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Effects of Enzymes, Chemicals, and Temperature on *Steinernema carpocapsae* Attraction to Host Plasma[1]

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Abstract: Migration of exsheathed infective juveniles of *Steinernema carpocapsae* to plasma of the host insect *Spodoptera litura* was not affected by treatments with the lectins concanavalin A, soybean agglutinin, or wheat germ agglutinin; with the enzymes neuraminidase, α-mannosidase, lipase, pronase, or phospholipase C; or with cetyl trimethylammonium bromide or spermidine. Treatment with sodium metaperiodate or sodium hypochlorite inhibited nematode attraction towards insect plasma; numbers of randomly wandering nematodes increased. Nematode migration towards the source of attraction was unaffected by temperatures below 33 C but was impaired at 35 and 37 C. The adverse effect of 5 mM and 10 mM $NaIO_4$ on migratory behavior was reversed 24 hours after rinsing with buffered saline. The effect of NaOCl on nematode behavior was slightly reversible at concentrations of 0.2 and 0.4% (v/v) but apparently irreversible at 0.6 and 1.0%. The effect of heat treatment at 35 and 37 C was reversible.

Key words: attraction, insect plasma, nematode surface coat, *Spodoptera litura, Steinernema carpocapsae,* temperature dependency.

Infective juveniles of the entomopathogenic nematode, *Steinernema carpocapsae,* parasitize larvae of many kinds of insects (18). They are not only attracted to the insect body, but also to various stimuli, for example, aqueous surface washes of *Galleria mellonella* larvae (22), CO_2 (8), insect body temperature (1,2), the symbiotic bacterium *Xenorhabdus nematophilus* (10,20), and insect fecal components (16,23). Recently, we demonstrated that infective juveniles were attracted to the plasma of three insect hosts, although the nature of active substance(s) in the plasma has not been determined (15).

Structurally, *S. carpocapsae* infective juveniles have clearly defined amphids (19), which are widely accepted as the main chemoreceptors in nematodes. On the sensillum membrane of receptors and on the cuticle surface, several investigators have so far demonstrated the presence of carbohydrates that are functional components in nematode-nematophagous fungus interactions (12,17,21) and chemotaxis (26,27).

In the present study, we examined the effects of enzyme, chemical, and temperature treatments on the attraction of *S. carpocapsae* to the plasma of the host insect, the common cutworm *Spodoptera litura.*

MATERIALS AND METHODS

Nematode: Steinernema carpocapsae (str. All) was maintained at 25 C using *Galleria mellonella* larvae (5). In order to use physiologically uniform infective juveniles for attraction tests, the following method was employed: 2 weeks after incubation of nematode-infected insects at 25 C, the insect cadavers that had been kept on filter paper strips moistened with 0.01% (v/v) formalin in test tubes were stored at 6–10 C for about 2 months. During the storage time, most of the juveniles exsheathed and became more responsive to insect plasma (15). After washing with distilled water, only those exsheathed juveniles that moved through a nylon mesh screen (32-μm aperture) were recovered for experimental use. They were washed three times in phosphate buffered saline (PBS) (20 mM potassium phosphate, 150 mM NaCl, pH 7.2) before use (4).

Insect plasma: Hemolymph of full-grown last instar larvae of *S. litura* was collected by puncturing the second prolegs and allowing the hemolymph to collect in a test tube partially immersed in ice water. Immedi-

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ately after collection, the hemolymph was centrifuged at 5,000*g* for 5 minutes at 4 C to remove hemocytes. The plasma was passed through 0.2-μm sterile filters to eliminate possible attractant effects due to bacterial contaminants. A few crystals of phenylthiourea were added to the plasma to prevent melanization. The plasma was stored at −70 C until use.

Test chemicals: Lectins (concanavalin A, soybean agglutinin, wheat germ agglutinin: Sigma Chemical Co., St. Louis, MO), enzymes (neuraminidase, α-mannosidase, lipase, pronase, phospholipase C from Seikagaku Kogyo Co., Tokyo, Japan), spermidine (Aldrich Chemical Co., Milwaukee, WI), cetyl trimethylammonium bromide (CTAB), sodium metaperiodate (Katayama Chemical Co., Tokyo, Japan), and sodium hypochlorite were used. The treatment conditions with lectins were basically those of Dunphy and Webster (4); neuraminidase, mannosidase, and lipase procedures were in accordance with Jansson et al. (11); and pronase procedure was that of Spiegel et al. (24). The experimental conditions used to examine the effects of the enzymes and chemicals on nematode attraction were as follows: Lectins, 500 μg/ml in PBS (pH 7.2) at 20 C; neuraminidase, 1 IU/ml in 10 mM sodium acetate buffer (pH 5.5) containing 4 mM $CaCl_2$ at 30 C; α-mannosidase (Jack bean), 2 IU/ml in 10 mM sodium citrate buffer (pH 4.5) at 25 C; lipase, 1,000 IU/ml in 10 mM Tris buffer (pH 7.7) at 30 C; pronase, 10 mg/ml in PBS (pH 7.2) at 37 C; phospholipase C, 10 IU/ml in PBS (pH 7.2) at 37 C; CTAB and spermidine at 500 mg/ml in distilled water at 25 C. The incubation time of the above-mentioned tests was 1 hour. In the case of sodium metaperiodate and sodium hypochlorite, dose-response relations were examined for different concentrations; nematodes were exposed to 0.005, 0.01, and 0.05 M sodium metaperiodate solutions for 1 hour at 25 C in the dark and to 0.2, 0.4, 0.6, 0.8, and 1% sodium hypochlorite (v/v) solutions for 10 minutes at 20 C. After treatment, the test solution was removed by centrifuging at 450 g for 1 minute using PBS buffer as washing solution. The procedure was repeated five times. For each chemical treatment, a parallel treatment was conducted on control nematodes using respective buffer solutions. Twenty-four hours after rinsing in PBS, all nematodes were examined for their capacity to be attracted towards insect plasma.

Temperature dependence experiments: Exsheathed juveniles were suspended in 1 ml distilled water and incubated at 20, 25, 30, 33, 35, and 37 C for 1 hour before the migration assay. Behavioral response was also investigated after the incubation of nematodes in distilled water at 30 C or at 37 C for 20, 40, and 60 minutes. The procedure for washing was the same as outlined above. To examine the recovery of attractant-recognition ability, all nematodes were examined 24 hours after being returned to 25 C.

Attraction test: The effect of the various chemicals on nematode attraction was measured using the assay previously detailed (15). Briefly, 0.6% agar in PBS was poured in 4-mm thick layer in each petri dish (45-mm-d). In a preliminary test, PBS buffer was found to be more suitable for the attraction test than sodium citrate, sodium acetate, Tris buffers, and distilled water. Two wells (5-mm-d, 2-mm-deep) were made in the agar on opposite sides and 1 cm from the central inoculation well (2-mm-d, 2-mm-deep). Fifty μl of insect plasma were lyophilized, dissolved in 50 μl of PBS, and then applied in one side well. The same volume of PBS was applied in the opposite well as a control. Two hours after the addition of solutions to the side wells, 1 μl of PBS containing 30 infective juveniles treated with chemicals was applied in the inoculation well. After nematodes were allowed to move for 2 hours at 25 C in the dark, the plates were exposed to chloroform vapor at 35 C for 10 minutes to kill the nematodes. The number of nematodes in the plasma well, the PBS well, and the inoculation well, and those located outside these wells were counted. Nematodes within 5 mm of the plasma well

were counted as being attracted to the source. All experiments were performed four times each with four replications.

Statistical analyses: Data expressed in percentage were transformed to arcsine values to ensure normality. The single-classification ANOVA test and Duncan's multiple-range test were employed for the statistical analyses. All data were presented as means ± standard error of means.

Results

Effect of enzyme and chemical treatments: The effects of enzyme and chemical treatments were evaluated by comparing the migration of nematodes treated with enzymes or chemicals to insect plasma with those incubated in the corresponding buffer solution (Table 1). Nematode migration to host plasma was not affected by treatment with concanavalin A, soybean agglutinin, or wheat germ agglutinin at a concentration of 500 µg/ml. Neuraminidase, mannosidase, lipase, pronase, and phospholipase C had no effect at tested concentrations. Cetyl trimethylammonium bromide and spermidine also had no influence. Treatment with sodium metaperiodate (0.005–0.01 M) significantly decreased nematode migration, with larger numbers of wandering nematodes not moving towards the source of attraction (Table 2). At the lowest concentration (0.005 M), the nematode migration decreased to 11.2% and was almost prevented by the rest of tested molarities (0.01 and 0.05 M). Twenty-four hours after rinsing with PBS, however, 41.5% of those treated with 0.05 M sodium metaperiodate regained their oriented movement. Treatment with sodium hypochlorite also significantly decreased the migratory behavior of the nematode at all tested concentrations (0.2–1.0%) and increased the numbers of wandering nematodes (Table 3). The higher the concentration of sodium hypochlorite, the greater the decrease in the attraction rates. At 0.4–1.0% concen-

Table 1. Effects of enzyme and chemical treatments on the migration of *Steinernema carpocapsae* infective juveniles toward *Spodoptera litura* plasma.

Treatment	Concentration	Percentage migration† to			
		S. litura plasma	Inoculation zone	PBS‡	Outside zones
Concanavalin A	500 µg/ml	82.5 ± 1.4 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.5 ± 1.4 d
Soybean agglutinin	500 µg/ml	82.7 ± 1.2 a	0.3 ± 0.7 bc	0.0 ± 0.0 c	17.0 ± 1.4 d
Wheat germ agglutinin	500 µg/ml	82.3 ± 1.9 a	0.0 ± 0.0 c	0.7 ± 0.8 bc	17.0 ± 2.4 d
Control (PBS, 20 C)		83.8 ± 3.5 a	0.0 ± 0.0 c	0.0 ± 0.0 c	16.2 ± 3.5 d
Pronase	10 mg/ml	82.3 ± 1.5 a	0.0 ± 0.0 c	0.4 ± 0.9 bc	17.3 ± 1.2 d
Phospholipase C	10 IU/ml	82.1 ± 1.2 a	0.0 ± 0.0 c	0.5 ± 0.6 bc	17.4 ± 1.5 d
Control (PBS, 30 C)		82.3 ± 1.0 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.7 ± 1.0 d
Neuraminidase	1 IU/ml	82.5 ± 2.0 a	0.0 ± 0.0 c	0.9 ± 1.0 bc	16.6 ± 2.8 d
Control (10 mM sodium acetate buffer, pH 5.5)		82.5 ± 2.7 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.5 ± 2.7 d
α-Mannosidase	2 IU/ml	82.3 ± 1.6 a	1.0 ± 1.9 bc	1.1 ± 1.6 bc	15.6 ± 3.1 d
Control (10 mM sodium citrate buffer, pH 4.5)		82.3 ± 2.0 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.7 ± 2.0 d
Lipase	1,000 IU/ml	82.1 ± 2.5 a	1.1 ± 1.3 bc	0.9 ± 1.0 bc	15.9 ± 3.8 d
Control (10 mM Tris buffer, pH 7.7)		82.8 ± 3.0 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.2 ± 3.0 d
CTAB	500 µg/ml	82.3 ± 2.4 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.7 ± 2.4 d
Spermidine	500 µg/ml	81.8 ± 1.2 a	0.0 ± 0.0 c	0.0 ± 0.0 c	18.2 ± 1.2 d
Control (distilled water, 25 C)		82.8 ± 1.8 a	0.0 ± 0.0 c	0.0 ± 0.0 c	17.2 ± 1.8 d

† Mean ± SE (standard error).
‡ Phosphate-buffered saline.
Values followed by the same letter are not significantly different according to Duncan's multiple-range test ($P = 0.05$).

TABLE 2. Effect of sodium metaperiodate on migration of infective juveniles of *Steinernema carpocapsae* toward *Spodoptera litura* plasma.

Concentration of $NaIO_4$	Percentage migration† to			
	S. litura plasma	Inoculation zone	PBS‡	Outside zones
	Immediately after treatment for 60 minutes§			
0.005 M	11.2 ± 3.8 a	0.8 ± 0.9 b	0.0 ± 0.0 e	88.0 ± 3.7 c
0.01 M	1.6 ± 1.1 b	0.9 ± 1.8 b	0.0 ± 0.0 e	97.5 ± 0.9 f
0.05 M	1.2 ± 1.6 b	1.2 ± 1.4 b	0.0 ± 0.0 e	97.6 ± 1.7 f
Control (H_2O)	82.2 ± 2.5 c	0.0 ± 0.0 e	0.0 ± 0.0 e	17.8 ± 2.5 a
	24 hours after being rinsed with PBS following the above treatments			
0.005 M	82.5 ± 1.6 c	0.9 ± 0.6 b	0.0 ± 0.0 e	16.6 ± 2.0 a
0.01 M	80.8 ± 1.7 c	0.4 ± 0.8 be	0.0 ± 0.0 e	18.8 ± 1.2 a
0.05 M	41.5 ± 6.5 d	1.5 ± 1.9 b	0.0 ± 0.0 e	57.0 ± 9.9 g
Control (H_2O)	82.7 ± 2.1 c	0.0 ± 0.0 e	0.0 ± 0.0 e	17.3 ± 2.1 a

Investigations were made immediately after the treatment with various concentrations of sodium metaperiodate for 60 minutes and 24 hours after rinsing with PBS following the treatments. Values followed by the same letter are not significantly different according to Duncan's multiple-range test ($P = 0.05$).

† Mean ± SE.

‡ Phosphate-buffered saline

§ Nematodes were all rinsed with PBS after treatment.

trations the behavior was almost prevented. Twenty-four hours after being rinsed in PBS, approximately 40 and 10% of nematodes treated with 0.2 and 0.4% sodium hypochlorite, respectively, regained their migratory behavior, but those treated with higher concentrations were not able to resume oriented movement.

Effect of temperature: The nematode's migratory behavior to insect plasma was unaffected by temperature below 33 C, but sharply declined after incubation at 35 and 37 C for 1 hour (Table 4). On the other hand, the treatments at 35 and 37 C significantly ($P < 0.05$) increased the number of wandering nematodes outside zones.

TABLE 3. Effect of sodium hypochlorite on migration of infective juveniles of *Steinernema carpocapsae* toward *Spodoptera litura* plasma.

Concentration of NaOCl	Percentage migration† to			
	S. litura plasma	Inoculation zone	PBS‡	Outside zones
	Immediately after treatments for 10 minutes§			
0.2%	8.6 ± 2.4 a	1.3 ± 2.5 be	0.0 ± 0.0 e	90.1 ± 4.1 f
0.4%	3.2 ± 2.3 b	1.4 ± 2.1 be	0.6 ± 1.2 be	94.8 ± 2.6 f
0.6%	2.1 ± 2.4 be	1.4 ± 2.9 be	0.9 ± 1.9 be	95.6 ± 1.1 f
0.8%	2.0 ± 1.4 be	1.6 ± 2.1 be	0.0 ± 0.0 e	96.4 ± 3.2 f
1.0%	1.3 ± 1.5 be	1.7 ± 2.0 be	0.0 ± 0.0 e	97.0 ± 2.5 f
Control (H_2O)	82.4 ± 3.2 c	0.0 ± 0.0 e	0.0 ± 0.0 e	17.6 ± 3.2 g
	24 hours after being rinsed with PBS following the above treatments			
0.2%	40.0 ± 8.8 d	0.8 ± 0.9 be	0.0 ± 0.0 e	59.2 ± 8.9 h
0.4%	9.7 ± 1.3 a	1.3 ± 0.9 be	0.0 ± 0.0 e	89.0 ± 2.0 f
0.6%	4.6 ± 0.4 b	1.5 ± 1.2 be	0.0 ± 0.0 e	93.9 ± 1.5 f
0.8%	3.9 ± 0.7 b	1.8 ± 0.6 be	0.0 ± 0.0 e	94.3 ± 0.2 f
1.0%	3.2 ± 1.5 b	1.9 ± 1.2 be	0.0 ± 0.0 e	94.9 ± 1.5 f
Control (H_2O)	83.5 ± 1.4 c	0.0 ± 0.0 e	0.0 ± 0.0 e	16.5 ± 1.4 g

Investigations were made immediately after treatment for 10 minutes and 24 hours after being rinsed with PBS following the treatments.

Values followed by the same letter are not significantly different according to Duncan's multiple-range test ($P = 0.05$).

† Mean ± SE.

‡ Phosphate-buffered saline.

§ Nematodes were all rinsed with PBS after treatment.

TABLE 4. Effect of temperature on migration of infective juveniles of *Steinernema carpocapsae* toward *Spodoptera litura* plasma.

Temperature (C)	Percentage migration to insect plasma†	
	Immediately after treatment	24 hr after being returned to 25 C
20	81.6 ± 1.0 a	83.1 ± 1.5 a
25	81.6 ± 2.6 a	82.8 ± 1.5 a
30	81.3 ± 0.9 a	81.4 ± 1.3 a
33	81.2 ± 1.4 a	81.9 ± 0.8 a
35	49.2 ± 8.2 b	81.2 ± 1.6 a
37	18.7 ± 4.2 c	79.4 ± 2.6 a

Values followed by the same letter are not significantly different according to Duncan's multiple-range test ($P = 0.05$).

† Migration was investigated immediately after and 24 hours after being returned to 25 C following treatment for 1 hour at the various temperatures. Migration percentage expressed as Mean ± SE.

When the nematodes were incubated at 30 C for varying periods of time, their capacity for oriented movement towards insect plasma was not decreased. After incubation at 37 C, the percentage migration to insect plasma decreased with increasing exposure time up to 60 minutes (Table 5). Temperature effects were reversible because chemotactic activity was restored 24 hours after return to 25 C.

TABLE 5. Migration of infective juveniles of *Steinernema carpocapsae* toward *Spodoptera litura* plasma, as affected by preincubation temperature before bioassay.

Time (minutes)	Percentage migration to insect plasma†	
	Immediately after treatment	24 hours after being returned to 25 C
	Preincubation at 30 C	
20	81.3 ± 2.7 a	83.0 ± 1.6 a
40	81.3 ± 2.0 a	82.6 ± 1.6 a
60	81.1 ± 1.2 a	81.6 ± 1.9 a
	Preincubation at 37 C	
20	76.1 ± 6.8 a	81.3 ± 1.3 a
40	52.0 ± 9.5 b	81.1 ± 2.1 a
60	16.2 ± 3.8 c	80.9 ± 1.7 a

Values followed by the same letter are not significantly different according to Duncan's multiple-range test ($P = 0.05$).

† Migration was examined immediately after and 24 hours after being returned to 25 C following the temperature treatment at 30 or 37 C for 20, 40, and 60 minutes. Migration percentage expressed as mean ± SE.

DISCUSSION

It has been suggested that chemical stimuli act on the binding sites of receptors on a restricted portion of the cuticle surface in nematodes (26). Removal or blockade of the binding site results in a decrease in the response of nematode to stimuli (11,13, 14). For instance, treatment of the free-living nematode *Caenorhabditis elegans* with the lectin concanavalin A reduced attraction to the food source, *Escherichia coli* (13). The same effect was observed after exposure of *C. elegans* to the enzymes mannosidase or sialidase (11). The existence of carbohydrate moieties was confirmed by use of fluorescein isothiocyanate-conjugated lectins in *S. carpocapsae,* in which D-mannose, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine were detected (4). However, no effects of enzymatic elimination of these carbohydrates on the in vivo insect hemocyte attachment to nematode cuticle were observed. In the present experiments, treatments with concanavalin A, soybean agglutinin, or wheat germ agglutinin, which bind specifically to D-glucose and D-mannose, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine, respectively, did not affect the migration of infective juveniles to host insect plasma. This is probably due to a failure of binding to receptors in the absence of specific sugars or due to failure of inhibiting reception after binding.

Under the present experimental conditions, neuraminidase, α-mannosidase, lipase, pronase, and phospholipase had no effect on nematode migration to insect plasma. The presence of neuraminidic acid, mannose, lipids, and surface proteins is probably not essential for the reception of the attractive agent from insect plasma. However, pretreatment by periodate ions, which oxidize polysaccharides or polysaccharide moieties of glycoproteins under certain conditions (9), almost abolished the nematode's attraction behavior. The fact that *S. carpocapsae* infective juveniles failed to move to the source of stimuli after periodate treatment but not after concanava-

lin A, wheat germ agglutinin, soybean agglutinin, mannosidase, and neuraminidase treatments indicates that saccharides other than the above carbohydrate moieties may be involved in mediating the nematode's attraction to host plasma. Sodium metaperiodate treatment under mild conditions resulted in inhibiting the binding of lectins to the cuticle of preparasitic *Meloidogyne incognita* second-stage juveniles and their eggs (25). Thus, it appears that oxidation of the cuticle surface saccharides interferes with the migration of *S. carpocapsae* to plasma. The discrepancy between *S. carpocapsae* and *C. elegans* (11,13), which could not respond to their food sources after lectin or enzyme treatments, may be due to structural differences of receptors between the nematode species or in the arrangement of carbohydrates associated with the chemosensory structures. The cationic detergent cetyl trimethylammonium bromide and the low molecular weight cationic protein spermidine, both of which have been reported to reduce the ability of juveniles of *Globodera rostochiensis* to receive stimuli (7), had no influence on the nematode's migration to insect plasma.

Sodium hypochlorite is an effective oxidizing agent and halogenates aromatic or aliphatic organic compounds (3). In the present experiment, sodium hypochlorite also inhibited nematode chemotaxis.This may be due to a damage of the sensory apparatus of the nematode. Disturbance of nematode attraction by sodium hypochlorite has also been demonstrated for *Heterodera schachtii* (6) and *Globodera rostochiensis* (7).

The results presented here indicate that the nematode's chemosensory moiety (moieties) involved in attraction is unaffected by temperature in the range 20–33 C. The nematode's attractive behavior declined after incubation at 35 and 37 C for 1 hour; however, movement of the nematode was not hampered by this temperature. Thus, it appears that the ligands on *S. carpocapsae* for chemical stimuli are not thermostable.

The behavior of infective juveniles towards the host insect plasma was modified by treatment with sodium metaperiodate and sodium hypochlorite, and also by exposure to high temperature. The behavior of the juveniles after treatment with 0.005 and 0.01 M sodium metaperiodate was restored 24 hours after being rinsed in PBS, whereas the nematodes treated with 0.05 M sodium metaperiodate regained only 41.5% of their activity. The reduced response after heat treatment was reversed 24 hours after the nematodes were returned to 25 C. Receptors on the nematode surface can be partly denatured by sodium metaperiodate or by high temperature, but the receptors seemed to restore to their normal functional capacity within 24 hours after treatment. In 0.2 and 0.4% sodium hypochlorite (v/v) treatments, a small percentage of nematodes regained their capacity for oriented movement, but those treated with higher concentrations failed to regain this ability, probably due to a change in the chemical structure of the receptors.

Thus, it is proposed that carbohydrate moieties on the cuticle and (or) amphids may play a role in mediating chemical signals from host insects. The site(s) of sodium metaperiodate action and the manner by which it acts at the molecular level require determination.

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