Peptides, Vol 9, pp 1403–1406 © Pergamon Press plc, 1989 Printed in the U S A 0196-9781/88 $3 00 + 00

Synthetic Peptide Analogs to Barnacle Settlement Pheromone

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Received 25 March 1988

TEGTMEYER, K AND D RITTSCHOF *Synthetic peptide analogs to barnacle settlement pheromone* PEPTIDES 9(6) 1403–1406, 1988 —Barnacle pheromone enhances the rate of settlement and metamorphosis of larvae of *Balanus amphitrite* Darwin Analogs to the heterogeneous pheromone peptides were sought Settlement assays were used to assess both the pheromone and the potential analogs The pheromone has a lower threshold of activity at a concentration of 0 2 μg BSA protein equivalence l^{-1} Treatment with carboxypeptidase eliminates biological activity Series of dipeptides were tested to determine if dipeptides could promote settlement Combinations of acidic, neutral, and basic amino acids in dipeptides were examined Specific small peptides can mimic barnacle pheromone Only peptides with a basic carboxy-terminal amino acid and either a neutral or a basic amino-terminal amino acid enhance settlement Six peptides were shown to mimic pheromone activity at concentrations comparable to the native molecule Some peptides were more potent than others The most effective peptides were L-leucyl-L-arginine and L-histidyl-L-lysine which had a lower threshold of settlement enhancement of $2\,0\times10^{-10}$ M and caused a 130% increase in settlement rate at $2\,0\times10^{-8}$ M Glycyl-glycyl-L-arginine, glycyl-L-histidyl-L-lysine, L-leucyl-glycyl-L-arginine and L-tyrosyl-L-arginine had thresholds between 2.0×10^{-8} M and $2\,0\times10^{-9}$ M Peptide pheromone analogs should be useful in determining the nature and mechanism of barnacle pheromone receptor interactions

Metamorphosis Pheromone Barnacle Crustacean Peptides Fouling

IT has long been recognized that the presence of conspecifics enhances the recruitment and settlement of new barnacles (5). Knight-Jones and Crisp (2) hypothesized that the reason for the aggregation of barnacles was due to chemical communication between the settled adult barnacles and the swimming larvae. More recently, pheromones responsible for aggregation have been discovered (9). Glycoproteins extracted from adult barnacle tissue (6,7), enhance the settlement of cyprids Compounds extracted from barnacles were found to be effective in terms of increasing cyprid settlement on a mg/l level. Barnacle settlement pheromone is a 3000 to 5000 dalton peptide that is released from living *Balanus* and is effective at μg/l concentrations (9)

Short-chained peptides have been shown to elicit responses in numerous different biological systems (3, 14, 16). Biological activity has specific compositional requirements Two biological systems in particular have shown absolute requirements for basic amino acids at the carboxy terminal The C3a human anaphalaxin response has been shown to have an absolute requirement for L-arginine at the carboxy terminus (3), while the crab pumping system has been found to require either L-lysine or L-arginine at the carboxy terminus (3) In the latter system, L-arginine carboxy-terminal peptides are at least 1,000-fold more potent than L-lysine carboxy-terminal peptides Fragmentary information on the chemical nature of barnacle settlement pheromone (the pheromone also attracts predatory snails) suggests that it may also have a requirement for a basic carboxy terminus (11).

This study was performed to determine if small synthetic peptides could mimic the native barnacle settlement pheromone We report that peptides with compositional restrictions similar to those of the C3a system peptide and crab pumping pheromone peptides mimic the activity of the barnacle settlement pheromone.

METHOD

Adult *Balanus amphitrite* (Darwin) barnacles were collected from pilings on Radio Island, NC Brooded nauplii were released by crushing the adults in sea water Nauplii were cultured to the settlement stage in 4 days (10). Upon transformation to the settlement stage, cyprid larvae were separated from untransformed nauplii and used immediately in biological assays.

Test solutions were made in 30–35 ppt sea water (identical in salinity to culture water) filtered to remove all particles greater than 100,000 daltons. The solutions were made immediately before use Carboxypeptidase A (10 BAEE units Sigma-C0261, 1000 units per ml) was added to 10 μl concentrated barnacle pheromone and diluted to 200 μl total volume with boiled 100,000 dalton filtered sea water. Controls included pheromone alone and carboxypeptidase alone Samples were incubated at 37°C for 2 hours. After incubation, 10 μl aliquots were removed and diluted with 100,000 dalton filtered sea water to a final concentration of 20 μg pheromone BSA equivalence per l, and run in the settlement

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TABLE 1

RESPONSE THRESHOLDS AND TEST RANGES FOR DIPEPTIDES OF VARYING COMPOSITIONS TESTED IN SETTLEMENT ASSAY FOR ABILITY TO ENHANCE SETTLEMENT OF DAY 0 CYPRIDS

Peptide	Range Tested	Range of Response
Neutral-Basic L-leucyl-L-arginine	2.0×10^{-8} to 2.0×10^{-12} M	2.0×10^{-8} to 2.0×10^{-10} M
Basic-Basic L-histidyl-L-lysine	2.0×10^{-8} to 2.0×10^{-12} M	2.0×10^{-8} to 2.0×10^{-10} M
Acid-Basic L-glutamyl-L-lysine	2.0×10^{-8} to 2.0×10^{-12} M	No Response
Basic-Neutral L-lysyl-glycine	2.0×10^{-8} to 2.0×10^{-12} M	No Response
Acid-Neutral L-glutamyl-glycine	2.0×10^{-8} to 2.0×10^{-12} M	No Response
Neutral-Neutral glycyl-L-serine	2.0×10^{-8} to 2.0×10^{-12} M	No Response
Basic-Acid L-arginyl-L-glutamic acid	2.0×10^{-8} to 2.0×10^{-12} M	No Response
Neutral-Acid glycyl-L-aspartic acid	2.0×10^{-8} to 2.0×10^{-12} M	No Response
Acid-Acid L-glutamyl-L-glutamic acid	2.0×10^{-8} to 2.0×10^{-12} M	No Response

TABLE 2

TEST AND RESPONSE RANGES FOR PEPTIDES THAT SHOWED SIGNIFICANT SETTLEMENT ENHANCEMENT IN SETTLEMENT ASSAY WITH DAY 0 CYPRIDS

Peptide	Range Tested	Range of Response
L-leucyl--glycyl-L-arginine	2.0×10^{-8} to 2.0×10^{-12} M	2.0×10^{-8} to 2.0×10^{-10} M
L-tyrosyl-L-arginine	2.0×10^{-8} to 2.0×10^{-12} M	2.0×10^{-8} M
glycyl-glycyl-L-arginine	2.0×10^{-8} to 2.0×10^{-12} M	2.0×10^{-8} to 2.0×19^{-9} M
glycyl-L-histidyl-L-lysine	1.6×10^{-8} to 1.6×10^{-12} M	1.6×10^{-8} to 1.6×10^{-9} M
L-histidyl-L-lysine	2.0×10^{-8} to 2.0×10^{-12} M	2.0×10^{-8} to 2.0×10^{-10} M

assay Test peptides were obtained from Sigma Chemical Company or were gifts from a collaborative effort on crab pheromones involving R. B. Forward and B Erickson. Peptides represented all combinations of neutral, basic and acidic amino acids at the amino and carboxy termini, both di-and tripeptides were used. Purity of peptides were monitored by TLC and HPLC (3) A partially purified pheromone solution was isolated from the sea water surrounding adult barnacles and was a gift of Robert G. Shepherd

Settlement assays (9) measured the relative percent settlement of cyprids Assay solutions were made up in duplicate in 5 cm polystyrene petri dishes, from 10^{-8} to 10^{-12} M concentration range. Thirty to 150 cyprids were added to each dish. The dishes were incubated for 22 hours at 28°C on a 15:9 LD cycle After incubation several drops of 10% formalin was added to kill the barnacles in the dish. Each dish was counted, keeping separate tally of settled and not-settled cyprids. Frequency analysis (13) and proportions test (15) were used to compare responses.

RESULTS

Partially purified barnacle pheromone elicits a significant response in the settlement assay. The dose-response curve is biphasic over a range of 3 orders of magnitude The lower threshold of response occurred at 0.2 μg BSA protein equivalence l^{-1}. Peak responses were at 10 $\mu g \cdot l^{-1}$. Concentrations above 100 $\mu g \cdot l^{-1}$ showed no enhancement of settlement activity. Assuming a molecular weight of 3–4 kD the lower threshold and peak response were 10^{-10} M and 10^{-6} M respectively. Significant responses, by frequency analysis ($G>3.84$, $p<0.05$), were at least 20% greater than control responses.

The importance of the carboxy terminus of native pheromone was determined by incubating pheromone with carboxy peptidase. Treatments containing carboxypeptidase A incubated pheromone settled at negative control levels (3% settlement $g<0.3$, $p>0.05$) and were significantly less than the positive pheromone control (23% settlement) $G(1)=10\ 584$, $p<0\ 01$, just two hours

Settlement assays were also used to determine if dipeptide analogs could mimic the activity of barnacle settlement pheromone. A series of dipeptides was tested in the settlement at assay concentrations from 2.0×10^{-8} M to 2.0×10^{-12} M These concentrations bracket the lower range of active native pheromone concentrations The dipeptide series includes all possible combinations of acidic, neutral, and basic carboxy and amino terminal amino acids. Peptides with a basic carboxy-terminal amino acid and either a neutral or a basic amino-terminal amino acid elicited a significant response from 10^{-8} M to 10^{-10} M. L-leucyl-L-arginine, a neutral-basic dipeptide, and L-histidyl-L-lysine, a basic-basic dipeptide, both had threshold concentrations of 2.0×10^{-10} M (Table 1). Additional peptides were tested in the settlement assay, including: two more neutral-basic dipeptides, one more basic-basic dipeptide, two neutral-neutral-basic tripeptide, and one neutral-basic-basic tripeptide.

All neutral-basic and basic-basic peptides tested have been found to elicit a significant response in the settlement assay. They are all effective at a concentration of 2.0×10^{-8} M Two of the peptides, glycyl-glycyl-arginine and glycyl-L-histidyl-L-lysine, have lower thresholds of 2.0×10^{-9} M. L-hystidyl-L-lysine and L-leucyl-glycyl-L-arginine have thresholds of 2.0×10^{-10} M (Table 2). Settlement seems to be linearly related to concentration for approximately 4 orders of magnitude of concentrations (Fig. 1). However, peptides including L-tyrosyl-L-arginine which have been tested at concentrations as high as 10^{-6} M show a biphasic dose-response curve with inhibition of settlement at high concentrations. The effective range of individual dipeptides is approximately 3 orders of magnitude of concentration.

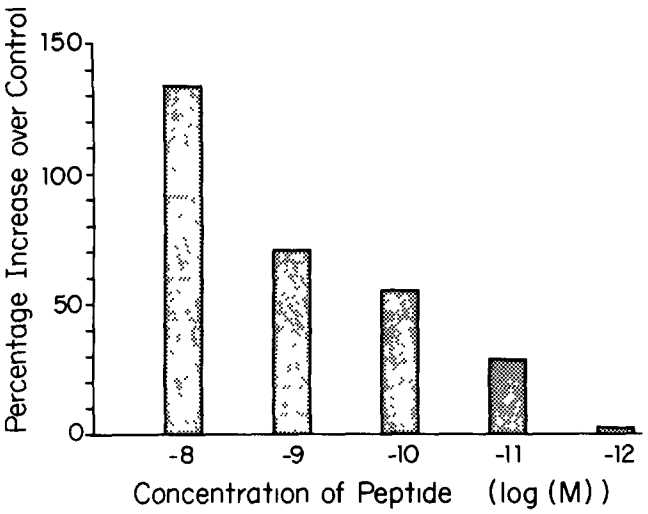

FIG 1 Dilution series data of the induction of metamorphosis in larval barnacles of L-histidyl-L-lysine Plotted is the percent increase over control (y axis) versus the log molar concentration of the settlement pheromone mimetic peptide in solution Concentrations of peptide greater than or equal to 10^{-10} M showed significant response at the $p<0$ 01 level

Among the peptides tested L-leucyl-glycyl-L-arginine and L-histidyl-L-lysine showed the strongest response in the settlement assay Peak response in the range tested was at 2.0×10^{-8} M, with a response 140% greater than control. L-histidyl-L-lysine was significantly active $g>6$ 6, $p<0.01$ between 2 0×10^{-9} M and 2.0×10^{-10} M (Fig 1)

DISCUSSION

A number of factors including cyprid age, flow, texture, and pheromones influence the rate of settlement and metamorphosis of cyprids (2). Barnacle settlement pheromone is released by juvenile and adult barnacles (9) The pheromone enhances the rate of settlement and metamorphosis of young cyprids As the cyprids age in the laboratory their settlement rates approach levels at which the pheromone causes little change in settlement (11).

Specific di- and tripeptides can mimic the effect of barnacle settlement pheromone. Peptides with basic carboxy-terminal amino acids and either a neutral or a basic amino-terminal amino acid promote barnacle settlement and metamorphosis. Peptides with histidine, lysine and arginine carboxy termini increased settlement rates. Other combinations of neutral, acidic, and basic carboxy- and amino-terminal amino acids failed to enhance settlement. Native barnacle pheromones are thought to be a heterogeneous group of 3000 to 5000 dalton peptides (9). Similarly, heterogeneity is observed with native leukocyte attractants of bacterial origin (12). However, it is possible that the molecule or molecules in the pheromone preparation that are ultimately responsible for the native pheromone activity are short peptides carried along with the larger peptides. There is precedence for such an occurrence with molecules that induce metamorphosis of *Haliotis rufescens* larva (8). Thus, the 3000 to 5000 dalton molecule could be 1) a carrier molecule, 2) native molecule whose information content is sequestered in the carboxy terminal residues. In nature, the rest of the molecule may have additional functions such as adhesion to surfaces or may also contain the information that alters larval behavior (9)

The C3a anaphylaxin response of the human complement system can be mimicked by pentapeptides with an absolute requirement for arginine at the carboxy terminal (14). Crab larval release behavior is stimulated by di- and tripeptides and has an absolute requirement for a basic amino acid at the carboxy terminal (arginine > lysine) and a neutral amino acids in the other positions of active peptide. Potency can be altered 12000-fold by choice of amino acid constituents (7). Carboxypeptidase studies were performed to assess the importance of the carboxy terminal in the activity of the barnacle pheromone Carboxypeptidase A, which cleaves at the amino end of lysine and arginine carboxy terminal residues, was chosen because of the dependence of the aforementioned studies on basic carboxy termini Carboxypeptidase was shown to eliminate pheromone activity Barnacle settlement pheromone analogs have less stringent requirements than the anaphlaxin or carb larval release system Analogs to barnacle settlement pheromone have the same requirement for a basic carboxy-terminal amino acid, but the amino terminus can be either a neutral or a basic amino acid

The presence of conspecifics is known to enhance settlement and metamorphosis in many benthic invertebrates Several of these phenomena have been shown to be caused by chemical cues. For their sheer diversity and specificity proteins and polypeptides are thought to comprise a large number of communication molecules Polypeptides are identified as pheromones in sand dollars (1) and as settlement cues for abalone (8) and oyster larvae (4) Evolution of peptide cues may have been facilitated by the preexistence of specific high information hydrolysis products due to the action of site specific proteases such as serine proteases

ACKNOWLEDGEMENTS

We would like to thank E Sessions and A R Schmidt for their assistance This work was supported in part by National Science Foundation grant OCE-86-03945, Office of Naval Research grant N-00014-86-K-0261, and by the GFT-AMHT Educational Foundation grant K-87-002

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