JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of Calcium-Alginate Gel as an Artificial Diet Medium for Bioassays on Common Cutworms

MASANORI MORIMOTO,* KAZUHIKO MATSUDA, YOSHINORI OHTA, TAKASHI IHARA, AND KOICHIRO KOMAI

Department of Agricultural Chemistry, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan

A calcium-alginate gel diet was developed for *Spodoptera litura* larvae, and its reliability as a carrier for incorporating antifeedants as well as insecticides was investigated. The alginate gel diet was prepared with a simple protocol, which does not involve any heating process. When tested using this diet, acephate, a *Bacillus thuringiensis* endotoxin formulation and rotenone reproducibly showed insecticidal activity against the larvae, while neem oil and scabequinone deterred the larval feeding effectively. However, not only the insecticidal activity of acephate but also the antifeedant activity of neem oil was reduced by replacing the alginate component by agar in the diet, suggesting the usefulness of the alginate gel diet as an assay tool for testing a broad range of samples against the larvae.

KEYWORDS: Bioassay method; calcium-alginate gel; artificial diet; insecticidal compounds; antifeedants; lepidopteran larvae

INTRODUCTION

A variety of insecticides have been used to protect plants from pests (*1*, *2*), while transgenic plants expressing insecticidal proteins from *Bacillus thuringiensis* have also been deployed to enhance crop protection efficacy (*3*, *4*). Even though these measures are effective, intensive efforts have also been made for the development of new insecticidal chemicals as well as for exploration of insecticidal proteins to prepare for the emergence of resistant species. To obtain reliable results the bioassay methods for these objectives have to be simple and stable. However, there have been many cases when samples were applied to test their activities on the surface of leaf disks as well as the artificial diet made from raw plant materials. Since, in such cases, the samples were not evenly distributed in the diet, and might be degraded or converted to other substances by either metabolic systems (*5*) or reactive molecules from plants (*6*), it is likely that the biological activities were either over- or undervalued.

To resolve these problems, we have developed for the *Spodoptera litura* larvae a novel alginate gel diet, which does not include any plant materials. It was possible to prepare the alginate gel diet at room temperature or lower, and to reproducibly evaluate antifeedant as well as insecticidal activities of several known compounds against the common cutworms. When tested at the same concentrations, the biological activities determined using this diet were higher compared with those determined using the diet that contains agar instead of alginate, suggesting that the alginate gel diet can be used as an assay tool for evaluating the pest-controlling activities of many synthetic and natural compounds.

MATERIALS AND METHODS

Insects. The common cutworms, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) were reared on an artificial diet (Insecta LF, Nihon Nosan Kogyo Co., Japan) in a controlled room environment at 26.5 °C and 60% humidity. This insect species is well-known as the worst pest damaging to a wide range of vegetables in Asia.

Test Chemicals. The novel biological assay method was evaluated using commercial insecticides and natural products. An organic phosphorus insecticide acephate and a natural insecticide rotenone from Derris (*Derris elliptica*) were purchased from Wako Chemical Industries, Ltd., and Sigma Chemical Co., Ltd., respectively. A biopesticide Bt was a commercial agent, Toaro flowable CT including insecticidal crystals, produced by the Sumitomo Chemical Takeda Agro Company, Ltd. The neem oil, including insect antifeedant azadirachtins from the fruit of the neem tree (*Azadirachta indica*) (*7*), was a gift from Daiho Perfumery Co., Ltd, while scabequinone was isolated from the tropical sedge, *Cyperus distans* L, as reported earlier (*8*).

Preparation of Calcium-Alginate Gel Diet. On the dried yeast (60 mg/well) distributed in each well of a 24-well plate, the basic solution (300 μL) was poured and mixed with the yeast powder. The basic solution consists of sodium alginate (Wako Chemical Industries, Ltd., viscosity 100∼150 cP, 1 g), sucrose (2 g), sodium ascorbate (0.4 g), sodium sorbinate (0.14 g), and methyl benzoate (0.3 g) in distilled water (30 mL). An aqueous sample solution (500 μL) was then poured into the dish. Lipophilic compounds were tested by dissolving in water by the addition of their DMSO:Tween 80 (1:1) stock solution (Final concentration of DMSO:Tween 80 (1:1) was 1 ∼ 10%). The solvent in this concentration range had no effect on the insect feeding. After gelation by the addition of 4% aqueous $CaCl_2$ solution (200 μL), a piece of the diet was placed on a paraffin sheet (ca. 4 cm^2), which was

* Author to whom correspondence should be addressed (telephone +81-742-43-1511; fax +81-742-43-1445; e-mail morimoto@nara.kindai.ac.jp).

10.1021/jf0497511 CCC: $27.50
Published on Web 06/25/2004

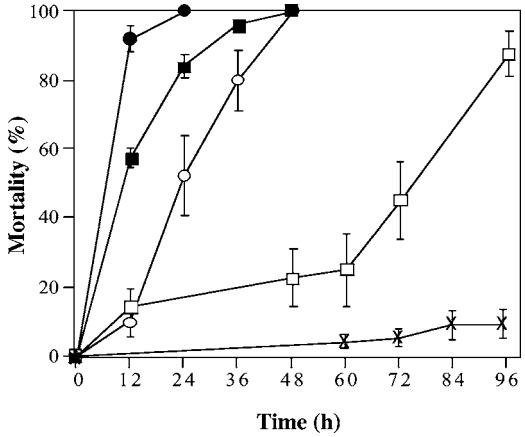

Figure 1. Time-dependent progress of insecticidal actions of acephate and a Bt agent tested using the calcium-alginate gel. Acephate was applied at 25 μg/mL against second (●) and third (■) instars of *S. litura* larvae, while the Bt agent was applied at 700 μg/mL against second (○) and third (□) instars of the larvae. Mortality of the third-instar larvae that infested the control gel diet is indicated by **X**. Ten *S. litura* larvae were used for replicate at each dose. Each point represents the mean ± SEM of 5 replicates.

placed on a moistened filter paper (Qualitative filter paper No. 1, Toyo Roshi Kaisha, Ltd.) at the center of a glass Petri dish (93-mm diameter).

An agar version of the gel diet was also prepared. Namely, 300 μL of the basic solution lacking sodium alginate and containing dry yeast powder was mixed with 500 μL of 3% agar solution containing the test sample and 200 μL of 4% aqueous $CaCl_2$ solution at 50 °C, and then the mixture was cooled to set in a water bath.

Assay Procedure. Ten second- or third-instar larvae of *S. litura*, which were starved for 24 h before the tests, were released in each dish containing a piece of the diet and finally covered with the lid of the glass Petri dish. The dishes containing the sample diet and larvae were kept under dark condition at 26.5 °C with 60% humidity during tests. The mortality of the larvae and the fresh weight of the diet were measured at every 12 h after the start of the experiments. The tests were multiplied over time at a range of concentrations. The mortality was judged by the loss of larval mobility, while antifeedant activity was evaluated by comparing the decrease in the diet weight with that of blank and control diets, which were placed in the absence of larvae and test chemicals, respectively, under the same conditions.

To evaluate the insecticidal and antifeedant activities of each test sample, LC_{50} and EC_{50} (μg/mL) values, concentrations required to kill half of the third-instar larvae tested and reduce their consumption of the diet by 50% compared with the control in terms of the feeding suppression index, respectively, were calculated by the Probit analysis (*9*) using a PC software (Microsoft, Excel ver. 5, macro program). The feeding suppression index (%) was calculated according to the following equation:

$$\text{Feeding suppression index (\%)} = [(\text{fresh weight of test diet} - \text{fresh weight of control diet})/(\text{fresh weight of blank diet} - \text{fresh weight of control diet})] \times 100$$

RESULTS AND DISCUSSION

It was possible to mix the alginate components with the sample solution and subsequently make the mixture set by the addition of $CaCl_2$ solution at room temperature or lower. The cutworms were able to grow normally on the gel diets at least for 4 days (**Figure 1**). When applied by mixing with the diets, a commercial insecticide acephate reproducibly showed high and rapid oral toxicity. The second-instar larvae died more quickly than the third-instar larvae, but both stages of the larvae died completely by 36 h after the start of the experiments when applied at 25 μg/mL (**Figure 1**). The LC_{50} value of acephate

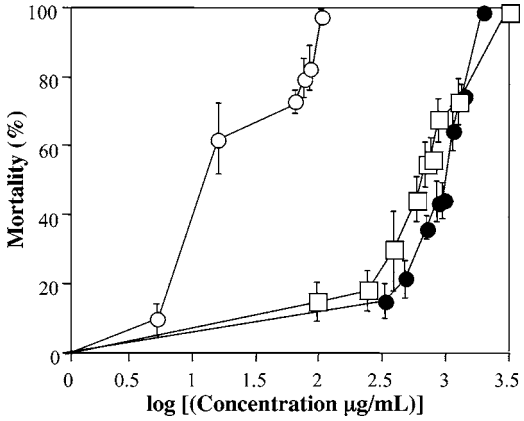

Figure 2. Dose-insecticidal activity relationships for acephate (○), rotenone (□), and a Bt agent (●) tested using the calcium-alginate gel bioassay method. Ten *S. litura* larvae (third instar) were used for replicate at each dose. Insecticidal activity of acephate and rotenone was determined at 24 h, while that of the Bt agent was determined at 48 h. Each point represents the mean ± SEM of 3–6 replicates.

Table 1. Evaluation of Insecticidal and Antifeedant Activities of the Chemicals by Calcium-Alginate Gel Bioassay against Third-Instar Common Cutworms (*S. litura*)

test chemical	N[a]	time (h)	insecticidal activity LC_{50}[b]	slope	CI[c]	antifeedant activity EC_{50}[b]	slope	CI[b]
acephate	3–5	24	12.8	2.29	11–15.1	ND[d]		
rotenone	3–6	24	656	2.20	430–10 53	ND		
Bt agent	3–5	48	911	2.85	686–12 77	ND		
neem oil	5	48	ND			120	1.02	66–181
scabequinone	4	48	ND			669	1.23	462–1034

[a] Numbers of experiments. [b] LC_{50} and EC_{50} values (unit: μg/mL) were obtained from means of 3–6 experiments by the Probit analyses. [c] CI: 95% confidence interval. [d] Not determined.

determined at 24 h for the third-instar larvae was 12.8 μg/ mL (95% confidence interval (CI): 11.0−15.1 μg/mL, **Figure 2** and **Table 1**). A Bt agent containing the insecticidal crystal proteins was also orally toxic, but acted more slowly than acephate in dose and insect-age dependent manners (**Figure 1**). When tested at 700 μg/mL, the Bt agent was able to kill half of the second-instar larvae within 48 h after the start of the experiment. However, a higher dose was required to be effective on the third-instar larvae with a LC_{50} of 911 μg/mL (CI: 686−1277 μg/mL, **Figure 2** and **Table 1**). Rotenone, a natural insecticide acting on respiration systems, exhibited a moderate insecticidal activity in a dose-dependent manner (**Figure 2**) with an LC_{50} value of 656 μg/mL (CI: 430−1053 μg/mL, **Table 1**).

In addition to the insecticidal compounds, the antifeedant activities of natural products and synthetic compounds were also evaluated. Even though insect antifeedants 2,3-dihydrobenzofurans (*10*) and chromones (*11*) were ineffective in deterring the feeding by the common cutworms due to unknown mechanisms (data not shown), several compounds tested were active as antifeedants. Neem oil and scabequinone did not kill the common cutworms within the doses tested, but inhibited the larval feeding in a dose-dependent manner (**Figure 3**). Neem oil was more potent in the antifeedant activity with an EC_{50} value of 120 μg/mL (CI: 66−181 μg/mL) than scabequinone with an EC_{50} value of 669 μg/mL (CI = 464−1034 μg/mL), when tested against the third-instar larvae (**Table 1**).

The insecticidal and antifeedant activities described above were obtained with good correlation coefficients (>0.97) in the Probit analyses (**Table 1**), suggesting that it is possible

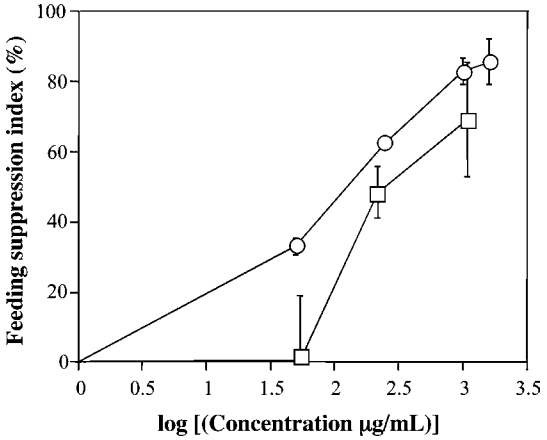

Figure 3. Dose-antifeedant activity relationships for neem oil (○) and scabequinone (□) tested using the calcium-alginate gel. Ten *S. litura* larvae (third instar) were used for replicate at each dose, and the feeding suppression index (%, see text for definition) was determined at 48 h. Each point represents the mean ± SEM of 3–5 replicates.

to evaluate using the alginate gel diet these two activities of various samples irrespective of their mode of actions. If necessary, the assay sensitivity can be enhanced by prolonging the test period as well as by employing younger insects for tests (see **Figure 1**).

As compared with the alginate gel diet, the diet containing the same concentration of agar instead of alginate (see Materials and Methods) required higher concentrations of samples to exhibit the same insecticidal and antifeedant actions. Acephate tested at 25 μg/mL for 24 h killed 92.0 ± 3.9% (mean ± SEM, $n = 4$) of the cutworms when applied using the alginate gel diet, while it killed 61.7 ± 6.1% ($n = 4$) of the larvae when applied using the agar diet. On the other hand, neem oil applied at 250 μg/mL inhibited the larval diet feeding by 62.7 ± 0.9% ($n = 4$), whereas no significant antifeedant action was seen even if the sample was applied at 500 μg/mL using the agar diet, indicating the superiority of the alginate gel diet over the agar counterpart.

In conclusion, we have developed a calcium-alginate gel diet and investigated its usefulness for bioassays against the common cutworms. Heating was not necessary to prepare the diet, and the biological activities obtained using this diet were reproducible. Thus, the alginate gel diet can be used as a carrier for a broad range of insect-controlling agents, even though it is necessary to improve the diet composition and to accumulate more data supporting the merit of the diet. Finally, we note that, if the antimicrobial preservatives are excluded, the diet may also be used to test pathogenicity to insects of living microorganisms from a variety of origins.

LITERATURE CITED

(1) Kagabu, S. Chloronicotinyl insecticides − discovery, application and future perspective. *Rev. Toxicol.* **1997**, *1*, 75−129.

(2) Sparks, T. C.; Crouse G. D.; Durst, G. Natural products as insecticides: the biology, biochemistry and quantitative structure−activity relationships of spinosyns and spinosoids. *Pest Manag. Sci.* **2001**, *57*, 896−905.

(3) Roush, R. T. Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? *Pestic. Sci.* **1997**, *51*, 328−334.

(4) Yamamoto, T. One hundred years of *Bacillus thuringiensis* research and development: Discovery to transgenic crops. *J. Insect Biotechnol. Sericol.* **2001**, *70*, 1−23.

(5) van Eerd, L. L.; Hoagland, R. E.; Zablotowicz, R. M.; Hall, J. C. Pesticide metabolism in plants and microorganisms. *Weed Sci.* **2003**, *51*, 472−495.

(6) Kubo, I.; Fujita, K.-I.; Kubo, A.; Nihei, K.-I.; Lunde, C. S. Mode of antifungal action of (2*E*)-alkenals against *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* **2003**, *51*, 3951−3957.

(7) Mordue, L. A. J.; Blackwell, A. Azadirachtin: an update. *J. Insect Physiol.* **1993**, *30*, 903−924.

(8) Morimoto, M.; Fujii, Y.; Komai, K. Antifeedants in Cyperaceae: Coumaran and quinones from *Cyperus* spp. *Phytochemistry* **1999**, *51*, 605−608.

(9) Finney, D. J. In *Probit Analysis*, 2nd ed.; Cambridge University Press: London, 1952.

(10) Morimoto, M.; Urakawa, M.; Fujitaka, T.; Komai, K. Structure−activity relationship for the insect antifeedant activity of benzofuran derivatives. *Biosci. Biotech. Biochem.* **1999**, *63*, 840−84.

(11) Morimoto, M.; Tanimoto, K.; Nakano, S.; Ozaki, Y.; Nakano, A.; Komai, K. Insect antifeedant activity of flavones and chromones against *Spodoptera litura*. *J. Agric. Food Chem.* **2003**, *51*, 389−393.

Received for review February 15, 2004. Revised manuscript received April 24, 2004. Accepted May 7, 2004. This study was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences from Bio-oriented Technology Research Advancement Institution.

JF0497511