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Tissue distribution of neutral deoxyribonuclease (DNase) activity in the mussel *Mytilus galloprovincialis*

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Abstract

The presence of neutral DNase activity in bivalves is reported for the first time. The enzyme activity in four tissues of the mussel *Mytilus galloprovincialis* was analyzed by three different methods (i) specific denaturating SDS-PAGE zymogram, (ii) sensitive single radial enzyme diffusion (SRED) assay and (iii) rapid and sensitive fluorimetric determination of DNase activity with PicoGreen. The fluorimetric assay was rapid and sensitive enough for determination of hydrolytic activity of dsDNA in mussel hepatopancreas, adductor, gills and mantle. Maximal activity in all mussel tissue extracts was obtained in the presence of Ca^{2+} and Mg^{2+} at pH 7.0 with dsDNA as substrate. The neutral DNase activity in mussel tissue decreases in order hepatopancreas, mantle > gills > adductor. The enzyme activity displays interindividual variability in particular tissue as well as variability among tissues within one specimen. In the hepatopancreas one to three distinct proteins expressing neutral, Ca^{2+}, Mg^{2+}-dependent, DNase activity were detected by denaturating SDS-PAGE zymogram. This heterogeneity of neutral nucleases involved in DNA hydrolysis in hepatopancreas could reflect interindividual variability in mussel food utilization and nutrient requirement.

Keywords: Deoxyribonuclease; Enzyme activity; Mussel; *Mytilus galloprovincialis*; Neutral DNase; Tissue distribution

1. Introduction

Enzymatic cleavage of nucleic acids is performed by the nucleic acid hydrolyzing enzymes: ribonucleases and deoxyribonucleases. A unique property of deoxyribonucleases (DNases) is the fact that they effectively hydrolyze the stable phosphodiester bond. DNases play an important role in metabolism of nucleic acids and maintenance of physiological DNA concentration in the body as well as in protection of organisms against xenobiotic nucleic acid (Baranovskii et al., 2004). The most studied human DNases are nonspecific DNase I, DNase II and phosphodiesterase I. DNase I and phosphodiesterase I are bivalent cations dependent and have an optimal neutral or alkaline pH. Phosphodiesterase I shows preference for single chain or denatured DNA, RNA substrates (Belli and Goding, 1994). DNase II has an acidic pH optimum and bivalent cations are not requirement for its activity.

DNase I occurs in many tissues of animals, plants and micro-organisms. Human DNase I is very well described including characterization of gene and the level of its expression in the body, structural and physicochemical characteristics, active site structure and mechanisms of hydrolysis, catalytic properties, stability, inhibition and polymorphism (Baranovskii et al., 2004). Its activity decrease in the following order: pancreas > kidneys > small intestine > liver, stomach > other organs. Besides mammalian the biochemical and molecular characterization of DNase I in aves (Nakashima et al., 1999), reptilia (Takeshita et al., 2003), amphibia (Takeshita et al., 2001) and pisces (Yasuda et al., 2004) have been reported. Among marine invertebrates the neutral DNase was purified and characterized from shrimp hepatopancreas (Chou and Liao, 1990) and sea urchin spermatozoa (Shastina et al., 2003). The metal ion requirements of DNase isolated from shrimp hepatopancreas and DNase purified from sea urchin spermatozoa are very similar to those of bovine DNase I. *In vivo* experiment with sea urchin spermatozoa revealed that endogenous neutral Ca^{2+},

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Mg^{2+}-DNase is responsible for the internucleosomal cleavage of chromosomal DNA (Shastina et al., 2003).

Acid DNase from marine mollusk *Chlamys islandica* was recently isolated (Øverbø and Myrnes, 2006) but to our knowledge there is only one report regarding activity of DNase with a neutral pH optimum in marine mollusks published 30 years ago (Rasskazov et al., 1975). Three bivalves, *Myzuhopecten yessoensis, Crenomytilus grayanus,* and *Spisula sachalinensis* have been investigated but none displayed neutral DNase activity (Rasskazov et al., 1975). Therefore, it is essential to elucidate the presence and activity of neutral DNase in bivalves. Mussels of the genus *Mytilus* are among the commonest of marine mollusks and constitute an important element in the ecology of coastal waters. They are important as food and fouling organisms, as well as biomonitors of coastal water quality. Exposure of mussel *Mytilus galloprovincialis* to environmental contaminants gives rise to cell cycle alteration (Bihari et al., 2003) and programmed cell death (apoptosis) (Mičić et al., 2001). Since endogenous neutral deoxyribonuclease, DNase I, has been regarded as a candidate endonuclease facilitating chromatin breakdown during apoptosis (Mannherz et al., 1995) it is expected that *Mytilus* sp. express DNase activity.

In the present study we analyzed the tissue distribution of neutral DNase activity in mussel *M. galloprovincialis* by (i) specific denaturating SDS-PAGE zymogram, (ii) sensitive single radial enzyme diffusion (SRED) assay and (iii) rapid and sensitive fluorimetric determination of DNase activity with PicoGreen. These procedures enabled the detection of neutral DNases hydrolytic activity in different mussel *M. galloprovincialis* tissues and detection of DNase isoenzymes in hepatopancreas.

2. Materials and methods

2.1. Materials

DNase I from bovine pancreas, calf thymus DNA (2000 Kunitz units/mg), protease inhibitor (PMSF), ethydium bromide, protein molecular mass standard, acrylamide, *N*,*N*˙-methylene-bisacrylamide, *N*,*N*,*N*′*N*′-tetramethylethylenediamine (TEMED) were obtained from Sigma-Aldrich, USA, agarose from Roth, Germany and fluorochrome dye PicoGreen® from Molecular Probes Inc., USA.

2.2. Mussel and preparation of tissue extracts

The mussel *M. galloprovincialis*, Lamarck 1819 (Mollusca: Bivalvia) average mass (10±2 g) and length (4±1 cm) were obtained from mariculture and kept in an aquarium with running seawater. Mussels were dissected and hepatopancreas, gills, adductor muscle and mantle removed. Tissue extracts were prepared by homogenizing the organs in lysing buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, 2 mM PMSF, pH 8) for 30 s on ice using a Teflon Potter homogenizer. Cell debris was sedimented by centrifugation at 10,000 g, for 30 min, at 4 °C and the protein content of the supernatants was determined (Lowry et al., 1951).

2.3. Zymogram method for DNAse

Detection of DNase isozymes in the gel was based on the zymogram method described by Lacks (1981) with modifications. Briefly, denaturating SDS-PAGE was performed in 7.5% gels using 0.1% SDS in running buffer. The gel solution (10 mL) consisted of 2.5 mL of mixture of 29.2% (w/v) acrylamide, and 0.8% (w/v) *N*,*N*′-methylene-bisacrylamide, 2 mL of 5× electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3), 4.9 mL water 500 μL standard DNA (2 mg/mL) and 50 μL 10% (w/v) ammonium persulphate. After addition of 20 μL of *N*,*N*,*N*′*N*′-tetramethyl ethylene diamine, gels were polymerized in Mini Protein II electrophoresis chambers (Bio-Rad, Munich, Germany). Protein samples dissolved in loading buffer (50% glycerol, 50% 1× electrophoresis buffer, bromophenol blue) were loaded on the gels and electrophoresis was ran for 3 h at 90 V. Afterwards, the gels were incubated in reactivation buffer (20 mM Tris, 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 10 μg/mL ethidium bromide, pH 7.3) at 27 °C for 24 h and photographed on a UV transilluminator. On incubating the gels after electrophoresis, DNase activity resulted in dark bands of hydrolyzed DNA on fluorescent background that did not stain with DNA binding dye, ethidium bromide. To obtain positive nuclease signal after 24 h of incubation the maximal amount of proteins that could be applied on the gel (100 μg proteins from hepatopancreas, gills and mantle) or could be extracted from adductor (60 μg proteins) was used.

2.4. Single radial enzyme diffusion (SRED)

Presence of DNase activity in extracts of different mussel tissues was detected by the single radial enzyme diffusion (SRED) method (Nadano et al., 1993). 50 mL of 0.5 g/L melted agarose in buffer (20 mM Tris, 5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.3), containing 5 mL DNA (2 mg/mL) and 500 μL ethidium bromide (1 μg/μL)) was poured into a horizontal plastic tray. After solidification at room temperature circular well (0.2 mm)

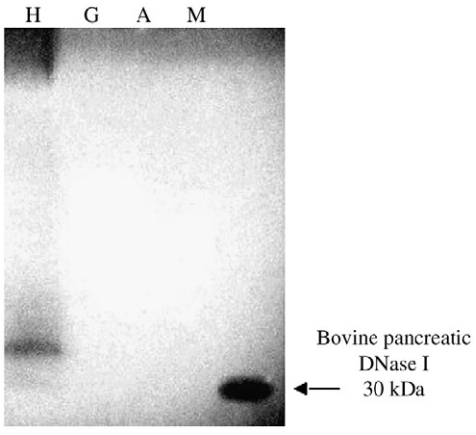

Fig. 1. Denaturating SDS-PAGE zymogram of neutral Ca^{2+}, Mg^{2+}-dependent DNase in mussel *Mytilus galloprovincialis* tissue extracts. H — hepatopancreas (100 μg proteins), G — gills (100 μg proteins), A — adductor (60 μg proteins), M — mantle (100 μg proteins) bovine pancreatic DNase I — (5 ng/well, 10^{-2}Kunitz units).