbance value of the negative controls was 0.0805 ± 0.0131, so the positive threshold was 0.0805 + 3*0.0131 = 0.1201 (Sutula et al., 1986). The detection period of the EGA-4A9-recognizable protein at 25 °C was 6.02 days when the positive threshold was put into the equation.

Field experiment

The main predator species collected from wheat fields were examined to qualitatively identify predator species feeding on *S. avenae* in wheat fields and to evaluate their relative importance using McAb EGA-4A9. Between 28 and 72% of coccinellids collected from the field were positive for English grain aphid protein by ELISA, and all collected ladybird beetle species were positive (Table 2), indicating that they preyed on *S. avenae* in wheat fields. The percentage positives of hoverflies, green lacewings, and predatory capsid bugs were all zero as expected because the monoclonal antibody we used was specifically developed to the semi-digested products of *S. avenae* in the guts of ladybird beetles. The total percentage positive predatory ladybird beetles changed with the change of *S. avenae* density (Figure 4). Correlation analysis indicated that the total percentage positives was significantly correlated with the density of *S. avenae* in wheat fields ($r = 0.7615$, $P = 0.004$).

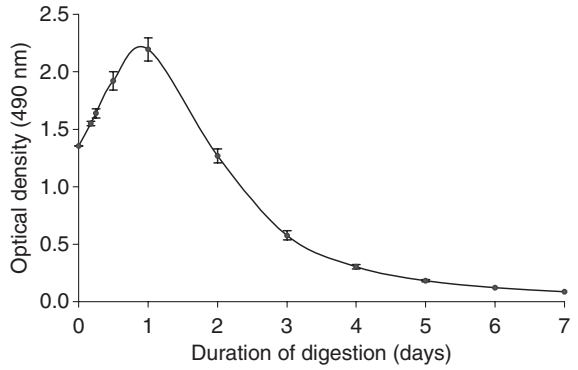

Figure 3 Relationship between absorbance values (mean ± SE) for detection of the epitope to which McAb EGA-4A9 binds and digestion time of *Sitobion avenae* proteins in the gut of *Adonia variegata*.

Discussion

Several monoclonal antibodies enabling recognition of arthropod crop pests have been developed (Greenstone et al., 1991; Hagler et al., 1993; Pang et al., 2001; Harwood et al., 2005; Fournier et al., 2006) since Lenz & Greenstone (1988) developed a monoclonal antibody against the arylphorin of *Helicoverpa zea* (formerly *Heliothis*) (Boddie). However, monoclonal antibodies against aphids have seldomly been developed, although many aphids are important pests in agricultural ecosystems. This may be due to the fact that aphids are digested too quickly in the predators' gut to be detected. We previously developed two highly species-specific monoclonal antibodies to *S. avenae* using a homogenate of *S. avenae* adults and nymphs as immunogen (Pang et al., 2006). These antibodies reacted strongly with the native antigen. However, they did not detect the aphid antigen in the gut of ladybird beetles within less than an hour of being consumed by ladybird beetles, probably because the epitope had become denatured. Symondson et al. (1999) encountered the same problem when they attempted to develop a species-specific antibody against the

Table 2 Percentage McAb EGA-4A9-positives of main predatory species in wheat fields

Predator species	No. tested	Positive number	% positive
Adonia variegata	234	147	62.8
Propylea japonica	86	39	45.3
Hippodamia tredecimpunctata	29	21	72.4
Coccinulea sinensis	43	12	27.9
Coccinella septempunctata	16	11	68.7
Adalia bipunctata	5	2	40
Chrysopa phyllochroma	63	0	0
Syrphus corollae	38	0	0
Deraeocoris punctulatus	34	0	0

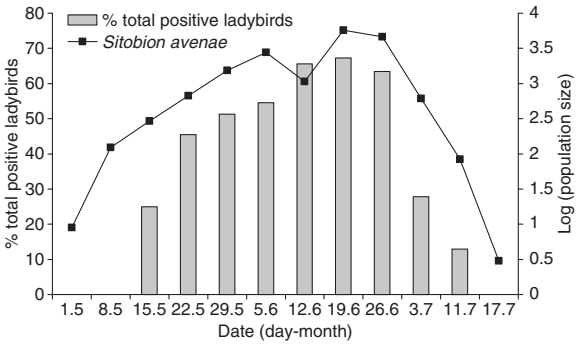

Figure 4 Time course of the total percentage McAb EGA-4A9-positives of ladybird beetles and of *Sitobion avenae* population size in wheat fields.

aphid *M. dirhodum*. They suggested as a strategy to immunize with proteins that have already been semi-digested in the gut of a predator, and tried to use slug proteins that had been digested for 6 h in the foreguts of carabid beetles as antigens, but no useful antibodies resulted. As far as we know, this is the first report on successful development of a monoclonal antibody for detection of prey-derived proteins in predators using semi-digested proteins present in the guts of predators as immunogen.

The monoclonal antibody EGA-4A9 we developed reacted strongly only with the ladybird beetles which had preyed on *S. avenae*, but it neither reacted with native *S. avenae* proteins nor with proteins of other arthropods or of predators which had either fed on other aphid species or had been starved. Furthermore, the experiment on detection period showed that the absorbance values for detection of the epitopes in the guts of ladybird beetles fed *S. avenae* first increased with time and then declined because the antigen continued to decay. The field experiment also showed that EGA-4A9 did not react against hoverflies, green lacewings, and predatory capsid bugs, just as in the cross-reactivity tests. These observations indicated that the antigen protein to which the monoclonal antibody EGA-4A9 bound was a semi-digested product of the wheat aphid in the gut of ladybird beetles.

The field experiment demonstrated that among the ladybird beetle species the population of *A. variegata* was the largest and the percentage of positive beetles was higher than that of the other species except for that of *Coccinella septempunctata*. However, it did not confirm that *A. variegata* was the most important predator for *S. avenae* in wheat fields because the rates of disappearance of the epitope in different predators were not compared. Nevertheless, this monoclonal antibody is useful to investigate the rate of predation of ladybird beetles on *S. avenae* in the field.

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