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Potentiation of Tentacle Ball Formation by a Trypsin-Like Protease and Accompanying Augmented Ingestion in Glutathione-Induced Feeding in *Hydra*

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ABSTRACT—Tentacle ball formation may be a component of sequential feeding behavior in *Hydra*. This behavioral response is elicited by reduced glutathione after exposure to trypsin for 5 min at concentrations ranging from 0.1 fg/ml to 1 μg/ml. Trypsin and thrombin potentiated this response more effectively than the other proteases examined. Trypsin significantly promoted the ingestion of dead, fixed shrimp attached to their tentacles in the presence of glutathione. In an actual feeding situation, a trypsin-like protease, released from living wounded prey, may potentiate tentacle ball formation, and as a result, the ingestion of prey would be promoted in co-operation with reduced glutathione. We found that an immunoreactive protein for the monoclonal antibodies J245 and J5 was reduced in size in animals treated with trypsin; >300 kDa in animals without trypsin vs. 250 kDa or 110 kDa depending on the extent of trypsin treatment. Thus, this protein that is immunoreactive with J245 and J5 is likely to be involved in the trypsin-dependent potentiation of tentacle ball formation and the promotion of ingestion.

INTRODUCTION

Several behavioral components have been reported to be involved in the reduced glutathione-induced feeding response of *Hydra* [23], i.e., tentacle waving, tentacle concert, tentacle writhing [22], tentacle ball formation [9], and mouth opening [23]. The specificity, threshold, and possible relationship of these behavioral components in the context of feeding have been partially studied [6, 19, 29]. Previously, we studied the behavioral response to reduced glutathione in *Hydra japonica* which showed tentacle ball formation of long duration, and found that tentacle ball formation was sensitively modulated by several biologically active substances, including catecholamines [10], platelet proteins [11], and growth factors from mammals [11, 13]. Moreover, this modulation occurred in such a way that even trace amounts of these factors could be discriminated from each other.

However, when the author moved from Fukuoka to Otsu, *Hydra* cultured in Otsu did not exhibit tentacle ball formation of long duration, despite the use of the same culture method, prey, and the original strain, which was also moved from the previous location. The hydra only demonstrated a short-duration response, which was no longer modulated by biologically active substances. According to personal communications from Drs. O. Koizumi, T. Fujisawa, and B. Marcum, the short-duration response typically prevails in cultured hydra, and this observation encompasses different strains. Thus, the response of hydra in the Fukuoka laboratory was of unusually long duration. We attempted to determine the precise conditions under which tentacle ball formation of long duration could be reproducibly observed in response to glutathione.

We previously developed several monoclonal antibodies for immunizing mice with Sepharose-binding proteins from hydra cultured in Fukuoka [12, 30]. Among these monoclonal antibodies, J245 and J5 depressed the response to 50 μM S-methylglutathione (GSM) [30]. In immunofluorescence studies, J245 was localized to the cnidocil apparatus and the apical part of the nematocytes, whereas J5 was localized just to the apical part of the nematocytes [30]. Behavioral and biochemical analyses have suggested that the common antigen for these two antibodies is a candidate for the receptor which is responsible for the response to 50 μM GSM (R5 receptor) [27]. In an immunoprecipitation study, we found that an approximately 220-kDa component in solubilized crude membrane proteins from hydra cultured in Fukuoka was labeled with a ^{35}S-photoaffinity derivative of glutathione [27]. Thus, we used J245 and J5 for the present study and investigated the relationship between the antigen protein and feeding behavior, with particular focus on tentacle ball formation.

We previously reported different patterns of J245-immunopositive proteins in a blotting analysis of an extract from hydra cultured in Fukuoka *vs.* hydra cultured in Otsu [15]. This observation suggested differential protein processing in the two locations, which appeared to be related to the altered behavioral response. In the present study, we found that the treatment of hydra with low concentrations of trypsin for a short period greatly changed the behavioral response to glutathione, and induced tentacle ball formation of long

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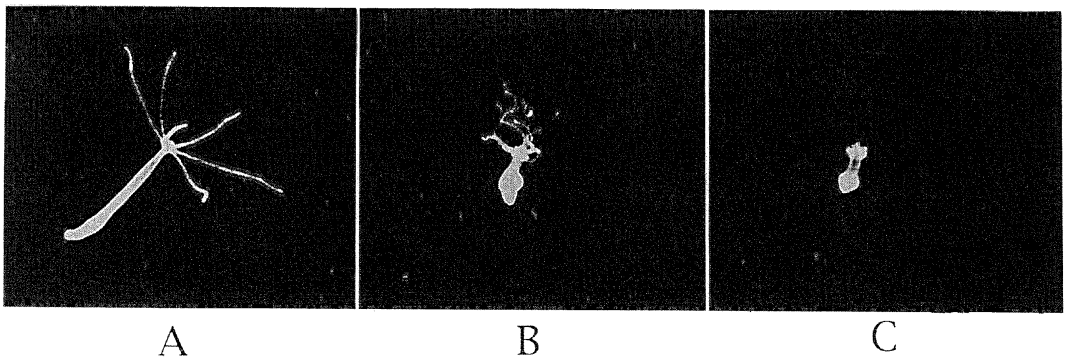

FIG. 1. The response of *Hydra* stimulated with GSM, with or without trypsin treatment. A) hydra without stimulation; B) hydra without trypsin treatment, 6 min after stimulation; C) hydra treated with trypsin (20 pg/ml), 5 min after stimulation. Without trypsin, the tentacles are less folded (compare B and C), and distinct tentacle ball formation is greatly reduced 5 min after stimulation in hydra that were cultured in Otsu.

duration (Fig. 1). This change was accompanied by the appearance of smaller antigens.

Furthermore, we performed an ingestion test using fixed, inert prey. Our results suggest that tentacle ball formation of long duration is related to the efficient ingestion of prey captured on the tentacles.

MATERIALS AND METHODS

Animals

Hydra japonica were cultured in a basal medium containing 0.1 g/l $NaHCO_3$, 0.005 g/l KCl, 0.05 g/l $CaCl_2 \cdot 2H_2O$, 0.015 g/l $MgCl_2 \cdot 6H_2O$, and 0.05 g/l EDTA·4Na [9]. The medium was made with water purified by reverse osmosis and ion-exchange resins (LaboionPure 12, Millipore, Bedford, MA, USA). Animals were fed daily with *Artemia salina* from China (distributed by Shintoa-Koeki, Tokyo, Japan), which were hatched in a solution of 30 g/l table salt (>99% NaCl, Japan Tobacco, Tokyo, Japan) after aeration for 2 days at 28°C. The medium was changed a few hours after feeding.

Hydra cultured in basal medium supplemented with additives

The effect of trace amounts of chemicals in the basal medium on the glutathione-induced response was examined to evaluate "location effects" on culture. Animals from mass cultures were transferred to the following 4 types of medium after feeding: i) basal medium, ii) basal medium supplemented with 1 ppm ammonium acetate, iii) basal medium with 1 ppm pyroglutamic acid, and iv) basal medium with both of these chemicals. Transferred animals were cultured for 4 days in each medium, with one feeding on the second day. These animals were then subjected to behavioral tests and used for protease assays, after homogenization (see below). The animals showed a new behavior after the 4-day culture period.

Evaluation of the behavioral response

Ten animals were transferred to 2 ml of 1 mM piperazine-N,N'-bis-(2-ethanesulfonic acid) sodium salt, and 1 mM $CaCl_2$, at pH 6.2 (PIPES buffer). After 5 min, they were stimulated with GSM at 5 different concentrations: 0.1, 0.3, 3, 10, and 50 μM. The response at these concentrations is comprised of R1-R5 components, respectively [11]. To stimulate hydra, concentrated GSM solution in a volume of 3–10 μl was applied with a glass pipette, and the solution was immediately agitated for 30 sec to give the final concentrations described above. The static charge on the distal part of the stimulating pipette remained negative by monitoring with a digital electrometer (model R8240, Advantest, Tokyo, Japan) equipped with a homemade Faraday cage. It was essential to stimulate the animals by using a negatively charged glass pipette to observe a reproducible response [14]. The number of animals which demonstrated tentacle ball formation was counted every minute, from 6 to 10 min after stimulation. The fraction of animals which showed this response was summed at each minute of the observation. This value was used as a quantitative measure of the response, and correlated well with the average duration of the response, which had been used to measure the response in previous experiments [9] (Fig. 2). The response from 6 to 10 min after stimulation was better correlated with the average duration of the response, and permitted clearer discrimination between long- and short-duration responses, than the response from 1 to 5 min after stimulation (Fig. 2). This measurement also required less time to determine than the average response duration, which required observation of the entire response.

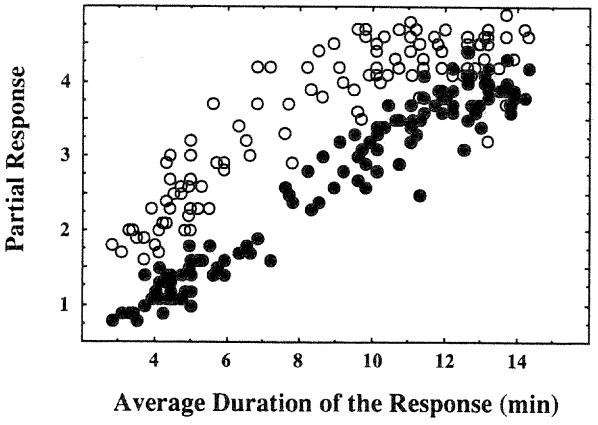

FIG. 2. The correlation between the average duration of the response and the partial response. The partial response was calculated from 1 to 5 min (open circle), or from 6 to 10 min (closed circle) after stimulation. The average duration of the response was calculated from the entire time course of the response as described in [9]. The partial response from 6 to 10 min after stimulation (correlation coefficient, 0.969; $r^2=0.939$) was more closely correlated with the average duration of the response than that from 1 to 5 min (correlation coefficient, 0.875; $r^2=0.766$).

Treatment of live hydra with protease for the behavioral assay

Less than 1000 animals that had been starved for 30–48 hr were transferred to 400 ml of 1 mM $NaHCO_3$ and 1 mM $CaCl_2$ (BC solution), and maintained in this solution for at least 0.5 hr at 21°C. A specified amount of protease was then added to the medium, which was then gently mixed. The proteases used included chymotrypsin from bovine pancreas (EC3.4.21.1) (Sigma, St. Louis, MO, USA), papain (EC3.4.22.2) (Sigma), protease K (EC3.4.21.14) (Boehringer, Mannheim, Germany), thermolysin (EC3.4.24.4) (Nacalai, Kyoto, Japan), thrombin from human plasma (EC3.4.21.5) (Mochida, Tokyo, Japan), and trypsin from bovine pancreas (EC 3.4.21.4) (Sigma). After 5 min or an alternate specified time, the medium was changed to fresh BC solution without protease, and hydra were kept in this solution at 21°C until subjected to the behavioral assay.

In experiments which examined the dose effect of proteases on the potentiation of the response, protease treatment was carried out in a dish with a diameter of 35 mm. Ten animals, which had been kept in BC solution, were transferred in 2 ml of BC solution containing a specified amount of protease, and incubated in this solution for 5 min. The protease solution was removed immediately and replaced by 2 ml of PIPES buffer after rinsing. The response was then examined. Stimulatory conditions are described in the relevant figures.

Ingestion of dead, fixed Artemia salina

Artemia salina (brine shrimp) were fixed in 2% paraformaldehyde, 50% ethanol, and 1 mM phenylmethanesulfonyl fluoride for 1 day, extensively washed with 50% ethanol, and then washed again with water for 2 days to prevent the release of small molecules, such as amino acids and reduced glutathione, and to prevent the release of endogenous proteolytic activity to the surrounding medium. Dead, fixed shrimp in 25 μl of 1 mM GSM and 30 ng/ml trypsin were applied to an animal in 2 ml of basal culture medium. One to six brine shrimp were attached to the tentacles of each animal by simply touching them to the tentacles. The animal was observed for 10 min under a binocular microscope, and the number of ingested shrimp was counted. In control experiments, either trypsin, GSM, or both were omitted.

Protein blotting analysis

About 10^5 animals were treated with 0.3 μg/ml trypsin for 0.5 hr, or 3 μg/ml trypsin for 1 hr. This was followed by the addition of 0.1 mM phenylmethanesulfonyl fluoride and 50 μM tosyl-L-lysinechloromethyl ketone to inactivate the enzyme. Even when untreated animals were used, these inhibitors were added to the medium before homogenization since medium which held a high density of the animals contained sufficient endogenous protease activities released from the animals that affected the result. Animals were homogenized with a teflon homogenizer in 50 ml of 10 mM Tris and 0.15 M NaCl, at pH 7.4 (TBS) containing 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM tosyl-L-lysinechloromethyl ketone, and 25 mM $NaMoO_4$. The homogenate was centrifuged at 1000 g for 10 min and the pellet was collected. After washing once with TBS, followed by a second wash with TBS containing 50% acetone, a 40-ml suspension of the pellet was layered on 40 ml of 50% sucrose in TBS, and centrifuged at 2×10^4 *g* for 30 min. The bottom pellet was recovered, washed once with 2 M NaCl and 0.05 M K_2CO_3, and then solubilized in 10 ml TBS containing 2% n-octyl-β-D-glucoside and 2 μg/ml DNase from bovine pancreas (Sigma, type II). This mixture was centrifuged at 2.5×10^4 *g* for 20 min. The supernatant was applied to a column packed with 6 ml Epoxy-Sepharose (Pharmacia, Uppsala, Sweden) which had been inactivated with 1 M ethanolamine, pH 8.0. Inactivated epoxy-Sepharose appeared to be more suitable for collecting the relevant antigen than unmodified Sepharose, which was originally used [30]. After extensive washing with TBS containing 0.5% Nonidet P-40, the bound proteins were eluted with a solution containing 7M urea, 0.05M K_2CO_3, and 0.5% Nonidet P-40. The eluted fractions were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE, 5% gel) with 2-mercaptoethanol as a reducing reagent. The proteins which had been separated were transferred to a polyvinylidenedifluoride membrane (Immobilon P, Millipore) with the use of a semi-dry blotting apparatus at 10 V for 1 hr [27].

The membrane was first incubated with 3% skim milk (Difco, Detroit, MI, USA) in TBS for 30 min, and then with monoclonal antibodies J245 or J5 (1:5000 dilution) in TBS containing 1% skim milk for 1 hr at room temperature. After 2 washes with TBS containing 0.05% Tween 20 (BioRad, Richmond, CA, USA) (TBS-T) for 10 min, the membrane was incubated with TBS containing 1% skim milk and anti-mouse IgG antibody conjugated with alkaline phosphatase (Gibco-BRL, Grand Island, NY, USA) at 1:5000 dilution for another 1 hr. The membrane was then washed 3 times with TBS-T followed by another wash with TBS, at 10 min for each wash. Immunoreactive bands were visualized with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.37 mM nitro blue tetrazolium in 0.1 M Tris, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 9.5. The molecular mass was estimated with reference to prestained marker proteins for SDS-PAGE (high range, BioRad).

Protease assay

Protease activity in the homogenates was examined with benzoyl-carboxy-Phe-Arg-MCA (MCA, 4-methylcoumarineamide), succinyl-Leu-Leu-Val-Tyr-MCA, and Arg-MCA as substrates (Peptide Institute., Osaka, Japan: [25, 31, 33]. These substrates release fluorescent 7-amino-4-methylcoumarin after hydrolysis, although the substrates themselves are not fluorescent. The supernatant obtained from the homogenate (10^4 *g*×20 min) was used as an enzyme source. The reaction mixture, which contained 120–150 ng protein and 5 nmole substrate in 1 ml TBS, was incubated for 10 min at 30°C, followed by the addition of 1 ml 0.1 M CH_3COOH and 0.1 M $ClCH_2COOH$ to stop the reaction [1]. After the addition of 1 ml water, fluorescence at 440 nm was determined following excitation at 340 nm using a fluorescence spectrophotometer (Shimadzu RF1500, Kyoto, Japan). Fluorescence of a blank reaction mixture was determined and subtracted from the total fluorescence. The blank reaction mixture had the same composition as the reaction mixture, except that the stop solution had been added immediately after the addition of the enzyme solution.

Other techniques

Protein was quantitated using the dye-binding method [3] with a kit obtained from BioRad (Protein Assay). Statistical analyses were performed using the StatView II software package (Abacus, Berkeley, CA, USA).

RESULTS

Potentiation of tentacle ball formation by proteases

The presence of different blotting patterns for crude membrane proteins suggested that proteolytic processing was involved in the change in the response of animals cultured in

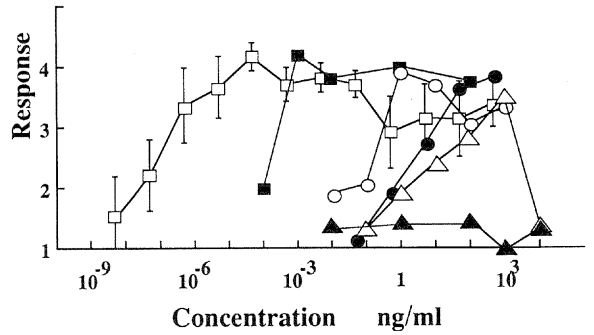

FIG. 3. The potentiation of tentacle ball formation by various proteases. The animals were treated with each protease for 5 min at specified concentrations, and the response to 10 μM GSM was then examined. (□), trypsin; (■), thrombin; (○), chymotrypsin; (●), papain; (△), thermolysin; (▲), protease K. The response is represented as the mean ± SD calculated from 4 independent trials. For clarity, the SD is shown only for trypsin data.

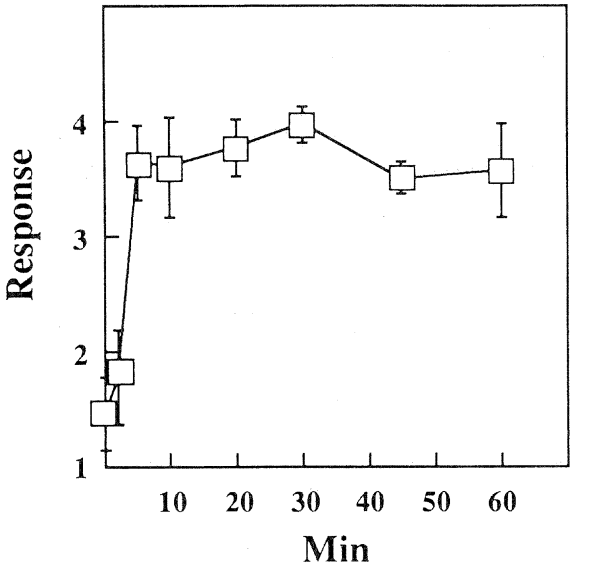

FIG. 4. The time course of potentiation of the response. The response of hydra to 10 μM GSM was examined after trypsin (100 pg/ml) was applied to hydra in BC solution. Data represent the mean ± SD from 4 independent trials.

different media or locations. Thus, the effect of protease treatment on the glutathione-induced response was examined in live animals. Treatment with proteases such as trypsin, chymotrypsin, papain, and thrombin at low concentrations in BC solution potentiated long-duration tentacle ball formation in hydra cultured in Otsu, which otherwise showed only a short-duration response (Figs. 1, 3). Potentiation was rapid, and 5 min of treatment at a concentration of 100 pg/ml trypsin was enough to produce a significantly longer response (Fig. 4). Figure 3 shows the concentration-dependence of the potentiation of tentacle ball formation by proteases. Trypsin was the most potent protease examined. Potentiation of the response was observed after 5 min of treatment with trypsin, at a wide range of concentrations from 1 fg/ml to 1 μg/ml. This observation suggested that the component responsible for the response was relatively resistant to over-digestion by trypsin.

The effect of protease inhibitors was examined to confirm whether this potentiation was due to the protease activity of these enzymes. The addition of leupeptin, a potent, specific trypsin-like protease inhibitor [34], to a reaction mixture containing trypsin effectively eliminated the potentiation (Fig. 5). In contrast, the addition of chymostatin, a potent chymotrypsin-like protease inhibitor [34], had no effect. This observation indicated that the effects were specific to some tryptic activity. In addition, similar effects were observed for potentiation by chymotrypsin. Considering that the potentiation by chymotrypsin occurred at a 10^6-fold higher concentration than that by trypsin (Fig. 3), it is likely that contamination of the chymotrypsin preparation by trypsin caused this potentiation.

More efficient ingestion of attached prey in the presence of trypsin

The effect of trypsin on the ingestion of dead, fixed prey attached to the tentacles was examined. The dead, fixed shrimp were more efficiently ingested in the presence of both GSM and trypsin than in the presence of GSM alone (Table I). The concentration of GSM used was near the maximum that hydra would be exposed to in a natural environment. Even at this concentration of GSM, ingestion was less efficient in the absence of trypsin. Trypsin alone at this concentration or without additional chemicals did not promote the ingestion of dead, fixed prey.

Protein blotting analysis of J245 and J5 antigens from hydra

Immunoreactive J245 proteins in the crude membrane fraction were compared in hydra cultured in Fukuoka and those cultured in Otsu to examine possible biochemical correlates with the different behavioral response to GSM in the two groups of animals. The distribution of immunoreactive proteins on the SDS PAGE gel differed in the two groups of animals (Fig. 6).

Changes in the size of the immunoreactive proteins following trypsin-treatment were detected by protein blotting analysis using J245 and J5 (Fig. 7). The antigens were isolated from crude membrane protein extracts with inactivated epoxy-Sepharose to determine the direct effects on the original antigen. J245 strongly visualized a protein of very large mass (>300 kDa on SDS PAGE) among proteins obtained from hydra homogenates without trypsin treatment. J5 revealed only diffuse bands. After treatment with 0.3 μg/ml trypsin for 0.5 h, J5 revealed distinct proteins of approximately 250 kDa which produced duplicate bands of similar molecular mass during the initial phase of color development. J245 showed no distinct bands for proteins from animals after moderate trypsin treatment. Instead, the smeared bands of smaller proteins increased. A protein of 110 kDa appeared with more extensive trypsin treatment (3 μg/ml, 1 hr). Both J245 and J5 revealed this component,

TABLE 1. Facilitation by trypsin of the GSM-induced ingestion of dead, and fixed *Artemia salina*

chemical	number of attached shrimp	number of tests	number of ingested shrimp	attached shrimp (total)	not ingested shrimp (total)	ingested shrimp (total)
none[a)]	1	1	0	28	28	0
	2	10	0			
	3	1	0			
	4	1	0			
trypsin[b)]	1	1	0	42	41	1
	2	5	0			
	3	1	0			
	4	4	1			
	6	2	0			
GSM[b)]	1	7	3	42	25	17[c)]
	2	11	8			
	3	1	1			
	4	1	1			
	6	1	4			
GSM & trypsin[b)]	1	1	0	46	15	31[c)]
	2	7	10			
	3	4	7			
	4	2	4			
	5	1	5			
	6	1	5			

[a)] Dead, fixed, and thoroughly washed shrimp (n=1–6, number of attached shrimp) were applied to hydra tentacles for the specified numbers of trials (number of tests).
[b)] Shrimp were applied in medium containing 25 μl of 30 ng/ml trypsin (trypsin), 1 mM GSM (GSM) or both.
[c)] The ratios of the number of ingested shrimp to the number of shrimp not ingested were significantly different from each other (chi-square test, $P<0.05$), indicating that hydra ingested more attached shrimp in the presence of both trypsin and GSM than with GSM alone. Hydra did not ingest attached shrimp in the absence of GSM ($P<0.01$).

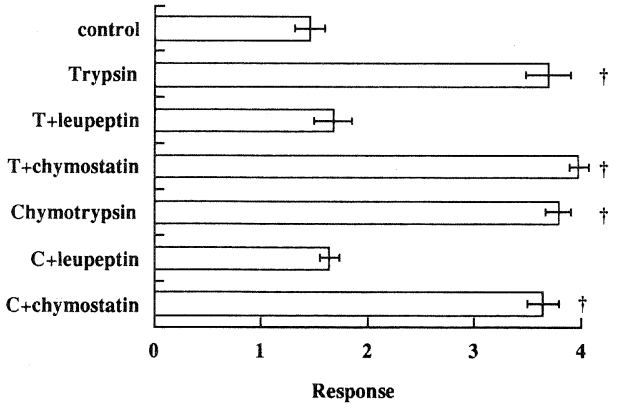

FIG. 5. The effect of specific inhibitors on potentiation of the response by proteases. The animals were treated with 10 pg/ml trypsin (T) or 10 ng/ml chymotrypsin (C) for 5 min in the presence or absence of inhibitors; 0.1 μg/ml leupeptin (+ leupeptin) or chymostatin (+chymostatin). The response to 10 μM GSM was then examined. (†) indicates a significant difference from the control response, as determined by one-way ANOVA, followed by multiple comparisons using Scheffe's method ($P<0.01$).

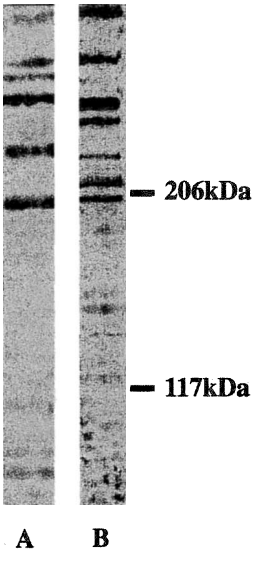

FIG. 6. Protein-blot analysis of crude membrane proteins in *Hydra* which were cultured in different locations. The protein samples consisted of an octylglucoside-solubilized extract of the crude membrane fraction. (A), Hydra cultured in Fukuoka; (B), Hydra cultured in Otsu.

and the band with J5 was more diffuse.

We did not detect the >300 kDa protein in either crude extract from Fukuoka hydra or in purified antigen from Otsu

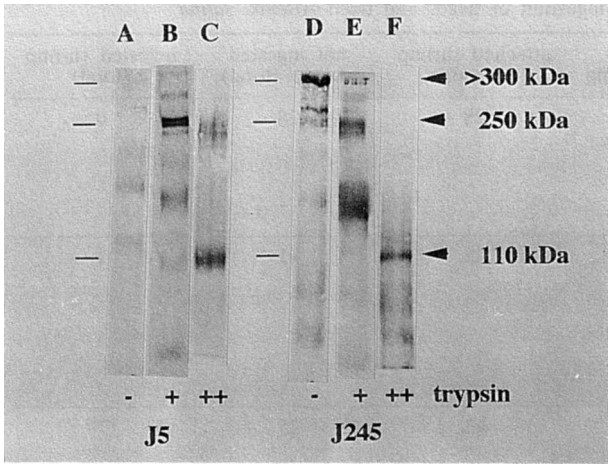

FIG. 7. Protein-blot analysis of Sepharose-binding proteins from hydra, with and without trypsin treatment. Small aliquots of the fractions from the Sepharose column were electrophoresed and blotted on a membrane. Proteins were visualized with mAb J5 (lanes A, B, and C) or mAb J245 (lanes D, E, and F). The proteins were extracted from animals without trypsin treatment (trypsin −; A, D), from animals treated with trypsin at 0.3 μg/ml for 0.5 h (trypsin +; B, E), and from animals treated with trypsin at 3 μg/ml for 1 h (trypsin ++; C, F). This photo shows a protein blot of fraction #11 from the column eluate. Similar results were observed for fractions #8 to #15, and sometimes up to fraction #25.

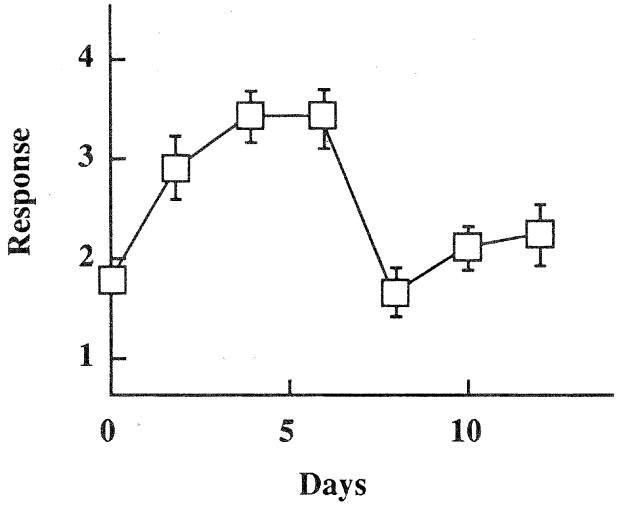

FIG. 8. A transient, but stronger response was noted in *Hydra* cultured in medium supplemented with 1 ppm ammonium acetate. The animals were transferred to basal medium supplemented with 1 ppm ammonium acetate at day 0, and cultured in this medium. The supplemented media were renewed every 2 days.

hydra treated with trypsin. Therefore, the disappearence of this protein appears to be closely related to the potentiation of tentacle ball formation.

Protease activity of hydra cultured in different media

Hydra that had been cultured in media supplemented with various chemicals including amino acids, organic acids,

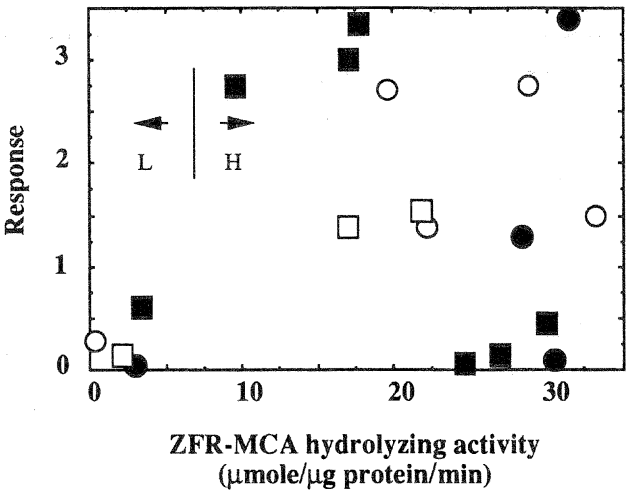

FIG. 9. The response to glutathione and the proteolytic activity of hydra which were cultured in different media. Animals were cultured in 4 different media (i - iv, see Materials and Methods) for 4 days. Both the response to 10 μM GSM and the ZFR-MCA hydrolyzing activity were examined simultaneously in the crude hydra homogenates. These values were plotted as follows: (○), medium i; (●), medium ii; (□), medium iii; (■), medium iv. Data were collected from 5 independent experiments. The vertical axis represents the response, and the horizontal axis represents hydrolyzing activity (μmole/μg protein/min). The bar in the figure delineates the point at which hydrolytic activity was divided into two groups: high (H) and low (L) activity.

amines, vitamins, and minerals normally used in the culture of mammalian cells were examined (data not shown). Among these chemicals, ammonium salts and pyroglutamic acid seemed to most effectively induce tentacle ball formation [15]. However, this effect was observed for only a short time, a few days after the initial chemical supplementation, even though supplemented medium was renewed every 2 days (Fig. 8).

The protease activity of the homogenate and tentacle ball formation in groups of hydra cultured for 4 days in different media were examined to determine if there was a relationship between the two processes (Fig. 9). When the protease activity of the homogenate was high, a long-duration response was observed more frequently (Fig. 10). This was true for all three substrates examined and for most of the responses elicited at different GSM concentrations. The response data were divided into two groups, high and low protease activity, regardless of the culture conditions. The response observed in animals with high protease activity was significantly longer than that in hydra with low protease activity, as determined by the paired Student's *t*-test (Fig. 10).

DISCUSSION

Potentiation of tentacle ball formation by a trypsin-like protease

Trypsin and thrombin effectively potentiated tentacle ball formation of long duration in response to GSM. This

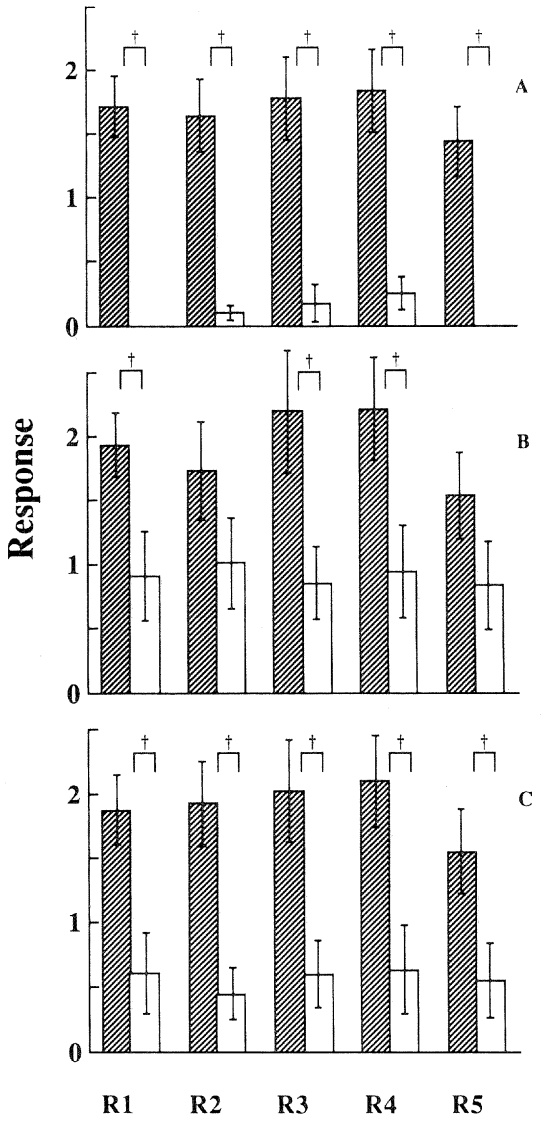

FIG. 10. The response to GSM and the protease activity in hydra homogenates. Protease activity, assayed using substrates ZFR-MCA (A), SucLLVY-MCA (B), and R-MCA (C), was examined in homogenates obtained from hydra cultured in 4 different media supplemented with chemicals. The response to 5 different concentrations of GSM was examined simultaneously in the corresponding animal groups. The vertical axis shows the response; hatched bar, mean response of animals which showed higher proteolytic activity (animal group H in Fig. 9); open bar, mean response of animals with lower proteolytic activity (animal group L). R1 - R5 represent the responses to 0.1, 0.3, 3, 10, and 50 μM GSM, respectively. The response data and the protease activity data from 5 independent trials for each animal group in the 4 different media (a total of 20 data pairs) were analyzed after dividing the hydra into high and low activity groups. The sample size in the high and low activity groups, respectively, was 16 and 4 for A, 9 and 11 for B, and 12 and 8 for C. (†) indicates a significant difference between the responses of the two groups of animals ($P<0.05$) by Student's paired *t*-test.

potentiation was inhibited by a specific inhibitor, leupeptin. No meaningful potentiation was observed when the reaction took place in PIPES buffer at pH 6.2 (unpublished observation). This result may be due to the fact that these proteases are more active at pH's ranging from 7 to 8, which further suggests that protease activity is important for potentiation of the response. Trypsin and thrombin cleave peptides at the carboxyl side of arginine residues [5]. This cleavage specificity, however, does not appear to correlate directly with the potentiation phenomena. For example, although papain also cleaves peptides at the carboxyl side of arginine residues, it was far less effective than trypsin or thrombin. Neither arginine endopeptidase (EC 3.4.21.40), nor lysylendopeptidase (EC3.4.21.50) potentiated the response at all (unpublished observation). Protease K, a non-specific protease [20], also did not potentiate the response. Potentiation of the response by proteases appears to be the result of complex interaction with a specific protein.

Tentacle ball formation appears to be an indispensable component of the feeding sequence

In the presence of trypsin, dead, fixed shrimp attached to the tentacles of hydra were more efficiently ingested. Considering the potentiation of tentacle ball formation by trypsin-like enzymes, tentacle ball formation may be an indispensable component of the entire feeding sequence in *Hydra*. The feeding sequence may be divided into three discrete steps; prey capture on the tentacles (nematocyst discharge), transfer to the mouth, and ingestion. The behavioral change induced by trypsin-like enzymes appears to be closely related to the second step: the transfer of the captured prey to the mouth. Tentacle ball formation, resulting from this behavioral change, makes the transfer of prey to the mouth more efficient.

Change in protein size with trypsin treatment

The protein of >300 kDa, which was revealed by J245, appeared to be closely related to the potentiation of tentacle ball formation. This component was not detected upon treatment with trypsin or in hydra cultured in Fukuoka. Indeed, components with lower molecular mass increased with trypsin treatment. The distribution of J245-immunopositive proteins in trypsin-treated hydra cultured in Otsu was different from that of hydra cultured in Fukuoka (cf. Figs. 6 and 7). A different, unknown, protease may be responsible for the digestion of the antigen in the Fukuoka hydra. At any rate, it is remarkable that the present antigen is easily truncated, and is changed in size by the external action of proteases.

Trypsin-like proteases have been reported to induce a feeding response in *Hydra* at higher concentrations than those used in the present study [18, 21]. It has been pointed out that proteases act on a component closely related to the mechanism for the glutathione reaction, since both glutathione and proteases induce similar behavior. It may be interesting to clarify the function of the >300 kDa J245 antigen in relation to the potentiation of tentacle ball formation.

Culture medium and the response

We first suspected that the differences in the behavioral response of hydra cultured in Fukuoka *vs*. Otsu were due to trace amounts of impurities in the culture medium from tap water or contamination from laboratory environment. Accordingly, the effect of trace amounts of ammonium acetate and pyroglutamic acid in the medium were examined. However, the addition of these compounds had no significant effect on behavior (Fig. 9). Next, the response was analyzed after dividing the data into two groups on the basis of protease activity. Although we do not know the relationship between the protease activity of the homogenate and that of external medium, it is likely that both activities are correlated to some extent because *Hydra* perpetually shed old cells into the medium [28].

Considering the present results, the long-duration response observed in Fukuoka was likely due to contamination with a trypsin-like enzyme, or possibly due to the aberrant expression of a similar protease induced for unknown reasons. This behavioral difference might also be related to the use of *Artemia salina* which were hatched in a table salt solution at a relatively high temperature (>26°C). Although further study is necessary, the conditions under which the brine shrimp are hatched also appear to affect the *Hydra* feeding response.

Possible biological significance of the protease-induced change in behavior

Efficient ingestion of dead, fixed shrimp in the presence of trypsin suggests that a trypsin-like enzyme plays a role in the ingestion of captured prey in actual feeding situations. When capturing free swimming prey, hydra attacks the prey with nematocysts. Desmoneme nematocytes are used to stick the prey to the tentacles, while stenotele nematocytes inject poison into the prey to stop motion [6, 24]. Wounded prey captured in this manner may release trypsin-like enzymes, in addition to releasing reduced glutathione. Trypsin-like proteases are found in most organisms, and proteases of this type are likely to be activated during the acute phase of tissue repair in wounded prey. We observed that thrombin and factor Xa (unpublished observation), indispensable proteases for blood-clotting in mammals, were also as effective potentiating tentacle ball formation as trypsin. Thus, a trypsin-like enzyme, as well as reduced glutathione, may play a role in determining whether or not the captured substrate is prey. Hydra is completely carnivorous and never eats dead prey [23]. We also noted that this trypsin-induced potentiation apparently requires a few minutes, during which the prey will likely stop moving. Thus, this delay may be advantageous for the engulfment of prey.

Proteases have been repeatedly reported to modify the responses of biological systems, and it is unclear whether these actions of proteases occur naturally. Examples of such modifications include the protease-induced modulation of rat macrophage activity in the binding of rabbit-IgG-sensitized sheep erythrocytes [2], the protease-enhanced respiratory burst activity of macrophages [4], the protease-induced formation of focal tight junctions in HT 29 adenocarcinoma cells [7], the protease-induced differentiation of human myeloid leukemic cells [8], the protease-induced constriction in rat bronchi [17], the protease-induced stimulation of mucous glycoprotein release from hamster tracheal ring organ culture [26], the stimulation of secretion from cultured bovine airway gland serous cells [32], and the regulation of flagellar motility in sperm by proteasomes [16]. With the exception of this last effct, these modifications are mediated through the direct action of proteases on a surface component of the relevant cells, as in the present change in the behavior of hydra in response to GSM.

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