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Deoxyribonucleases (DNases) in the Cortex and Endosome from the Marine Sponge *Tethya aurantium*

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Summary - The presence and activity of deoxyribonucleases in the cortex and endosome sections from the sponge, sea orange, *Tethya aurantium* were investigated. The maximal enzyme activity in sponge homogenate was detected at pH 4.27, pH 7.0 and pH 8.5-8.75. Among different specimens, several distinct patterns of neutral DNase isozymes were observed in the cortex section. In each investigated specimen the highest neutral DNase activity belongs to high molecular weight proteins (up to75 kDa). The acid DNases showed low level of enzyme activity. In the endosome section the acid DNase activity was up to 10 times higher than in the cortex and the presence of DNase II – like protein was detected. Neutral DNase expressing the highest enzyme activity in all investigated specimens has a molecular weight of 20 kDa and belongs to DNase I – like family. The results indicate that the activity of neutral and acid DNases is related to sponge sections and their biological functions. The cortex, as a sponge section communicating with the environment, express high interindividual variability and heterogeneity of neutral DNases while the endosome section, where the intracellular digestion is localized, is a site of high acid DNase activity.

Keywords: Marine sponge, *Tethya aurantium*, acid DNase, neutral DNase, cortex, endosome

INTRODUCTION

The sponges (phyllum Porifera) exist as a loose aggregation of cells. They have no defined organs and body functions are performed by the activities of cells acting more or less independently. The sponges possess a well-constructed and complex network of water-conducting channels and choanocyte chambers, which are lined with the flagellated choanocyte cells. This system processes a water mass corresponding to a sponge volume every 5 s (Vogel, 1994) allowing the supply of the cells with the oxygen, food and elimination of toxic gasses and substances. Bacteria, single celled algae and other food particles from the

filtered water are captured by phagocytosis and endocytosis within the choanocyte chambers located within the inner part of the sponge, the endosome. Digestion is entirely intracellular, occurring in food vacuoles of choanocytes and amoebocytes. In some sponges the outer part, cortex, is well developed. As a protective device (Burton, 1928), the cortex is found to be particularly thick and well structured in sponge species living in shallow waters subject to strong currents and high light intensities (Sará, 1987).

Sponges, as recognised model organisms in molecular evolutionary studies, are the source of the most ancient metazoan proteins (Gamulin et al., 2000). Until present, 42 phylogenetically conserved proteins from four marine sponges (Porifera) were described. There are only few reports on DNases in marine sponges. Heicke and Schmidt (1969) described DNase protein of about 62 kDa in *Verongia aerophoba*, Rasskazov et al. (1974) described acid (pH 4.5- 5.0), neutral (pH 7.5) and alkaline (pH 8.1- 8.5) DNases in *Spongia* genus and more recently, Shpak et al. (2008) suggested the presence of DNase II homolog among translated sequences of the demosponge *Reniera*.

Deoxyribonucleases (DNases) are enzymes responsible for the hydrolytic cleavage of dsDNA in many tissues of animals and plants as well as in microorganisms. A unique property of DNases is the fact that they effectively hydrolyse the phosphodiester bond, the most stable chemical bond found in biological molecules (Baranovskii et al., 2004). DNases, as DNA degrading enzymes, play an important role in the maintenance of physiological DNA concentration in the body as well as in the protection of organisms against xenobiotic nucleic acids. The most studied DNases are human non-specific neutral and acid DNases belonging to the DNase I and DNase II family, respectively. Enzymes of the neutral DNase family require a neutral pH optimum and the presence of bivalent metals, Mg^{2+} and Ca^{2+}, for their hydrolytic activity. For the human neutral DNase I structural and physicochemical characteristics, active site structure and mechanisms of hydrolysis, catalytic properties, stability, inhibition, gene expression and polymorphism were described (Baranovskii et al., 2004). DNase I, acting as a digestive enzyme of the gastrointestinal tract, displays the highest activities in digestive glands (Lacks, 1981), contributing to the supply of oligonucleotides (Rudolph, 1994). Besides for mammalian, the biochemical and molecular characterisation of neutral DNase has been reported for aves (Nakashima et al., 1999), reptilia (Takeshita et al., 2003), amphibia (Takeshita et al., 2001) and pisces (Yasuda et al., 2004). Enzymes of the acid DNase II family are characterised by acidic pH optimum and lack of activator requirement (Baranovskii et al., 2004). They are highly conserved with ubiquitous tissue

distribution, located in lysosmes and primarily involved in engulfment–mediated DNA degradation (Evans and Aguilera, 2003). Their origin may be related with the origin of phagotrophic feeding (Shpak et al., 2008). Beside extensive studies on DNase II enzymes in mammals, few reports about the presence of acid DNase activity in invertebrates are available (Rasskazov et al., 1975, Hedgecock et al., 1983, Øverbø and Myrnes, 2006, Fafanđel et al., 2008). DNase II enzymes perform a variety of functions from digestion of ingested DNA to more specialised roles in the toxin of the sea star *Acanthaster* (Shiomi et al., 2004) and in the venoms of rattle snakes (Shpak et al., 2008).

In the present study we analysed the presence and the activity of neutral and acid DNases in the cortex and in the endosome sections of the sponge, sea orange, *Tethya aurantium*.

MATERIALS AND METHODS

DNase I from bovine pancreas, DNase II (Type IV) from porcine spleen, phenyl methyl sulphonyl fluoride (PMSF), ethydium bromide, protein molecular mass standard, acrylamide, bisacrylamide and polyclonal (PcAb) alkaline phosphatase-coupled goat secondary antibodies were obtained from Sigma-Aldrich, USA, agarose from Roth, Germany and fluorochrome Picogreen® from Molecular Probes Inc., USA. Goat polyclonal antibody raised against a peptide mapping within an internal region of DNase I of mouse origin and goat polyclonal antibody raised against a peptide mapping within an internal region of DNase II of human origin were obtained from Santa Cruz Biotechnology, Inc. USA.

Specimens of *Tethya aurantium* (Porifera, Demospongiae, Hadromerida, Tethyidae) were collected by scuba diving in the vicinity of Rovinj (Northern Adriatic Sea, Croatia) from the depth of 28 - 35 m. The sponges were kept in a tank with running seawater and processed within 2 hours. The cortex and endosome sections were separated and cut into small pieces (5 g) that were homogenized on ice in lysing buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, 2 mM PMSF, pH 8) with Potter-Elvjhem homogenizer. Cellular debris were eliminated by centrifugation at 10 000 g, for 30 min, at 4 °C and the protein content in the supernatants was determined by Lowry et al., 1951.

To detect the presence of neutral and acid DNase activity in extracts from the sponge cortex and endosome the single radial enzyme diffusion (SRED) assay was used (Nadano et al., 1993). For detection of neutral DNases 150 mg agarose was melted in 13.5 ml of neutral buffer (20 mM Tris, 5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.0), 1.125 ml DNA (2 mg/ml) and 0.375

ml ethidium bromide (2 mg/ml) were added. To detect acid DNase activity, acidic buffer (50 mM EDTA, 50 mM sodium acetate, pH 5.5) was used. The agarose was poured into horizontal petry dishes and after solidification at room temperature circular wells (0.2 mm) were incised in the gel. 2.5 μl of sponge extracts containing 17 μg/μl of cortex proteins, and 20 μg/μl of endosome proteins were dispensed into circular wells. Incubation was performed in a moist chamber at 37 °C for 24 h until well-defined dark circles of hydrolyzed DNA were visible after illumination with UV light (312 nm). The specific enzyme activity was calculated as diameter of dark circle per mg of proteins.

Fluorimetric determination of DNase activity in sponge extracts with PicoGreen® (Choi and Szoka 2000, Bihari et al., 2007) was preformed to detect the levels of DNase activities in different pH conditions, since SRED assay requires at least 24 h of incubation. This method is based on the ability of the PicoGreen® dye to enhance its fluorescence when bound to double stranded DNA. Each reaction mixture was prepared in a 96-well fluorescence microtiter plate and contained 10 μl of the substrate DNA (0.2 μg), 10 μl of cortex or endosome extract and 80 μl of neutral (20 mM Tris, 5 mM $CaCl_{2,}$ 5 mM $MgCl_{2,}$ pH 6.5-9.0) or acidic (50 mM EDTA, 50 mM sodium acetate, pH 3.75-6.0) reaction buffer. The plate was sealed with aluminium foil and incubated at 37 °C for 30 minutes. After the incubation period, 100 μl of PicoGreen® reagent (50 μl of Picogreen® dye in 10 ml of TE buffer containing 10 mM Tris, 1.0 mM EDTA, pH 7.3) were added to each reaction mixture. Fluorescence intensity was measured at excitation of 485 nm and emission at 520 nm in a Fluoroscan Ascent microplate reader (Labsystem, Finland). The enzyme activity was defined as a change in the amount of the dsDNA- PicoGreen® complex (Delta fluorescence) due to hydrolytic cleavage of DNA by the DNase present in the sample. The fluorescence change was calculated by subtracting the measured fluorescence of the reaction mixture, containing cortex homogenate of individual sponge specimen, from the blank value obtained with the reaction mixture, containing heat-denatured homogenate (95 °C, 15 min).

For the detection of sponge proteins expressing neutral DNase activity denaturating SDS–PAGE zymogram (DPZ) was performed with sponge exctracts. It is an electrophoretic technique that uses DNA copolymerized with the polyacrylamide as the nuclease substrate (Lacks, 1981). Protein extracts (170 μg) and bovine pancreatic DNase I (2.5 ng) dissolved in loading buffer (50 % glycerol, 50% 1× electrophoresis buffer, bromophenol blue) were loaded on the gel and the electrophoresis was performed for 120 min at 90 V in the electrophoretic

buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). The gels were incubated in a reactivation buffer (20 mM TRIS, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 µg/ml ethidium bromide, pH 7.0) at 37 °C overnight.

For the detection of sponge proteins expressing acid DNase activity native PAGE zymogram was used (Napirei et al. 2004) Protein extracts (2.5 - 200 µg) dissolved in loading buffer (50 % glycerol, 50% 1× electrophoresis buffer, bromophenol blue) were loaded on the gel and the electrophoresis was performed for 120 min at 90 V in electrophoretic buffer (25 mM Tris, 250 mM glycine, pH 8.3). The gels were incubated in a reactivation buffer (50 mM EDTA, 50 mM sodium acetate, 10 µg/ml ethidium bromide, pH 4.25) at 37^{o}C. UV illumination of DPZ and NPZ gels revealed DNase activity as dark bands of hydrolysed DNA on fluorescent background that did not stain with a DNA binding dye (ethidium bromide).

For the immunodetection of DNase I and DNase II sponge homologues sponge protein extracts containing 30 µg of total proteins per lane were subjected to electrophoresis in 12 % polyacrylamide gels prior to electrotransfer to PVDF – Imobilon P membranes. Since DNases are among phylogenetically conserved proteins, membranes were incubated with either goat antibody (1 : 750) raised against mouse DNAse I or goat antibody (1 : 750) raised against human DNAse II. Following incubation period of 2 h at room temperature and subsequent washing steps, the blots were incubated with alkaline phosphatase-coupled goat secondary antibodies (1: 1500). After the addition of alkaline phosphatase substrates, (nitroblue tetrazolium and bromochloroindolyl phosphate) immunoprecipitates were dried and scanned (Hewlet Packard ScanJet 6100).

RESULTS AND DISCUSSION

The preliminary detection of the DNases activity in the sponge *Tethya aurantium* was performed by the SRED assay under previously reported conditions for invertebrate neutral DNases at pH 7.0 (Bihari et al., 2007) and acid DNases at pH 5.5 (Fafanđel et al., 2008). The presence of neutral and acid DNases activity revealed by dark circles of hydrolysed DNA was detected in both sponge cortex and endosome (Fig. 1.). Sponge neutral DNase activity was observed after 24 h of incubation (Fig. 1a). Sponge acid DNase activity after 24 h of incubation was detected only in endosome (Fig 1b). Longer incubation (72 h) was required for its activity in the cortex as well as for the detection of DNase II (5 ng) from porcine spleen

(Fig. 1c). The prolonged incubation required for porcine spleen DNase II is a consequence of inadequate reaction mixture pH since the optimal pH for its activity is 4.6. Similarly, it is very likely that pH 5.5 was not optimal for sponge acid DNase activity. Therefore a pH dependence curve was generated by the rapid and sensitive fluorimetric determination in microplates with Picogreen® (Fig. 2). The highest DNases activities were detected at pH 4.25 in the acid pH region (3.75 – 6.0), at pH 7.0 in the neutral pH region (pH 6.5 - 8.0) and between pH 8.5 - 8.75 in the alkaline pH region (pH 8.0-9.0). In the sponge *T. aurantium* acid and neutral DNases were in the same range of pH optima as in marine invertebrates reported by Rasskazov et al., (1975), Øverbø and Myrnes (2006) and Fafanđel et al., (2008) while the pH optima of the sponge *T. aurantium* alkaline DNase was higher than previously described (pH 8.1 – 8.5) for the phylum Spongia (Rasskazov et al., 1975).

Neutral DNase - To investigate the interindividual variability of the neutral DNase activity the SRED assay was performed for ten different *T. aurantium* specimens (Fig. 3.). There was no visible uniformity in the level of enzyme activity among different specimens in both, sponge cortex and endosome. Variability of DNase specific activity was lower in the endosome (200- 320 mm/ mg proteins) than in the cortex (188- 588 mm/ mg proteins). The level of enzyme activity in both sponge sections was characteristic of each specimen. In specimen No. 5 the specific enzyme activity was 2.5 times higher in the cortex than in the endosome while in specimen No. 7 the enzyme activity was 2.5 times higher in the endosome than in the cortex. The observed difference in DNase activity between sponge cortex and endosome could reflect interindividual variation in DNase isozymes. Likewise, the variability in DNase activity between different sponge specimens could display interindividual variations in sponge food utilization as well as variations in DNase isozymes (Takeshita et al., 2000, Bihari et al., 2007).

In contrast to the SRED assay, the DPZ allows the differentiation between nucleases of different migration behaviour. Therefore, in addition to the individual specificity of neutral DNase activity in sponge sections, the heterogeneity of isozymes in the cortex and endosome was determined (Fig. 4). Heterogeneity of DNases was elucidated throughout dark bands of hydrolysed DNA. Among the cortex samples several bands from 20 to 75 kDa displayed neutral DNase activity and no common protein band was observed (Fig. 4a). The distribution and activity of isozyme bands were specific for each specimen. Despite the difference in mobility, all the bands correspond to multiple enzymes or isoenzymes with the same requirements on both Mg^{2+} and Ca^{2+} ions; pH optimum of 7.0 for their activity and double

stranded DNA as substrate. Similarily, different isozyme patterns of human serum neutral DNase phenotypes were specified by Kishi et al. (1990). It is very likely that multiple bands in the cortex of all specimens correspond to polypeptides of different composition and length, and are the product of multiple genes or alternative protein processing (Lacks, 1980).

In the endosome samples protein bands of 20 and 25 kDa were common for 6 of 7 sponge samples (Fig. 4b). The two most prominent bands (20 and 25 kDa) may represent different forms of neutral DNases, which could arise from variation in the carbohydrate moiety attached to the enzyme. Such variation has been found in bovine pancreatic DNase I (Salnikow et al., 1970). Immunodetection by polyclonal antibody raised against mouse DNase I revealed two protein bands of 20 and 25 kDa in cortex samples and one band in endosome samples as immunopositive (Fig. 4c). The expression level of immunopositive bands was higher in the cortex than in the endosome. In 4 of 7 cortex samples the slower migrating protein band was stronger. It should be emphasised that neutral DNases expressing the highest enzyme activity in cortex samples are not from the DNAse I-like family, but rather belong to high-molecular weight DNases already described in marine sponge *Verongia aerophoba* (Heicke and Schmidt, 1969). In endosome samples neutral DNases expressing the highest enzyme activity belong to DNase I-like enzymes.

Acid DNase - To investigate interindividual variability of acid DNase activity the SRED assay of ten different *T. aurantium* specimen was performed at pH 4.25 (Fig. 5.). For both, sponge cortex and endosome, there was no interindividual variability in the level of acid DNase activity among different sponge specimens. In all ten investigated specimens the specific enzyme activity was higher in the endosome than in the cortex. An attempt to reveal heterogeneity of acid DNase isozymes by DPZ resulted with no visible bands with DNase activity (data not shown). That could be the consequence of denaturating conditions and the enzyme capacity to renaturate during DPZ (Liao, 1985), therefore NPZ was performed. Dark areas of hydrolysed DNA revealed one or two distinguished bands displaying DNase activity in the cortex and endosome (Fig. 6a). A faster migrating protein band was present with different intensity in both sections of all 7 investigated specimens. A slower migrating protein band was absent in some cortex (lane 2 and 5) samples. For a better comparison between DNase activity in the sponge cortex and endosome, different amounts of protein of each section were applied in NPZ (Fig. 6b). The minimum amount of protein necessary to obtain a positive nuclease signal after 24 h of incubation was specified as a measure of acid DNase activity. In the specimen No. 1 the minimal amount of proteins required for a positive

nuclease signal was the same in both sponge sections while in specimen No 7 the minimal amount of proteins required for a positive nuclease signal was approximated as ten times higher in the cortex than in the endosome section. Since the acid DNase is a lysosomal enzyme involved in the engulfment-mediated DNA degradation and sponges have an entirely intracellular digestion occurring in food vacuoles of amoebocytes in the endosome, the high activity of acid DNases was expected.

Immunodetection by a polyclonal antibody raised against human DNase II following native PAGE did not reveal any positive band (data not shown) while positive signals after a denaturating SDS-PAGE were detected in both sections of all investigated specimens. Two immunoreactive bands of 20 and 25 kDa were found in the cortex samples and one immunoreactive band in the endosome samples of all investigated specimens (Fig. 6c). It is very likely that both immunopositive protein bends represent sponge DNase II–like protein and that the slower migrating band occurring in the cortex represent the same protein with post-translational modifications.

Taken together the results indicate strongly that the activity of DNA degrading enzymes in the sponge *Tethya aurantium* is related to sponge sections and their functions. The cortex, as a sponge section communicating with the environment, express high interindividual variability of enzyme activity as well as heterogeneity of neutral DNases, while the endosome section, where the intracellular digestion is localized, is a site of high acid DNase activity.

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FIGURE CAPTION

Figure 1. SRED gel plate, demonstrating the dark circles produced by diffused DNases from the sponge *Tethya aurantium*. A) neutral Ca^{2+}, Mg^{2+}- dependent DNase: S – 5 ng of DNase I from bovine pancreas, C – cortex (85 μg of proteins/ well), E – endosome (125 μg of proteins/ well), B) acid DNase, 24 h incubation: S – 5 ng of DNase II from porcine spleen, C – cortex (85 μg of proteins/ well), E – endosome (125 μg of proteins/ well), C) acid DNase, 72 h incubation: S – 5 ng of DNase II from porcine spleen, C – cortex (85 μg of proteins/ well), E – endosome (125 μg of proteins/ well).

Figure 2. pH dependence curve of sponge cortex DNases. One sponge specimen was analysed.

Figure 3. SRED assay of neutral Ca^{2+}, Mg^{2+}- dependent DNases (pH 7.0) in sponge *Tethya aurantium* cortex (specific activity 188- 588 mm/ mg proteins) and endosome (specific activity 200- 320 mm/ mg proteins).

Figure 4. Interindividual difference of neutral Ca^{2+}, Mg^{2+}- dependent DNase activity expression among sponge *Tethya aurantium* specimens. A) Denaturating SDS- PAGE zymogram of neutral DNase activity in the cortex and B) in the endosome. M – molecular weight markers, S –bovine pancreatic DNase I (2,5 ng/well). Lanes 1 to 7 represents different specimens of *Tethya aurantium* (170 µg proteins/well each) and corresponds to specimens number 2, 4, 3, 5, 6, 7, 10, respectively. C) – Immunodetection of sponge DNase I-like proteins in C- cortex and E- endosome.

Figure 5. SRED assay of acid DNases (pH 4.25) in sponge *Tethya aurantium* cortex (42 µg proteins/well) and endosome (50 µg proteins/well).

Figure 6. Interindividual difference of acid DNAse activity expression among sponge *Tethya aurantium* specimens. A) Native PAGE zymogram (NPZ) of acid DNase activity in the cortex and the endosome. Lanes 1 –7 represents different specimens of *Tethya aurantium* (170 µg proteins/well). B) Determination of minimum amount of protein necessary to obtain a positive DNase signal in NPZ for two sponge specimens. C) – Immunodetection of sponge DNase II-like proteins in C- cortex and E- endosome after SDS-PAGE.

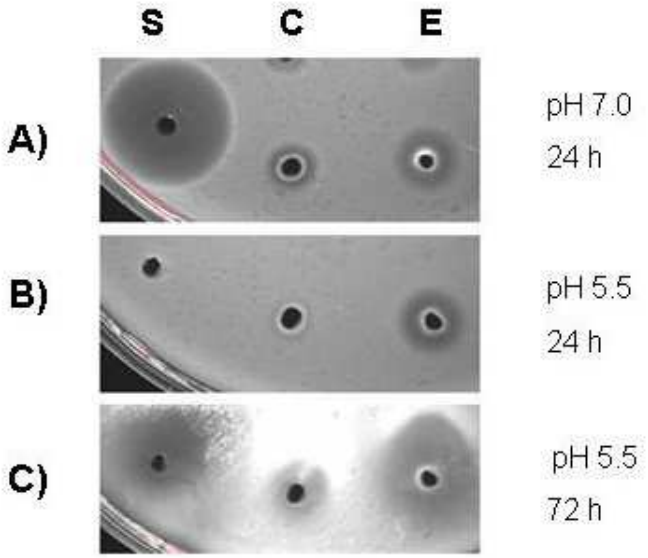
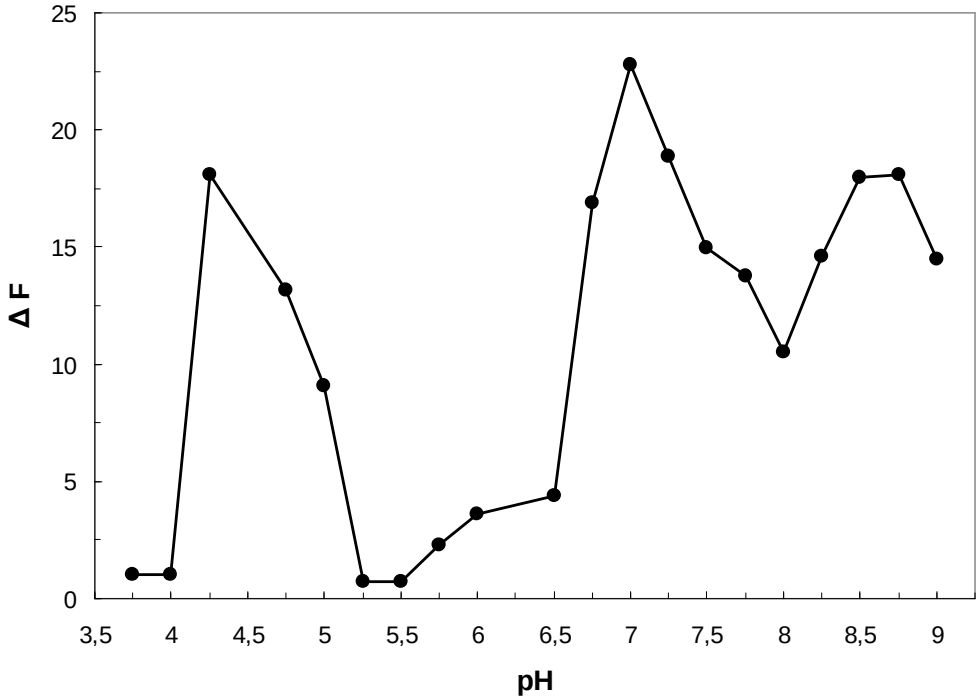


Figure 1.



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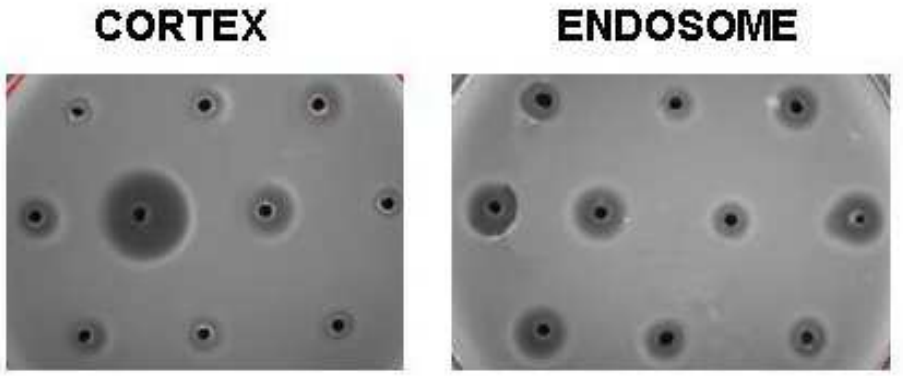
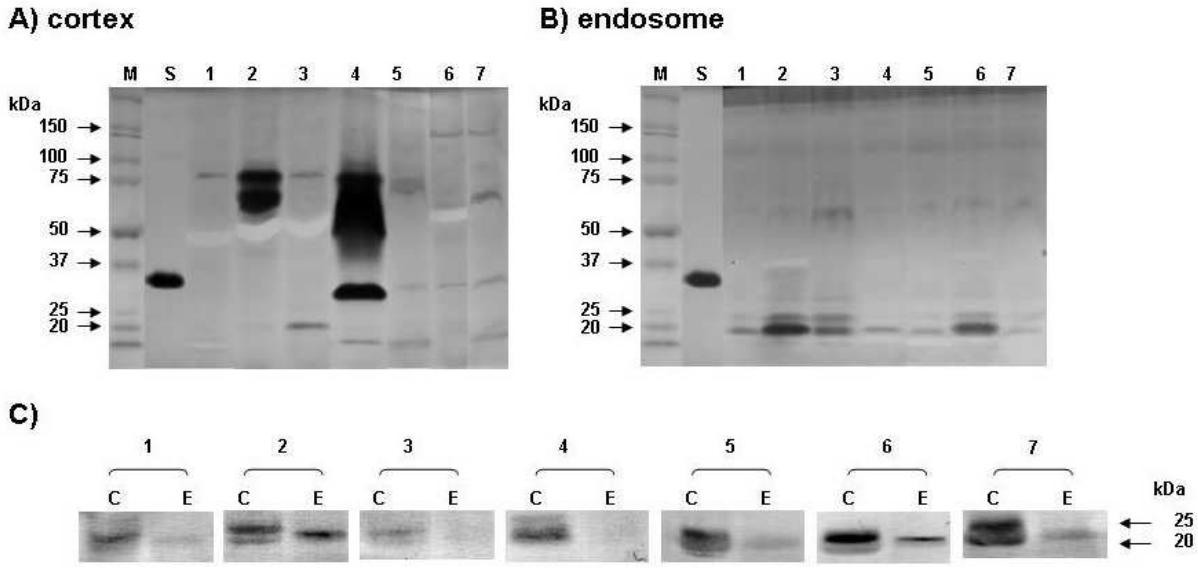


Figure 3.


A) cortex
B) endosome
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C)
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Figure 4.

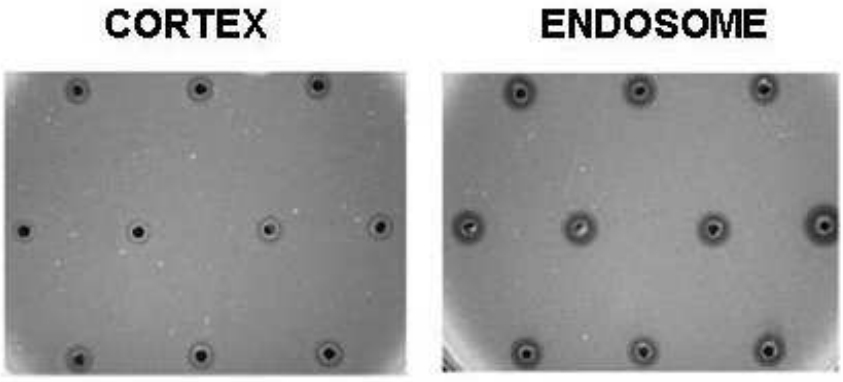
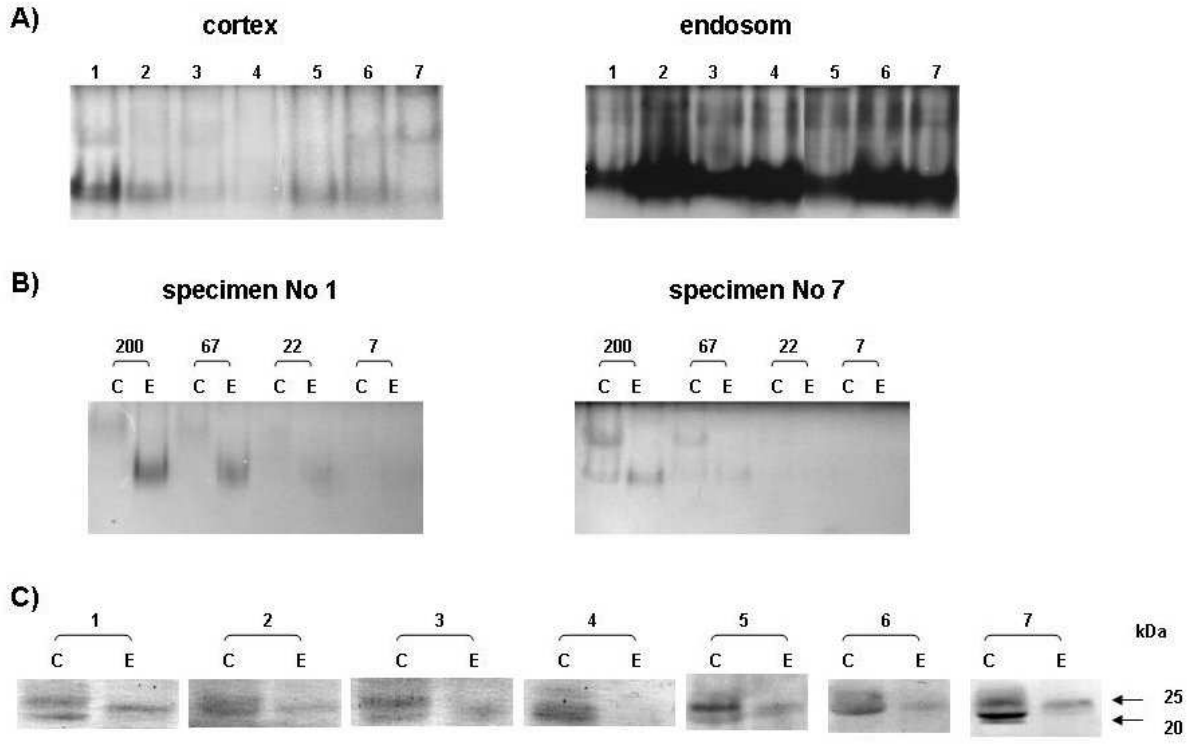


Figure 5.


A)
cortex
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specimen No 1
specimen No 7
200 67 22 7
C E C E C E C E
C)
1 2 3 4 5 6 7
C E
kDa
25
20

Figure 6.

Fafanđel, Maja; Ravlić, Sanda; Smodlaka, Mirta; Bihari, Nevenka. Deoxyribonucleases (DNases) in the cortex and endosome from marine sponge Tethya aurantium. // Russian Journal of Marine Biology. 36 (2010) , 5; 383-389

Deoxyribonucleases (DNases) in the Cortex and Endosome from the Marine Sponge *Tethya aurantium*

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