ANTIMICROBIAL PROPERTIES OF MESOHYLAR GRANULAR CELLS OF *HALISARCA DUJARDINI* JOHNSTON, 1842 (DEMOSPONGIAE, HALISARCIDA)

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ABSTRACT

It was shown that granular mesohylar cells of *Halisarca dujardini* Johnston, 1842 contain cationic peptides and proteins. As it is known that such agents are the molecular factors of resistance to bacteria and lowest fungi, the proposed toxic activity of these peptides and proteins was studied. We have shown that some isolated protein and peptide fractions have antimicrobial activity against *Escherichia coli* and *Listeria monocytogenes*. Molecular weights of antimicrobial substances were about 6.2 kDa for peptide fractions and about 14 - 25 kDa for protein fractions. These results suggest an important biological role of the cationic peptides and proteins in defense processes of adults and larvae of *H. dujardini*.

KEY WORDS

Sponges, *Halisarca dujardini*, antimicrobial activity, granular cells.

INTRODUCTION

The survival of all animal species from Protozoa to Vertebrata including human in environment enriched with pathogenic microorganisms was only possible by the emergence in evolution of mechanisms providing defense from infection deseases (BOMAN, 1991). Since sponges do not have a closed circulatory system, their immune reactivity has to be restricted to cell-mediated defense reactions (MÜLLER *et al.,* 1999). Defense mechanisms of sponges are very diversified. Sponges of this group synthesize and (or) accumulate toxic metabolites in secretory cells.The Mediterranian sponge *Crambe crambe* (Demospongiae, Poecilosclerida) can be cited as an example. Crambin, a toxic metabolite, is accumulated in its sphaerulous cells, and is thought to be used by the sponge as a defensive agent against bacteria and predators (URIZ *et al.,* 1996a). The same protective function is assured by avarol, a secondary metabolite of *Dysidea avara* (Demospongiae, Dendroceratida) (URIZ *et al.,* 1996b). Toxic substances have been found in the secretory cells of many other sponge species, for example: ent-furodysinin from *Dysidea fragilis* (Dendroceratida) (MARIN *et al.,* 1998), tritherpene glycosides from *Erylus formosus* (Geodiidae) (KUBANEK *et al.,* 2000). Earlier, we have found out cationic peptides and proteins in eosinophylic granular cells of *Halisarca dujardini* (KRYLOVA *et al.,* 2002). Such

peptides and proteins are known to be molecular factors of resistance to bacteria and to the lowest fungi in different animal groups (KOKRYAKOV, 1999). Remarkably, these eosinophilic granular cells (or eosinophilic amoebocytes) of maternal sponge penetrate developing embryos and remain in their bodies until the first metamorphosis stages (KOROTKOVA & ERMOLINA, 1982; ERESKOVSKY & GONOBOBLEVA, 2000).

The aim of the present work was to investigate the possible role of granular mesohylar cells in defense mechanisms of adults and larvae of the demosponge *Halisarca dujardini.*

MATERIALS AND METHODS

Samples of *Halisarca dujardini* Johnston, 1842 were collected at the Kandalaksha Bay, White Sea in the sublittoral zone. Sponges were collected with algal substrate and maintained in aquaria at a temperature of 10° C. Sponges were separated from algal substrate and used for obtaining pure fraction of eosinophilic amoebocytes or fixed in 20 % acetic acid and stored at 4 - 5° C for cationic peptides and proteins extraction.

Obtaining pure eosinophilic amoebocytes (EA) fraction

Cell fractionation was conducted on Percoll gradients, according to the following procedure (modified from SALOMON *et al.,* 2001): a fresh sponge was cut into small pieces, approximately 4 mm in diameter, to give a total volume of 2 ml, rinsed several times in artificial sea-water free of calcium and magnesium (CMF-ASW) with EDTA (300 mM NaCl, 20 mM KCl and 25 mM EDTA) to eliminate the aggregation factors released by the cells and dissociated by gentle pipetting at temperature ranging 12 to 14° C. The resulting cell suspension was carefully layered onto the top of the Percoll gradients.

Discrete Percoll gradients were prepared in 15 ml tubes by adding, from bottom to top, 2 ml of the following solutions of Percoll in CMF-ASW: 45, 40, 35, 30, 25, 20 and 10 %. After 10 min of centrifugation at 1000 rpm, cell fractions were accumulated at interfaces between successive densities along the gradient. Clearly separated fractions were identified and numbered from top to bottom of the gradient. Each fraction was isolated individually by aspiration into a pipette and rinsed from Percoll in CMF-ASW without EDTA. An aliquot of each fraction was observed by phase contrast technique to determine cell type in fraction.

EA fraction was fixed in 20 % acetic acid for cationic peptides and proteins extraction. Further acetic extract was tested for antimicrobial activity.

Extraction of cationic peptides and proteins from adult sponges and larvae

Fixed sponges were centrifuged for 1 h at 5000 rpm at 4° C. The supernatant (E_0) was ultrafiltrated through "Amicon" YM-1 membrane, which retains components with molecular masses larger than 1 kDa. Pellet was homogenized and centrifuged for 1.5 h at 15000 rpm at 4° C. The supernatant (E_1) was also undergone to ultrafiltration. All samples were dialyzed against Spectra/Por membrane with a cut-off limit of 1 kDa.

Cationic peptides and proteins analysis

The spectrum of cationic peptides and proteins in both extracts, was evaluated by electrophoresis in acetic acid / urea buffer system (PANYIM & CHALKLEY, 1969) in 12.5 % polyacrylamide gel plates. The approximate molecular weights were determined by disc-gel electrophoresis in the presence of sodium dodecyl sulfate (SCHÄGGER & VON JAGOW, 1987) in 16 % polyacrylamide gel plates. The kit of the molecular weight marker (Sigma, USA) contained the following proteins: insulin (3000 Da), trypsin inhibitor from bovine pancreas

(5800 Da), lysozyme (14600 Da), lactoglobulin (20300 Da), carboanhydrase (29000 Da) and ovalbumin (46000 Da).

Investigation of antimicrobial properties of proteins and peptides

Antimicrobial activities of cationic peptides and proteins were determined by the methods proposed by LEHRER *et al.* (1991). As target for antimicrobial activity, cultures of the following microorganisms were used: *Escherichia coli* (gram-negative), strain ML-35p and *Listeria monocytogenes* (gram-positive), strain EGD.

Fractionation of peptides and proteins

E_0 and E_1 were concentrated using a Speed Vac CS-18 centrifuge and then fractionated by HPLC on "Beckman" device. Separation was performed on Altech C-18 column (4.6 x 250 mm; Macrosphere sorbent 300 C 4, diameter of particles is 5 mkm) by an acetonitrile gradient (0 - 60 %) in 0.1 % trifluoroacetic acid.

RESULTS

A functional test of the saltless extract (E_0) containing the cationic peptides and proteins from *Halisarca dujardini* tissues showed antimicrobial activity against *Escherichia coli* and *Listeria monocytogenes*. Then we fractionated the total protein extract by HPLC and each fraction from 5 to 40 was tested for antimicrobial activity against *E. coli* and *L. monocytogenes*. The results showed antimicrobial activity against gram-negative *E. coli* in fractions 34 - 39. Fraction 39 has the most potent effect. Only this fraction showed antimicrobial activity against gram-positive *L. monocytogenes* (Fig. 1). The results of SDS electrophoresis indicated that all active fractions were heterogeneous and contained major component with molecular weight about 6.2 kDa and minor component with molecular weight about 25 kDa (Fig. 2).

No antimicrobial activity was detected in E_1 extract.

Six distinct fractions were obtained from the Percoll gradients. They were numbered 1 - 6 from top to bottom of the gradient. Fraction 5 was determined as eosinophilic amoebocytes (EA) fraction. Fraction 6 was constituted by spherulous cells and not dissociated cell conglomerates. Fractions 1 - 4 contained a mixture of other cell types of *H. dujardini*.

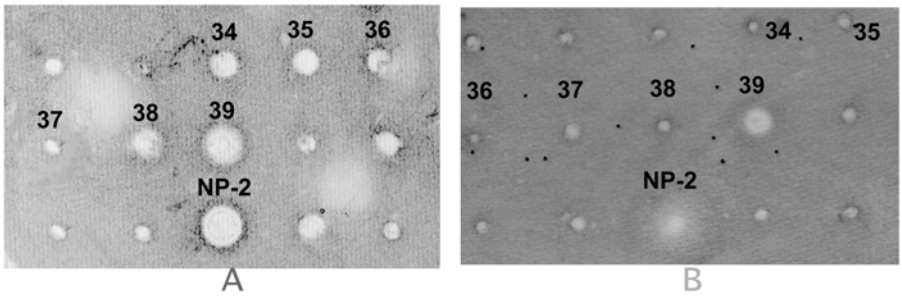

Fig. 1. Antimicrobial activity of fractions 34 - 39 after HPLC of the total saltless extract from *H. dujardini* against *E. coli* (**A**) and *L. monocytogenes* (**B**). NP-2 - Rabbit defensin NP-2.

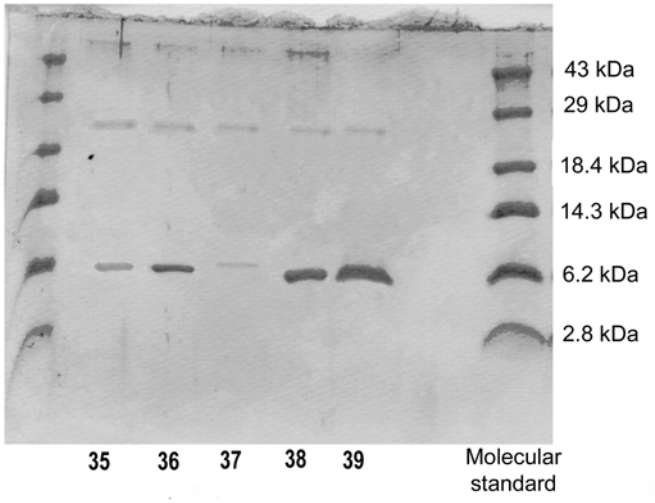

Fig. 2. Results of SDS-PAGE of fractions 34 - 39 of the total saltless extract from *H. dujardini*.

Functional test of the EA acetic extract showed antimicrobial activity against *E. coli* and *L. monocytogenes in vitro*. The lysis zone on the plates with gram-negative *E. coli* was larger than that with gram-positive and *L. monocytogenes* (Figs 1A,B). Acetic extract of other *H. dujardini* cell fractions was used as control. No antimicrobial activity was detected in control.

DISCUSSION AND CONCLUSIONS

All living organisms need effective defense mechanisms because of constant danger of pathogen invasion. Group of cationic peptides plays an important role in defense from pathogenic microorganisms in vertebrates. These peptides are characterized by low molecular weights and amphiphility (KOKRYAKOV, 1999). Besides the direct involvement of these peptides in disactivation of microbial pathogenes they are known to have a number of additional functions as regulatory molecules. In particular such peptides, at low concentrations, can influence all proliferation (KOKRYAKOV, 1999).

Similar molecules have been found in invertebrate animals. Investigation on these substances allows us to compare the composition of characteristic peptides and proteins in different systematic groups of invertebrates. At present, there are few data about Porifera. Our results suggest that peptides and proteins we have studied do not belong to any known group of antimicrobial substances. Such a supposition we have made on the basis of peptides and proteins molecular weights and electrophoretic mobility in the acid buffer system. It is important to note that cationic peptides and proteins composition in cells studied is characterized by high heterogeneity, i.e. extract fractions are not homogenous.

The antimicrobial activity showed by several fractions is comparable with that of rabbit defensin NP-2. On the basis of our results we suggest that these peptides and

proteins take part in sponge defense reactions. Migration of eosinophilic amoebocytes into embryos and larvae (ERESKOVSKY & GONOBOBLEVA, 2000) leads to defense of young organisms from bacterial and fungal pathogens and it may be considered as a kind of progeny care.

However, it is possible that antimicrobial defense of progeny is not the only explanation of maternal cell migration. As it was mentioned above, most of the cationic peptides are polyfunctional. Probably, maternal molecules influence embryo cells like factors activating proliferation. Indeed, the increase of proliferative activity of embryo cells is observed after maternal cell penetration into the embryo. Degranulation of the eosinophilic amoebocytes was not observed and, at present we cannot prove a morphogenic influence of this cell category.

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