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Current Pharmaceutical Biotechnology, 2016, *17*, 000-000

Antimicrobial Activity of Molluscan Hemocyanins from Helix and Rapana Snails

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Abstract: For the first time the antimicrobial activities of hemocyanins from the molluscs *Rapana venosa* (RvH) and *Helix aspersa* (HaH) have been tested. From the hemolymph of the garden snail *H. aspersa* one structural subunit (βc-HaH) and eight functional units (FUs, βc-HaH-a to βc-HaH-h) were isolated, and their N-terminal sequences and molecular weights, ranging between 45 and 65 kDa, determined. The antimicrobial test of the hemocyanins against different bacteria showed that only two FUs from Rapana, RvH1-b and RvH1-e, exhibit a low inhibition effect against *Staphylococcus aureus*. In contrast and surprisingly, the structural subunit βc-HaH of *H. aspersa* not only shows strong antimicrobial activities against *S. aureus* and the likewise Gram-positive *Streptococcus epidermidi*s, but also against the Gram-negative bacterium *Escherichia coli*. We suggest that this subunit therefore has the potential to become a substitute for the commonly used antibiotics against which bacterial resistance has gradually been developed.

Keywords: Antibacterial activity, hemocyanins, *Helix aspersa* (HaH), *Rapana venosa* (RvH), *Staphylococcus aureus*, *Streptococcus epidermidis*, *Streptococcus pyogenes*, *Escherichia coli*.

1. INTRODUCTION

Antimicrobial peptides (AMPs) are part of the natural defense system of the organisms that contain them and have very broad range of activities against different microorganisms [1, 2]. Several peptides/proteins from molluscs and arthropods have been found to exhibit a broad spectrum of microbial activities against Gram+ and Gram- bacteria, and yeast [3, 4]. The first reported protein, achacin, was isolated from the mucus of the Giant African snail *Achatina fulica*. It has a molecular mass of 150 kDa and is composed of 2 subunits [5]. Other antibacterial proteins have been mostly isolated from the hemolymph, such as carcinin from the shore crab *Carcinus maenas* [6]. Also the hemocyan in the hemolymph of the Giant African snail appears to be active against bacteria, even 6 species being susceptible [7]. Moreover, three bacteriostatic glycoproteins, aplysianin P, A, and E, with molecular masses of 60-320 kDa, were identified in the sea hare *Aplysia kurodai* [8]. All these data are welcome news in view of the increasing phenomenon of resistance of bacteria against existing antibiotics, as they open the way to the development of new compounds in the fight against pathogenic bacteria [9, 10].

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Recently, we identified four novel proline-rich peptides, with molecular masses between 3000 and 9500 Da, from the hemolymph of the marine snail *Rapana venosa*, showing strong antimicrobial activities against the Gram+ bacterium *Staphylococcus aureus* and the Gram- bacterium *Klebsiella pneumonia* [11]. In the present paper we aimed to investigate whether hemocyanin, the major protein in the hemolymph of the garden snail *Helix aspersa*, and/or its subunits have antibacterial properties. We carried out a comparative study with the subunits of the hemocyanin from the marine snail *R. venosa*, as well as with those of another garden snail, *Helix lucorum*, thereby enlarging our knowledge on the bioactivities of hemocynins in general.

Since the purification and characterization of *H.aspersa* hemocyanin (HaH) and of its subunits has not been reported before, these experiments will firstly be outlined.

2. METHODS

2.1. Purification of Hemocyanins

The snails *Rapana venosa* (Rv) and *Helix aspersa* (Ha) were collected in Bulgaria. Rv hemocyanin (RvH) and its two structural, as well as its eight functional units were isolated from the hemolymph as described before [12-14]. Ha hemolymph was isolated and the hemocyanin component purified as mentioned [15]; the blue protein pellet was resuspended

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in 50 mM Tris/HCl stabilizing buffer, pH 7.5, containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$.

2.2. Quaternary Structure of Ha Hemocyanin

The quaternary structure of native HaH, was analyzed using a FEI Tecnai Spirit G2 transmission electron microscope operated at 120kV. Unbinned images were recorded on a Gatan Ultracscan Model 895**,** 4k x 4k CCD-camera. Electron micrographs were routinely recorded at an instrumental magnification of 49000.

2.3. Isolation of Structural Subunit β_C-HaH

Native HaH was first dialysed for 4–5 days against 10 mM sodium acetate buffer, pH 5.2, at 4°C. The precipitated β_C-subunit (β_C-HaH) was obtained after 30 min of centrifugation at 4°C and 15000 g, and the pellet redissolved in 0.1 M sodium acetate buffer, pH 5.8. The subunit was initially purified by anion-exchange chromatography on a DEAE-Sepharose CL-6B column using a stepwise gradient from 0.0–1.0 M NaCl in 50 mM Tris–HCl buffer, pH 8.0, at 1.5 ml/min. The collected fraction were desalted by ultrafiltration (30 kDa Amicon® PM membranes), and dialysed against 0.1 M phosphate buffer, pH 6.5, at 4°C. After concentration on a 100 kDa Amicon® PM membrane, the subunit was purified subjected to a final gel filtration step on a Sephacryl S 300 column.

2.4. Isolation of Functional Subunits of β_C-HaH

FUs were obtained by limited proteolysis of β_C-HaH with TPCK-trypsin, used at a ratio (w/w) of 400/1, in 50 mM Tris, pH 7.5, containing 1 mM EDTA, for 4 h at 37°C, The resulting components were separated on an anion exchange DEAE Sepharose CL-6B column (HR 10/10, Pharmacia), mounted in an FPLC chromatographic system, using a stepwise gradient of NaCl (0–1.0 M) in 50 mM Tris–HCl buffer, pH 8.2. The functional units were then concentrated and additionally purified using an HPLC system ~~on~~ equipped with a Nucleosil 7 C_{18} column (250 mm×10 mm; Macherey-Nagel, Germany), equilibrated with buffer A (H_2O containing 0.1% TFA). They were isolated by a linear gradient from 5% solvent A (0.1% TFA in water) to 100% solvent B (0.085% TFA in AcN) within 50 min, at a flow rate of 1.5 $ml.min^{-1}$, with detection at 280 and 214 nm.

2.5. Biochemical Characterization of FUs

N-terminal amino acid sequences of isolated HPLC fractions, after drying and subsequently dissolving them in 40% methanol/1% formic acid, were determined by automated Edman degradation in a 494 Pulsed Liquid Protein Sequencer (Applied Biosystems, Foster City, CA).

The molecular masses of the fractions were determined using an Autoflex™III, High-Performance MALDI-TOF, System (Bruker Daltonics), around 50 pmol of each being dissolved in 0.1% (v/v) TFA and applied to the target. The matrix used was α-cyano-4-hydroxycinnamic acid. Protein standards were applied to calibrate the mass scale. The masses assigned to the amino acid residues are of average values.

2.6. Antibacterial Assays of the Hemocyanins and their Isoforms

The antimicrobial activities of the molluscan hemocyanins from *Rapana venosa* and *Helix aspersa* and of the artropodan hemocyanin from *Carcinus aestuarii,* as well as of their isoforms (RvH1 and β_c-HaH) and their functional units (FUs), were tested against a series of Gram-positive (*Staphylococcus aureus*, *Enterococcus faecium, Staphylococcus epidermidis, Streptococcus pyogenes*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The samples were qualitatively tested according to the liquid growth inhibition assay. Briefly, 10 µl of the samples were mixed with 6 ml of a mid-logarithmic phase culture of bacteria in poor broth nutrient medium (1% dextrose)**,** with definite OD. Microbial growth was assessed by an increase in the MacFarland (McF) value after incubation for 24 h at 37°C. The protein's nutrient medium with the bacterial culture alone was used as a control. One unit McF corresponds to a number of $3x10^8$ cells/ml. The turbidity was measured using a DENSIMAT (BioMerieux, France).

A second antibacterial test was performed as a microdilution assay in 96 well plates. Again, 10 µl from each peptide were incubated for 24 h with the bacterial culture in Tryptic Soy Broth (TSB) medium, in a total volume of 120 µl. The initial OD_{600} of the bacterial culture was 0.4 – 0.6. Medium without bacteria was used to verify the conditions of the test, and the bacterial culture without any peptide was the negative control. Three different concentrations of each sample (10 µM, 6.5 µM and 1.25 µM) were tested.

3. RESULTS

3.1. Purification of *H. aspersa* Hemocyanin and its Structural Subunit β_C-HaH

After purification of the native hemocyanin from *H. aspersa* hemolymph and dissociation against 0.13 M Glycine buffer, pH 9.0, three isoforms were identified by electrophoresis (data not shown). One of these isoforms, the β_C-subunit, was obtained *via* sedimentation and purification by anion-exchange chromatography, as detailed in the Methods section. The isoform β_C-HaH was further analyzed by 5% native polyacrylamide gel electrophoresis, showing a single band with a mass of around 450 kDa (data not shown).

Native HaH was investigated by electron microscopy, revealing mostly didecamers in side view and some dissociated subunits (Fig. **1**).

3.2. Isolation of Functional Units of β_C-HaH

Limited proteolysis of β_C-hemocyanin with TPCK-trypsin yielded several multi-unit fragments, as well as individual FUs. Fig. (**2**) shows the result of the initial separation by anion exchange chromatography. Re-chromatography of the fractions on the same column allowed to characterize eight functional units (FUa to FUh), having masses as determined by SDS/PAGE, ranging from 47 to 65 kDa (data not shown). The more precise molecular masses of the isolated fractions were determined by MALDI-MS. The presence of two ions at m/z 47796.7 $(M+H)^+$ and 63303.4 $(M+H)^+$ in fraction 5 (Fig. **3**) revealed that at least this fraction still needed to be purified further. In fact, all fractions

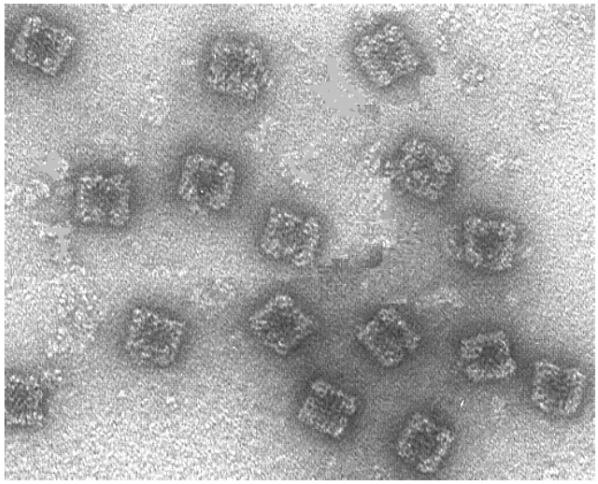

Fig. (1). Electron microscopy of negatively-stained native *H. aspersa* hemocyanin, showing didecamers in side view.

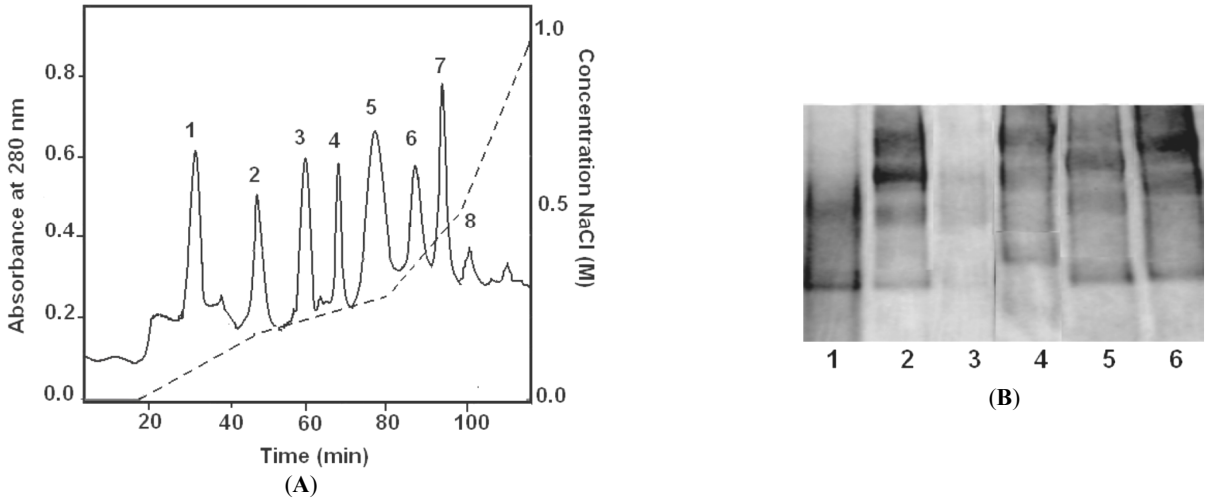

Fig. (2). A) FPLC chromatogram of β_C-HaH structural subunit after limited proteolysis with trypsin. Chromatographic conditions: see Methods. **B**) SDS- electropherogram of the isolated fractions shown in Figure 2A, starting from fraction 2 up to fraction 7.

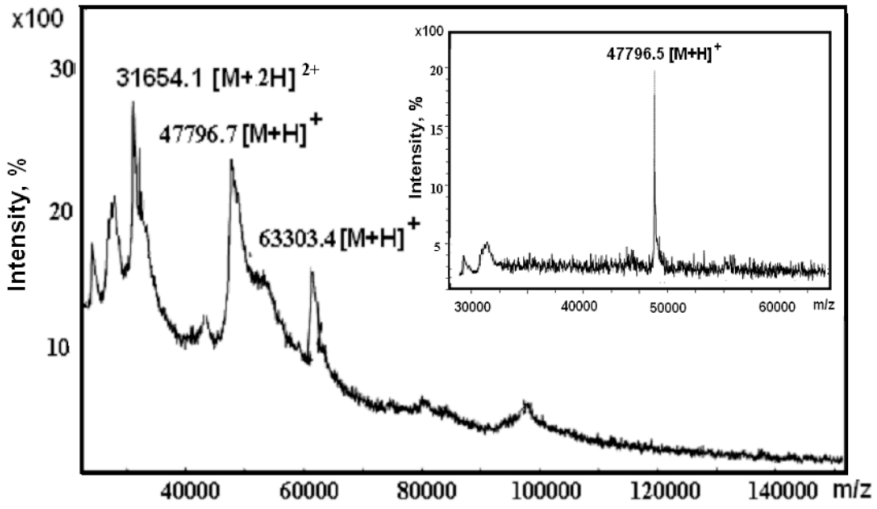

Fig. (3). Mass spectrum of the two major compounds of peak fraction **5** in the chromatogram of Fig.2; the mass at 31654.1 $(M+2H)^{2+}$ is the doubly charged value of the peak with the highest mass. The value of 47796.5 is the mass of the protein in the neigbouring fraction 6 (Figure 2A).

FUs	Amino acid sequences
β-HlH-a	**VRKNVDKLTKDELY**DL**QR**ALRDVVA
β-HaH-a	EL**VRKNVDKLTKDELY**N**LQK**
β-HlH-b	HEHEFHEGVSV**RKNVD**RL**T**VEEVAEI
β-HaH-b	DHP**R-NVD**HM**T**SHD
β-HlH-c	TESRL**RK**E**VD**H**LT**AE**E**TLE**L**RH
β-HaH-c	ELV**RK**N**VD**K**LT**QD**E**LYD**L**QK
β-HlH-d	Y**GQEYRP**L**VTAGSHVR**H**NLE**H**LSAGE**
β-HaH-d	D**GQEYRP**Q**VTVGSHVR**F**NLE**D**LSAGE**
β-HlH-e	**HG**D**RAP**L**LVRKNV**R**SLSPLE**N**YHLV**
β-HaH-e	**HG**G**RAP**P**LVRKNV**E**SLSPLE**K**YHLV**K
β-HlH-f	VPLNK-I**RRN**ID**SLE**ERDIQ**SL**QT**AL**
β-HaH-f	ETVASYV**RRD**LE**SL**S**E**GEVE**SL**RA**AL**
β-HlH-g	VPGDSV**RKN**V**N**D**L**TDSEVANLRAALRDV
β-HaH-g	VETTPNNI**R**H**N**L**N**S**L**EERD
β-HlH-h	**NL**VRK**SV**N---**SL**TLGEASNLK**Q**ALR
β-HaH-h	R**NL**ESL**SV**GEVE**SL**RSAFLAI-Q**Q**DHT

Fig. (4). Alignments of N-terminal sequences of the isolated isoforms from β-*Helix aspersa* with those of functional units from *Helix lucorum*. Conserved amino acid residues are shown in fat lettering.

were further purified by re-chromatography on the same column, and additionally, by an HPLC system, equipped with a Nucleosil C18 column (data not shown). The N-terminal sequences of each of the purified fractions were found to have varying degrees of similarity with the corresponding FUs of *H. lucorum* Hc (Fig. **4**). The highest similarities are those between the functional units a, d and e of both hemocyanins [14,16]. Remarkably, even in the units with low similarity, a typical Arg-Lys-Asn motif appears to occur.

3.3. Antimicrobial Activity of some Structural and Functional Subunits of Molluscan Hemocyanins

In this study, the structural subunit of molluscan hemocyanin *R. venosa* (RvH1), five FUs (a to e) of both the structural subunits RvH1, as well as one subunit (β_c-HaH) and eight FUs (a to h) of β_c-HaH were tested against different species of Gram-positive (*S. aureus*, *E. faecium, S. epidermidis* and *S. pyogenes*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria.

The preliminary results about antibacterial activity of the above mentioned compounds against *S. aureus*, *E. faecium,* and *S. pyogenes* showed that, in the liquid growth inhibition assay, only the two structural units β_c-HaH and RvH1 posses inhibitory effects on cell growth (Fig. **5 A-C**). βc-HaH causes a fairly pronounced inhibition of over 65% against *S. aureus* and 51% against *S. pyogenes.* Some limited antimicrobial effect (35%) was observed after treatment of *S. pyogenes* with subunit RvH1.

We subsequently tested the effect of βc-HaH against the two Gram-negative bacteria and compared the results with those for the three Gram-positive bacteria *S. aureus*, *E. faecium* and *S. epidermidis.* We observed a very high inhibition effect of βc-HaH on *E. coli* and *S. epidermidis* and nearly as much (60%) on *S. aureus* (Fig. **6**). There was no inhibitory effect after treatment of the above mentioned Gram+ and Gram- bacteria with any FU of HaH and RvH.

To determine the minimum antimicrobial concentration of β_c-HaH causing the antibacterial effect, bacterial suspensions of *S. aureus*, *S. epidermidis, E. coli,* and *P. aeruginosa* were incubated with decreasing concentrations from 10 μM to 1.25 μM of β_c-HaH. It was found that the Gram-positive bacteria *S. aureus*, *S. pyogenes*, and *S. epidermidi*s and the Gram-negative bacterium *E. coli* exhibit about 60-65% affinity for the subunit at a concentration of 6.5 μM.

4. DISCUSSION

Most organisms contain body fluids, such as the hemolymph of mollusks, that are complex mixtures of biochemically and pharmacologically active compounds [17]. We recently discovered that, besides its oxygen-transporting function, the well-characterized *R. venosa* hemocyanin has also antiviral and antitumor activities [17-19] and that it presumably takes part in the defense system of the snail against bacteria. In this context we carried out a deeper study of the antimicrobial properties of the Hc from *R. venosa* and of the recently isolated hemocyanin from the hemolymph of garden snail *H. aspersa*.

Several molluscan hemocyanins are known to have one or two isoforms and their gene sequences have been determined [19-21]. An additional third isoform was only identified in the hemocyanins of *H. lucorum* and *Helix pomatia* [16, 22]. Three isoforms were also identified, after dissociation and purification, by electrophoresis of the native hemocyanin from *H. aspersa*. The one we mainly focussed on in this work is b_c-HaH.

Studies on the structure of HaH show several similarities with other molluscan Hcs. The electron microscopic analysis of the native protein confirms that, although the hemocyanins from Vetigastropoda (*Haliotis*), Caenogastropoda (*Rapana*), Heterobranchia (*Helix*) and bivalve Nucula contain one, two or three isoforms respectively, the native Hcs nevertheless occur as didecamers [22, 23]. We now found that

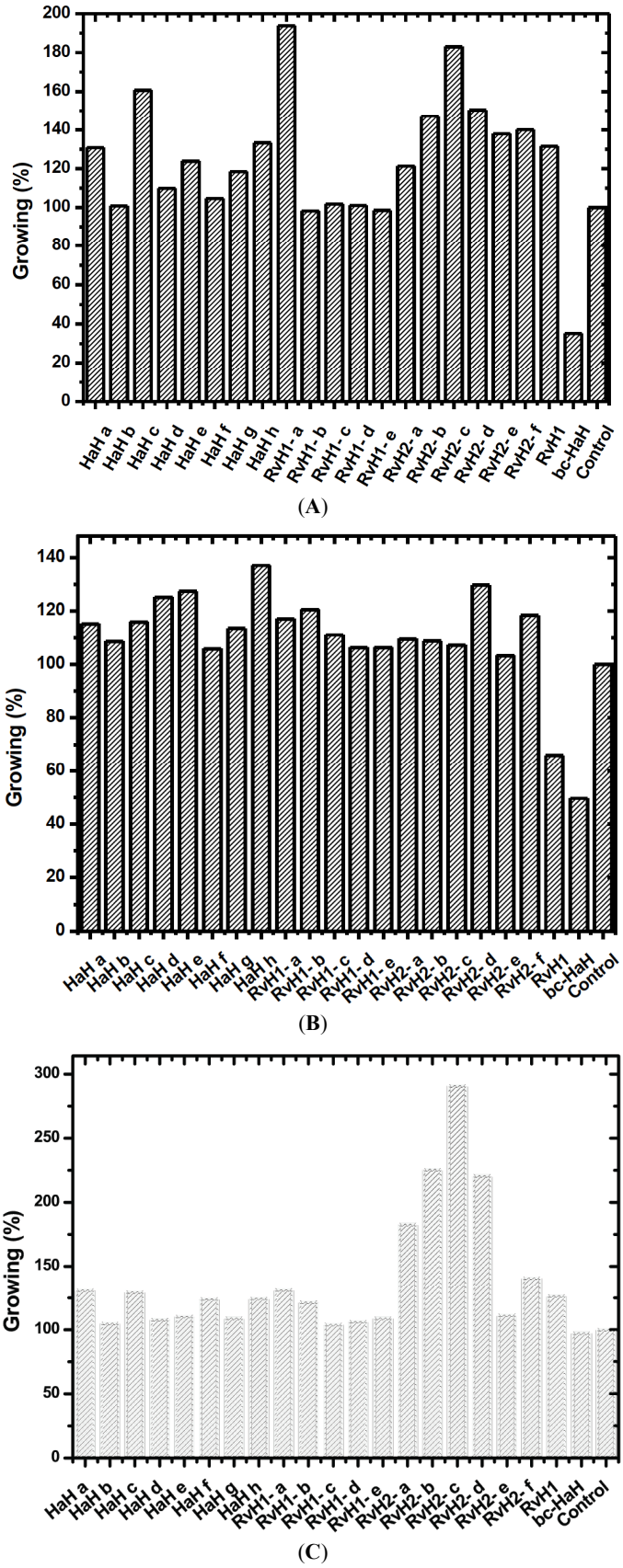

Fig. (5). Antimicrobial activity of the structural and functional subunits of *R. venosa* (RvH1) and *H. aspersa* (βc-HaH) hemocyanin at a concentration of 10 µM against the Gram-positive bacteria *Staphylococcus aureus* (**A**), *Streptococcus pyogenes* (**B**), and *Enterococcus faecium* (**C**). Some 10 µl of the samples at a concentration of 6.5 µM was analyzed using the liquid growth inhibition assay (see Methods).

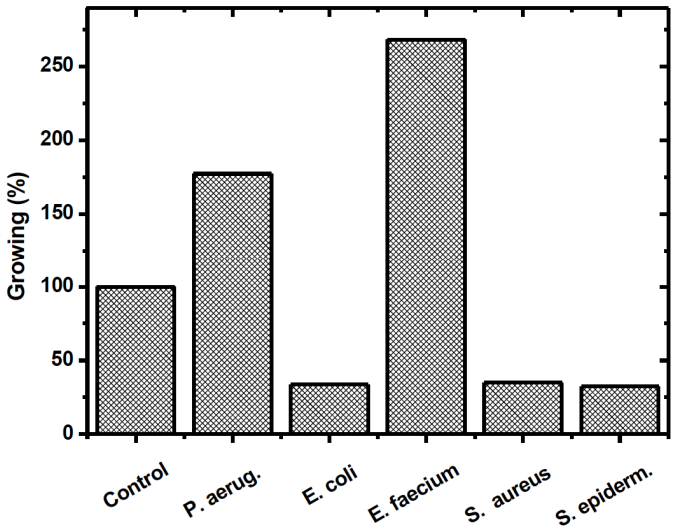

Fig. (6). Antimicrobial activity of the structural subunit of *H. aspersa* (βc-HaH) at a concentration of 6.5 μM against the Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and the Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecium,* and *Staphylococcus epidermidis*.

the structural subunit β_c of *H. aspersa* hemocyanin contains eight functional units (FU-a to FU-h), with masses between 47 and 65 kDa, as was found for the subunits of *Rapana* and *H. lucorum*, in contrast to the hemocyanins from *Nautilus pompilius* and *Octopus dofleini* which consist of only seven FUs. Alignment of the N-terminal sequences of the isolated FUs of β_c-HaH with the known sequences of the HlH-subunits [14] now shows fairly strong homology for only three from the eight FUs (HaH-a, HaH-d and HlH-e).

As regards the antimicrobial properties, it is known that the host immune system recognizes pathogen-associated molecular patterns and develop various counter actions, such as the release of antimicrobial peptides and the synthesis of highly toxic reactive oxygen species (ROS). Earlier studies have shown that the respiratory proteins hemoglobins in vertebrates, and hemocyanins in invertebrates, which generate ROS as part of a powerful antimicrobial strategy [24,25]. In crabs, antimicrobial peptides reported include scygonadin from *Scylla serrata* [26], crustin from *Scylla paramamosain* [27], and the two peptides PsHCt1 and PsHCt2 located at the C-terminus of the crustacean hemocyanin from *Panulirus interruptus* [28]. Besides peptides, also proteins appear to have antibacterial effects, such as for example carcinin from *Carcinus maenas* [6] and C-reactive protein 2 (CRP2) from the plasma of the horseshoe crab [29], more in particular against *Escherichia coli* K1, *P. aeruginosa,* and *S. aureus*. Moreover, the interaction between CRP2 in the native physiological state and hemocyanin seems to be enhanced upon *Pseudomonas* infection. It was therefore proposed that rCRP2 and its interacting partners contribute to effective bacterial clearance [29].

Hemocyanins are principally known as oxygen-carrying glycoproteins, but have antitumor [16,17,30,31] and antiviral efficacies as well [32]. A high level of hemocyanin was found to circulate upon infection, suggesting that the protein complex is involved in the prevention of automelanization of the blood [33]. Recently, we reported that seven peptides from the hemolymph of *R. venosa* snail exhibiting antimicrobial activities against two bacterial strains, a Gram-positive (*Staphylococcus aureus*) and a Gram-negative (*Klebsiella pneumoniae*) [11] species. In continuation of these investigations we now tested the antibacterial activities of the structural isoforms RvH1 and RvH2 from *Rapana venosa* and β_c-HaH from *Helix asperta*, as well as of their functional subunits, against several Gram-positive (*S. aureus*, *E. faecium, S. epidermidis, S. pyogenes)* and Gram-negative bacteria (*E. coli* and *P. aeruginosa*). These species were chosen because they are known to be opportunistic microorganisms causing several types of infections which exponentially increase in hospitals and clinical environments, resulting in thousands of deaths worldwide per year. From all fractions tested, we found that only the structural subunits β_c-HaH and RvH1 have an antimicrobial effect. A minimal antimicrobial concentration of 6.5 μM of β_c-HaH exhibits, within 24 h, a highest affinity for the Gram+ bacteria *S. aureus, S. pyogenes* and *S. epidermidi*s (for 60, 51 and 65%, respectively), but can nevertheless act at the same concentration on the Gram- bacterium *E. coli* (65 %). Moreover, an effect of 31.5% was observed after treatment of *S. pyogenes* with subunit RvH1. Results also show that at 6.5 μM, β_c-HaH is capable of causing a very high and rapid reduction in bacterial density within 24 h.

CONCLUSION

Aside of the structural characterization of structural subunit HaH we provide with this work also additional information about the defense system of molluscan Hcs from *R. venosa* and *H. aspersa*. We showed for first time that the structural subunits RvH1 and β_c-HaH serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens. β_c-HaH was found to be the most promising and effective protein inhibiting Gram-

positive (*S. aureus* and *S. epidermidis*) and the Gram-negative bacterium *E. coli* at a concentration of 6.5 μM. The *Staphylococcus* species are becoming increasingly resistant to many commonly used antibiotics including penicillins, tetracycline etc. For this reason, the study of new natural products such as the Hcs from *Rapana* and Helix with antimicrobial activity is of great importance for potential medical application.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinstchaft (DFG-STE 1819/5-1/2012), Germany, the Bulgarian Ministry of Education, projects TK01-496/2009, ДНТС/01/6 and "Young researchers" DMU 03/26, grant BG051PO001-3.3.06-0025 financed by the European Social Fund and Operational Programme Human Resources Development (2007 – 2013) and co-financed by Bulgarian Ministry of Education, Youth and Science, and by the collaborative project VS.025.12N between the Flemish Fund for Scientific Research FWO and the Bulgarian Academy of Sciences BAS. We also thank to Dr. Maren Paulmann and Prof. Birgit Schittek from Eberhard-Karls-Universität, Hautklinik, Sektion Dermatologische Onkologie, Tübingen, for their support in the antimicrobial analyses.

REFERENCES

[1] Peng, K.; Kong, Y.; Zhai, L.; Wu, X .; Jia, P. ; Liu, J. and Yu, H. Two novel antimicrobial peptides from centipede venoms. *Toxicon,* **2010,** *55*(2-3), 274-279.

[2] Lee, S.Y; Lee, B.L. and Soderhall, K. Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*. *J Biol Chem*., **2003**, *278*, 7927-7933.

[3] Rameshkumar, G.; Ravichandran, S.; Kaliyavarathan, G. and Ajithkumar T.T. Antimicrobial Peptide from the Crab, *Thalamita crenata* (Latreille, 1829). *World J Fish and Marine Sciences,* **2009,** *1*(2), 74-79.

[4] Chernish, S.; Kim, S.I.; Beckeer, G.; Pleskach, V.A.; Filatova, N.A.; Anikin, V.B.; Platonov, V.G. and Bulet, P. Antiviral and antitumor peptides from insects. *Proc Natl Acad Science USA,* **2002,** *99*(20), 12628-12632.

[5] Iguchi, S.M.; Aikawa, T. and Matsumoto, J.J. Antibacterial activity of snail mucus mucin. *Comp. Biochem. Physiol.*, **1982**, *72A*, 571-574.

[6] Brockton, V.; Hammond, J.A. and Smith, V.J. Gene characterization, isoforms and recombinant expression of carcinin, an antibacterial protein from the shore crab, Carcinus maenas. *Mol. Immunol.,* **2007,** *44,* 943-949.

[7] Jummai, A.T.; Okoli, B.J. Antimicrobial potentials of hemocyanin. *Research J Engin and Applied Sciences,* **2013,** *2*(6) 446-449.

[8] Kamiya, H.; Muramoto, K. and Yamazaki, M. Aplysianin-A an antibacterial and antineoplastic glycoprotein in the albumen gland of a sea hare, *Aplysia kurodai. Experientia,* **1986,** *42,* 1065-1067.

[9] Tincu, J.A. and Taylor, S.W. Antimicrobial Peptides from Marine Invertebrates. *Antimicr, agents and Chemoth*, 2004, 48(10), 3645–3654.

[10] Pathan, F.K.; Venkata, D.A. and Panguluri, S.K. Recent patents on antimicrobial peptides. *Recent Pat DNA Gene Seq*, **2010,** 1,10-16.

[11] Dolashka, P.; Moshtanska, V.; Borisova, V.; Dolashki, A.; Stevanovic, S.; Dimanov, T. and Voelter, W. Antimicrobial proline-rich peptides from the hemolymph of marine snail *Rapana venosa*. *Peptides*, **2011**, 32(7), 1477-1483.

[12] Dolashka-Angelova, P.; Stefanovic, S.; Dolashki, A.; Devreese, B.; Tzvetkova, B.; Voelter, W.; Van Beeumen, J. and Salvato, B. A challengig insight on the structural unit 1 of molluscan *Rapana venosa* hemocyanin. *Arch Biochem Biophys,* **2007**, *459*(1), 50-58. *Peptides,* **2011,** *32*(7), 1477-1483.

[13] Dolashki, A.; Velkova, L.; Atanasov, B.; Hristova, R.; Voelter, W.; Stevanovic S.; Schwarz H.; Di Muro, P.; Dolashka-Angelova, P. Reversibility and "pH-T phase diagrams" of *Rapana venosa* hemocyanin and its structural subunits. *Biochim Biophys Acta*, **2008**, *1784*(11), 1617-1624.

[14] Velkova, L.; Dimitrov, I.; Schwarz, H.; Stevanovic, S.; Voelter, W.; Salvato, B.; Dolashka-Angelova, P. Structure of hemocyanin from garden snail *Helix lucorum. Comp Biochem Physiology* B, **2010**, *157* (1) 16-25.

[15] Antonova, O.; Dolashka, P.; Toncheva, D.; Rammensee, H-G, Foetenmeyer, M. and Stevanovic, S. *In vitro* antiproferative effect of *Helix aspersa* hemocyanin on multiple malignent cell lines. *Z Naturforsch* **2014**, *69*, 325-334.

[16] De Smet, L.; Dimitrov, I.; Debyser, G.; Van Beeumen, J.; Dolashka-Angelova P. and Devreese, B. The cDNA sequence of three hemocyanin subunits from the garden snail *Helix lucorum*. *Gene,* **2011,** *487*(2), 118-128.

[17] Coates, C.J.; Nairn, J. Diverse immune functions of hemocyanins. Review. *Developm and Compar Immun* **2014,** *45,* 43-55.

[18] Antonova, O., Yossifova, L., Staneva, R., Stevanovic, S., Dolashka, P., Toncheva, D. Changes in the gene expression profile of the bladder cancer cell lines after treatment with *Helix lucorum* and *Rapana venosa* hemocyanin. JBUON **2015**, *20*(1), 180-187.

[19] Dolashka, P.; Velkova, L.; Iliev, I.; Beck, A.; Dolashki, A.; Yossifova, L.; Toshkova, R.; Voelter, W. and Zacharieva, S. Antitumor Activity of Glycosylated Molluscan Hemocyanins via Guerin ascites tumor. *Immunol Investig,* **2011,** *40*(2), 130-149

[20] Lieb, B.; Altenhein, B.; Markl, J.; Vincent, A.; van Olden, E.; van Holde, K.E. and Miller, K.I. Structures of two molluscan hemocyanin genes: significance for gene evolution. *Proc Natl Acad Science USA,* **2001,** *98*(8), 4546-4551.

[21] Bergmann, S.; Lieb, B.; Ruth, P. and Markl, J. The hemocyanin from a living fossil, the cephalopod Nautilus pompilius: protein structure, gene organization, and evolution. *J Mol Evol*, **2006,** *62*(3), 362-374.

[22] Lambert, O.; Boisset, N.; Taveau, J.C.; Préaux, G. and Lamy, J.N. Three-dimensional reconstruction of the alpha D and beta C-hemocyanins of *Helix pomatia* from frozen-hydrated specimens. *J Mol Biol*, **1995,** *248*(2), 431-48.

[23] Dolashka-Angelova, P., Schwarz, H., Dolashki, A., Beltramini, M., Salvato, B., Schick, M., Saeed, M., Voelter, W. Oligomeric stability of *Rapana venosa* hemocyanin (RvH) and its structural subunits. *Biochim Biophys Acta* **2003,** *1646* (1-2) 77-85.

[24] Kawano, T.; Pinontoan, R.; Hosoya, H.; Muto, S. Monoamine-dependent production of reactive oxygen species catalyzed by pseudoperoxidase activity of human hemoglobin. *Biosc Biotechnol Biochem*, **2002,** *66,* 1224–1232.

[25] Iwanaga, S. The molecular basis of innate immunity in the horseshoe crab. *Curr Opin Immunol*, **2002,** *14,* 87-95.

[26] Wang, K.J.; Huang, W.S.; Yang, M.; Chen, H.Y.; Bo, J.; Li, S.J. and ***Wang,*** G.Z. A male-specific expression gene, encodes a novel anionic antimicrobial peptide, scygonadin, in *Scylla serrata*. *Mol Immunol*, **2007,** *44,* 1961-1968.

[27] Imjongjirak, C.; Amparyup, P.; Tassanakajon, A.; Sittipraneed, S. Molecular cloning and characterization of crustin from mud crab *Scylla paramamosain*. *Mol Biol Rep*, **2009,** *36,* 841-850.

[28] Decker, H. and Jaenicke E. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. *Devel Compar Immunol*, **2004,** *28,* 673-687.

[29] Tan, S.S.; Ng, P.M.; Ho, B. abnd Ding, J.L. The antimicrobial, properties of C-reactive protein (CRP), *J Endotoxin Res* **2005,** 11(4) 249-256.

[30] Aarntzen, E.H.; De Vries, I.J.; Lesterhuis, W.J.; Schuurhuis, D.; Jacobs, J.F.; Bol, K.; Schreibelt, G.; Mus, R.; De Wilt, J.H.; Haanen, J.B.; Schadendorf, D.; Croockewit, A.; Blokx, W.A.; Van Rossum, M.M.; Kwok, W.W.; Adema, G.J.; Punt, C.J. and Figdor, C.G. Targeting CD4(+) T-helper cells improves the induction of antitumor responses in dendritic cell-based vaccination. *Cancer Res.*, **2013,** *73*(1), 19-29.

[31] Boyanova, O.; Dolashka, P.; Toncheva, D.; Rammensee, H-G. and Stevanovic, S. In vitro effect of molluscan hemocyanins on CAL-29 and T-24 bladder cancer cell lines. *Biomed Reports,* **2013,** *1,* 235-238.

[32] Dolashka, P.; Velkova, L.; Shishkov, S.; Kostova, K.; Dolashki, A.; Dimitrov, I.; Atanasov, B.; Devreese, B.; Voelter, W. and Van Beeumen, J. Glycan structures and antiviral effect of the structural subunit RvH2 of Rapana hemocyanin. *Carboh Research,* 2010, *345*,16, 2361-2367.

[33] Ayres, J. S. and Schneider, D.S. A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. *PLoS Biol*, **2008,** *6*(12), 2764-2773.

Received: September 02, 2014 Revised: October 18, 2014 Accepted: December 11, 2014