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Purification and Characteristics of Ca^{2+}, Mg^{2+}- and Ca^{2+}, Mn^{2+}-Dependent and Acid DNases from Spermatozoa of the Sea Urchin *Strongylocentrotus intermedius*

V. V. Shastina, N. I. Menzorova, Yu. T. Sibirtsev*, and V. A. Rasskazov

Pacific Institute of Bioorganic Chemistry, Far East Division, Russian Academy of Sciences, pr. 100 let Vladivostoku 159, Vladivostok 690022, Russia; fax: 7 (4232) 314-050; E-mail: sibir@piboc.dvo.ru

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Abstract—Ca^{2+},Mg^{2+}- and Ca^{2+},Mn^{2+}-dependent and acid DNases were isolated from spermatozoa of the sea urchin *Strongylocentrotus intermedius*. The enzymes have been purified by successive chromatography on DEAE-cellulose, phenyl-Sepharose, Source 15Q, and by gel filtration, and the principal physicochemical and enzymatic properties of the purified enzymes were determined. Ca^{2+},Mg^{2+}-dependent DNase (Ca,Mg-DNase) is a nuclear protein with molecular mass of 63 kD as the native form and its activity optimum is at pH 7.5. The enzyme activity in the presence of bivalent metal ions decreases in the series (Ca^{2+} + Mg^{2+}) > Mn^{2+} = (Ca^{2+} + Mn^{2+}) > (Mg^{2+} + EGTA) > Ca^{2+}. Ca,Mg-DNase retains its maximal activity in sea water and is not inhibited by G-actin and N-ethylmaleimide, whereas Zn^{2+} inhibits the enzyme. The endogenous Ca,Mg-DNase is responsible for the internucleosomal cleavage of chromosomal DNA of spermatozoa. Ca^{2+},Mn^{2+}-dependent DNase (Ca,Mn-DNase) has molecular mass of 25 kD as the native form and the activity optimum at pH 8.5. The enzyme activity in the presence of bivalent metal ions decreases in the series (Ca^{2+} + Mn^{2+}) > (Ca^{2+} + Mg^{2+}) > Mn^{2+} > (Mg^{2+} + EGTA). In seawater the enzyme is inactive. Zinc ions inhibit Ca,Mn-DNase. Acid DNase of spermatozoa (A-DNase) is not a nuclear protein, it has molecular mass of 37 kD as a native form and the activity optimum at pH 5.5, it is not activated by bivalent metal ions, and it is inhibited by N-ethylmaleimide and iodoacetic acid. Mechanisms of the endonuclease cleavage of double-stranded DNA have been established for the three enzymes. The possible involvement of DNases from sea urchin spermatozoa in programmed cell death is discussed.

Key words: sea urchin, spermatozoa, Ca^{2+},Mg^{2+}-dependent DNase, Ca^{2+},Mn^{2+}-dependent DNase, acid DNase, DNase II, apoptosis

The internucleosomal cleavage of chromatin by endogenous nucleases is a significant biochemical manifestation of programmed cell death (apoptosis) [1]. At present, deoxyribonucleases (DNases) involved in this process are intensively searched for and studied. As a result, two types of DNases have been found. The first type DNases are active only in the presence of bivalent metal ions and their pH optimum is in the alkaline region. These DNases include Mg^{2+}-dependent DNase [2] and the most numerous group of Ca^{2+},Mg^{2+}-dependent DNases [3-7]. The second type DNases are cation-independent acid DNases with the pH optimum in the acidic region [8-10]. The involvement of Ca^{2+},Mg^{2+}-dependent DNases in apoptosis is suggested based on their ability to produce fragments with terminal 3′-OH/5′-P-groups and on their high sensitivity to Zn^{2+}, which inhibits the internucleosomal cleavage of chromatin [4, 7]. Acid DNases produce DNA fragments with terminal 5′-OH/3′-P-groups and their sensitivity to Zn^{2+} is lower [11]. However, acidification of the intercellular medium to pH 6.5 during apoptosis occurs in some cells that results in the post-translational modification of proteins and activation of acid DNases [10]. It was recently suggested that at different stages of apoptosis different DNases should be involved which should be specific to structural features of different chromatin regions (rosettes, loops, and the linker region of nucleosomes) and cleave them. This results in the cleavage of chromatin into high-molecular-weight fragments of 50-300 kb and low-molecular-weight fragments (the internucleosomal cleavage in the range of 200 bp) [12]. It was also suggested that DNases involved in apoptosis should differ depending on the cell type and factors initiating apoptosis [5, 10]. Thus, up to now there is no consensus on the types of DNases involved in apoptosis, their number, and molecular mechanisms of the cell chromatin fragmentation by endonucleases.

* To whom correspondence should be addressed.

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Sea urchin spermatozoa were chosen for studies of DNases based on current concepts on biochemical processes in spermatozoa. The spermatozoon is considered a simplified model of the cell deprived of many functions inherent in somatic cells; therefore, it is suggested to have no high DNase activities. Moreover, sea urchin spermatozoa have no lysosomes responsible for utilization of nucleotide substances. On the other hand, in male gonads of sea urchins a negative selection of sex cells is reported to occur during all studies of spermatogenesis, up to the complete resorption of all unreleased spermatozoa after the termination of the reproductive cycle [13]. Based on these data, we suggested that apoptosis should play an important role in the resorption of male sex cells and DNases found in spermatozoa should be involved in this process. Note that so far DNases of male sex products have been described only in a few papers. Thus, bovine seminal plasma was shown to contain Ca^{2+},Mg^{2+}-dependent DNase and human seminal plasma was shown to contain both acid DNase II and DNase I [3, 14, 15]. However, directly in male sex cells acid DNase II was first detected in spermatozoa of mud loach (*Misgurnus fossilis* L.) [16]. The authors suggested that this enzyme should be involved in negative selection of mature sex cells. However, in studies on the distribution of DNases in rabbit reproductive organs neither Ca^{2+},Mg^{2+}-dependent nor acid DNase were found in the spermatozoa [17]. We have preliminarily shown that spermatozoa of sea urchin *Strongylocentrotus intermedius* contain three different DNases, two of which depend on bivalent metal ions and have the optimum activity at pH values of 7.5-8.5 and the third DNase which is independent of bivalent metal ions and has its activity maximum at pH 5.5 [18].

The purpose of this work was to develop a method for purification of DNases of sea urchin spermatozoa detected by us and to determine their principal physicochemical and enzymatic properties.

MATERIALS AND METHODS

Enzyme source, substrates, and reagents. Spermatozoa of the sea urchin *S. intermedius* were used which were obtained during autumn sexual maturity of the males. Sperm release was stimulated by shaking the animals, the sperm was collected into sea water, the spermatozoa were washed twice free of the seminal plasma with cold sterile sea water using centrifugation (1400*g*, 20 min) to precipitate the cells, and the spermatozoa precipitated were frozen and stored at −25°C. To purify the enzyme and determine their properties the following reagents were used: DEAE-cellulose DE-52 (Whatman, England); an highly efficient FPLC system with Superdex 75HR and Source 15Q columns (Amersham Pharmacia Biotech, Sweden); phenyl-Sepharose, standard proteins with molecular weights from 14.4 to 97 kD (Pharmacia, Sweden); agarose, phenylmethylsulfonyl fluoride (PMSF), BSA, EDTA, glycerol, SDS, Tris, dithiothreitol (DTT), Coomassie R-250, acrylamide (Sigma, USA); EGTA (Serva, Germany); DNA of λ phage and a supercoiled DNA pBR322 (SibEnzym, Russia); DNA from salmon milt (Vector, Berdsk, Russia), DNase I (Worthington Biochemical, USA); G-actin from rabbit muscle was kindly presented by N. S. Shelud'ko (Institute of Marine Biology, Vladivostok). Agarose gels were photographed using a Herolab E.A.S.Y. Win 32 Systems setup (Germany).

Determination of enzymatic activities. Activities of the three enzymes were qualitatively determined by their ability to catalyze the transition of λ phage DNA into low-molecular-weight fragments or of the supercoiled DNA pBR322 (form I) into the open ring and the linear forms (forms II and III, respectively) of DNA. The incubation mixture (20 μl) contained 1 μg DNA of λ phage or the DNA pBR322, 1-3 μl of the enzyme preparation, and the appropriate buffer. The hydrolysis products were separated by electrophoresis in 1% agarose gel, visualized with ethidium bromide, and photographed (method 1).

Activities of the enzymes were estimated quantitatively by the yield of oligonucleotides produced during hydrolysis of native DNA. DNA which had not been cleaved by the enzyme was precipitated with 0.5 M $HClO_4$, and the amount of acid-soluble products (to 8-12 nucleotides) in the supernatant fluid was determined at 260 nm [19]. The incubation mixture (100 μl) contained the appropriate buffer, 16 μg DNA of salmon milt, and 1-10 μl of the enzyme preparation of various concentrations (method 2). The enzyme amount which catalyzed the production of 1 optical unit (260 nm) of oligonucleotides by DNA hydrolysis for 1 h at 37°C was taken as the activity unit of DNase. Activities of the isolated enzymes were determined using the reaction mixtures of the following composition: for A-DNase: 40 mM sodium acetate buffer (pH 5.5), 1 mM EDTA; for Ca,Mg-DNase: 5 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 5 mM $MgCl_2$; for Ca,Mn-DNase: 10 mM Tris-HCl (pH 8.5), 2 mM $CaCl_2$, 3 mM $MnCl_2$. The concentration of DNA was determined by absorption at 260 nm, the protein concentration was determined as described in [20].

Purification of DNases. The purification procedures were performed at 4°C. Spermatozoa (7 g) were lysed in a glass homogenizer on an ice bath in buffer T (10 mM Tris-HCl (pH 8.0)) in the presence of 1 M NaCl, 1 mM DTT, 1 mM EDTA, 0.5% Triton X-100, and 0.5 mM PMSF. The resulting lysate was clarified by centrifugation at 14,000*g* for 20 min and then dialyzed against buffer T. After dialysis, the lysate was clarified once more by centrifugation at 14,000*g* for 20 min and then applied onto a column (2.5 × 15 cm) with DEAE-cellulose DE-52 preliminarily equilibrated with T buffer. The column was washed with T buffer (300 ml), and the enzymes were eluted with a gradient of 0-0.6 M NaCl (2 × 350 ml) in T

buffer (pH 7.5). The DNase activities were determined in all fractions. The fractions containing the activities of A-DNase and Ca,Mn-DNase (0.07-0.16 M NaCl) were combined (Fig. 1a, activity peak *1*), supplemented with ammonium sulfate to the concentration of 1.8 M, and applied onto a column (1.1 × 8.5 cm) with phenyl-Sepharose equilibrated with buffer TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) containing 1.8 M ammonium sulfate. A-DNase was eluted with 0.9 M ammonium sulfate in buffer TE. Ca,Mn-DNase was eluted with a linear gradient of 0.8-0.3 M ammonium sulfate (2 × 40 ml) in buffer T (Fig. 1b). Each fraction was dialyzed against buffer T (pH 7.5); the enzymatic activities in the resulting fractions were determined by method 1. The fractions containing the activities of A-DNase (0.9 M ammonium sulfate) (Fig. 1b, activity peak *1*) and of Ca,Mn-DNase (0.7-0.6 M ammonium sulfate) (Fig. 1b, activity peak *2*) were combined, dialyzed, concentrated, and applied onto Superdex 75 HR 10/30 columns equilibrated with 0.1 M NaCl in 10 mM phosphate buffer (pH 7.2). The proteins were eluted with the same buffer. Yields of the enzymes in the resulting fractions were determined by analysis of hydrolysis products of phage λ DNA by method 1. The fractions containing DNase activities were combined, dialyzed, concentrated, introduced into 40% glycerol, and stored at −20°C.

The fractions with Ca,Mg-DNase obtained by elution from DEAE-cellulose (0.2-0.28 M NaCl) (activity peak *2*) were combined, supplemented with ammonium sulfate to the concentration of 1.8 M, and applied onto a

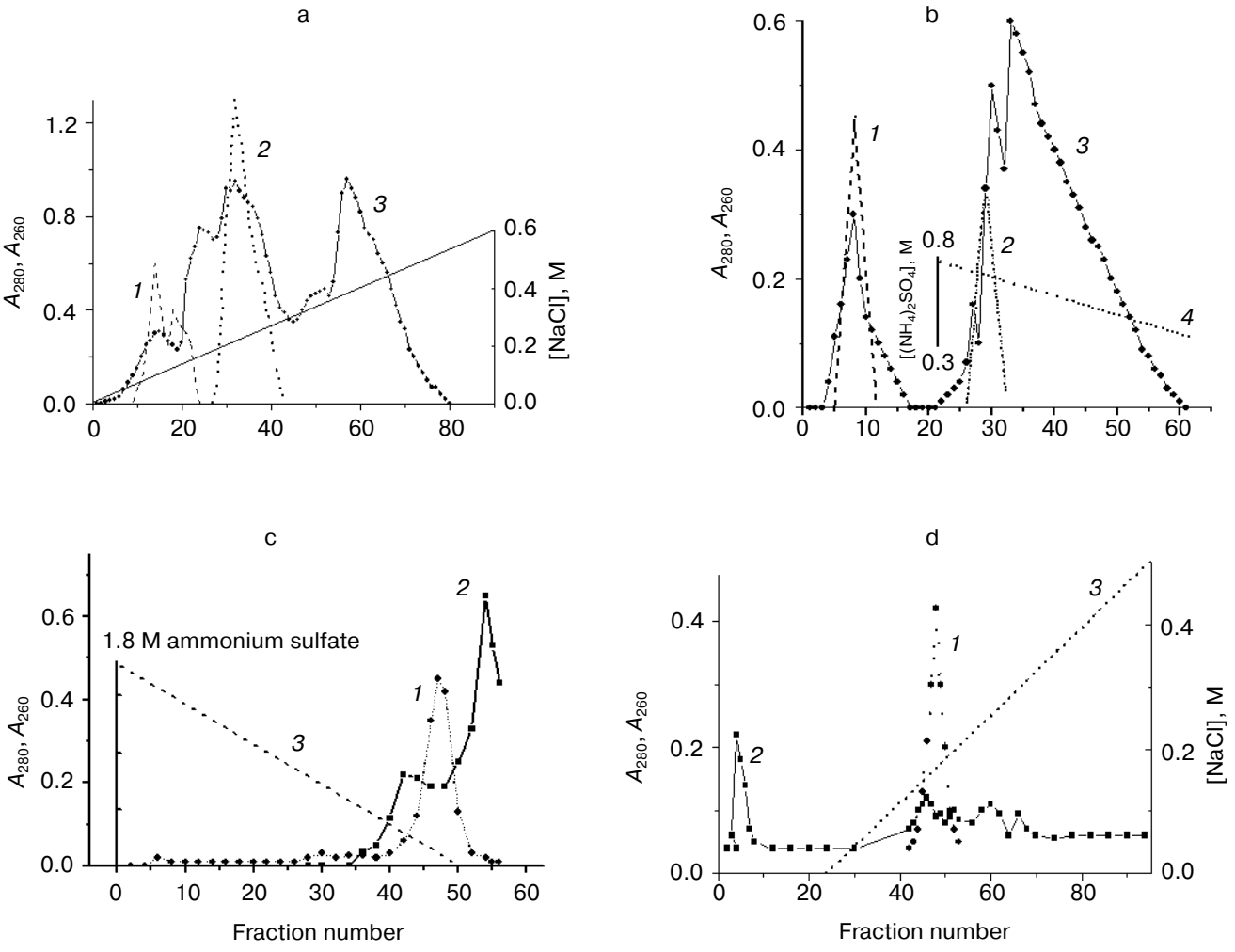

Fig. 1. Elution profile of DNases from sea urchin spermatozoa on DEAE-cellulose (a), phenyl-Sepharose (b, c), and Source 15Q PE (d). a) Activities of A-DNase and Ca,Mn-DNase (peak *1*) (*1*), Ca,Mg-DNase (peak *2*) (*2*), protein yield at 280 nm (*3*). b) Activities of A-DNase (peak *1*) (*1*) and Ca,Mn-DNase (peak *2*) (*2*), protein yield at 280 nm (*3*), concentration gradient of $(NH_4)_2SO_4$ (*4*). c) Activity of Ca,Mn-DNase (*1*), protein yield at 280 nm (*2*), concentration gradient of ammonium sulfate (*3*). d) Activity of Ca,Mg-DNase (*1*), protein yield at 280 nm (*2*), concentration gradient of NaCl (*3*). The enzyme activities were determined at 260 nm by increase in the amount of acid-soluble oligonucleotides resulting during hydrolysis of high-molecular-weight DNA.

column (1.1 × 8.5 cm) with phenyl-Sepharose equilibrated with 1.8 M ammonium sulfate in buffer TE; the enzyme was eluted with a linear gradient of ammonium sulfate (1.8-0 M) in buffer T (2 × 40 ml). The enzymatic activity in the fractions was determined by method 2 (Fig. 1c). The fractions containing the DNase activity were combined and concentrated on a column with phenyl-Sepharose (column volume 1.5 ml), and the protein was eluted with buffer T. The resulting preparation of Ca,Mg-DNase was applied onto a Source 15Q 4.6/100 column equilibrated with 10 mM phosphate buffer (pH 7.2). The protein was eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. The yield of the enzyme was determined by method 2. The fractions containing the DNase activity (0.15-0.2 M NaCl) (Fig. 1d) were combined, dialyzed, concentrated, and applied onto a Superdex 75 HR 10/30 column equilibrated with 10 mM phosphate buffer (pH 7.2) containing 0.1 M NaCl. The protein was eluted with the same buffer. The yield of the enzyme in the resulting fractions was determined by method 1.

Determination of catalytic properties of the enzymes. The optimum pH values of activities of the isolated enzymes were determined using the following buffer solutions: sodium acetate (pH 4.0-6.2); imidazole-HCl (pH 6.2-7.8); Tris-HCl (pH 7.1-9.2). The effects of dithiothreitol, N-ethylmaleimide, iodoacetic acid, and G-actin were determined after the preliminary incubation of these reagents (20 min) with the enzyme preparation in the corresponding concentrations. The effects of ionic strength and different bivalent metal cations on activities of the isolated enzymes were determined under their appropriate optimum conditions.

Determination of molecular masses of the enzymes. Molecular masses of the isolated enzymes were determined by gel filtration on Superdex 75HR columns which were preliminarily calibrated with standard proteins in 10 mM phosphate buffer (pH 7.2) containing 0.1 M NaCl.

Determination of the enzyme location. The nuclei were isolated from spermatozoa using our modification of the method described in [21]. The freshly isolated spermatozoa (1 g) were washed twice with buffer A (10 mM Tris-HCl (pH 7.5), 3 mM $CaCl_2$, 0.3 mM PMSF, 0.25 M sucrose) by centrifugation at 2000*g* for 10 min. The precipitate was resuspended in buffer A, homogenized in a glass homogenizer in an ice bath, and precipitated at 2000*g* for 10 min. This precipitate was dissolved in four volumes of buffer A in the presence of 1% Triton X-100, homogenized again in a glass homogenizer, and kept at 4°C for 30 min. The resulting nuclei of spermatozoa were washed twice with buffer A, precipitated at 2000*g* for 10 min, and then lysed in 10 mM Tris-HCl buffer (pH 7.5) in the presence of 1 mM EDTA, 1 mM DTT, 0.3 mM PMSF, 0.5% Triton X-100, and 1 M NaCl. The lysate of the nuclei was clarified by centrifugation at 15,000*g* for 40 min. The resulting preparation was dialyzed and used for determination of the enzyme activities with DNA of λ phage by method 1.

The enzyme location was also determined by electrophoresis of the lysates in 14% polyacrylamide gel containing DNA polymerized into it (100 μg/ml) (the SDS-PAGE-DNA method) as described earlier [22]. Because heating to 100°C completely denatures Ca,Mg-DNase, the enzyme samples under study before placing onto the gel were kept at 25°C for 30 min in the presence of 0.1% SDS. After separation of the proteins, SDS was washed off the polyacrylamide gel with 10 mM Tris-HCl buffer (pH 7.5) at 37°C by three changes of the buffer (each session, 1 h) with gentle mixing. Then the gel was placed into the reaction mixture to detect Ca,Mg-DNases and incubated for 16-24 h at 37°C. The DNA uncleaved inside the gels was stained with ethidium bromide and photographed under UV irradiation.

Determination of involvement of Ca,Mg-DNase in the *in vivo* internucleosomal cleavage of chromosomal DNA. Freshly prepared spermatozoa (10^6 cells) were incubated at 37°C for 1 h in 100 μl of natural seawater. The spermatozoa were lysed by addition of the equal volume of 20 mM Tris-HCl buffer (pH 7.5), 20 mM EDTA, 2% SDS. The resulting lysates were deproteinized with the same volume of mixture phenol–chloroform (1 : 1 v/v) (chloroform was supplemented with isoamyl alcohol, 24 : 1 v/v) and then centrifuged for 6 min in a microcentrifuge. An alternative treatment of samples with proteinase K and RNase A was not markedly more advantageous; therefore, later we used just the phenol method. The resulting samples were analyzed by electrophoresis in 1.2% agarose gel. The DNA uncleaved inside the gels was stained with ethidium bromide and photographed under UV irradiation.

RESULTS

Purification of DNases from sea urchin spermatozoa. Shaking of sea urchins to stimulate the release of the sex cells initiated the leaving only of mature spermatozoa. The viability of the spermatozoa was monitored with a microscope by their mobility and by their ability to fertilize mature oocytes of sea urchin. As a rule, our collection of sea urchin spermatozoa ensured 98% fertilization of the mature oocytes. The double washing of the spermatozoa with sterile seawater removed the seminal plasma, while the spermatozoa remained intact. The use of 1 M NaCl and 0.5% Triton X-100 as a lysing mixture provided the maximal specific activities of the enzymes under study in the cell lysates.

Chromatography of the specimens on a column with DEAE-cellulose allowed us to eliminate nucleotide substances and most concomitant proteins, and to separate Ca,Mg-DNase by elution with 0.2-0.28 M NaCl from Ca,Mn-DNase and A-DNase eluted with 0.07-

0.16 M NaCl (Fig. 1a). The use of phenyl-Sepharose in the second stage of the purification allowed us to separate acid DNase eluted with 0.9 M ammonium sulfate from Ca,Mn-DNase eluted with 0.7-0.6 M ammonium sulfate (Fig. 1b). Ca,Mg-DNase was eluted from phenyl-Sepharose with 0.2-0 M ammonium sulfate (Fig. 1c) that resulted in tenfold purification of the enzyme. The subsequent high performance ion-exchange chromatography on a Source 15Q PE column resulted in removal of many basic concomitant proteins and in preparation of Ca,Mg-DNase with specific activity of 5500 units/mg. The yield of the enzyme was determined in the region of 0.15-0.2 M NaCl (Fig. 1d). After gel filtration on Superdex 75 HR columns, the specific activities of the three enzymes were significantly increased: that of A-DNase to 4700, that of Ca,Mg-DNase to 11,000, and the activity of Ca,Mn-DNase to 70 units/mg. The results of purification of the three DNases are presented in Table 1.

Because the activity of Ca,Mn-DNase was low compared to the high activities of Ca,Mg-DNase and A-DNase, it was impossible to assess the yield of this enzyme in the first stages of purification. Therefore, the table lacks data on the specific activity of Ca,Mn-DNase in the extract and after the chromatography on a column with DEAE-cellulose. Thus, the purification scheme used provided the isolation of three individual DNases without admixtures of other nucleases.

Determination of molecular masses. The molecular masses of Ca,Mg-DNase, Ca,Mn-DNase, and A-DNase determined by gel filtration were 63, 25, and 37 kD, respectively.

pH optimum of the enzyme activities. The maximum activity of Ca,Mg-DNase in the presence of 1 mM $CaCl_2$ and 5 mM $MgCl_2$ was found at pH 7.5 (Fig. 2). In the presence of only Ca^{2+}, Mg^{2+}, or Co^{2+} the enzyme activity was low. Note that on addition of Mn^{2+} the enzyme activity was ~30% and, as in the presence of Co^{2+}, the pH optimum was displaced to acidity and became less pronounced. Thus, the pH optimum of Ca,Mg-DNase significantly depended on the kind of activating bivalent cations. Ca,Mn-DNase had no clear pH optimum; it was active over a wide range of pH from 6.5 to 9.5, with a slight increase at pH 8.5. The pH optimum of acid DNase was characterized by a clear activity peak at pH 5.5 (Fig. 3).

Effects of bivalent metal ions. Mg^{2+}, Ca^{2+}, and Mn^{2+} had weak activating effects on Ca,Mg-DNase and Ca,Mn-DNase (Table 2). However, in the concurrent presence and at the strictly determined ratio of Ca^{2+} and Mg^{2+} or Ca^{2+} and Mn^{2+} the activities of the enzymes were maximal with a clear synergism. In this case the activity of Ca,Mg-DNase and of Ca,Mn-DNase was increased 14-fold and more than 5-fold, respectively. Consequently, the enzymes isolated were Ca^{2+},Mg^{2+}- and Ca^{2+},Mn^{2+}-dependent DNases. Note, that the activity of Ca,Mg-

Table 1. Purification of DNases from spermatozoa of the sea urchin *S. intermedius*

Stage of purification	DNase	Protein, mg	Specific activity, unit/mg	Yield, %
Extract	A-DNase	142	5.5	100
	Ca,Mg-DNase	142	15.2	100
	Ca,Mn-DNase	142	—	—
DEAE-cellulose	A-DNase	11	50	70
	Ca,Mg-DNase	10.5	118	66
	Ca,Mn-DNase	11	—	—
Phenyl-Sepharose	A-DNase	0.13	1900	32
	Ca,Mg-DNase	0.71	1400	45
	Ca,Mn-DNase	0.35	20	—
Sourse 15Q PE	Ca,Mg-DNase	0.11	5500	27
Superdex 75 HR	A-DNase	0.035	4700	21
	Ca,Mg-DNase	0.023	11000	11
	Ca,Mn-DNase	0.013	70	—

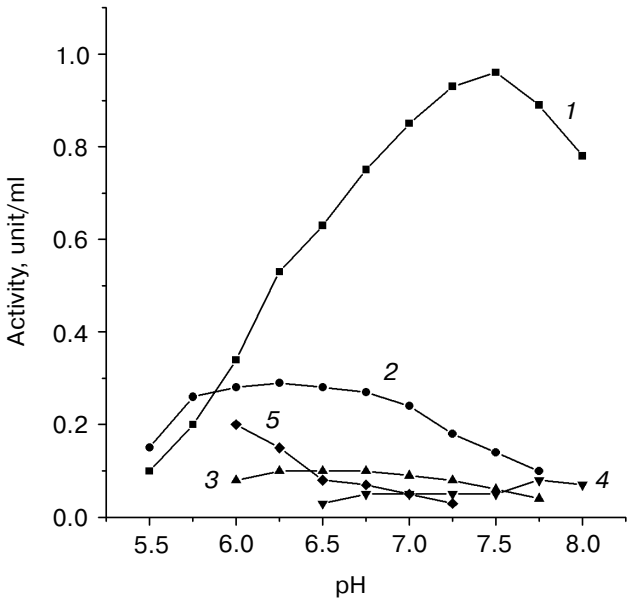

Fig. 2. Activity dependence of Ca,Mg-DNase on pH of the buffer in the presence of 5 mM bivalent metal ions: Mg^{2+} + 1 mM Ca^{2+} (*1*), Mn^{2+} (*2*), Mg^{2+} + 0.5 mM EGTA (*3*), Ca^{2+} (*4*), Co^{2+} (*5*). For the determination of activity see the legend to Fig. 1.

DNase was strongly inhibited by Zn^{2+}. The initial activity of Ca,Mg-DNase was 50% inhibited by 10^{-6} M zinc ions that was 50 times less than was required for inhibition of Ca,Mn-DNase ($5 \cdot 10^{-5}$ M).

A-DNase from sea urchin spermatozoa was not activated by bivalent metal ions, and some ions inhibited the enzyme (Table 3). The enzyme activity was mainly inhibited by magnesium and zinc ions. A weak activation of the enzyme in the presence of EDTA seemed to be due to its ability to bind bivalent metal cations in the reaction mixture.

Effect of ionic strength. The dependence of the activities of DNases on ionic strength was a specific feature of these enzymes. The greatest was the effect of NaCl on the activity of Ca,Mn-DNase (Fig. 4). In the presence of 75 mM NaCl the enzyme activity was decreased by 50%. The activities of Ca,Mg-DNase and A-DNase were decreased by 50% in the presence of 175 and 150 mM NaCl, respectively. It should be specially noted that the retention in natural seawater of the enzymatic activity by Ca,Mg-DNase from sea urchin spermatozoa, which is a typical metal-dependent enzyme, was an unusual property. However, Ca,Mn-DNase and A-DNase were inactive in natural seawater.

Effects of inhibitors. The reaction of DNases to specific inhibitors is a significant characteristic of these enzymes that determines its classification. Thus, iodoacetic acid which is a specific inhibitor of a certain group of acid DNases II decreased by 50% the activity of A-DNase from spermatozoa but at a relatively high concentration of the inhibitor (0.4 mM) (Table 4). The polyamine spermidine, which affects the conformation of DNA, inhibited the activity of A-DNase more strongly than the activity of Ca,Mg-DNase: A-DNase was 50% inhibited at the spermidine concentration 1.6-fold lower than the concentration inhibiting Ca,Mg-DNase. Note that G-actin from rabbit muscles, which fails to inhibit the activities of Ca^{2+},Mg^{2+}-dependent DNases [3], also had no effect on the Ca,Mg-DNase from sea urchin spermatozoa. N-Ethylmaleimide, which blocks sulfhydryl groups in the active site of some enzymes and thus inhibits their activities, had no effect on Ca,Mg-DNase from spermatozoa. A preincubation of the enzyme with dithiothreitol which promotes the recovery of disulfide bonds in protein molecules did not change the enzyme activity. Thus, sulfhydryl groups and disulfide bonds were not functionally important for the activity of Ca,Mg-DNase. But it is necessary to note a high sensitivity of A-DNase to N-ethylmaleimide, which suggests the presence of free sulfhydryl groups in this enzyme.

Action mechanism. The mechanism of enzymatic cleavage of duplex DNA allows us to more definitely discuss the *in vivo* function of enzymes. The hydrolysis kinetics of the DNA pBR322 by the isolated enzymes was studied, and it was suggested that Ca,Mg-DNase and A-DNase should cleave the supercoiled form of DNA without accumulation of form II. Significant amounts of form III DNA and of shorter fragments were produced even with the presence in the incubation medium of a certain amount of the initial form I of DNA (Fig. 5). Consequently, the enzymes acted by the single-hit mech-

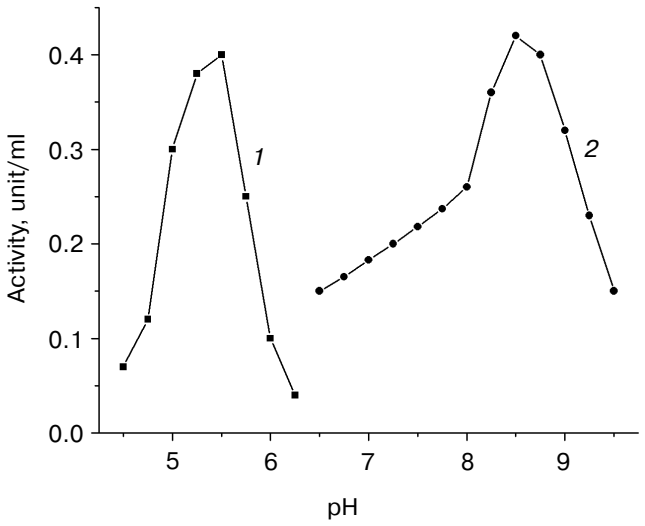

Fig. 3. Dependence of activities of A-DNase (*1*) and Ca,Mn-DNase (*2*) on pH of the buffer under the optimum conditions. For the determination of activity see the legend to Fig. 1.

Table 2. Effects of bivalent metal ions on activities of Ca,Mg-DNase and Ca,Mn-DNase from spermatozoa of the sea urchin *S. intermedius*

Metal ions	Concentration, mM	Activity of DNases*, %	
		Ca,Mg-DNase	Ca,Mn-DNase
(0.5 mM EGTA) + Mg^{2+}	1	—	10
	2	—	6
	3	6	—
	5	7	—
Ca^{2+}	2	—	0
	5	5	—
Mn^{2+}	1	8	10
	3	15	18
1 mM Ca^{2+}+Mg^{2+}	3	82	—
	5	100	—
2 mM Ca^{2+}+Mg^{2+}	3	—	73
	4	—	70
2 mM Ca^{2+}+Mn^{2+}	1	15	80
	2	15	100
(Mg^{2+} + Ca^{2+})** + Zn^{2+}	0.001	59	—
	0.002	18	—
	0.01	0	—
(Ca^{2+} + Mn^{2+})** + Zn^{2+}	0.01	—	83
	0.05	—	43
	0.1	—	21

* Relative to the maximum activity under the optimum conditions (see "Materials and Methods").
** Optimal concentrations of Ca^{2+} + Mg^{2+} or Ca^{2+} + Mn^{2+}.

Table 3. Effects of some ions and reagents on the activity of A-DNase from spermatozoa of sea urchin *S. intermedius*

Ions, reagents	Concentration, mM	Activity*, %
Without additions	—	85
EDTA	1.0	100
Ca^{2+}	5.0	98
Mn^{2+}	5.0	94
Mg^{2+} + Ca^{2+}	5.0 + 1.0	68
Mg^{2+} + EGTA	5.0 + 0.1	68
Zn^{2+}	3.0	45
Sodium acetate	175	50

* The activity was determined in 40 mM sodium acetate buffer (pH 5.5).

Table 4. Effects of inhibitors on the activities of Ca,Mg-DNase and A-DNase from spermatozoa of the sea urchin *S. intermedius*

Inhibitor	Concentration	Activity of DNases*, %	
		Ca,Mg-DNase	A-DNase
Iodoacetic acid	0.2 mM	100	65
	0.4 mM	100	53
	0.8 mM	96	23
	1.2 mM	95	15
	2.0 mM	93	6
Spermidine	0.1 mM	84	81
	0.15 mM	73	31
	0.2 mM	51	4
	0.3 mM	14	2
G-Actin	15 μg/ml	100	95
	100 μg/ml	100	70
N-Ethylmaleimide	0.1 mM	—	50
	0.5 mM	—	20
	1 mM	100	0
	4 mM	100	0

* The activities of DNases were determined under the optimum conditions for the enzyme activities (see "Materials and Methods").

anism and could concurrently cleave both DNA chains without producing single-strand breaks. As a result, both forms I and II (as a rule, the latter is in small amount present in the initial DNA) were immediately transformed into the linear form III with is subsequently cleaved into smaller fragments.

The cleavage kinetics of the DNA pBR322 by Ca,Mn-DNase suggested another action mechanism of this enzyme. At first the cleavage of the supercoiled DNA was accompanied by accumulation of both form II and form III, then their amounts decreased with an increase in the number of shorter fragments. Obviously, during the hydrolysis single-strand breaks in the opposite DNA chains were accumulated and overlapped, and this resulted in conversion of form II into the linear form III with subsequent cleavage into shorter fragments. Thus, the cleavage of two DNA chains by the enzyme occurred by the double-hit mechanism.

For comparison, we present the cleavage kinetics of the supercoiled DNA by pancreatic DNase I, which is a

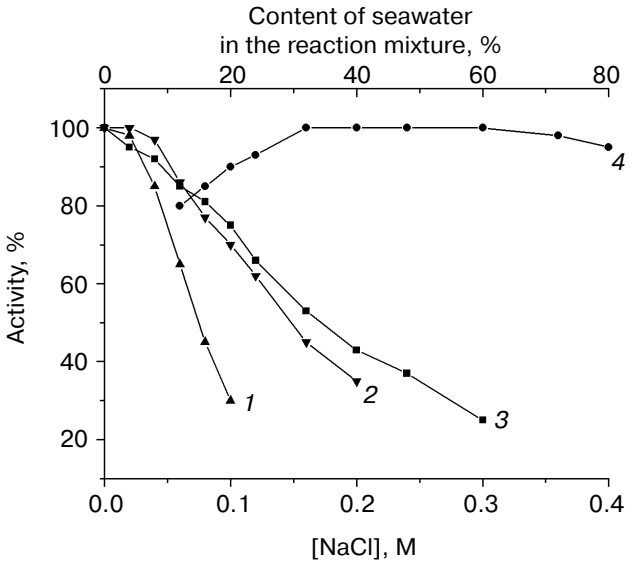

Fig. 4. Dependence of activities of Ca,Mg-DNase, Ca,Mn-DNase, and A-DNase on the concentration of NaCl under the optimum conditions and the effect of natural seawater on the activity of Ca,Mg-DNase: *1*) Ca,Mn-DNase; *2*) A-DNase; *3*) Ca,Mg-DNase; *4*) the activity of Ca,Mg-DNase in natural seawater. For the determination of activity see the legend to Fig. 1.

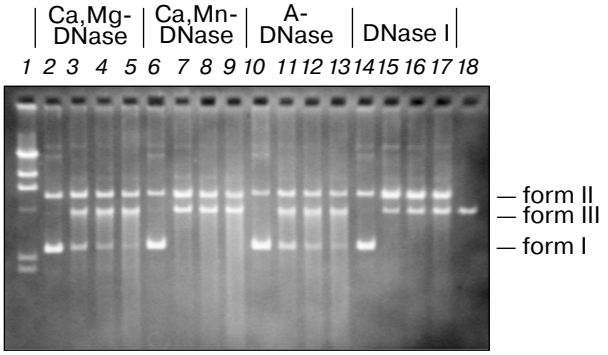

Fig. 5. Hydrolysis kinetics of DNA pBR322 by Ca,Mg-DNase, Ca,Mn-DNase, and A-DNase from sea urchin spermatozoa and by DNase I under the optimum conditions: *1*) hydrolyzate of λ phage DNA by restrictase *Hind*III; *2*, *6*, *10*, *14*) DNA without the enzyme (control); *3*, *7*, *11*, *15*) 10 min of incubation; *4*, *8*, *12*, *16*) 20 min of incubation; *5*, *9*, *13*, *17*) 30 min of incubation; *18*) hydrolyzate of DNA pBR322 with restrictase *Eco*RI.

typical DNase with the double-hit action mechanism [23]. Figure 5 shows the accumulation of form II DNA along with an insignificant increase in the amount of form III, because for production of form II a single break is required, while for conversion into the linear DNA form and shorter fragments a significant number of single-strand breaks in opposite chains are necessary.

Location of the enzymes. On determination of DNase activities in the lysate of nuclei from sea urchin spermatozoa by method I, Ca,Mg-DNase was detected in the nuclei, while no A-DNase was found there (data not presented). The determination of the activities in polyacrylamide gel after the separation of protein preparations in SDS-PAGE-DNA also revealed the presence of Ca,Mg-DNase in the nuclei (Fig. 6). Note that the mobility of the protein band with the activity of Ca,Mg-DNase isolated from spermatozoa by purification was the same as the mobility of the band which characterized the enzyme activity in the nuclear lysate. These data suggested that Ca,Mg-DNase is located in the nucleus and that A-DNase is an extranuclear protein.

Involvement of Ca,Mg-DNase in the *in vivo* internucleosomal cleavage of chromosomal DNA. On incubation of sea urchin spermatozoa in seawater, their chromosomal DNA was cleaved by endogenous DNase with production of DNA fragments with periodically decreasing molecular masses (Fig. 7). The degree of hydrolysis increased with time that showed this cleavage to be enzymatic, and 0.5 mM $ZnCl_2$ inhibited this process. Because chromosomal DNA of spermatozoa was hydrolyzed in natural seawater, i.e., under conditions of the maximum activity of Ca,Mg-DNase, it was suggested that endogenous nuclear Ca,Mg-DNase of spermatozoa should be responsible for the specific cleavage of DNA. A significant excess *in vivo* of the sensitivity threshold of the enzyme to Zn^{2+} as compared to the data presented in Table 2 seemed to be due to the enzyme action *in vivo* in natural seawater. The use of reference DNA with periodicity of 100 base pairs (bp) allowed us to assess molecular masses of the resulting DNA fragments. Molecular

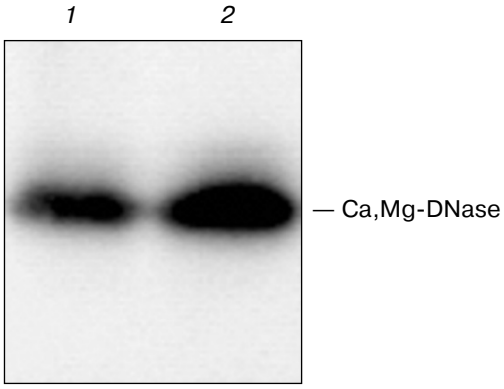

Fig. 6. Determination of DNase activities in SDS-PAGE-DNA after electrophoresis of the nuclear lysate (*1*) and of Ca,Mg-DNase isolated from spermatozoa by purification (*2*).

masses of the fragments of the chromosomal DNA (lanes *1-4*) corresponded to 260, 520, 760, and 1040 bp, respectively. This is in agreement with literature data that the nucleosome of sea urchin spermatozoa contains 260 ± 26 bp of DNA [24]. Consequently, Ca,Mg-DNase was responsible for cleavage of the chromosomal DNA at internucleosomal linker regions.

DISCUSSION

Thus, we have succeeded in isolation from sea urchin spermatozoa of three DNases with different enzymatic and physicochemical properties. The properties of Ca,Mg-DNase are especially interesting. On one hand, the synergism in increasing the activity in the presence of both Ca^{2+} and Mg^{2+} and the resistance of this enzyme to sulfhydryl reagents resemble DNases from the group of the pancreatic DNase I. Unlike Ca,Mg-DNase from spermatozoa, enzymes of this group are not nuclear proteins but are located in the endoplasmic reticulum of cells of secretory organs, are glycoproteins with molecular mass of 30-35 kD, exist in several isoforms with p*I* 3.5-4.5, and are inhibited by G-actin [14, 25, 26]. On the other hand, Ca,Mg-DNase from spermatozoa cannot be assigned to the group of so-called "Ca^{2+},Mg^{2+}-dependent" DNases which have been found in the nuclei of cells of various tissues. DNases of this group are inhibited by SH-reagents, polyamines (spermine and spermidine), but are not inhibited by G-actin, and are basic proteins with molecular mass of 25-45 kD [3, 27, 28]. The totality of such properties of Ca,Mg-DNase of spermatozoa, as the location in the nucleus, the resistance to G-actin and N-ethylmaleimide, the inhibition by spermidine, and also p*I* < 7 (by behavior on DEAE-cellulose) allows us to assign this enzyme to another, still not numerous group of nuclear Ca^{2+},Mg^{2+}-dependent DNases isolated from sea urchin embryos [29], human spleen lymphocytes [30], and hepatopancreas of prawn and crab [31, 32]. Unlike the main group of Ca^{2+},Mg^{2+}-dependent DNases, the enzymes of this group are acidic proteins with molecular mass of 45-57 kD and are not inhibited by N-ethylmaleimide. An unusual feature of the two Ca^{2+},Mg^{2+}-dependent DNases of this group isolated from embryos [29] and spermatozoa of sea urchin *S. intermedius* should be especially noted: they retain the maximum enzymatic activity in natural seawater the ionic strength of which corresponds to NaCl concentration of 0.45 M. The internucleosomal cleavage of nuclear DNA of spermatozoa by Ca,Mg-DNase in seawater is the most significant biochemical manifestation of involvement of DNases in apoptosis. The inhibition by zinc ions of the internucleosomal cleavage of nuclear DNA by Ca,Mg-DNase found by us is another biochemical manifestation which suggests the involvement of this enzyme in apoptosis [7, 28].

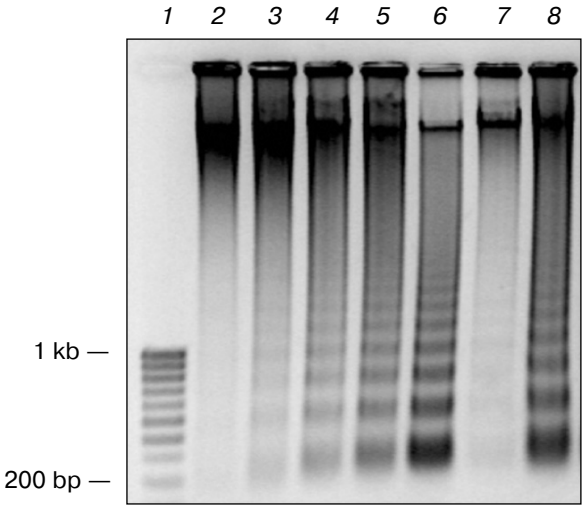

Fig. 7. Cleavage kinetics of chromosomal DNA from spermatozoa by endogenous Ca,Mg-DNase in seawater at 37°C and the effect of zinc ions: reference DNA (*1*), without incubation (*2*), on incubation for 5 (*3*), 15 (*4*), 30 (*5*), and 60 min (*6*); incubation for 60 min in the presence of 0.5 mM (*7*) and 0.1 mM $ZnCl_2$ (*8*).

Some Ca^{2+},Mg^{2+}-dependent DNases involved in the cleavage of chromosomal DNA during apoptosis should also be included into the group of nuclear DNases. Such is the DNase γ the activity of which is inhibited by Zn^{2+} but not inhibited by G-actin, and which can be detected in the cell nuclei of rat spleen, thymus, liver, and kidneys after initiation of apoptosis [33]. Enzymes similar to the DNase γ are also found in frog hemopoietic cells [34]. Enzymes isolated from the bovine seminal plasma [3] and the recombinant Ca^{2+},Mg^{2+}-dependent DNase DNAS1L3 [7] can also be included in this group of nuclear DNases.

Thus, on consideration that Ca,Mg-DNase of spermatozoa is a nuclear enzyme with high activity and its properties significantly coincide with those of the enzymes involved in apoptosis, it was suggested that the single-hit action mechanism of this DNase should be realized at the stage of internucleosomal fragmentation of DNA in the course of apoptotic negative selection of sex products during spermatogenesis. It was also suggested that the incubation of spermatozoa in seawater should model the natural processes when spermatozoa released into seawater retain for 17 h their ability to fertilize and then seemed to be eliminated by apoptosis because by this time their metabolism had changed.

Ca,Mn-DNase is another enzyme isolated from sea urchin spermatozoa, and its properties are significantly different from those of Ca,Mg-DNase. The presence of

several DNases in the cell is rather common. Thus, in nuclei of rat thymocytes four endonucleases were found [35] which varied in the specific effects on native and denatured DNA and also on the linker region of chromatin nucleosomes. Four nucleases were isolated from cell nuclei of rat liver: two Ca^{2+},Mg^{2+}-dependent, Mn^{2+}-dependent, and acid DNases [36]. These Ca^{2+},Mg^{2+}-dependent DNases had different molecular masses and displayed different synergism in the presence of Ca^{2+} and Mg^{2+}.

Ca,Mn-DNase of spermatozoa differs from Ca,Mg-DNase by degree of activation with Mg^{2+} or Mn^{2+} in the presence of Ca^{2+}, by degree of inhibition by Zn^{2+}, and by the mechanism of cleavage of double-stranded DNA; it has a significantly lower molecular mass and is inactive in seawater. Because of a low content of Ca,Mn-DNase in spermatozoa and its low specific activity, we failed to isolate this enzyme in sufficient amount and to determine the effect of inhibitors. However, the findings suggest that by enzymatic properties and molecular mass Ca,Mn-DNase of spermatozoa is most like endonucleases isolated from nuclei of human, calf, and rat thymus [27]. Similar to the enzyme from spermatozoa, these DNases display the maximum activity in medium which contains both Ca^{2+} and Mn^{2+}. Ca^{2+} and Mg^{2+} activate the enzymes significantly more weakly, although according to tradition authors have assigned them to Ca^{2+},Mg^{2+}-dependent DNases. These DNases are virtually inactive in medium containing only Ca^{2+} or Mg^{2+}. Similar to the enzymes isolated from nuclei of human, calf, and rat thymus [27], Ca,Mn-DNase from spermatozoa is active in over a wide range of pH from 6.0 to 8.5 with a slightly pronounced optimum at pH 8.0-8.5; addition of 100 mM NaCl decreases by 70-80% the activities of these endonucleases. Their molecular masses are in the range of 20-30 kD [27]. However, DNases from chromatin of the human, calf, and rat thymus cells are basic proteins, while Ca,Mn-DNase from sea urchin spermatozoa is an acidic protein. Unfortunately, in most studies on Mn^{2+}-dependent DNases [37, 38] no data are presented about the combined effect of Ca^{2+} and Mn^{2+}, although Ca^{2+} plays a significant role in the most important regulatory processes of cell metabolism.

The functions of Ca^{2+},Mg^{2+}-dependent and acid DNases including their involvement in apoptosis are rather sufficiently studied, while there are only a few studies of the functional role of Ca^{2+},Mn^{2+}- and Mn^{2+}-dependent DNases. Thus, Mn^{2+}-dependent DNase of rat liver chromatin was shown to be a constituent of the replicative complex and to correspondingly influence the replicative synthesis of DNA [39]. At present, there are no publications about the role of Ca^{2+},Mn^{2+}-dependent DNases as components of apoptosis. However, the wide distribution of Mn^{2+}-dependent DNases and the presence of Ca^{2+},Mn^{2+}-dependent DNases in chromatin of rat and calf thymus [27] suggest the involvement of these enzymes in apoptosis. This can occur, in particular, by the mechanism suggested for mitochondrial DNase G from *Caenorhabditis elegans* which during apoptosis leaves the mitochondria and translocates into the nucleus [40]. It seems that on the first stage of apoptosis when the number of breaks in DNA is low, the double-hit action mechanism of Ca,Mn-DNase of spermatozoa can result in production of high-molecular-weight fragments with molecular masses of 50-300 kD [12]. To make more definite conclusions on the functional role of Ca,Mn-DNase of spermatozoa, further studies are required, in particular, the enzyme location has to be determined.

By majority of features studied, A-DNase isolated from spermatozoa belongs to the widely distributed type of acid DNases II. The enzymes of this type isolated from various sources are mainly different in molecular mass, subunit composition of the enzyme molecule, mode of cleavage of double-stranded DNA, and location in the cell. A-DNase isolated from sea urchin spermatozoa is suggested to be either an acrosomal enzyme or to be located in a perinuclear space. The functional role of acid DNases is under discussion in relation with their location. Some authors believe acid DNases to be involved in apoptosis [8] during which they are translocated from the cytosol into the nucleus [9]. During the post-translational modification of the protein, a 10-kD polypeptide is split off the proenzyme with the molecular mass of 40 kD that results in an active form of the enzyme [41].

The molecular mass of 37 kD found for A-DNase coincides with the molecular mass of the enzymes isolated from salmon oocytes [42], salmon milt [43], mucous membranes of human stomach and cervix uteri [44]. These enzymes consist of two similar subunits, and their molecular masses determined by gel filtration are in the range of 36-38 kD [44]. The non-lysosomal location of A-DNase of sea urchin spermatozoa suggests another functional role of this enzyme in male sex cells as compared to lysosomal enzymes.

It was earlier shown that during spermatogenesis of sea urchin *S. intermedius* accessory cells of the gonad are filled with the sex cells which are resorbed and then completely eliminated [13]. Endogenous A-DNase of spermatozoa seems to be involved in this process together with alkaline DNases, and this finally provides a negative selection of sex cells during spermatogenesis of the sea urchin *S. intermedius*.

The probable location of A-DNase in the perinuclear space of the cell suggests its protective function: it can prevent the penetration of alien DNA into a spermatozoon during its residence in seawater. A protective function of acid DNases was suggested by some authors [45].

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