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Induction of larval attachment and metamorphosis in the serpulid polychaete *Hydroides elegans* by dissolved free amino acids: isolation and identification

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ABSTRACT: The calcareous tube-building polychaete *Hydroides elegans* (Haswell) is a major fouling organism in tropical waters around the world. In the marine environment, larvae of *H. elegans* rapidly settle and metamorphose in response to acceptable surface-bound bio-organic films. In addition to biological inducers, previous investigations by our group have indicated chemical inducers for larval settlement and metamorphosis in *H. elegans*. Crude samples derived from adult *H. elegans* and colonies of the bryozoan *Bugula neritina* induced a high percentage of larval metamorphosis in laboratory assays. Here, we describe a bioassay-guided isolation and purification of larval metamorphic inducers originating from these samples. Biologically active HPLC-purified fractions of both samples were composed of free amino acids. The entire free amino acid composition was determined by quantitation with *ortho*-phthaldialdehyde (OPA) and was found to be comprised of aspartic acid, glutamic acid, serine, histidine, glycine, arginine, alanine, asparagine, glutamine and taurine in concentrations ranging from 0.2 µM (histidine) to 5.6 µM (taurine). In the laboratory assay, the larval metamorphic response to an artificially prepared sample in identical concentrations of these amino acids was very similar to the natural isolates.

KEY WORDS: *Hydroides elegans* · *Bugula neritina* · Amino acid · Polychaete · Bio-organic film · Metamorphic inducer · Larvae

INTRODUCTION

The life cycle of many benthic marine invertebrates includes a planktonic larval phase. Free swimming larvae must locate acceptable substrata to attach onto in order to begin their benthic life phase. The larvae of sessile marine invertebrates attach and metamorphose in response to a wide range of stimuli during the course of substratum selection (reviewed by Crisp 1974, Pawlik 1992, Rodriguez et al. 1993). Generally, the fact that a settlement cue is required may indicate that larval settlement patterns are not completely random. It has been suggested that in terms of a hierarchy of different stimuli, physical factors of the marine environment and physical properties associated with the substratum are subordinate to biological and chemical surface characteristics and signals (LeTourneux & Bourget 1988, Pawlik 1992). Many marine invertebrate larvae are believed to respond to cues associated with the surface of conspecific individuals. Larval attachment on or close to conspecific adults has both advantages and disadvantages, such as maximization of reproductive success, protection against predators, and competition for space and food if the aggregation exceeds certain densities. Nevertheless, owing to the prevalence of gregariousness in hard-substratum marine communities, the benefits of this phenomenon seem to outweigh the costs (Pawlik 1992).

The calcareous tube-building polychaete *Hydroides elegans* (Haswell) is a major fouling organism in Hong Kong waters and is abundant in tropical and

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subtropical coastal fouling communities. Larval attachment and metamorphosis in *H. elegans* is induced by acceptable bio-organic films (Hadfield et al. 1994, Lau & Qian 1997, Qiu & Qian 1997, Walters et al. 1997) whereas larval settlement patterns are explained by passive larval deposition in crevices of roughness elements, followed by selective attachment to an acceptable bio-organic film (Walters et al. 1997). Early investigations in our laboratory into factors that induce larval attachment and metamorphosis other than natural biofilm and physical environmental determinants were the first attempts to explain gregarious larval settlement patterns of *H. elegans* via chemical inducers. In laboratory assays, Bryan et al. (1997) observed that a homogenate of conspecific juveniles and adults of *H. elegans* from Hong Kong waters induced 39 and 81% larval metamorphosis after periods of 48 and 96 h, respectively. A homogenate of calcareous tubes alone was biologically inactive. Bryan et al. (1997) suggested that the assumed chemical inducer originated from the worm body itself. Moreover, Bryan et al. (1998) observed that *H. elegans* preferentially colonized the surfaces of the bryozoan *Bugula neritina* rather than a variety of other surfaces available in the field, such as subtidal rocks, a cement pier and nylon ropes. In laboratory experiments, larvae of *H. elegans* settled and metamorphosed on branches of *B. neritina* and on the bottom of dishes containing a naturally released exudate of *B. neritina*. The extraction of the leachate and subsequent bioassay analysis of the extract resulted in 74% larval metamorphosis after 96 h exposure. Bryan et al. (1998) suggested a chemically mediated relationship between larval settlement of *H. elegans* and compounds present in the leachate of *B. neritina*.

The objective of this study was to verify the existence of chemical larval inducers in sources that have been reported to contain bioactive metamorphic cues triggering larval attachment and metamorphosis in *Hydroides elegans*. We intended to gain a deeper understanding of potential chemical factors inducing larval settlement in *H. elegans* by isolation, elucidation and subsequent comparison of bioactive chemical components putatively present in the homogenate of *H. elegans* adults and in leachate of *Bugula neritina*. Owing to the distinct settlement behavior of larvae of *H. elegans* in laboratory assays, i.e. the larval settlement rate is effectively enhanced in the presence of a settlement cue as compared to filtered seawater (FSW) controls (Bryan et al. 1997), we attempted a bioassay-guided isolation procedure. The bioassay procedure in this study was similar to the methodology previously applied by Bryan et al. (1997) and Bryan et al. (1998) and was specifically designed to correlate the larval metamorphic response with the efficacy of a chemical inducer.

MATERIALS AND METHODS

Larval culture and bioassay procedures. Adult *Hydroides elegans* were obtained from 2 sources. Worms were collected in the field from February to July, when monospecies aggregations were abundant on submerged rafts at a fish farm in Port Shelter Bay, Hong Kong (22°19'N, 114°16'W). From August to January, when the reproductive activity of *H. elegans* decreased in the field due to low salinity caused by heavy rainfalls (Qiu & Qian 1997), a laboratory broodstock was used. The adults were induced to spawn according to the method described in Bryan et al. (1997) and Qiu & Qian (1997). Using this procedure, we obtained batches of larvae with mixed parentage and low variation in larval size for bioassays. In some batches, 4 d old larvae were reared in FSW (0.22 µm) containing antibiotics (2.5×10^{-4} M streptomycin and 10×10^{-5} M penicillin) to the competent stage.

Bioassays under sterile conditions were performed following the detailed methodology described in Bryan et al. (1997) and Bryan et al. (1998). Metamorphic competence was determined by both examination of larval morphology (Wisely 1958) and a fast preliminary bioassay with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) according to the methods described in Pechenik & Qian (1998) and Qian & Pechenik (1998). In addition to the methods cited, samples were generally filter sterilized (0.22 µm) prior to their investigation in the bioassay. Unless otherwise stated, bioassays were performed without the addition of antibiotics. When metamorphic compounds were investigated in the presence of antibiotics (2.5×10^{-4} M streptomycin and 10×10^{-5} M penicillin), antibiotic-treated larvae were used in the bioassay.

Sample preparation I: homogenate of *Hydroides elegans* adults. Three hundred adult worms of mixed sexes were individually separated from their tubes with forceps under a dissecting microscope. Only individuals that were not damaged by this procedure were collected for sample preparation. The worms were transferred into a 50 ml plastic vial containing 10 ml of water (Millipore, Milli-Q Reagent-Water-System) and stored at −20°C. The defrosted sample was homogenized and centrifuged at 4000 rpm ($1935 \times g$) for 10 min. The aqueous supernatant was decanted and the precipitate was subsequently extracted 3 times with 1 ml water in consecutive steps. Supernatants were combined and freeze-dried, yielding 192 mg of lyophilisate. The dried extract was stored at −80°C until use. An aliquot of the extract was adjusted to the concentration of 0.1 mg lyophilisate ml^{-1} FSW. In order to evaluate whether the metamorphic response was concentration dependent, a dilution series of 0.1, 0.01,

0.001 and 0.0001 mg lyophilisate ml^{-1} FSW was bioassayed. The amino acid composition and concentration in the aqueous extract of homogenate of adults of *H. elegans* in the concentration of 0.1 mg lyophilisate ml^{-1} FSW was determined by precolumn derivatization with *ortho*-phthaldialdehyde (OPA).

Sample preparation II: *Hydroides elegans* conditioned seawater. ***Laboratory:*** A cluster of a monospecies colony of *H. elegans* (1850 tubes with a gray deposit embedded in between, 28 g) collected from a fish farm in Port Shelter Bay in April 1998 was placed in a glass beaker with 140 ml FSW for 1 min. While we gently swirled the cluster, carefully avoiding any damage to tubes or injury to worms, the colony fell into smaller subcompartments and released parts of the deposit. After precipitation of suspended material, a 30 ml aliquot was sampled, filter sterilized (0.22 µm), subdivided into 5 replicates and immediately bioassayed. One ml of the sample was stored at −80°C. A control was prepared by performing the same procedure with FSW.

Field: At the same site, 25 ml aliquots of seawater were sampled in sterilized syringes in direct proximity (<0.5 cm) to aggregations of *Hydroides elegans* adults in 0.5 m water depth (10 replicates). Seawater control samples were taken in a distance of 1 m to the aggregation in the same water depth. Samples were immediately filter sterilized (0.22 µm), frozen in liquid nitrogen and subsequently stored at −80°C. The amino acid composition and concentration in *H. elegans* conditioned FSW and the control samples was determined by precolumn derivatization with OPA.

Sample preparation III: *Hydroides elegans* gamete conditioned seawater. Tubes containing sexually mature individuals were carefully cleaned in FSW and gently cracked with forceps under a dissecting microscope. One individual (whilst in the broken tube) was placed in a cell well (Corning 25820) containing 1 ml FSW. When we touched the operculum slightly, gametes were generally released from individuals in a viscous string through the posterior end. After specimens had been removed, potentially water-soluble compounds in the germ cells' covering fluid were dissolved by stirring. Suspended gametes were centrifuged (4500 rpm [2449 × *g*], 3 min) and the clear supernatants were filter sterilized and stored at −80°C. The amino acid composition and concentration in the germ cells' covering fluid of 10 males and 10 females in 10 ml FSW each was determined by precolumn derivatization with OPA.

Sample preparation IV: *Bugula neritina* conditioned seawater. Colonies of *B. neritina* (500 ml volume displacement in a graduated cylinder filled with FSW) were freshly collected in February 1997 at the same site as *Hydroides elegans*. They were placed in 4 l beakers containing 3 l FSW for a period of 60 min at 23°C, enriching the water with a bright purple leachate (Bryan et al. 1998). The leachate was filtered (0.22 µm) and stored at −80°C until use. An aliquot of this sample was 1/10 diluted with FSW and immediately bioassayed along with *H. elegans* gamete conditioned FSW. The amino acid composition and concentration in 1/10 diluted *B. neritina* conditioned FSW was determined by precolumn derivatization with OPA.

Adjustment of sample concentrations during bioassay-guided isolation. Different isolation procedures were performed in order to evaluate their application for (1) concentration of the bioactive component, and (2) separation of biologically inactive sample components. After an isolation procedure with a bioactive sample had been performed, the distribution of sample components in the resulting isolates was unknown. The resulting isolates were concentrated and redissolved in the same volume of FSW as the preceding sample in order to adjust the concentration of bioactive compounds to be equivalent to that in the original sample. By investigating these isolates in the bioassay, it was possible to evaluate whether bioactive compounds were (1) enriched in a single isolate (very similar metamorphic response to the treatment with the preceding sample), or (2) distributed in more than 1 isolate (lower metamorphic response than the treatment with the preceding sample).

Bioassays with the aqueous extract of adult *Hydroides elegans* homogenate (see 'Sample preparation I') indicated a maximum metamorphic response at a concentration of 0.1 mg lyophilisate ml^{-1} FSW. Therefore, isolates were adjusted to the same concentration of bioactive compounds as in the aqueous extract in order to compare the metamorphic responses of isolates obtained from different purification procedures.

Isolation and purification procedure I: solid-liquid extraction (SLE). To determine whether the metamorphic inducer was selectively extractable from the lyophilisate of the aqueous extract of adult *Hydroides elegans* homogenate, an aliquot of 5 mg lyophilisate was extracted 3 times with 3 ml methanol for 30 min at 8°C in consecutive steps periodically agitating and sonicating the mixture. The solvent extracts were combined and dried in nitrogen (methanol extract, ME). The methanol extraction residue (MER) was dried under vacuum. Both the ME and the MER were completely redissolved in 50 ml FSW and bioassayed. As a blank control, 9 ml methanol was dried in a rotor evaporator and the potential residue was redissolved in 50 ml FSW (MeOH blank).

Isolation and purification procedure II: solid-phase extraction (SPE). Alternatively, SPE was performed to divide the aqueous extract into fractions of different

polarity. One ml of the aqueous extract (32 mg lyophilisate ml^{-1} water) was applied to a preconditioned reversed phase octadecylsilane extraction column (J. T. Baker, 3 ml volume, 500 mg sorbent weight). Processing the column by vacuum, it was firstly eluted with 3 ml water (polar fraction) and subsequently with 3 ml methanol (nonpolar fraction). Fractions were dried in vacuum, redissolved in 1 ml water and subsequently bioassayed.

Isolation and purification procedure III: ultrafiltration. To estimate the molecular weight of bioactive compounds, an aliquot of the aqueous extract of adult *Hydroides elegans* homogenate at a concentration of 0.1 mg lyophilisate ml^{-1} water was ultrafiltered (Amicon Ultrafiltration cell, 350 ml; 500 Dalton Diaflo membrane YC05). The ultrafiltrate (<0.5 kDa) and the filter residue (>0.5 kDa) were freeze-dried, redissolved in the same volume of FSW as the unfiltered aliquot and subsequently bioassayed.

Isolation and purification procedure IV: liquid chromatography. High-performance liquid chromatography (HPLC), gel-filtration chromatography (GFC) and thin-layer chromatography (TLC) were applied to isolate bioactive samples originating from *Hydroides elegans* and *Bugula neritina*.

One ml of the aqueous extract of adult *Hydroides elegans* homogenate (32 mg lyophilisate ml^{-1} water) was solid-phase extracted. The resulting polar fraction was ultrafiltered prior to HPLC and GFC in order to prevent retention of high molecular weight compounds at the column entrance and to improve chromatographic resolution by reducing the complexity of the sample composition. The 0.5 kDa ultrafiltrate of the aqueous extract of adult *H. elegans* homogenate was concentrated, redissolved in 1 ml water and further separated in a series of 5 chromatographic steps by HPLC and GFC.

HPLC was carried out with a Waters HPLC system connected to a photodiode array detector (Waters 996). Depending on the sample volume, reversed-phase columns of analytical (Merck, LiChrospher-100, RP 18 endcapped, 250 × 4 mm, particle size 5 µm) and semi-preparative scale (CS, Nucleosil-100, RP 18 endcapped, 250 × 10 mm, particle size 10 µm) were provided. The following mobile phases were either applied isocratically or as binary linear gradients: Milli-Q water (pH 5.5), water/0.1% trifluoroacetic acid (TFA) (pH 2.1) and water/methanol (40/60 v/v). All solvents and solvent mixtures were degassed for at least 30 min prior to usage and during chromatography (Helium, 10 ml min^{-1}). Chromatographic parameters are given in the figure legends.

GFC was performed with polyacrylamide gel (BIO-RAD, Bio-Gel-P2, fractionation range: 100 to 1800 Da) packed in a Pharmacia C-16 column (60 × 1.6 cm). The column was attached to the HPLC system described above. Helium saturated Milli-Q water was used as the mobile phase. Chromatographic parameters are given in the figure legends.

HPLC and GFC samples were fractionated according to the elution profile in the real time plot of the chromatogram. Fractionation criteria were peak minima between resolved peaks and the change in detection of distinct UV spectra within unresolved peaks (see Figs. 4A to 8A, fractionation steps indicated by vertical dotted lines). Sampled fractions were dried by rotor evaporation or freeze-drying, redissolved in FSW and bioassayed. The selection of mobile phases and additional buffers was limited to the eluents mentioned above because they could be completely removed without leaving any residues of buffer salts. The metamorphic compounds were purified and isolated by repeated chromatography of bioactive fractions and variation of chromatographic elution parameters (bioassay-guided fractionation).

TLC was performed on precoated layers of silica gel-60 (Merck, 20 × 20 cm, 0.2 mm) for qualitative sample analysis with specific postchromatographic staining reactions. Aqueous sample solutions were spotted onto the layer with a microliter syringe. Three mobile phase systems were used for chromatographic development: n-propanol/water (70/30 v/v), 1-butanol/99.8% acetic acid/water (80/20/20 v/v/v), n-propanol/25% ammonium hydroxide (70/30 v/v) (Stahl 1969). TLC was carried out in a separation chamber for ascending development. Spots were visualized with the following reagents: iodine vapor (general reagent for unsaturated carbon bonds), ninhydrin-collidine reagent (for primary amines, amino acids, oligopeptides), chlorine/*o*-tolidine (for nitrogen-containing compounds that are convertible to chloramines), sulfuric acid (general reagent for oxidizable compounds), and anisaldehyde-sulphuric acid (for sugars, steroids, terpenes) (Stahl 1969, Jork et al. 1990). Additionally, samples were developed on separate layers and stained in the sequence: (1) ninhydrine-collidine and (2) chlorine /*o*-tolidine. After derivatization, visible spots were immediately marked for the calculation of retention factors (Rf), presented as hRf = Rf × 100. Characteristics like spot shape, resolution, and color were noted for the comparison with standards.

Free amino acid analysis. Qualitative and quantitative analysis of dissolved free amino acids (DFAA) was performed by precolumn derivatization with OPA using reversed-phase HPLC (Lindroth & Mopper 1979). The HPLC system described above was equipped with a fluorescence detector (Waters 474, excitation wavelength 330 nm, emission wavelength 418 nm) and a LiChrospher-100, RP-18e column (250 × 4 mm, particle size 5 µm, Merck). Gradient elution at 1.1 ml min^{-1} was

performed as follows: Solvent A, tetrahydrofuran/methanol/0.05 M sodium acetate (1/19/80 v/v/v); solvent B, methanol/0.05 M sodium acetate (8/2 v/v); elution profile: 0 to 15% B within 1 min, 15% B for 8 min, 15 to 30% B within 1 min, 30% B for 4 min, 30 to 50% B within 4 min, 50% B for 2 min, 50 to 100% B within 12 min, 100 to 0% B within 3 min, and 15 min re-equilibration. Amino acids and standard solutions in concentrations of 50, 10, 1, 0.1 µmol l^{-1} were obtained from Sigma Chemical Co. or prepared in water. DFAA were identified by spiking with standards. Quantitation was performed with the external standard method. Calibration curves based on peak area were generated over 2 points corresponding to standards covering the DFAA concentration range of the sample under investigation (Millenium software, v2.15).

All solvents and chemicals used in this study were reagent grade and water was Milli-Q quality. Unless otherwise stated, seawater was 0.22 µm filtered.

Isolation and purification procedure V: *Bugula neritina*. A bioassay-guided separation procedure like the one described in this study for the adult *Hydroides elegans* homogenate was applied to *B. neritina* leachate. Flash chromatography with XAD-2 (glass column, 30 × 3 cm) was performed according to the methods described in Bryan et al. (1998).

Statistical analysis. The data on percentage of larval metamorphosis in response to experimental treatments were arcsine-transformed before statistical analysis was carried out. Replicates in which no larvae metamorphosed were given the value of 1/4n (n = number of larvae in a single treatment) to improve the arcsine transformation (Zar 1996). The normality assumption was verified with Shapiro-Wilk's *W*-test (Shapiro & Wilk 1965). To analyze the data, 1-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$) was performed. Data that did not meet the normality assumption of parametric analysis were analyzed using nonparametric statistics. This was done by transforming values to ranks and then applying 1-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$) to the data (Conover & Iman 1981). In the final experiment, data were analyzed by 2-way ANOVA with equal replicates to compare larval metamorphic responses to biologically active samples in assays with and without presence of antibiotics. Percentage data in these 2 different treatments were arcsine-transformed and compared with Mann-Whitney *U*-test (Zar 1996). The figure legends state which statistical analysis was used in individual cases. The data presented in all figures are not transformed.

RESULTS

Larval metamorphosis in response to samples originating from *Hydroides elegans* and *Bugula neritina*

After 48 h, the aqueous extract of adult *Hydroides elegans* homogenate in the relative concentration of 0.1 mg lyophilisate ml^{-1} FSW induced 64% larval metamorphosis (Fig. 1A). This response was significantly higher than in dilution treatments with 0.01, 0.001 and 0.0001 mg lyophilisate ml^{-1} FSW and the FSW control ($p < 0.05$, Tukey's test). No significant difference in percentage of larval metamorphosis was observed among the treatments of 0.01, 0.001, and 0.0001 mg lyophilisate ml^{-1} FSW and the FSW control ($p > 0.07$, Tukey's test). The FSW samples conditioned with *H. elegans* and *Bugula neritina* colonies in the laboratory induced a significantly higher percentage of larval metamorphosis after 48 h than the FSW control (Fig. 1B, $p < 0.005$, Tukey's test). No significant difference in percentage of larval metamorphosis was observed between the seawater sample gained in close proximity to a *H. elegans* colony in the field and the FSW control (Fig. 1B, $p = 0.97$).

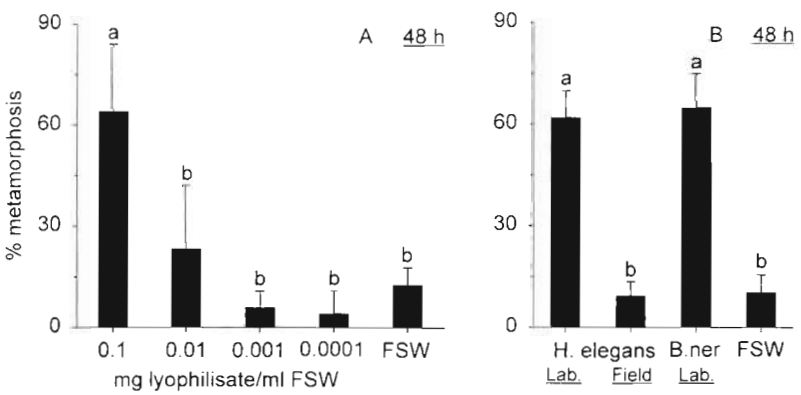

Fig. 1. Percentage of larval metamorphosis after 48 h in response to samples originating from *Hydroides elegans* and *Bugula neritina*. (A) Mean percentage in response to 4 different concentrations of the aqueous extract of adult *H. elegans* homogenate. Data plotted are means + SD of 6 replicates. (B) Mean percentage in response to FSW (filtered seawater, 0.22 µm) conditioned with *H. elegans* and *B. neritina* colonies in the laboratory (lab.) and a seawater sample taken in close proximity to a *H. elegans* colony in the field (field). Data plotted are means + SD of 5 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.0001$)

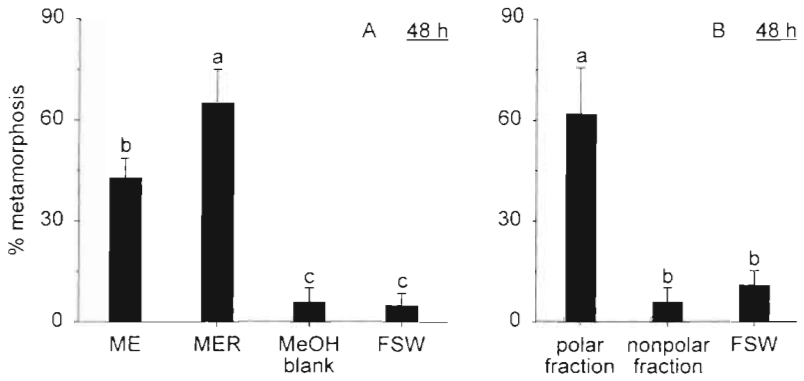

Fig. 2. Percentage of larval metamorphosis after 48 h in response to solid-liquid and solid-phase fractions of aqueous extract of adult *Hydroides elegans* homogenate. (A) Mean percentage in response to the methanol extract (ME) and the methanol extraction residue (MER) of freeze-dried aqueous extract of adult *H. elegans* homogenate. A concentrated sample of the extraction solvent served as a blank control (MeOH blank). (B) Mean percentage in response to the polar (water) and the nonpolar (methanol) fraction from solid-phase extraction of the aqueous extract of adult *H. elegans* homogenate. Data plotted are means + SD of 5 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.0001$)

Larval metamorphosis in response to solid-liquid and solid-phase extracts of the aqueous extract of adult *Hydroides elegans* homogenate

Both the MER and the ME induced significantly higher percentages of larval metamorphosis than the MeOH blank and the FSW control after 48 h (Fig. 2A, $p < 0.001$, Tukey's test). The MER induced 65% of larval metamorphosis, which was significantly higher than the 43% induced by the ME ($p < 0.05$, Tukey's test). No significant difference in percentage of larval metamorphosis was observed between treatments of the MeOH blank and the FSW control ($p = 0.98$, Tukey's test), indicating that potential impurities in the extraction solvent did not affect the bioassay result. The polar fraction induced 62% of larval metamorphosis after 48 h, which was significantly higher than the percentages of larval metamorphosis induced by the nonpolar fraction and the FSW control (Fig. 2B, $p < 0.001$, Tukey's test). Larval metamorphosis induced by the nonpolar fraction was statistically the same as in the FSW control ($p = 0.37$, Tukey's test). These results indicate that SPE in contrast to SLE was a suitable method to isolate biologically inactive, nonpolar sample components.

Larval metamorphosis in response to the ultrafiltrate and filter residue of an aqueous extract of adult *Hydroides elegans* homogenate

The ultrafiltrate (<0.5 kDa) induced 39% of larval metamorphosis after 48 h, which was significantly higher than the percentages of larval metamorphosis induced by the filter residue (>0.5 kDa) and the FSW control (Fig. 3, $p < 0.05$, Tukey's test). The percentage of larval metamorphosis in the treatment with the filter residue was statistically the same as in the FSW control ($p = 1.0$, Tukey's test). These results indicate that ultrafiltration was a suitable method to isolate biologically inactive sample components with a molecular weight higher than 500 Da.

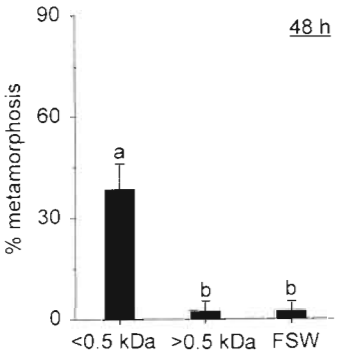

Fig. 3. Mean percentage of larval metamorphosis after 48 h in response to the ultrafiltrate (<0.5 kDa) and the filter residue (>0.5 kDa) of the aqueous extract of adult *Hydroides elegans* homogenate. Data plotted are means + SD of 4 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.05$)

Bioassay-guided chromatography — larval metamorphosis in response to HPLC and GFC fractions

Hydroides elegans

In the first chromatographic step by HPLC, bioactive compounds in the aqueous extract of adult *Hydroides elegans* homogenate were separated (Fig. 4A). Fraction F-1A (1.5 to 7.0 min) correlated with isochratic aqueous elution; Fraction F-2A (7.0 to 17.0 min) correlated with isochratic elution of methanol/water (60/40 v/v). Both fractions were bioassayed (Fig. 4B). After 48 h, Fraction F-1A induced a significantly higher percentage of larval metamorphosis (40%) than Fraction F-2A and the FSW control ($p < 0.001$, Tukey's test). The percentage of larval metamorphosis in the treatment with Fraction F-2A was statistically the same as in the FSW control ($p = 0.89$, Tukey's test), indicating that metamorphic compounds were exclusively present in Fraction F-

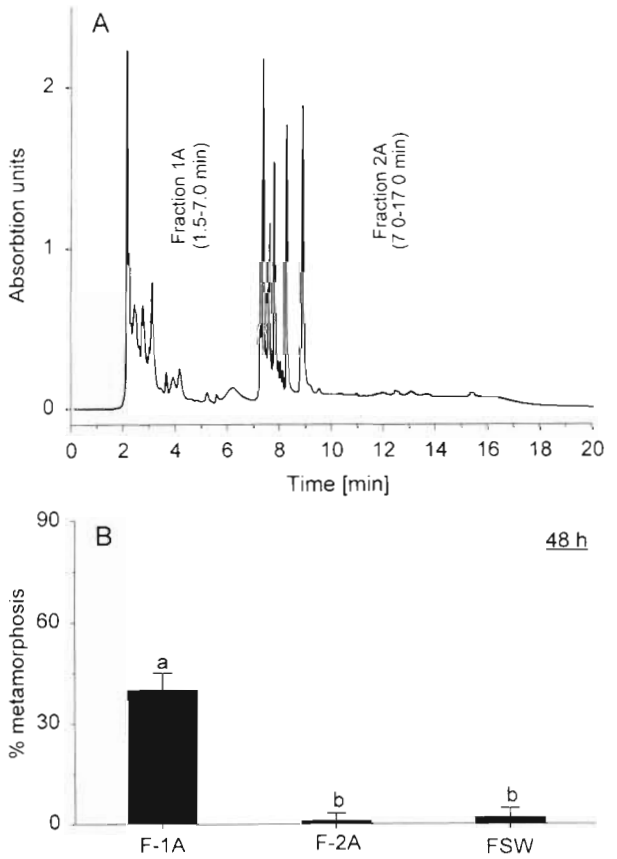

Fig. 4. (A) First chromatographic step (HPLC). Sample volume: 75 µl; column: LiChrospher-100, RP 18 endcapped, 250 × 4 mm, particle size 5 µm; detection: PDA 200–400 nm; mobile phase: A water, B methanol/water (60/40 v/v), 0–6 min 100% A, 6–16 min 90% B, 16–20 min 100% A; flow: 1 ml min^{-1} Fractionation profile is indicated by vertical dotted lines. (B) Mean percentage of larval metamorphosis after 48 h in response to Fractions F-1A and F-2A of the first chromatographic step. Data plotted are means + SD of 5 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.001$)

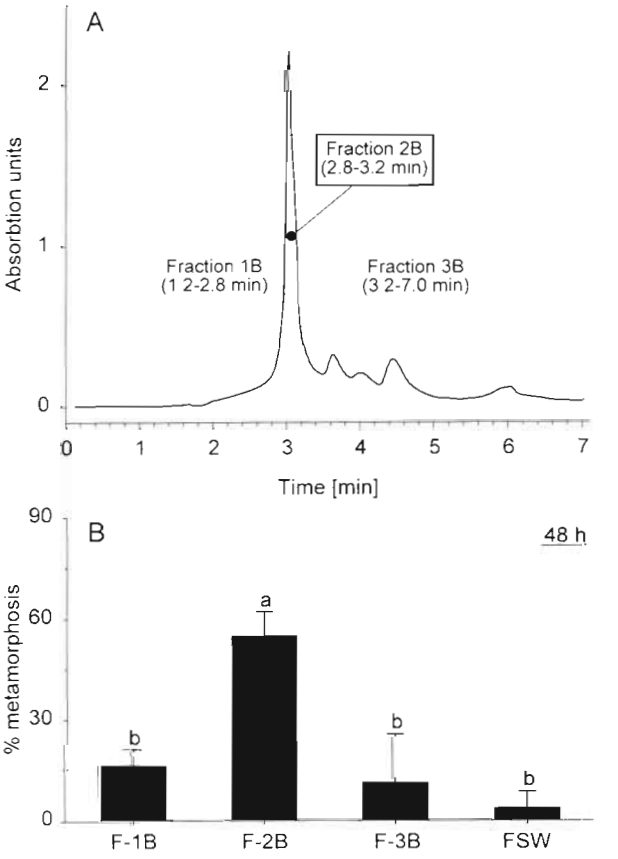

Fig. 5. (A) Second chromatographic step (HPLC). Sample volume: 500 µl; column: Nucleosil-100, RP 18 endcapped, 250 × 10 mm, particle size 10 µm; detection: PDA 200–400 nm; mobile phase: A water, B methanol/water (60/40 v/v), 0–6 min 100% A, 6–16 min 90% B, 16–20 min 100% A; flow: 6 ml min^{-1} Fractionation profile is indicated by vertical dotted lines. The part of the chromatogram from 7 to 20 min is not displayed. (B) Mean percentage of larval metamorphosis after 48 h in response to Fractions F-1B to F-3B. Data plotted are means + SD of 4 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.001$)

1A. In the second chromatographic step, Fraction F-1A was rechromatographed and the 3 resulting subfractions, F-1B to F-3B, were bioassayed (Fig. 5A,B). After 48 h, Fraction F-2B (2.8 to 3.2 min) induced significantly higher percentage of larval metamorphosis (55%) than Fractions F-1B (1.2 to 2.8 min) and F-3B (3.2 to 7.0 min) and the FSW control ($p < 0.05$, Tukey's test). The percentage of larval metamorphosis in treatments of Fractions F-1B and F-3B was statistically the same as in the FSW control ($p > 0.17$, Tukey's test), indicating that metamorphic compounds were mainly present in Fraction F-2B. In the third chromatographic step, Fraction F-2B was rechromatographed with mobile phases of different pH-values; water (pH 5.5) and water/0.1% TFA (pH 2.1) (Fig. 6A). Fraction F-2B was separated at pH 2.1 into 3 sub-fractions for subsequent bioassays (Fig. 6B). After 48 h, Fraction F-2B.1 (2.5 to 3.2 min) induced a significantly higher percentage of larval metamorphosis (46%) than Fractions F-2B.2 (3.2 to 6.0 min) and F-2B.3 (6.0 to 9.5 min) and the FSW control ($p < 0.05$, Tukey's test). The percentage of larval metamorphosis in treatments of Fractions F-2B.2 and F-2B.3 was statistically the same as in the FSW control ($p > 0.1$, Tukey's test), indicating that metamorphic compounds were exclusively present in Fraction F-2B.1. In the fourth chromatographic step by GFC, Fraction F-2B.1 was rechromatographed and separated into 5 sub-fractions for subsequent bioassays (Fig. 7A,B). After 48 h, Fraction F-2B.1.4 (245 to 280 min) induced a significantly higher percentage of larval metamorphosis than Fractions F-2B.1.1 (80 to

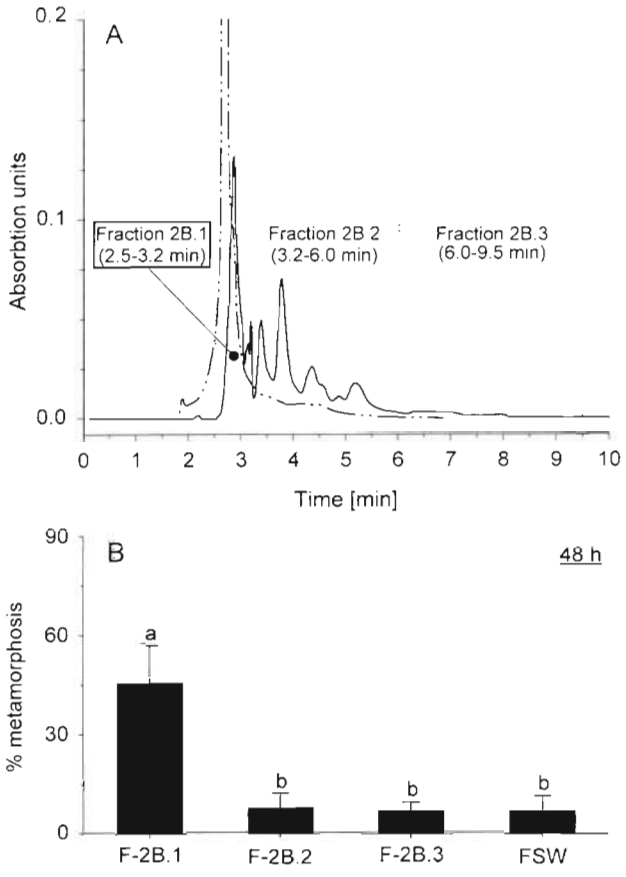

Fig. 6. (A) Third chromatographic step (HPLC). Sample volume: 50 µl; column: LiChrospher-100, RP 18 endcapped, 250 × 4 mm, particle size 5 µm; detection: PDA 200–400 nm; mobile phase: A water, B water/0.1% TFA; 0–10 min 100% A (dash-dotted line) and 0–10 min 100% B (solid line); flow: 1 ml min^{-1}. Fractionation profile is indicated by vertical dotted lines. (B) Mean percentage of larval metamorphosis after 48 h in response to Fractions F-2B.1 to F-2B.3 of the third chromatographic step. Data plotted are means + SD of 5 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.05$)

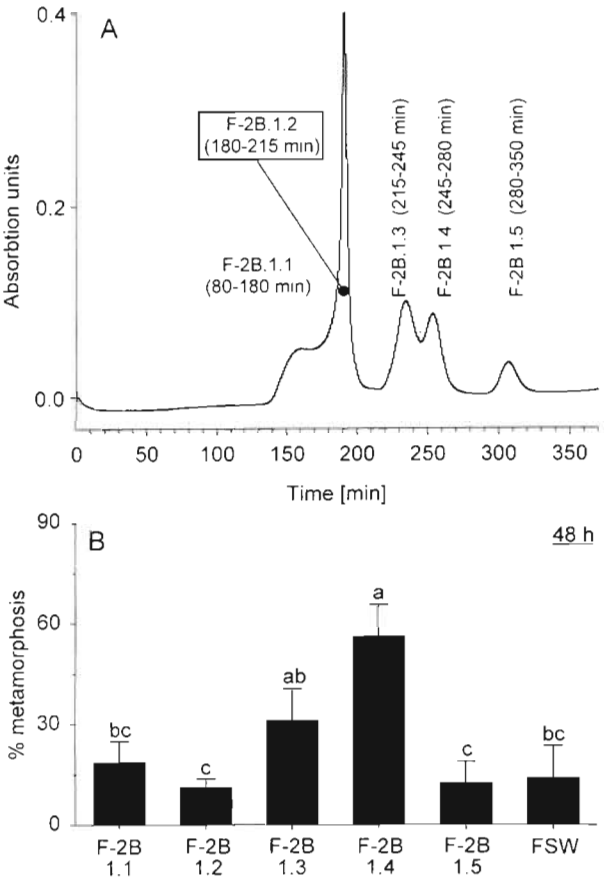

Fig. 7. (A) Fourth chromatographic step (GFC). Sample volume: 500 µl; column: Bio-Gel-P2, 100-1800 Da, 60 × 1.6 cm, particle size 45–90 µm; detection: PDA 200–400 nm; mobile phase: water; flow: 0.2 ml min^{-1} Fractionation profile is indicated by vertical dotted lines. (B) Mean percentage of larval metamorphosis after 48 h in response to Fractions F-2B.1.1 to F-2B.1.5 of the fourth chromatographic step. Data plotted are means + SD of 4 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.001$)

180 min), F-2B.1.2 (180 to 215 min) and F-2B.1.5 (280 to 350 min) and the FSW control ($p < 0.05$, Tukey's test). No significant difference in larval metamorphosis was observed between treatments with Fractions F-2B.1.3 (215 to 245 min) and F-2B.1.4 (245 to 280 min) ($p = 0.65$, Tukey's test). Therefore, metamorphic compounds were present in both Fractions F-2B.1.3 and F-2B.1.4. A comparison of mean percentage of larval metamorphosis of Fractions F-2B.1.3 (31%) and F-2B.1.4 (56%) with the elution profile between 215 and 280 min (overlapping signals, Fig. 7B) indicated that metamorphic compounds were predominantly present in Fraction F-2B.1.4. In the fifth chromatographic step by HPLC, the combined Fractions F-2B.1.3 and F-2B.1.4 were rechromatographed and separated into 3 sub-fractions for subsequent bioassays (Fig. 8A,B). After 48 h, the larval metamorphic response to Fractions F-2B.1.4.1 (2.4 to 4.2 min) and F-2B.1.4.2 (4.2 to 5.6 min) was significantly higher than to Fraction F-2B.1.4.3 (5.6 to 8.0 min) and the FSW control ($p < 0.05$, Tukey's test). Percentage of larval metamorphosis in the treatment with Fraction F-2B.1.4.3 was statistically the same as in the FSW control ($p = 0.97$, Tukey's test), demonstrating that metamorphic compounds were distributed in Fractions F-2B.1.4.1 and F-2B.1.4.2. In a repetition of this experiment, the signal between 2.4 and 5.6 min was sampled in a single fraction, F-2B.1.4.(1+2), and subsequently bioassayed. After 48 h, this fraction induced 52% of larval metamorphosis, which was not

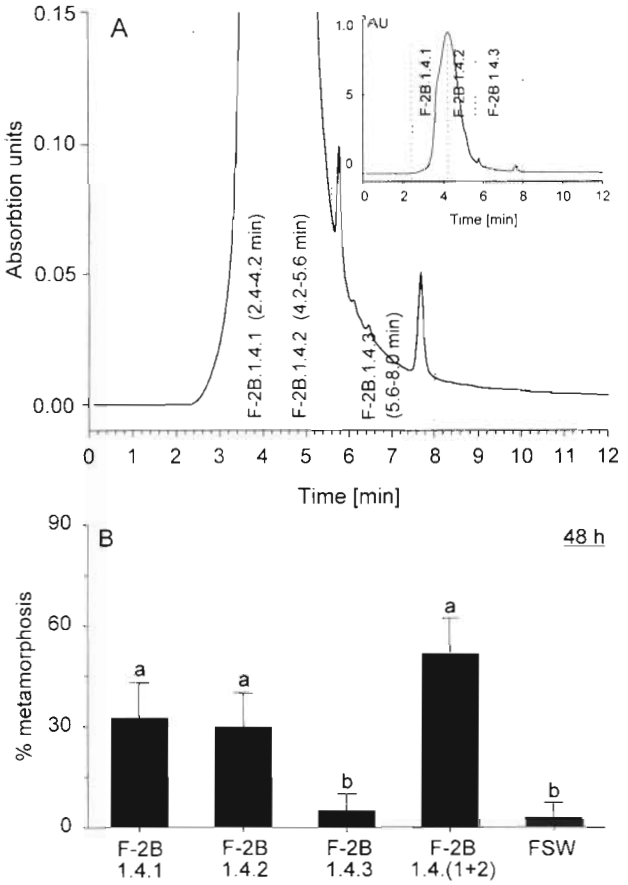

Fig. 8. (A) Fifth chromatographic step (HPLC). Sample volume: 100 µl; column: LiChrospher-100, RP 18 endcapped, 250 × 4 mm, particle size 5 µm; detection: PDA 200–400 nm; mobile phase: water; flow: 1 ml min^{-1}. Fractionation profile is indicated by vertical dotted lines. This figure represents a magnification (0.0 to 0.15 absorption units, AU) of the complete chromatogram displayed in the upper right corner. (B) Mean percentage of larval metamorphosis after 48 h in response to Fractions F-2B1.4.1 to F-2B1.4.3 and F-2B1.4.(1+2). Data plotted are means + SD of 5 replicates. Data that are significantly different are indicated by different letters above the bars (1-way ANOVA, $p < 0.001$)

significantly different from Fractions F-2B.1.4.1 and F-2B.1.4.2 ($p > 0.27$, Tukey's test).

Bugula neritina

The application of a bioassay-guided separation procedure like the one described in this study for adult *Hydroides elegans* homogenate to *Bugula neritina* leachate revealed a bioactive fraction with comparable chemical and biological characteristics. The biological activity was restricted to a HPLC fraction with chromatographic retention values similar to Fraction F-2B.1 (Fig. 6A). The amino acid composition and concentration in this HPLC fraction was determined by precolumn derivatization with OPA. Detailed chromatographic data are not published in this study.

Qualitative analysis of separated TLC spots

The bioactive HPLC Fractions F-2B.1.4.1 and F-2B.1.4.2 were further separated by TLC. The mobile phase n-propanol/25% ammonium hydroxide was found to reveal optimal chromatographic resolution. Separate visualization with the reagents ninhydrin-collidine and chlorine/*o*-tolidine indicated compounds with primary amino functions and/or intramolecular amide-bonds. The other staining methods were negative. Consecutive application of ninhydrin-collidine and chlorine/*o*-tolidine revealed no spots. This result suggested that the isolate did not contain compounds with intramolecular amide bonds. Fractions F-2B.1.4.1 and F-2B.1.4.2 revealed identical chromatographic results (Table 1). Fraction F-2B.1.4.3 was not stained with any of the reagents. By detailed comparison of the retention data and characteristic staining reactions with those of free amino acids (L-isomers), we identi-

Table 1. Results of the separation of Fractions F-2B.1.4.1 to F-2B.1.4.3 by thin-layer chromatography. Values are presented as retention factors (hRf) and spot colors revealed by different visualization reagents (see 'Materials and methods'); reference staining reactions of free amino acids (L-Asp: aspartic acid; L-Glu: glutamic acid; Gly: glycine; Tau: taurine) are given for comparison

Fraction/ reference	TLC solvent: n-propanol/25% ammonium hydroxide (70/30 v/v) hRf Spot 1	hRf Spot 2	hRf Spot 3	hRf Spot 4
F-2B.1.4.1	15 blue[a]/violet[b]	19 violet[a]/yellow[b]	35 brown[a]/yellow[b]	54 violet[a]/yellow[b]
F-2B.1.4.2	15 blue[a]/violet[b]	19 violet[a]/yellow[b]	35 brown[a]/yellow[b]	54 violet[a]/yellow[b]
F-2B.1.4.3	–[c]	–[c]	–[c]	–[c]
L-Asp	15 blue[a]/violet[b]			
L-Glu		19 violet[a]/yellow[b]		
Gly			35 brown[a]/yellow[b]	
Tau				54 violet[a]/yellow[b]

[a]Visualization with ninhydrin-collidine; [b]visualization with chlorine/*o*-tolidine; [c]no visualization.

Table 2. Qualitative and quantitative DFAA analysis of bioactive samples. No DFAA were detected in the FSW controls within the detection limit of 0.013 µM. Concentrations of analyzed samples given in footnotes. Asp: aspartic acid; Glu: glutamic acid; Ser: serine; His: histidine; Gly: glycine; Arg: arginine; Ala: alamine; Tyr: tyrosine; Met: methione; Val: valine; Phe: phenylalanine; Ile: isoleucine; Leu: leucine; Lys: lysine; Asn: asparagine; Gln: glutamine; Tau: taurine; Trp: tryptophan. nd: not determined

Amino acid	*Hydroides elegans*					*Bugula neritina*	
	Adult worm homogenate		Colony	Gametes		Colony	
	F-2B.1.4.(1+2)[a] 10^{-6} M	0.5 kDa-filtrate[a] 10^{-6} M	Conditioned FSW[b] 10^{-6} M	Eggs[c] 10^{-6} M	Sperm[c] 10^{-6} M	Conditioned FSW[b] 10^{-6} M	HPLC fraction[d] 10^{-6} M
Asp	1.12	1.35	0.34	0.25	0.56	1.33	0.92
Glu	0.76	0.82	2.87	0.57	1.52	2.00	1.60
Ser	1.3	1.35	1.93	0.28	0.43	1.16	0.21
His	0.2	0.22	0.63	0.80	0.22	2.99	2.26
Gly	4.94	5.31	3.62	2.52	19.00	3.75	3.05
Arg	1.02	1.14	0.53	nd	nd	0.95	0.52
Ala	2.05	2.27	1.58	0.74	1.18	4.31	3.23
Tyr	nd	0.96	0.47	nd	nd	0.73	nd
Met	nd	0.30	0.14	nd	nd	0.41	nd
Val	nd	0.87	0.41	nd	nd	1.58	nd
Phe	nd	0.92	0.27	nd	nd	0.62	nd
Ile	nd	0.72	0.17	nd	nd	1.05	nd
Leu	nd	1.40	0.42	0.19	0.19	1.73	nd
Lys	nd	1.11	nd	0.19	0.46	0.55	nd
Asn	0.43	0.53	0.57	0.31	0.32	0.46	0.26
Gln	0.86	0.97	0.38	0.29	0.29	1.26	1.00
Tau	5.62	5.98	2.97	2.40	2.14	8.12	7.54
Trp	nd	0.17	nd	nd	nd	nd	nd

[a]0.1 mg lyophilisate of the aqueous extract of adult *Hydroides elegans* homogenate per milliliter FSW; [b]according to sample preparation in 'Materials and methods'; [c]gametes of 1 animal per 1 ml FSW; [d]same concentration of bioactive compounds corresponding to 1/10 diluted conditioned FSW of *Bugula neritina* colony

fied the separated spots as aspartic acid, glutamic acid, glycine and taurine.

Dissolved free amino acid analysis — bioassays with quantified samples

In Table 2, the result of qualitative and quantitative DFAA analysis of the isolate, i.e. Fraction F-2B1.4.(1+2) is compared with the corresponding data of the aqueous extract of adult *Hydroides elegans* homogenate, the male/female germ cells' covering fluid and FSW samples conditioned with live colonies of *H. elegans* and *Bugula neritina* in the laboratory. No DFAA were determined in the seawater samples taken in close proximity to a *H. elegans* colony in the field (detection limit: 0.013 µM).

An artificially prepared amino acid sample (AAS) was prepared in the same concentration and amino acid composition as quantified in the natural isolate F-2B1.4.(1+2) (Table 2). The AAS was assayed along with the natural isolate and the 0.5 kDa ultrafiltrate of the aqueous extract of adult *Hydroides elegans* homogenate (0.5 kDa filtrate) both with antibiotics and without (Fig 9A). After 48 h, no significant difference in

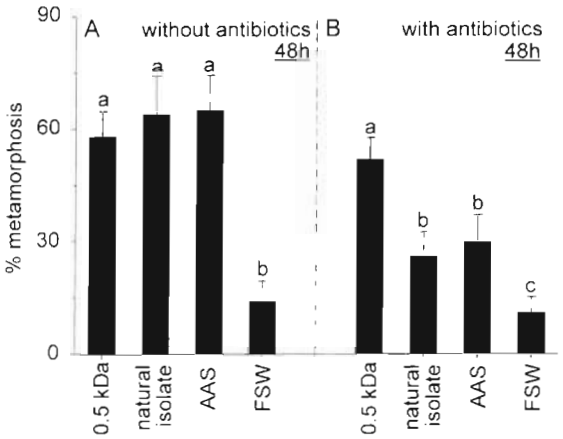

Fig. 9. (A) Mean percentage of larval metamorphosis after 48 h in response to 0.5 kDa filtrate of the aqueous extract of adult *Hydroides elegans* homogenate (0.5 kDa), Fraction F-2B.1.4.(1+2) (natural isolate) and the artificially prepared amino acid sample (AAS). (A) Without and (B) with antibiotics. One-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$) was performed with ranked data of the non-antibiotic and antibiotic treatments separately ($p < 0.0001$). Data plotted are means + SD of 5 replicates. Data that are significantly different are indicated by different letters above the bars

larval metamorphosis was observed among the treatments without antibiotics ($p > 0.62$, Tukey's test). They induced a significantly higher percentage of larval metamorphosis than the FSW control ($p < 0.05$, Tukey's test). In the antibiotic treatment, the 0.5 kDa filtrate induced 52% of larval metamorphosis, which was significantly higher than the metamorphic rate induced by the natural isolate and the AAS ($p < 0.005$, Tukey's test) and by the FSW control ($p < 0.0001$, Tukey's test). There was no significant difference in percentage of larval metamorphosis between the natural isolate and the AAS ($p = 0.62$, Tukey's test). Statistical analysis by 2-way ANOVA revealed a significant influence of antibiotics on larval metamorphic rates ($p < 0.0001$). A comparison of larval metamorphic rates of each sample with its antibiotic treatment demonstrated significant differences between the natural isolate and the AAS ($p < 0.01$, Mann-Whitney *U*-test), whereas statistic similarity between the treatments of the 0.5 kDa filtrate of aqueous extract of adult *H. elegans* homogenate was observed ($p = 0.17$, Mann-Whitney *U*-test).

DISCUSSION

Early investigations in our research group into factors other than natural biofilm that induce larval attachment and metamorphosis of *Hydroides elegans* have indicated chemical inducers present in the leachate of *Bugula neritina* (Bryan et al. 1998) and the aqueous extract of the homogenate of conspecific adults (Bryan et al. 1997). In laboratory bioassays, these crude samples induced competent larvae of *H. elegans* to attach and metamorphose into fully developed juveniles after 48 h. Partial purification of these samples revealed the molecular weight of inductive compounds in both samples to be lower than 10 kDa (Bryan et al. 1997, 1998).

In the present study, we elaborated a chromatographic separation procedure in order to verify the existence of chemical larval metamorphic inducers for *Hydroides elegans* in these samples. The separation was guided by an established and generally accepted bioassay procedure modified from Maki et al. (1988). By conducting sample preparation methods such as solid-phase extraction and subsequent ultrafiltration, we separated polar water-soluble, bioactive compounds with a molecular weight lower than 0.5 kDa. Through a series of different chromatographic procedures we ultimately purified a sample fraction (isolate) that induced 64% of larval metamorphosis in the bioassay (Fig. 9A). This metamorphic response was similar to bioassay results with the aqueous extract of adult *H. elegans* homogenate at the most effective concentration of 0.1 mg lyophilisate ml^{-1} FSW (58%, Fig. 9A). Owing to identical dilution factors, this result suggested that bioactive compounds were nearly completely enriched in the final isolate F-2B.1.4.(1+2) (Fig. 8B). Variances in metamorphic rates of bioactive fractions throughout the HPLC analysis were presumably caused by differences in larval responsiveness of different batches of larvae.

With reference to our chromatographic results, we hypothesized that polar low molecular weight compounds such as sugars, amino acids or short oligopeptides constituted the isolate. Chemical elucidation of the isolate was finally achieved by further TLC analysis. Specific postchromatographic derivatization reactions with the concentrated isolate indicated compounds with primary amino functions and excluded the presence of unsaturated carbon and intramolecular amide bonds. This result suggested that the bioactive isolate solely consisted of primary amines and/or amino acids. This indication was verified by TLC comparison of the isolate with synthetic amino acids. As shown in Table 1, the 4 amino acids glycine, glutamic acid, aspartic acid, and taurine were definitely identified. Quantitation of these DFAA with the more sensitive OPA method revealed additional DFAA in the isolate. Table 2 summarizes the qualitative and quantitative amino acid composition of the isolate and the aqueous extract of adult *Hydroides elegans* homogenate. A comparison indicates that the elaborated separation procedure selectively enriched polar and charged DFAA, excluding those with aromatic and nonpolar, aliphatic side chains.

The larval metamorphic response to the AAS was very similar to the results for the natural isolate, i.e. Fraction F-2B.1.4.(1+2) (Fig. 9A). Obviously, the metamorphosis inductive activity in the AAS was exclusively determined by the DFAA provided (Table 2). Owing to the same percentage of larval metamorphosis, the overall activity in the 0.5 kDa filtrate of adult *Hydroides elegans* homogenate was apparently limited to the amino acid composition quantified in the natural isolate (Fig. 8B). According to this result, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, lysine and tryptophan (all of them characterized by aromatic and nonpolar, aliphatic side chains) did not contribute to the bioactivity in assays with the 0.5 kDa filtrate of adult *H. elegans* homogenate.

The bioassay performed in this study was specifically designed to avoid the development of a potential bioactive microbial film in the assay vessels. Sterile petri dishes were used and samples under investigation were filter sterilized (0.22 µm) prior to each bioassay. Other bioassay attempts inevitably resulted in the buildup of a visible bioactive organic film in the assay dish within 24 h (authors' obs.). These attempts included established procedures, e.g. bioassaying larvae

along with adult specimens or dissected parts as well as smearing crude animal homogenate onto microscope slides. These techniques are not applicable for a selective investigation of potential chemical larval metamorphic inducers, particularly if larvae settle in response to bio-organic films, as is the case with *Hydroides elegans*.

Despite the performance of mostly sterile bioassays, we could not entirely rule out the potential buildup of a bacteria film in assay dishes and its influence on larval settlement rates. Provided that there is a possibility that bacteria may be inoculated into assay dishes along with larvae, we assumed that an addition of antibiotics significantly diminished larval metamorphic rates. Therefore, experiments with bioactive samples were analogously performed with antibiotics. In our experiments, antibiotics had no significant effect on percentage of larval metamorphosis in assays with 0.5 kDa filtrate of adult *H. elegans* homogenate (see Fig. 9A,B), whereas they lowered the metamorphic rate in treatments with the natural isolate and the AAS about 30%. Obviously, these samples were still bioactive in the presence of antibiotics. The apparent influence of antibiotics on a presumptive larva-bacteria film-DFAA interaction is the topic of a further investigation (Beckmann et al. unpubl.).

As outlined above, Bryan et al. (1997) hypothesized the aqueous extract of adult *Hydroides elegans* homogenate to be a natural source of conspecific larval metamorphic inducer. Evidently, the homogenate does not naturally occur in this state. It seems likely that a variety of proteinacious amino acids is released as a consequence of tissue homogenization. The identified DFAA may therefore only be ecologically relevant as a metamorphic cue for larvae of *H. elegans* when they are naturally released in sufficient concentrations. There was no significant larval metamorphic response to a seawater sample gained in close proximity to an aggregation of *H. elegans* in the natural environment. This sample did not contain detectable amounts of DFAA. However, our results demonstrated biological activity in seawater conditioned with a living *H. elegans* colony cluster in laboratory conditions. The metamorphic larval response to this sample was almost the same as to 0.5 kDa filtrate and the isolate of the aqueous extract of adult *H. elegans* homogenate (Figs. 1B & 9A). Compared to the isolate, the conditioned seawater contained all DFAA in nearly the same concentration range (Table 2). Spawning individuals likely release plumes of DFAA into the surrounding environment as we also determined amino acids in the germ cells' encasing body fluid (Table 2). Possibly, additional sources naturally associated with *H. elegans* colonies contribute to the net release of amino acids. Hence, fecal pellets in the gray deposit embedded in the interstices of tubes in the colony may provide a reservoir. Moreover, we assume that amino acids are released as metabolites of phytoplankton (Hellebust 1974) and zooplankton (Webb & Johannes 1967) attached to the colony. The source of DFAA is therefore obviously not restricted to individuals of *H. elegans* alone.

The results of laboratory bioassays with 1/10 diluted leachate of *Bugula neritina* colonies were very similar to those with the 0.5 kDa filtrate and the isolate of adult *Hydroides elegans* homogenate (Figs. 1B & 9A). Therefore, we hypothesized the leachate's bioactivity to be based on DFAA as well. The analysis revealed a very similar amino acid composition in 1/10 diluted leachate of *B. neritina*, in 0.5 kDa filtrate of adult *H. elegans* homogenate at 0.1 mg lyophilisate ml^{-1} FSW and in FSW conditioned with an *H. elegans* colony (Table 2). The biological activity was restricted to a HPLC fraction with chromatographic retention values like those of Fraction F-2B.1 (Fig. 6A) and revealed an identical amino acid composition in similar concentrations to those of Fraction F-2B.1.4.(1+2) (Table 2).

Our findings confirm those of Bryan et al. (1998) in the sense that leachate of *Bugula neritina* contains a bioactive chemical inducer for the induction of larval attachment and metamorphosis of *Hydroides elegans*. However, we found that bioactive metabolites from leachate of *B. neritina* were not bound to amberlite XAD-2 resin, a non-polar adsorbent. In contrast to their results, this indicated a hydrophilic nature of these compounds. Repeating the flash-chromatographic step through a column packed with XAD-2, we determined only the aqueous eluent to be bioactive rather than the methanol eluent containing compounds bound on the stationary phase (Harder & Qian unpubl. results). In analogy with Bryan et al. (1998), we determined the methanol extract of freeze-dried leachate to be bioactive as well. However, when we redissolved the methanol extraction residue in Milli-Q water, we determined a distinctly higher metamorphic response in comparison to the bioactivity of compounds present in the methanol extract. In both samples, the biological activity was caused by DFAA, as proven by bioassay-guided HPLC analysis (Harder & Qian unpubl. results). According to our results, we therefore conclude that the larval metamorphic response determined in the methanol extract of leachate of *B. neritina* and in the XAD-2 methanol eluent by Bryan et al. (1998) was caused by residual amino acids, finally enriched to efficacious concentrations. Provided that lipophilic compounds in the leachate of *B. neritina* display inducers for larval attachment and metamorphosis, we should have determined a bioactive HPLC fraction with certainly longer retention values than we actually observed. In conclusion, we cannot confirm that inductive compounds originating from *B. neritina* and *H.*

elegans have a fundamentally different structure, as proposed by Bryan et al. (1998).

Bryan et al. (1997) and Bryan et al. (1998) hypothesized that the aqueous homogenate of *Hydroides elegans* adults and the exudate of *Bugula neritina* colonies contain a chemical inducer for larval attachment and metamorphosis of *H. elegans*. In this study, we found that a composition of polar and aliphatic DFAA (see Table 2) constituted the biological activity of these samples in laboratory assays. However, our results highlight 2 important issues which question the ecological significance of the identified DFAA as natural chemical larval metamorphic inducers for *H. elegans*. (1) Colonies of *H. elegans* and *B. neritina* may not be the sole sources of amino acids in the natural environment. It seems very reasonable to suspect that amino acids from every potential source will trigger larval attachment and metamorphosis in laboratory assays as long as polar, aliphatic amino acids are provided in micromolar concentrations. (2) Detectable amounts of DFAA are absent in close proximity to aggregations of *H. elegans* in the marine environment.

In order to determine if DFAA act as a chemical cue for larval attachment and metamorphosis in the natural environment it is very important to know the mode of action of the complex of polar aliphatic amino acids on competent larvae of *Hydroides elegans*. The results of a detailed analysis of the mechanism of action of DFAA on larvae of *H. elegans* will be presented in a subsequent publication (Beckmann et al. unpubl.).

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