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Hedistin: A novel antimicrobial peptide containing bromotryptophan constitutively expressed in the NK cells-like of the marine annelid, *Nereis diversicolor*

Aurélie Tasiemski*, David Schikorski, Françoise Le Marrec-Croq, Christelle Pontoire-Van Camp, Céline Boidin-Wichlacz, Pierre-Eric Sautière

Laboratoire de Neuroimmunologie des Annélides CNRS FRE 2933, SN3 1er étage, USTL 59655 Villeneuve d'Ascq, France

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Abstract

A novel antimicrobial peptide, named hedistin was identified from the coelomocytes of *Nereis diversicolor*. Hedistin shows no obvious similarities with other known peptides and constitutes the first antimicrobial peptide containing bromotryptophans demonstrated in annelids. cDNA and mass spectrometry analysis revealed that, upon bacteria challenge, this peptide is secreted following processing of a precursor containing a signal peptide and prosequences. Hedistin was shown to possess an activity against a large spectrum of bacteria including the methicillin resistant *Staphylococcus aureus* and *Vibrio alginolyticus*. The gene was demonstrated to be constitutively and exclusively expressed in circulating NK cells like known to play an important role in the immunity of the sand worm. These data contrast with those observed in another annelid, the leech, in which genes coding for antimicrobial peptides are upregulated in a specific tissue and peptides are rapidly released into the hemolymph after septic injury.

Keywords: Bromotryptophan; Antimicrobial peptide; Annelids; Invertebrate immunity; NK cells like

1. Introduction

Antimicrobial peptides (AMPs) have been reported to be involved in many aspects of innate host defense including functions in phagocyte, local and systemic killing of microbes in invertebrates and vertebrates. AMPs constitute a large group of molecules, which can be subdivided into different classes according to their structure and their amino acid composition [1]. In marine organisms, numerous AMPs have been characterized not only to increase our knowledge on innate immunity but also for the development of possible treatment of bacterial infections affecting aquaculture. Among them are the cysteine-rich peptides of the horseshoe crab [2], mussels [3] and shrimps [4], and the α-helical peptides isolated from ascidians [5,6]. All are constitutively produced by circulating hemocytes and provide local, systemic and/or intracellular reactions in response to bacterial challenge. Although many AMPs have been characterized in marine metazoans, few are reported

*Corresponding author. Tel.: +333 20 33 59 57; fax: +333 20 43 40 54.
E-mail address: aurelie.tasiemski@univ-lille1.fr (A. Tasiemski).

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to contain bromotryptophan residues [7,8]. Bromination of tryptophan which is described as a result of post-translational modifications [9,10] seems to be typical of organisms living in seawater like tunicates. For example, mammalian cathelicidins, a family of AMPs, do not contain bromotryptophan while hagfish cathelicidins do [11].

Nereis diversicolor is a marine annelid, which lives in estuary sediments rich in microorganisms and toxic agents resulting from pollution. Their abundance in this type of environment suggests these worms have developed efficient immunodefense and detoxification strategies. An important cellular activity of three subpopulations of granular coelomocytes was demonstrated to play a role on the immune defense: the type 1 (G1), the type 2 (G2) and the type 3 granulocytes (G3) [12,13]. When worms are immunized with bacteria, G1 granulocytes produce and release into the coelomic fluid a bacteriostatic protein (MPII) that inhibits the growth of *Escherichia coli* and *Micrococcus kristinae* [14]. G2 cells were shown to play a role in the encapsulation process by possessing the capacity for phenoloxidase synthesis and by producing melanine in the form of a thick fluid when activated to encapsulate foreign bodies [15]. G3 granulocytes are involved in the first step in recognizing the "non-self" character of an implant and were also shown to present high cytotoxic activity against foreign cells by a contact-dependent process [16,17].

The evidence that *Nereis* coelomocytes exert cytotoxic and antimicrobial activities has generated interest to identify the molecules responsible of these actions. Moreover, only few data described the participation of AMPs in the anti-infectious response of annelids [18–23]. Hitherto, our studies have been focused on annelids belonging to a recent group *i.e.* the achaeta [24,25]. In this context, we report here the isolation and the complete molecular characterization from the granulocytes, of a novel AMP containing bromotryptophan in a coelomate annelid. Combining in situ hybridization (ISH), Northern-blot and mass spectrometry analysis, we investigated to study the implication of this peptide named hedistin in the immune defense of *Nereis*.

2. Experimental procedures

2.1. Animals

The polychæta annelids *N. diversicolor* were collected at Petit Fort Philippe (France) and at Baie d'Authie (France). Challenged animals were injected with a cocktail containing 10^7 colony forming unit (CFU)/ml of heat killed *E. coli D31* and *Micrococcus luteus IFO12708*.

2.2. Peptide purification

Coelomic fluid from 2000 animals was collected by puncturing the coelomic cavity, in Alsever Solution (AS: 27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.5) on ice.

AS is used as an anticoagulant, which also prevents cell lysis. Coelomocytes which were separated from plasma by centrifugation (800*g*, 15 min, 4 °C), were suspended in 50 mM Tris buffer pH 8.7 containing 50 mM NaCl and homogenized using dounce apparatus. After centrifugation (8000*g*, 20 min, 4 °C), the supernatant was brought to pH 3.5 using HCl 1 M. The pellet containing cellular organelles was extracted in 2 M acetic acid by sonication (3 × 30 s) at medium power on ice-cold water bath and centrifuged (8000*g*, 20 min, 4 °C). Supernatant was then prepurified by solid-phase extraction on a 35 cc C18 Sep-Pak Vac cartridge (10 g, Waters Associated), equilibrated in acidified water (0.05% trifluoroacetic acid (TFA)). The peptides were eluted with 10 and 60% of acidified acetonitrile (ACN). The fraction eluted with 60% of ACN was lyophilized and reconstituted with MilliQ water before subjection to reversed-phase high-performance liquid chromatography (RP-HPLC). All the following HPLC steps were carried out on a Perkin Elmer series 200 HPLC system with a variable wavelength detector. The column effluent was monitored by absorbance at 225 nm.

First step—The 60% Sep Pak fraction was subjected to RP-HPLC on a Sephasyl C4 column (250 × 10 mm, 214TP510 Vydac). Elution was performed at 30 °C with a gradient of ACN in 0.05% TFA from 2 to 62% over 60 min at 1.5 ml/min. The eluted fractions were hand collected in polypropylene tubes, dried, reconstituted in HPLC grade water and tested for antimicrobial activity.

Second step—The active fractions were further separated on a Sephasyl C18 column (250 × 4.1 mm 218TP54, Vydac) with a biphasic gradient of acidified ACN from 2% to 30% over 10 min and from 30% to 60% over 100 min at 1 ml/min. Fractions were collected and treated as above. The purity assessment and the molecular weight determination were carried out by matrix-assisted laser desorption/ionization-time of flight mass

spectrometry (MALDI-TOF-MS) on a Voyager DE Pro (Applied Biosystems).

2.3. Peptide characterization

Edman degradation—NH_2-terminal amino acid sequencing of the purified peptide was performed on a pulse-liquid automatic peptide Perkin Elmer Life Science/Applied Biosystems Procise cLC-492 microsequencer.

Peptide digestion—2 pmol of pure peptide were digested with 0.2 μg of sequence grade modified trypsin (Promega) in 25 mM ammonium bicarbonate buffer pH 8.6 for 6 H at 37 °C. Trypsin digests were then fractionated on a narrowbore C18 reversed phase column (150 × 2 mm, HPI Waters) with a gradient of acidified ACN from 5% to 65% over 60 min at 0.2 ml/min. Purified fragments were then analyzed by MALDI-TOF MS or by Electrospray Ionization-Tandem Mass Spectrometry (ESI-MS-MS) and were submitted to Edman degradation.

MALDI-TOF-MS—Analyses of peptides or trypsin digests were performed on a Voyager DE Pro (Applied Biosystems) in reflector or linear mode. One microlitre of peptide or trypsin digests was spotted on 1 μl of dried α-cyano-4-hydroxycinnamic acid (HCCA) (10 mg/ml). About 300 laser shots were accumulated to obtain the final spectrum. Mass measurements were then finalized after peak smoothing and calibration using either external calibrants or using the two autolysis trypsin fragment 2211.1 and 842.51.

ESI-MS-MS—After desalting the sample on a C18 Zip Tip (Millipore), samples were loaded into nanoES capillaries (Protana) using a 5 μl on-column syringe. The capillaries were inserted into an Applied Biosystems Q-STAR Pulsar (Q-TOF-MS) using an ion spray source. Doubly charged peptides were selected, fragmented by N_2 collision, and analyzed by ESI-MS-MS. The sequences were manually assigned.

2.4. Peptide synthesis

A synthetic peptide was produced by replacing the two bromotryptophan residues of the native hedistin by two unmodified tryptophan residues. Both synthesis and purification were performed by MilleGen (Labege).

2.5. Antimicrobial assays

2.5.1. Microorganisms

Gram negative: *E. coli* D31, *Acinetobacter baumanii*, *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, *Salmonella typhimurium* and *Haemophilus influenzae*.

Gram positive: *M. luteus IFO12708*, *Micrococcus nishinomiyaensis*, *Bacillus megaterium*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus* and *Staphylococcus epidermitis*.

2.5.2. Antimicrobial tests

After each purification step, antibacterial activity was monitored by a liquid growth inhibition assay as described by Bulet et al. [26]. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined according to the method of Bulet et al. also [26].

2.6. Chemotaxis assay

Cells for coelomocyte migration assays were obtained by harvesting coelomic liquid, which was mixed with an equal volume of AS. Migration was measured according to the protocol of Raftos et al. [27].

2.7. Incubation medium and cell contents analysis of bacterial stimulated coelomocytes

Coelomocytes from 50 *Nereis* were collected in AS and incubated with 2.5×10^8 CFU/ml of heat killed bacteria for 0 and 6 h at room temperature. Incubations without bacteria were performed in the same conditions as controls.

After centrifugation (2000*g*, 10 min), cell pellets and supernatants of both challenged and unchallenged coelomocytes were acidified with HCl as described above and were desalted using Zip Tip pipette tips containing C18 reversed phase (Waters). MALDI-TOF-MS analyses were performed in positive linear mode using 4HCCA in sample preparation as previously described. Synthetic hedistin was used as external calibrant.

2.8. cDNA cloning

cDNA for hedistin was cloned using two steps PCR amplification.

Step 1: *Reverse Transcriptase PCR*: Total RNA from whole *Nereis* was extracted using Trizol (Life Technologies). RNA (3 μg) was transcribed into single stranded cDNA using olido(dT)$_{18}$-adaptator primer, 5′-CGAGTCGACATCGATC G(T)$_{18}$-3′ (Kit superscript Tm, GIBCO/BRL, protocol of the manufacturer). One-fourth of the reaction was amplified by PCR using oligo(dT)$_{34}$ adapter primer and a pool of degenerate sense oligonucleotide pool whose sequence is deduced from Ala6-Thr12 with a designed 5′ flanking sequence 5′-GAATTCGC(A/T/G/C)GG(A/T/G/C)A(A/G)GT(A/T/G/C)C(A/T/G/C)GG(A/T/G/C)AC-3′ cDNA cloning. PCR was performed for 30 cycles using one unit of Taq polymerase (Appligene quantum) in 1.5 mM of $MgCl_2$. The cycling parameters were: 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min.

Step 2*: Rapid amplification of 5′cDNA end.* Reverse-transcription was performed using antisense oligonucleotides 5′-CTCATCTGCATCT CTCGTAGTT-3′ deduced, respectively, from the hedistin cDNA sequence previously obtained. After first strand cDNA synthesis and addition of a poly-d(A) tail at its 3′ end using a terminal transferase (GIBCO, protocol of the manufacturer), PCR was performed with an oligod(T) anchor primer and internal antisense primers deduced from the cDNA obtained in step 1: 5′-GAAGTCATTATTCACCT GA GAATC-3′. PCR parameters were identical to those described in step 1.

All PCR products were sub-cloned into pGEM-T easy vector (Promega) and several different cDNA clones were sequenced.

2.9. In situ hybridization

Unchallenged *Nereis* were fixed overnight in a 0.1 M sodium phosphate buffer pH 7.4, containing 4% paraformaldehyde (weight/volume). After dehydration, animals were embedded in paraplast and 8 μm sections were cut, mounted on poly-L-lysine coated slides and stored at 4 °C until use.

Probes: Digoxygenin (DIG)-UTP-labeled antisense and sense riboprobes were generated from linearized cDNA plasmids by in vitro transcription using RNA-labeling-kit (Roche). DIG-labeled riboprobes (40–100 ng per slide) were diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 10*x* Denhardt's solution, 0.5 mg/ml tRNA from *E. coli*, 0.1 M dithiothreitol (DTT) and 0.5 mg/ml salmon sperm DNA.

Hybridization: Riboprobes were hybridized to tissue section and detected as previously described [24]. Control for ISH consisted in replacing antisense riboprobe with sense riboprobe. RNase control sections were obtained by adding a pre-incubation step with 10 μg/ml RNase A prior to hybridization.

2.10. Northern-blot

Nereis were crushed in Trizol (2 ml/5 animals) using a rotor stator homogenizer. Total RNA was extracted according to the manufacturer's protocol (Life Technologies). Twenty microgram of total RNA and size markers were separated on a 1% agarose gel electrophoresis containing 0.02 M sodium phosphate buffer pH7.2, 8.5% formaldehyde and transferred to Hybond N membrane (Amersham). After checking RNA transfer efficiency under UV, membrane was heated 2 h at 80 °C.

Hedistin probe was synthesized from a 384 pb PCR fragment amplified with the sense oligonucleotide 5′-ATGTTAGGTGCTTGGTTGG CTGGGA-3′ deduced from the hedistin cDNA sequence previously obtained and oligo(dT)$_{34}$ adapter primer (see above for the PCR conditions). The product of five PCR was mixed and then purified from 1% agarose gel using the Wizard PCR preps DNA purification system (Promega) and was then (DIG)-UTP-labeled by random priming using the Ready-to-go DNA labeling kit (Pharmacia). Hybridization and detection were performed as described in [24].

3. Results

3.1. Hedistin purification

To isolate peptides responsible for antimicrobial activities of the body fluid of *Nereis*, coelomocytes from 2000 animals were collected and submitted to an acidic extraction and a prepurification on a RP cartridge (see experimental procedures). The 60% Sep-Pak fraction was subjected to RP chromatography on a C4 column (Fig. 1A). Each individually collected peak was lyophilized, reconstituted in water and tested for the presence of antibacterial activity. Biological assays were arbitrarily performed with two microorganisms (*E. coli* and *M. luteus*) commonly used in antibacterial tests, without regard to the pathogenicity to *Nereis*. One fraction eluted at approximately 50% ACN was found to be active against *M. luteus*. This fraction

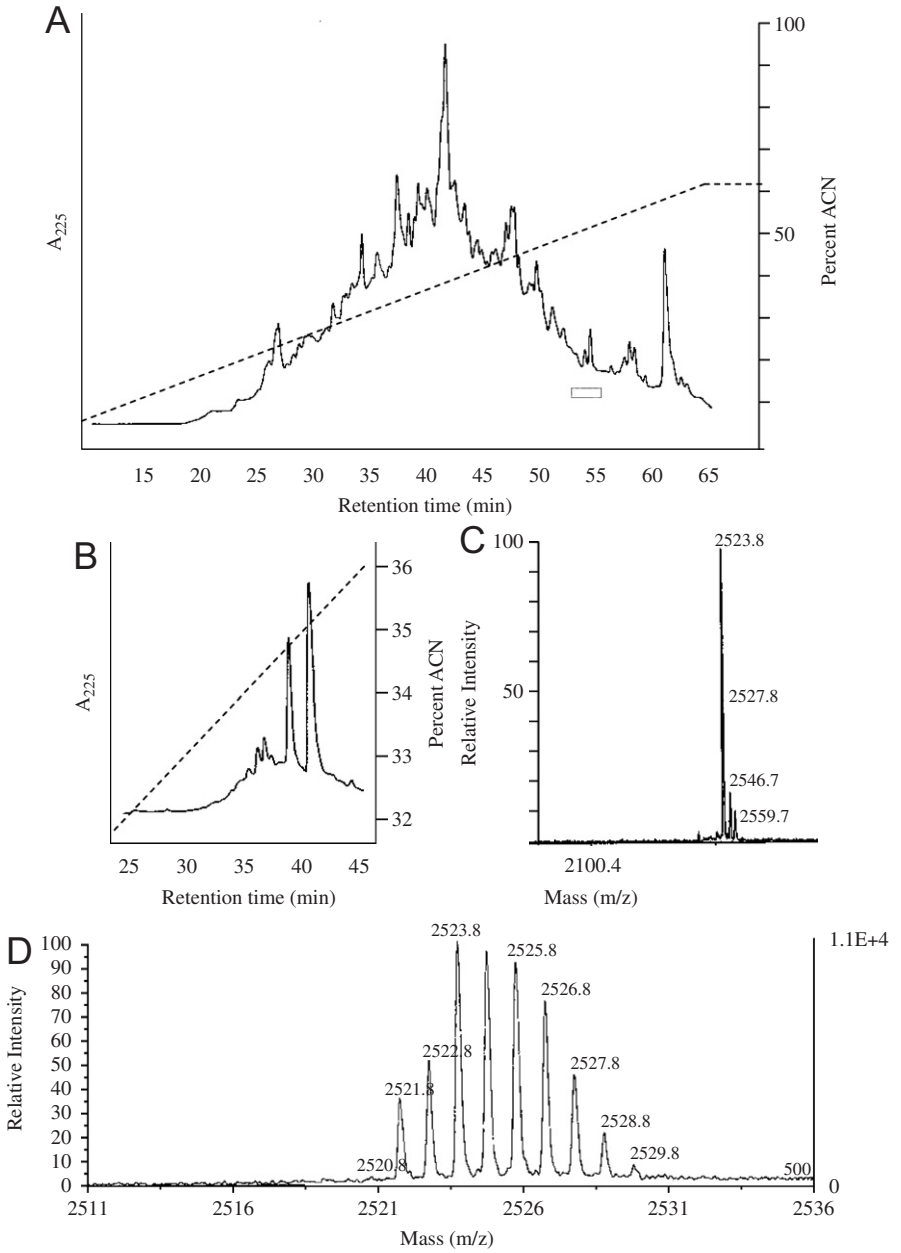

Fig. 1. Hedistin purification from the acidic extract obtained from coelomocytes of *N. diversicolor*. (A) After prepurification by solid phase extraction, the 60% ACN-eluted fraction was lyophilized and then subjected to RP-HPLC on a C4 column. Elution was performed at 1.5 ml/min with a gradient of 1% ACN/min. Antibacterial activity against *M. luteus* was detected in a fraction eluted about 50% ACN (rectangle). (B) This fraction was further purified by RP-HPLC on a C18 column with a gradient of 0.2%/min. The active fraction was eluted at 35%ACN. (C) MALDI mass spectrum of the purified peptide acquired in positive reflector mode shows a major peak at m/z 2523.8. (D) Isotopic distribution obtained by zoom-scan of the MALDI mass spectrum presented in C shows that the exact m/z value of hedistin is 2521.8 [M + H]+. Note this unusual isotopic distribution, which signals the presence of bromines in the hedistin sequence.

was further purified by RP chromatography on a C18 column following the protocol reported in the experimental procedures section and was eluted at 35% ACN (Fig. 1B). Mass measurement by MALDI-TOF in reflector mode recorded a species at m/z 2521.8 with an isotopic distribution characteristic of the presence of bromines (Fig. 1C and D).

3.2. Peptide mapping and characterization of the post translational modifications

Edman degradation of the purified fraction indicated a partial N-terminal sequence LGAX-LAXKVAGXXATYX where X stands for undetermined amino acid residue. Nor the amino acid residues in positions 4, 7, 12 neither the C-terminal sequence could be assigned by this approach. To do so and based on the presence of a lysine residue, trypsin digestion of the purified peptide was used. This yielded two fragments, which were isolated by RP-HPLC and analyzed by a combination of MALDI-MS, Edman degradation and ESI-MS/MS (Fig. 2). One of the two peptidic fragments gave the sequence LGAXLAGK by Edman degradation and a molecular mass measured at 893.3 $[M+H]+$ in reflector mode (Fig. 2A onset). The second one gave the sequence VAGTVATYAXNR and a monoisotopic molecular mass of 1386.6 $[M+H]+$ (Fig. 2B onset). For both fragments, a mass difference of 265 Da between the mass recorded and the mass deduced from the known sequence, suggested that the undetermined amino acid might correspond to a bromotryptophan. The presence of bromines on tryptophan residues was suggested by typical isotopic distribution obtained by MALDI-MS analysis of the native peptide in reflector mode (Fig. 1D).

To confirm the amino acid sequences of the octapeptide and the dodecapeptides the double charged ions at m/z 447.3 and 693.9, respectively were isolated and fragmented by ESI-MS in Q-Star instrument (Fig. 2A and B). Analyses of isotopic patterns of fragment ions were consistent with the amino acid sequences previously determined and confirmed the presence of one bromotryptophan residue in each fragment.

The sum of these data establishes the N-terminal sequence of the antimicrobial peptide as LG AW_{Br}LAGKVAGTVATYAW_{Br}NR. It appeared that the calculated mass of this N-terminal peptidic portion was lower by 261.68 Da than the measured mass of the native molecule at 2521.8 $[M+H]+$ indicating that it does not represent the full sequence. Data bank analysis (BLAST program in Swiss-Prot) revealed no obvious sequence similarities of this peptidic portion with other known peptides or proteins. This novel antimicrobial peptide containing bromotryptophan residues isolated from the hediste *N. diversicolor* was named hedistin.

3.3. cDNA and structure of hedistin precursor

To fully identify the amino acids sequence of hedistin, cDNA was cloned using two steps PCR-based approach with total RNA from whole *Nereis* as template (Fig. 3). The sequence deduced from the cDNA allowed to precise the C-terminal sequence of the active peptide obtained by mapping. It appeared that biochemically calculated mass of the native peptide was 155 Da in excess with the mass of the peptide deduced from the cDNA (2521–2366 = 155 Da). This difference in mass confirmed once again the presence of two bromines ($2 \times 79-2 = 156$) on the tryptophan residues 4 and 18 and also indicated the presence of a C-terminal amide (156–155 = 1 Da). Consequently the complete sequence of hedistin is LGAW_{Br}LA GKVAGTVATYAW_{Br}NRYV-$CONH_2$. Hedistin is a cationic peptide (calculated pH of 9.7). The precursor sequence was also deduced from the cDNA sequence (Fig. 3). From the amino acids sequence of preprohedistin four regions can be readily distinguished (i) a putative hydrophobic signal peptide (Met1-Ala18) as revealed by Signal PVI software analysis (ii) a cationic region (Leu19–Arg59) (iii) the active hedistin plus an extraglycine at the COOH end (Leu60–Val81) and (iv) the terminal region (Lys83–Lys110) also rich in basic residues. The presence of signal peptide leads to the notion that mature peptides may be generated through conventional-processing mechanisms and can then be released to the extracellular medium.

3.4. Biological activities of the native and the synthetic peptides

Antimicrobial activities of native and synthetic hedistin were assessed by liquid growth inhibition assay (Table 1). Both forms showed an equivalent bactericidal activity against the tested Gram positive bacteria *M. luteus* and *M. nishinomiyaensis* (MIC 0.4–0.8 μM–MBC 1.6 μM). Since synthetic hedistin was chemically produced without bromine, we suggest that bromination of tryptophan residues does not play a role in the antibacterial activity. Tests performed with the synthetic hedistin revealed the molecule to be active against a large spectrum of Gram-positive bacteria including methicillin-resistant *S. aureus* (MIC 3–6 μM–MBC 12 μM). Interestingly, when tested with different Gram-negative microorganisms, hedistin was active especially on marine bacteria *V. alginolyticus*. No cytotoxicity of

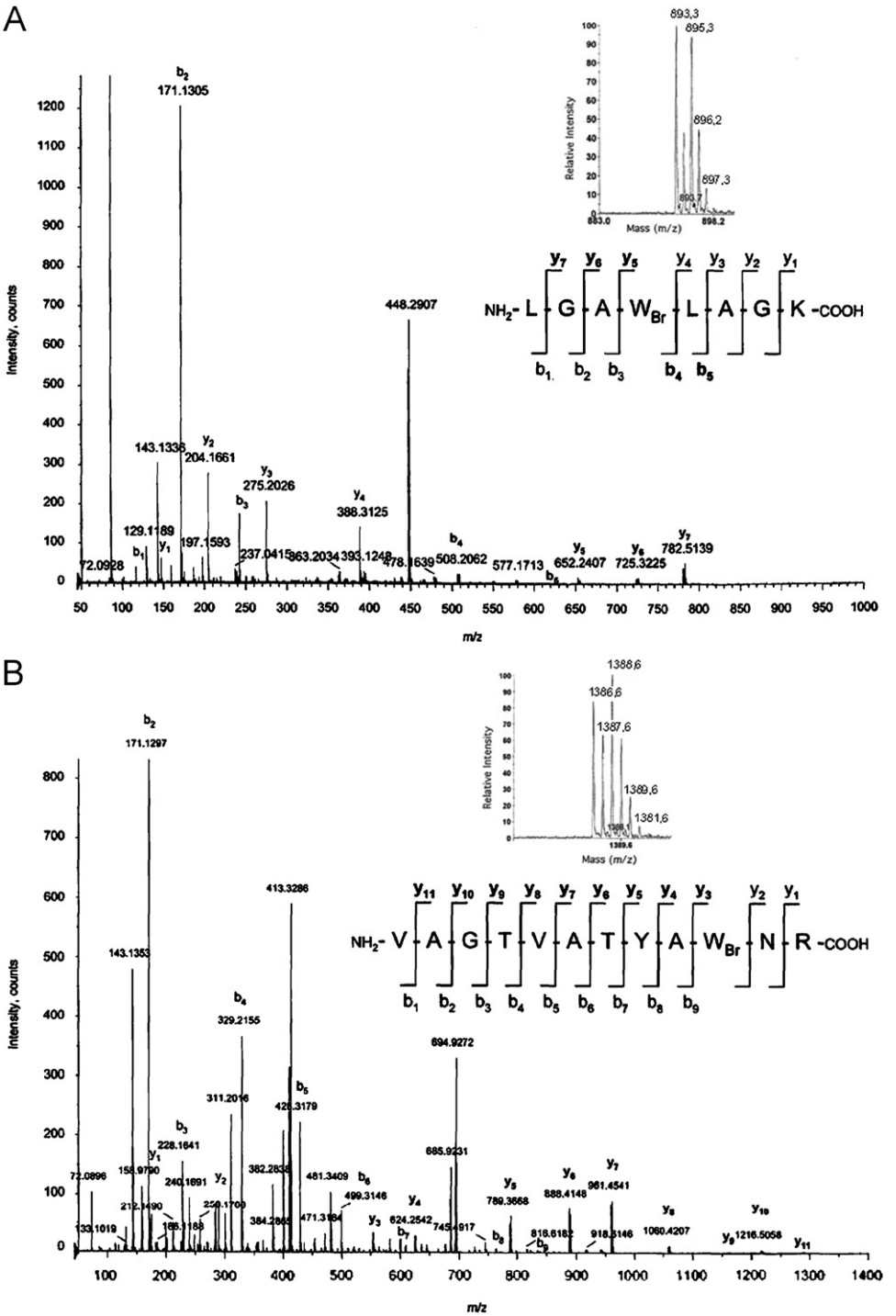

Fig. 2. Peptide mapping and characterization of the posttranslational modifications of hedistin. Trypsin fragments of hedistin (octopeptide and dodecapeptide) were purified and analyzed by ESI-MS-MS or MALDI-TOF-MS (insets). The fragment ions of the doubly charged octopeptide (m/z 447.3) and dodecapeptide (m/z 693.9) are presented in (A) and (B), respectively. The sequences of these fragments and the fragment b- and y-ions found in the fragment ion spectra are noticed. The distinctive isotopic distribution of the singly charged ion at m/z 893.3 (inset A) or 1386.6 (inset B) indicates the presence of bromine. The ion series harboring bromotryptophan residues are noticed in bold.

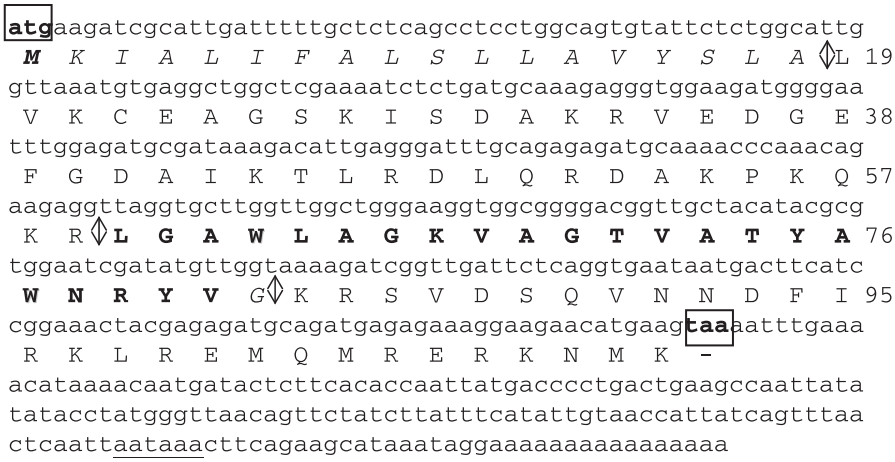

Fig. 3. Nucleotide sequence of hedistin cDNA. Complete sequence of cDNA was obtained by reverse transcription PCR using degenerated oligonucleotide primers deduced from the biochemical sequence. Deduced amino acid sequence of the open reading frame is shown under the nucleotide sequence. Initiator and stop codons are framed. The polyadenyation site is underlined. The double head arrows indicate the cleavage sites for processing of the preprohedistin. Active hedistin is in bold.

Table 1
Antimicrobial activity spectrum of synthetic hedistin compared with that of native hedistin

	Synthetic peptide		Native peptide	
	MIC	MBC	MIC	MBC
Gram negative bacteria				
Acinetobacter baumannii	>25	—	NT	
Aeromonas hydrophila	>25	—	NT	
Alcaligenes faecalis	>25	—	NT	
Enterobacter aerogenes	>25	—	NT	
Enterobacter cloacae	>25	—	NT	
E. coli D31	>25	—	NT	
Pseudomonas aeruginosa	>25	—	NT	
Vibrio alginolyticus	0.8–1.6	Bacteriostatic	0.8–1.6	
Salmonella typhimurium	>25	—	NT	
Haemophilus influenzae	>25	—	NT	
Gram positive bacteria				
Micrococcus luteus	0.4–0.8	1.6	0.4–0.8–	1.6
Micrococcus nishinomiyaensis	0.4–0.8	1.6	NT	
Bacillus megaterium	>25	—	NT	
Staphylococcus aureus	3–6	12.5	NT	
Staphylococcus haemolyticus	3–6	12.5	NT	
Staphylococcus saprophyticus	3–6	12.5	NT	
Staphylococcus epidermitis	3–6	12.5	NT	

Activities not tested or not detected at the highest concentration of 25 μM are indicated in the table by NT and >25 μm, respectively. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) are expressed as final concentration in μM.

either hedistin forms was observed against *Nereis* coelomocytes.

Since a variety of recent studies suggest that some AMPs are involved in other biologically functions, hedistin was also tested for chemoattraction of *Nereis* coelomocytes. No chemoattractant activity was detected with the native or the synthetic hedistin.

3.5. Transcriptional profiles after bacterial challenge and localization of hedistin mRNA

The gene expression pattern of hedistin during immune response was investigated by Northern-blot (Fig. 4A). An analysis of the RNA level in *Nereis* was assessed at 0, 3, 6 and 24 h after injection of a mix of killed *E. coli* and *M. luteus*. As illustrated on Fig. 4A, no enhancement of the basal transcription level was observed suggesting a constitutive expression of hedistin gene. To better understand the physiological role of hedistin, localization of the gene expression site was investigated by ISH. Fig. 4B gives results obtained with the DIG dUTP-labeled antisense probe on unchallenged *Nereis* sections. The gene is strongly and exclusively expressed in coelomocytes evenly distributed in the whole coelomic cavity. These are referred as the type 3 granulocytes according to their small size, their high nucleoplasmic ratio and the presence of few rounded granules in their cytoplasm (Fig. 4B) [12,28]. G3 cells are also called NK cells like because of their natural cytotoxicity [29]. Indeed, when mixed with *Arenicola* sp. coelomocytes or vertebrate erythrocytes, NK cells like release the content of their granules onto the foreign cells, which undergo lysis [12]. Pore-like structures have been observed on the membrane of lysed cells suggesting that a pore forming protein such as perforin might be operating in the *Nereis* system.

Even if the level of transcript does not increase after bacteria challenge, we noticed that "hedistin containing coelomocytes" accumulated around infection sites as shown on the encapsulation picture (Fig. 4B). This argues in favor of a local participation of hedistin in the antiinfectious response.

3.6. Peptide exocytosis

The presence of a putative signal peptide sequence in hedistin precursor indicated that the mature peptide might correspond to a secreted molecule. Mass spectrometry analyses were then used to study the eventual effects of bacteria on hedistin exocytosis (see experimental procedures and Fig. 5). Coelomocyte contents and cell free incubation mediums were prepurified and analyzed for the presence of hedistin by mass spectrometry. MALDI-TOF measurement in linear mode of the coelomocyte contents showed the presence in any conditions, of a molecule at a m/z of 2523.8 ± 0.5 which is in agreement with the molecular mass of the active hedistin (Fig. 1C). This suggests storage of the peptide into the coelomocytes. Interestingly, the mass corresponding to hedistin was detected in the cell incubation medium 6 h after bacterial challenge only. No mass at the value of interest was observed in the incubation medium of unchallenged coelomocytes at $t = 0$ and 6 h. This argues in favour of an exocytosis process, which occurs specifically after bacterial challenge. This mechanism does not seem to be instantaneous since hedistin was not detected in the incubation medium immediately collected ($t = 0$ h) after bacteria addition.

4. Discussion

The present study described the purification, the characterization and the gene expression site of a novel antimicrobial peptide from the coelomocytes of the hediste *N. diversicolor*. The peptide called hedistin showed potent microbicidal activities against a broad range of bacteria and no chemoattractant activities. To our knowledge, it represents the first antimicrobial peptide possessing bromotryptophan residues evidenced in annelids. The gene is constitutively expressed in NK cells like and the peptide is released into the local environment upon bacterial stimulation.

Bromotryptophan was first reported in conus peptide toxins as a post-translational modification [10]. AMPs characterized from the ascidian *Styela clava* [7] and from the hagfish *Myxine glutinosa* [11] was also found to contain bromotryptophan. It appears that molecules from marine organisms generally involve bromine substitution at position 6 of the indole ring. On the basis of these observations, we hypothesize an incorporation of bromine at position 6 of the hedistin tryptophans [9,10]. As suggested by Craig et al. [10], the large distribution of brominated product in the marine environment presumably arises through a broad spectrum of bromoperoxydases in marine organisms. Thus the presence of bromotryptophan in *Nereis* could be the result of an adaptation involving the recruitment of an enzymatic system already used by numerous marine organisms, in a specialized defense way against microorganisms.

In addition to bromotryptophan, the hedistin primary structure includes a C-terminal amidation. The presence of a Ct amide instead of a free valine residue increases the net cationic charge and consequently the electrostatic attraction to target membrane like the negative charged bacteria

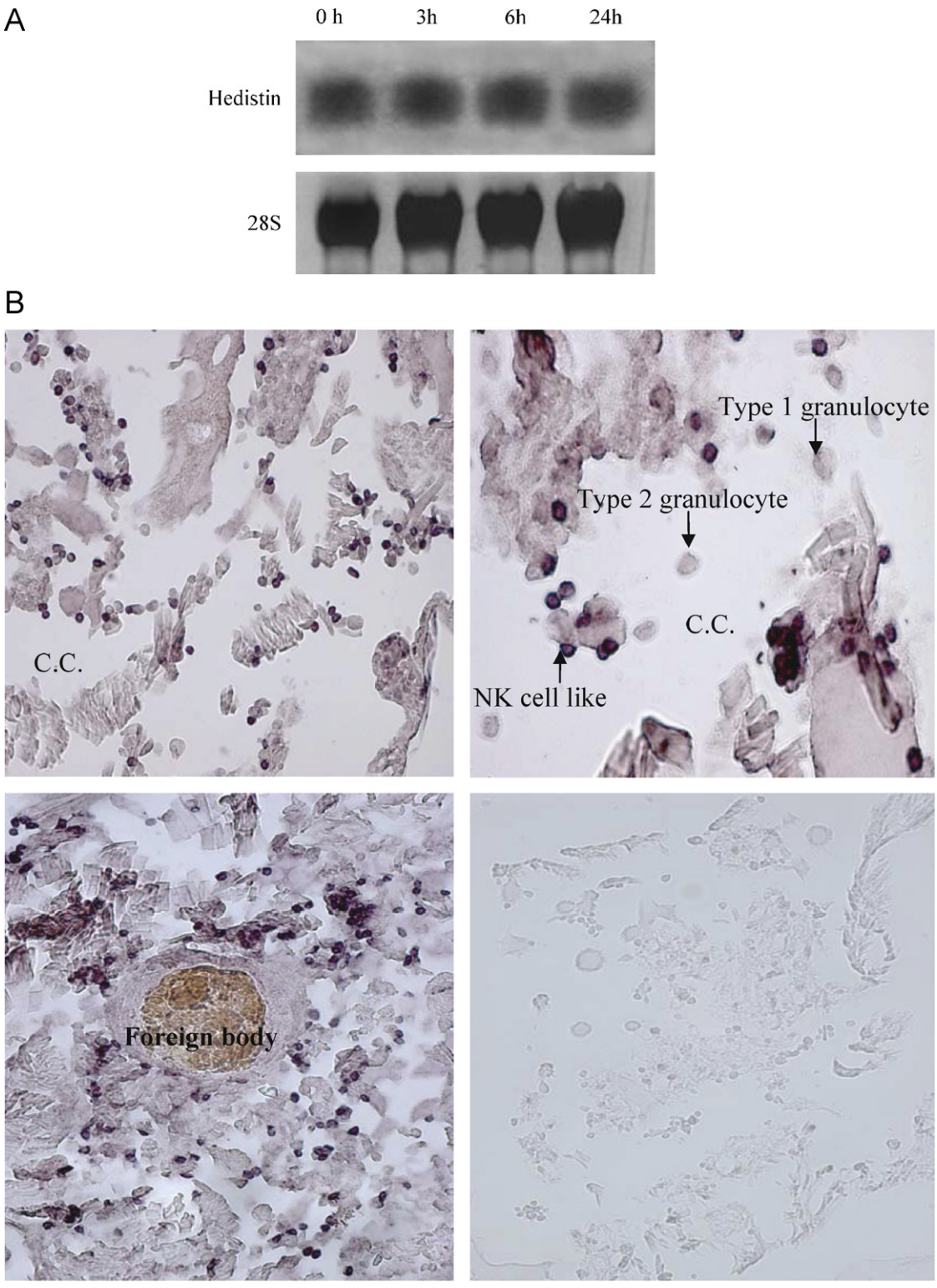

Fig. 4. Analysis of hedistin gene expression. (A) Northern-blot analysis of RNA from adult *Nereis* extracted at different times (0, 3, 6, 24 h) after challenge with killed bacteria. (B) ISH with digoxygenin dUTP-labeled hedistin riboprobes on sections of unchallenged adults. The signal was exclusively detected in NK cells like largely distributed in the whole coelomic cavity (CC) (upper left and right). The labeled coelomocytes accumulated around foreign body (bottom left). No hybridization was noticed with the sense riboprobe confirming the specificity of the labeling obtained with the antisense riboprobe (bottom right).

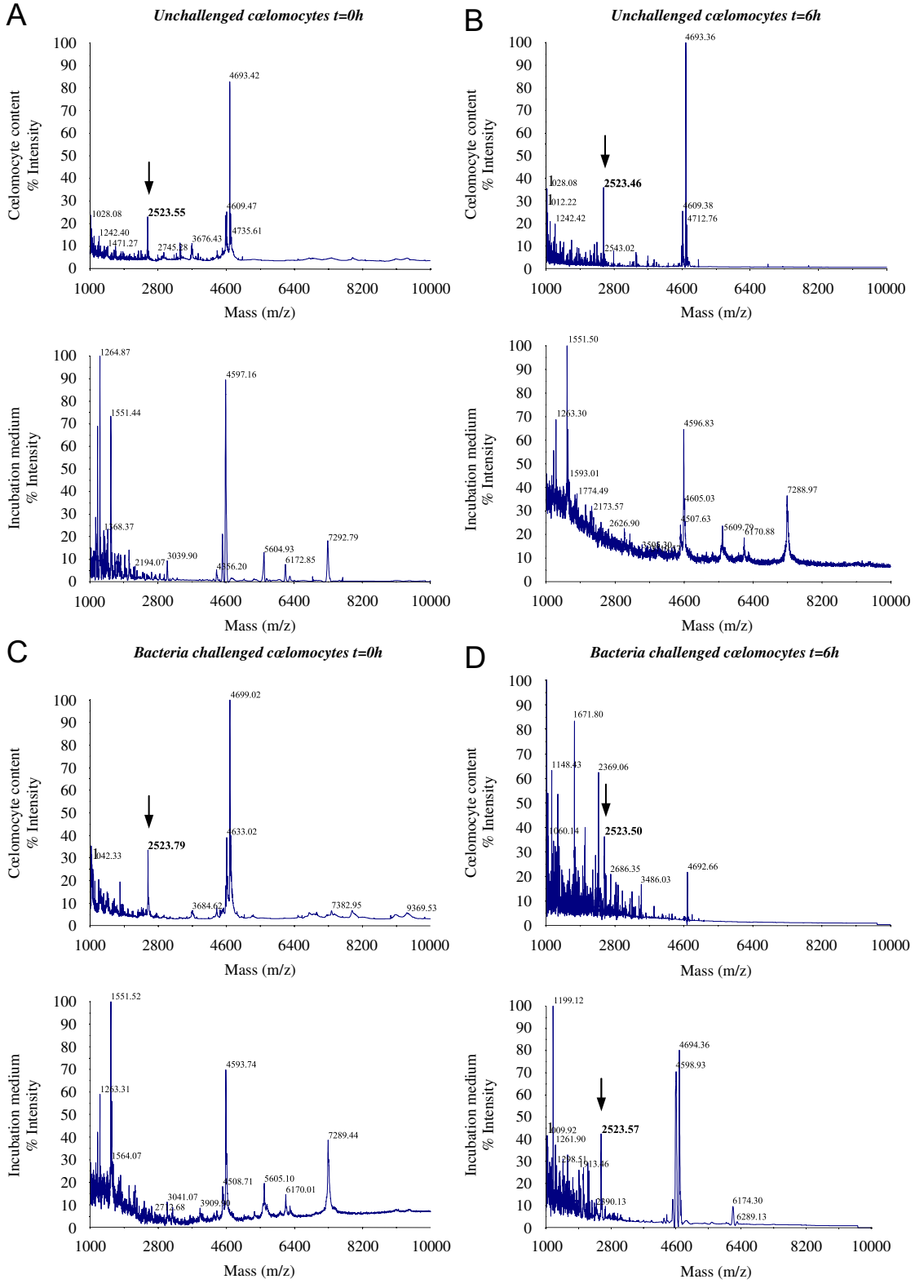

Fig. 5. Analysis of hedistin exocytosis was investigated by performing mass spectrometry measurements on cell contents and on incubation mediums of coelomocytes challenged with killed bacteria for 0 and 6 h. Analyses of the cell contents and the incubation mediums of unchallenged coelomocytes were performed as controls. The m/z value of 2523.8 ± 0.5 $[M+H]^+$ in linear mode corresponds to mature hedistin.

membrane [30–32]. This suggests that C-terminal amidation of hedistin might be implicated in its bactericidal properties. Moreover, as for the hagfish cathelicidins, the C-terminal amidation and the unusual amino acid bromotryptophan could make hedistin a poorer substrate for endogenous proteolytic enzyme by providing resistance to C-terminal exopeptidase and to proteolysis for steric reasons, respectively [11]. Such protease resistance could extend the lifetimes of hedistin *in vivo* sustaining antimicrobial activity.

Assays performed with the synthetic peptide devoid of bromines instead of the native hedistin did not affect its antibacterial properties demonstrating that bromination is not important for hedistin activities. Hedistin kills almost all tested Gram-positive bacteria including methicillin-resistant *S. aureus*. Among the panel of tested Gram-negative bacteria, only the growth of the marine *V. alginolyticus*, which is a causative agent of episodes of mass mortality of larvae of bivalves in commercial hatcheries was inhibited in presence of the peptide [33]. This could be attributable to the capacity of *Vibrio* to extensively degrade native cuticle collagen of *Nereis* [34]. Vibrial collagenase would help bacteria entrance into the worm body, making the mechanical defense barrier of the cuticle inefficient against *Vibrio* invasion. Thus, hedistin synthesis would follow from the adaptation of *Nereis* immune defense towards bacteria of its environment.

Hedistin gene is produced as a precursor containing a signal peptide and the active peptide surrounded by two cationic regions whose functions are still unknown. According to the preprohedistin and the hedistin sequences, the generation of active hedistin from its precursor must involve at least five post translational reactions, presumably in the following temporal sequence: (i) cleavage at the Ala18–Leu19 site to remove the prepeptide, (ii) cleavage at the monobasic site Arg59–Leu60 to free the proregion (iii) formation of the COOH-terminal amide with concomitant loss of one glycine residue, (iv) bromination of the tryptophan residues and (v) cleavage of the Val81–Gly82 bond to yield hedistine. The two cleavage reactions may in turn involve one or more splits by endo-and/or exopeptidases at proprotein convertase like sites. Moreover, the presence of a signal peptide suggests that the active peptide could be secreted. Analyses performed by mass spectrometry showed that upon stimulation by microbial substances only, coelomocytes release into the plasma the active peptide, which could then exert its antimicrobial properties in the immediate cell environment.

Our data establish that the NK cells-like of the sandworm *N. diversicolor* constitutively expressed the gene encoding an extensively modified antimicrobial peptide called hedistin. Hedistin-containing coelomocytes migrated and accumulated around infectious site where the presence of bacterial motifs triggers the release of the active peptide into the local environment. These results are reminiscent of those reported for marine organisms like the shrimp *Penaeus vannamei* [4], the mussel *Mytilus galloprovincialis* [35] and the horseshoe crab, *Tachyplesus tridentatus* [2]. In *M. galloprovincialis*, microbial challenge provokes the release of the antimicrobial peptide MGD1 from the hemocytes into the plasma. In *T. tridentatus*, bacterial stimulation triggers the degranulation and the release of different immune molecules such as AMPs and coagulogen substances.

In annelids, even if different AMPs have been characterized, only very few data described their participation in the anti-infectious response. The gene encoding the proline-rich lumbricin I from the oligochaeta annelid *Lumbricus rubellus* was shown not to be induced by bacterial infection [23]. In the Asian earthworm *Pheretima tschiliensis*, PP-1 a lumbricin1 analog was demonstrated to be synthesized in the body wall only [19]. Its localization in the mucus of the epidermis suggested a role in the mucosal defense. In the polychaeta annelid *Arenicola marina*, two isoforms of arenicin, a novel antimicrobial peptide containing a single disulfide bridge was isolated from the coelomocytes but its participation in the immune defense was not discussed [21]. In achaeta annelids, we obtained evidence for an enhancement of transcription levels of two genes encoding two new antibacterial peptides: theromacin and theromyzin in a specific tissue that could be assimilated to the fat body of *Drosophila melanogaster* [24]. These peptides are released into the coelomic fluid where they could exert their antibacterial properties by a systemic action. In addition to their participation in the systemic response, theromacin and theromyzin may have a role in the mucosal defense as supported by the immunodetection of both peptides in the intestine epithelium and the epidermis. These data contrast with those reported in the present study. Moreover neither theromacin nor theromyzin genes were amplified when using *Nereis* cDNA as

template for RT-PCR suggesting that these AMPs does not exist in *Nereis*. Reciprocally, no hedistin transcript was detected from cDNA of the leech *Theromyzon tessulatum* (data not shown).

In conclusion, two modes of fighting infections by different AMPs appear to exist in annelids (i) rapid transcriptional upregulation of the genes coding for AMPs, mainly in a specific tissue, after septic injury and rapid release into the hemolymph of the antimicrobial compounds in the leech *T. tessulatum* and (ii) constitutive production and storage of the antimicrobial substances, particularly in coelomocytes, and release of the peptides into the coelomic fluid after immune challenge in polychaeta and oligochaeta annelids.

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