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Separation and Characterization of Deoxyribonucleases from Hepatopancreas of Freshwater Snail in Normality and under *in vivo* Model Intoxication

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Abstract—Deoxyribonucleases (DNases) differing in subcellular localization and specificity towards native and denatured substrates and in products of cleavage of endogenous DNA were isolated for the first time from the hepatopancreas of freshwater snail by differential sedimentation and preparative isoelectrofocusing. It was found that treatment with phenol activates most of the investigated DNases, especially in lysosomes, and induces two new DNases of lysosomal origin. The possible different participation of certain DNases of the snail hepatopancreas in the regulation of DNA degradation under intoxication is discussed.

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The existence of enzymes as a set of multiple forms is a consequence of their molecular evolution directed to more finely tuned regulation of metabolism under constantly differing environment. Investigation of the structural and functional diversity of homological proteins and enzymes in aquatic organisms clarifies biochemical mechanisms of adaptations of these organisms and provides a background for using these phenomena for testing of the quality of water sources. We previously investigated a complex of acid phosphatases of the freshwater snail *Viviparus viviparus* L. and revealed its high sensitivity to toxic compounds that are present in the environment of the mollusks [1, 2]. We also demonstrated that different toxicants activate *in vivo* a number of deoxyribonucleases (DNases) in the hepatopancreas of the freshwater snail that are not revealed under normal conditions [3]. The spectrum of the proteins possessing DNase activity depends both on the nature of the toxic agent (i.e. specificity of its toxic action) and on the strength (concentration and duration) of its damaging effect, this suggesting the adaptive character of the observed changes [3].

In the present work we investigated in detail the polymorphism and some physicochemical and functional properties of DNases of the freshwater snail that is a widely spread representative of freshwater macrozoobenthos and one of the most suitable objects for biochemical testing of the quality of freshwaters [2, 4].

MATERIALS AND METHODS

Mollusks. Freshwater snails (*Viviparus viviparus* L., Viviparidae, Architaenioglossa, Gastropoda) were collected in the riverside area of the Vyaz' river (village Tishkovo, Moscow Region). The mollusks were acclimatized to the laboratory conditions for 1 month. The mollusks (no more that 200 individuals) were kept in an aquarium of 60 liters at 15-17°C under moderate natural illumination, 24-h aeration, and with a regular (1 time in 3 days) replacement of 1/3 volume of the water with settled tap water. The aquarium contained higher plants that were usual for the natural surrounding of the freshwater snail.

Model toxic conditions. Experimental animals (15 individuals) were placed into separate glass vessels without foreign flora or fauna containing 1 liter of phenol solution (0.02 mg/liter). The solution was prepared on the aquarium water by multiple dilutions of the original

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solution according to the accepted standards [5]. Simultaneously, groups of control mollusks were placed into vessels with pure aquarium water. Other conditions (temperature, illumination, aeration) were constant. Duration of the exposure was 48 h.

Extraction of proteins. The hepatopancreas was removed from the mollusks by vivisection, washed with several volumes of 0.15 M NaCl solution, and homogenized in the cold (0°C) for 5 min by grinding in a porcelain mortar with 0.5% Triton X-100 aqueous solution (10 ml per gram wet weight of the tissue). The homogenate was centrifuged (30 min, 10,000*g*, 4°C), and the resulting supernatant was used for determination of the DNase activity. The extracts were kept at −18°C no more than 30 days. Protein content in the extracts was determined by the Lowry method.

Determination of the deoxyribonuclease activity. The DNase activity was determined spectrophotometrically by measuring the accumulation of the acid products of DNA degradation [6]. The optimal pH value and composition of the buffer solution were chosen by measuring the activity of DNases in the original hepatopancreas extract within the ranges of pH 4.0-5.6 (50 mM Na-acetate buffer), 5.0-7.4 (50 mM phosphate-citrate buffer), and 7.2-8.2 (50 mM Tris-HCl). No significant activity was detected under neutral and alkaline conditions. The maximal DNase activity in the hepatopancreas extract was detected at pH 4.6. The DNase activity was thus subsequently measured at pH 4.6 and 37°C. We used a commercial preparation of high molecular weight DNA from chicken erythrocytes ("exogenous DNA"; Reanal, Hungary) and a preparation of high molecular weight DNA isolated from the investigated mollusks ("endogenous DNA", see below) as substrates. To determine the DNase activity, 0.3% (sometimes 0.1%, w/v) DNA solution was prepared using deionized water. The solution was used immediately (native DNA) or after denaturation of the DNA by heating in boiling water for 15 min with subsequent quick cooling on melting ice (denatured DNA). The unit of the DNase activity was determined as the amount of the enzyme that increased the absorption at 260 nm by 1 unit for 1 h of incubation. The specific activity was calculated as units of activity per mg protein. Statistical analysis of the results was made according to Student [5].

Separation of deoxyribonucleases. Analytical separation of DNases was performed by electrophoresis in polyacrylamide gel columns (4 mm in diameter and 8 cm in length) in an alkaline medium using a graduated buffer system as described by Davis [7]. The optimal concentration of the separating gel for the separation of DNases was found to be 7.2% (w/v), the ratio of methylene bis-acrylamide concentration to the sum of monomers constituting 2.6%. Samples of proteins (100-200 μg) mixed with an equal volume of 40% (w/v) of sucrose solution were applied to the columns of the gel. Electrophoresis was run at 4°C. The conditions were: 10-15 min at 1.5 mA per column for concentrating of the proteins and 90-100 min at 2.5 mA per column for separating. The DNase activity in the gel was revealed as described in [8]. A denatured DNA preparation from chicken erythrocytes was used as the substrate for the DNases. The substrate (0.4 mg per gel) was added to the solution for the separating polyacrylamide gel. After the electrophoresis, gel columns were washed with distilled water and treated according to the original protocol [8] using 50 mM sodium-acetate buffer, pH 4.6.

Preparative separation of DNases was performed sequentially using differential centrifugation of the hepatopancreas homogenate and isoelectrofocusing (IEF) of separate subcellular fractions.

Subcellular fractionation was performed by the procedure described by Sawant et al. [9] that was modified for the hepatopancreas of mollusks [1]. The extracted organs (2-3 g) were minced using scissors, washed twice with 0.15 M NaCl, and homogenized with a 5-fold volume of 0.32 M sucrose in a Potter–Elvehjem homogenizer for 4-5 min at 0°C. The homogenate was filtered through the double layer of a kapron tissue. Subsequent centrifugation at 0°C yielded sequential fractions of cell debris (20 min, 750*g*), nuclei (20 min, 1200*g*), mitochondria (20 min, 4500*g*), and lysosomes (30 min, 20,000*g*). The supernatant was used as the cytosol fraction. The fractions were resuspended in 2 ml of 0.25% Triton X-100, freeze–thawed three times, homogenized with a Potter–Elvehjem homogenizer, and centrifuged for 30 min at 10,000*g* at 0°C. The supernatants were dialyzed against 1000-fold volume of bidistilled water overnight. The purity of the isolated fractions was analyzed enzymatically according to the standard protocols [9].

Preparative isoelectrofocusing of the proteins extracted from the fractions of lysosomes and cell debris was performed in a granulated Ultrodex gel (LKB, Sweden) using ampholytes of working pH range of 3-10 (ICN, USA) and 4-6 (LKB). We used an LKB Multiphor device for horizontal electrophoresis equipped with a Power Pac 1000 power supply (Bio-Rad, USA). The electrodes were wet with 1 M H_3PO_4 and 1 M NaOH. The protein extract was diluted with bidistilled water to achieve concentration of 2-4 mg/ml, and 3 ml of the solution was applied to the gel. IEF was run at 4°C at constant amperage (15 mA) for 1.5 h and at constant voltage (800 V) for the subsequent 15 h until the current achieved the level of 2-3 mA. After finishing IEF, the gel was separated into 30 fractions. Proteins were eluted from the fractions with 0.15 M NaCl for 2 h at 4°C. After the pH values of the eluates were determined, they were dialyzed overnight against 1000-fold volume of bidistilled water.

Isolation and purification of DNA from the freshwater snail. DNA was isolated using the phenol–chloroform method [10]. Muscle tissue of the foot of snails (1 g) was minced using scissors, washed twice with 0.15 M NaCl solution, and homogenized at 0°C using a Potter–

Elvehjem homogenizer with 12 ml of freshly prepared extracting solution (0.1 mM Tris-HCl, 1.25 mM EDTA, 2 mM NaCl, 0.025% SDS, 0.1 mg/ml proteinase K, pH 8.0). The homogenate was incubated at 65°C for 1 h, supplemented with an equal volume of chloroform saturated with distilled water, mixed thoroughly for 1 min, and centrifuged (10 min, 10,000*g*, 4°C). The upper aqueous phase was placed into a tube and supplemented with 0.8 volume of isopropanol, mixed carefully, and left for 30 min at room temperature. The precipitate was separated by centrifugation (10,000*g*, 10 min, 4°C), washed sequentially with 70 and 96% (by volume) ethanol, and dried for 1-2 h at room temperature. The dried precipitate of DNA was dissolved overnight at room temperature in 500 μl of DNase-free deionized water The solution of unpurified DNA was supplemented with 5 μl of 1% (w/v) solution of RNase A (Pharmacia, USA) and incubated for 1 h at 37°C to remove ribonucleic acids. Then the DNA was purified by treatment with phenol (twice), phenol–chloroform mixture (1 : 1), and chloroform and by reprecipitation with 96% ethanol in the presence of 2.5 M ammonium acetate. The DNA precipitate was removed by centrifugation at 10,000*g* for 5 min at 4°C, washed sequentially with 70 and 96% ethanol, dried in air, and dissolved in 200 μl of sterile deionized DNase-free water. Concentration and purity of the DNA preparation was determined spectrophotometrically by measuring the absorption at 260 and 280 nm [11]. The DNA preparation was used as the substrate for the investigation of DNases.

Analysis of the products of DNA hydrolysis. The isolated DNA was hydrolyzed in the presence of the DNases obtained by the subcellular fractionation and isoelectrofocusing. The incubation mixture (200 μl) contained 3-6 mg of DNA (depending on the DNase activity of the preparation) and 20 μl of a DNase preparation obtained by IEF in 50 mM Na-acetate buffer, pH 4.6. The time of incubation varied from 10 min to 6 h at 37°C. The reaction was stopped by the addition of 10 μl of 5 M NaCl and 500 μl of 96% (by volume) ethanol. The mixture was incubated at –18°C for 2 h and then centrifuged for 10 min at 14,000*g*. The supernatant was removed completely; the pellet was dried at 37°C for 40 min, supplemented with 20 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and left overnight at room temperature to dissolve. The resulting solutions was mixed with 4 μl of sample buffer (50 mM Tris-HCl, 5 mM EDTA, 25% glycerol, 2 mg/ml xylene cyanole, 2 mg/ml bromophenol blue, pH 8.0) and analyzed by electrophoresis in 0.8% agarose gel with TAE buffer (2 M Tris, 1 M CH_3COOH, 50 mM EDTA, pH 8.0) in the presence of ethidium bromide at 100 V/cm for 1.5 h. The DNA bands were visualized in UV light (302 nm) using a Gel Doc 1000 system (Bio-Rad). A sample where the DNase preparation was added after the cessation of the reaction was used as the control. We used a Gene Ruler 1 kb DNA Ladder (Pharmacia) as the molecular weight marker.

RESULTS AND DISCUSSION

The subcellular fractionation yielded preparations of freshwater snail DNases localized in cytosol (postlysosomal fraction), lysosomes, and cell debris (here and below, the term "DNases" refers to proteins exhibiting DNase activity and differing in charge (p*I*) and molecular weight, and as a consequence, in electrophoretic mobility). The DNase activity determined in the subcellular fractions under normal conditions and after the treatment with phenol is presented in Table 1. It is seen that after exposure to phenol, the DNase activity in the hepatopancreas of mollusks increases almost two-fold. Under normal conditions, the highest activity is observed in the postlysosomal fraction, while after exposure to phenol the highest DNase activity is revealed in the cell debris. Slight nuclease activity was revealed also in the nuclear and mitochondrial fractions, which might be explained by insufficient washing of these fractions from cell fragments formed during the homogenization, as well as by the heterogeneity of the heavy cell fractions that can be completely separated only with the use of special approaches [12]. For example, "heavy" mitochondria are large capsules filled with usual mitochondria gemmated into the cell. They contain some amount of cell content with characteristic enzymes. While aging, the "heavy" mitochondrion can transform into the autophagosome, and after accumulation of acid hydrolases, into the secondary lysosome [12]. Considering this, the fraction designated as the cell debris is heterogeneous and can include any structures exceeding the size of the cell nucleus, for example, secondary lysosomes (hetero- and autophagosomes), cell fragments, intact cells, and cell groups. This fraction exhibits the most variety in the bands of the DNase activity in the electrophoregram (Fig. 1). Besides, this fraction exhibits the highest (almost 4-fold) increase in the total DNase activity after exposure to phenol (Table 1), this being likely due to an

Table 1. DNase activity in subcellular fractions of freshwater snail hepatopancreas in normality and after exposure to phenol (0.02 mg/liter, 48 h)

Subcellular fraction	Activity, units/mg protein	
	control	experiment
Cell debris	3.6 ± 0.3	13.6 ± 0.2
Nuclei	2.0 ± 0.3	2.1 ± 0.6
Mitochondria	3.3 ± 0.1	4.8 ± 0.2
Lysosomes	7.4 ± 0.1	7.6 ± 0.4
Postlysosomal fraction	10.6 ± 0.2	13.2 ± 0.8
Total homogenate	6.0 ± 0.4	11.3 ± 0.4

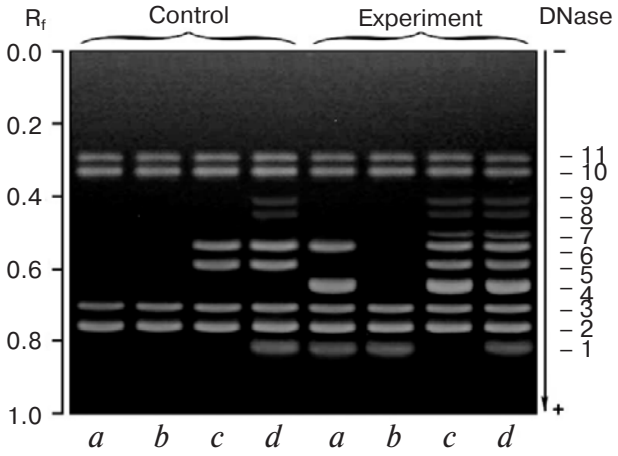

Fig. 1. Electrophoregrams of DNases from subcellular fractions of freshwater snail hepatopancreas in normality and under *in vivo* model intoxication. Control, control mollusks; experiment, mollusks after exposure to phenol (0.02 mg/liter, 48 h). *a*) Total homogenate; *b*) postlysosomal fraction; *c*) lysosomal fraction; *d*) cell debris; 1-11) DNases.

increase in destructive processes in the cells and to formation of large autophagosomes and secondary lysosomes that are concentrated in the indicated fraction during centrifugation.

As seen from Fig. 1, the spectrum of proteins exhibiting DNase activity includes both lysosomal and postlysosomal components. In normality, the set of DNases is represented by nine areas of DNase activity with R_f values of 0.82, 0.76, 0.71, 0.59, 0.54, 0.45, 0.41, 0.33, and 0.29. The corresponding DNases were designated as 1-11, respectively. DNases 1, 5, 6, 8, and 9 are not detected in the homogenates of the hepatopancreas, while they are revealed in the separate fractions obtained under the same conditions (Fig. 1). This fact can be accounted for a low activity of the indicated enzymes in the cells of the hepatopancreas that can be detected only after partial purification and concentration. According to previously obtained data [3], DNases 1, 5, 6, 8, and 9 are present in freshwater snails affected by exposure to different toxic compounds. Evidently, intoxication results in a more pronounced activation of the indicated components of the DNase complex that can be detected by electrophoresis. This is supported by the analysis of the DNases isolated from the mollusk tissues after exposure to phenol (Fig. 1). Electrophoresis of the crude extract of the hepatopancreas revealed three additional bands of DNase activity compared to the control with R_f values of 0.82, 0.65, and 0.54 corresponding to DNases 1, 4, and 6, respectively. Besides, each subcellular fraction of the experimental mollusks contained an increased number of proteins exhibiting DNase activity compared to the control that was due to the toxic action of phenol on the organism. For example, four additional DNases were revealed in the lysosomes of the experimental group, being absent in this fraction of the control (Fig. 1), and two of them (DNases 4 and 7) were found in none of the subcellular fractions of the control group. Their expression is evidently induced by intoxication. The fraction of cell debris contains the maximal number of DNases: 11 in the experimental group, and 9 in the control. Postlysosomal fraction of the hepatopancreas of the experimental mollusks contains DNase 1 that in normality is present only in the fraction of cell debris (Fig. 1). This might point to the liberation of this enzyme from the subcellular structures into the cell matrix under stress conditions connected with intoxication.

Preparative isoelectrofocusing allowed isolation of different DNases characterized by constant values of isoelectric points (p*I*) and electrophoretic mobility (R_f), the latter being determined by electrophoresis in polyacrylamide gel (Table 2). Some DNases, being clearly separated in polyacrylamide gel, exhibit very similar or the same isoelectric points, which did not allow isolation of the corresponding individual preparations. In these cases, the total DNase activity in the preparations was determined.

The data on the nuclease activity of the isolated DNases towards chicken DNA ("exogenous") and DNA isolated from tissues of freshwater snails ("endogenous") indicate that the various DNases prefer different substrates (Table 2). DNases 1 and 2-3 (combined preparation of the corresponding DNases) hydrolyzed mainly

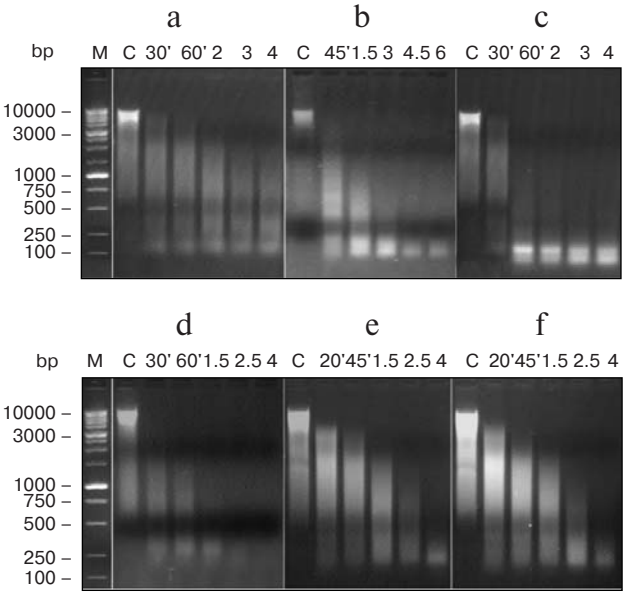

Fig. 2. Electrophoregrams of the products of DNA hydrolysis obtained under the action of different DNases from freshwater snail hepatopancreas: a) DNase 1; b) DNases 2-3; c) DNase 4; d) DNases 5-6; e) DNases 8-9; f) DNases 10-11; C) control (DNA without treatment with DNases). Time of the reaction of hydrolysis is indicated on the top (20-60 min or 1-6 h); M) molecular weight markers, the size of fragments being indicated on the left (bp).

Table 2. Some properties of DNases isolated from the hepatopancreas of the freshwater snail

DNase	Localization in cell	R_f in 7.2% polyacrylamide gel	pI	DNase activity towards different substrates × 10, units		
				exogenous denatured DNA	exogenous native DNA	endogenous native DNA
1	lysosomes	0.82	4.3	0.46 ± 0.03	0.08 ± 0.00	0.0
2	lysosomes, cytosol	0.76	4.5	1.69 ± 0.05	0.29 ± 0.06	0.0
3	lysosomes, cytosol	0.71	4.5			
4	lysosomes	0.65	5.4	0.37 ± 0.04	0.33 ± 0.05	0.47 ± 0.08
5	lysosomes	0.59	5.1	1.55 ± 0.04	1.67 ± 0.13	2.48 ± 0.42
6	lysosomes	0.54	5.1			
7	lysosomes	0.51	–	–	–	–
8	lysosomes	0.45	5.3	0.30 ± 0.03	1.11 ± 0.01	1.81 ± 0.23
9	lysosomes	0.41	5.3			
10	lysosomes, cytosol	0.33	5.5	0.36 ± 0.03	1.41 ± 0.10	2.63 ± 0.57
11	lysosomes, cytosol	0.29	5.4			

Note: Activity of DNases: 2 and 3, 5 and 6, 8 and 9, 10 and 11, was designated as sum for each pair. Dash – no data.

denatured DNA. DNases 8-9 and 10-11 exhibit a more pronounced affinity to the native substrates. The fractions containing DNases 4 and 5-6 exhibit virtually the same activity towards native and denatured DNA preparations. Interestingly, the isolated DNases exhibit a strict correlation between their electrophoretic mobility and substrate specificity: with decrease in R_f value, the activity decreases towards denatured DNA and increases towards native DNA, especially that of endogenous origin.

Analysis of the products of the hydrolysis of endogenous DNA by separate DNases and their groups demonstrated that the DNases of the freshwater snail are characterized by different nature and rate of degradation of substrates (Fig. 2). DNases 2-3, 4, 8-9, and 10-11 are likely endonucleases, this being indicated by the accumulation of oligonucleotides of fixed length in the reaction mixture. For example, DNases 2-3 yield fragments of 100 bp that are likely to be final products of the hydrolysis considering the long time of the hydrolysis (6 h) (Fig. 2b). DNase 4 also cleaves the substrate into oligonucleotides of 100 bp (Fig. 2c), and products of the reaction catalyzed by DNases 8-9 and 10-11 are oligonucleotides of approximately 200 bp (Fig. 2, e and f). The action of DNase 5-6 results in degradation of DNA to fragments that are not detected by the method employed (Fig. 2d). DNase 1 hydrolyzes DNA very slowly, gradually decreasing the molecular weight of the substrate during the reaction (Fig. 2a). In this case, the final product cannot be revealed because in the time necessary to complete the reaction the enzyme is inactivated. At the same time, DNase 1 exhibits enhanced activity towards partially hydrolyzed DNA: the summarized action of DNases 1-3 results in the complete degradation of the substrate after 4 h of incubation, this suggesting that DNase 1 is an endonuclease cleaving DNA completely only in the presence of other DNases.

Thus, the hepatopancreas of the freshwater snail contains a number of catalytically active proteins possessing DNase activity and differing in cell localization, physicochemical properties (pI and R_f values), and substrate specificity. The largest contribution to the DNase activity under normal conditions is made by DNases 2, 3, 10, and 11. They are localized in both lysosomes and cytosol and efficiently hydrolyze DNAs of different origin and structure (exogenous, endogenous, native, and denatured).

Changes in biochemical parameters of the DNases under conditions of toxic action are mainly connected with the increase in the DNase activity or induction *de novo* of a number of lysosomal DNases (Fig. 1). DNases 5 and 6 exhibit a high specific activity but have no pronounced substrate specificity (Table 2). They have the ability to completely hydrolyze DNA (Fig. 2) and thus represent typical digestive enzymes of the lysosomal complex that is especially characteristic for the liver of different animals [12]. The increase in their activity as a result of intoxication (for example, DNase 6 is revealed in the total protein extract of the hepatopancreas under intoxication, but not in normality (Fig. 1)) indicates a growing intensity of auto- and heterophagy in the hepatopancreas cells of the mollusks. Induction of the activity of DNase 1 (presumably an exonuclease) under toxic conditions can promote more efficient degradation of the damaged DNA with subsequent involvement of the additional material into metabolism. DNases 4, 8, and 9 activated by the stress evidently play a certain role in the adaptation to a

toxic exposure. Since they exhibit no unique properties, they probably duplicate the enzymes that are typical for normal conditions, providing the necessary level of metabolism under stress conditions. Actually, considering the action on DNA, DNase 4 virtually does not differ from DNases 2-3, and DNases 8-9 do not differ from DNases 10-11 (Fig. 2). But considering the recently obtained data that one of the characteristic features of apoptosis in both mollusks and mammals is internucleosomal fragmentation of DNA [13-16], it cannot be excluded that the activation of DNases 8-9 cleaving DNA of the mollusks into the fragments of approximately 200 bp (the size of nucleosomes) points to their participation in programmed cell death of hepatopancreas cells induced by intoxication.

In conclusion, it should be noted that the complex of DNases of the freshwater snail hepatopancreas possessing wide possibilities of action on DNA can efficiently cleave the substrates under both normal and stress conditions and can play a significant role in the regulation of DNA degradation in different states of the organism. A high sensitivity and heterogeneity of DNases of the freshwater snail makes it possible to use them as a biochemical test system for characterization of toxic properties of water: changes in the electrophoretic spectra and total activities of DNases can serve as a marker of intoxication of mollusks [17].

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