Journal of Cellular Biochemistry 67:304–315 (1997)

ARTICLES

Identification of a Cysteine Protease Responsible for Degradation of Sperm Histones During Male Pronucleus Remodeling in Sea Urchins

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Abstract We have identified a 60-kDa cysteine protease that is associated with chromatin in sea urchin zygotes. This enzyme was found to be present as a proenzyme in unfertilized eggs and was activated shortly after fertilization. At a pH of 7.8–8.0, found after fertilization, the enzyme degraded the five sperm-specific histones (SpH), while the native cleavage-stage (CS) histone variants remained unaffected. Based on its requirements for reducing agents, its inhibition by sulfhydryl blocking compounds and its sensitivity to the cysteine-type protease inhibitors (2S,3S)-*trans*-epoxysuccinyl-L-leucyl-amido-3-methylbutane-ethyl-ester (E-64 d), cystatin and leupeptin, this protease can be defined as a cysteine protease. Consistently, this protease was not affected by the serine-type protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and pepstatin. The substrate selectivity and pH modulation of the protease activity strongly suggest its role in the removal of sperm-specific histones, which determines sperm chromatin remodeling after fertilization. This suggestion was further substantiated by the inhibition of sperm histones degradation in vivo by E-64 d. Based on these three lines of evidence, we postulate that this cysteine protease is responsible for the degradation of sperm-specific histones which occurs during male pronucleus formation. J. Cell. Biochem. 67:304–315, 1997.

Key words: male pronucleus; cysteine protease; histones; chromatin; sea urchins

Sperm chromatin is tightly packed by a set of nuclear basic proteins that interact with DNA within the sperm nucleus. These proteins are quite diverse, in both structure and composition, ranging from cysteine-rich protamines in mammals to sperm-specific histones in sea urchins [Ausio, 1995]. Following fertilization, the sperm nucleus decondenses and transforms into the male pronucleus, which then fuses with the female pronucleus to re-establish the diploid genome of the embryo. Such decondensation implies a remodeling of sperm chromatin determined by the removal of sperm basic nuclear proteins and their replacement by histones from maternal origin [Ecklund and Levine, 1975; Das et al., 1975; Philpott et al., 1991; Philpott and Leno, 1992; Itoh et al., 1993; Katagiri and Ohsumi, 1994]. It was reported initially in batracians that these protein transitions were promoted by nucleoplasmin, a thermostable pentameric acid protein [Laskey et al., 1978; Earnshaw at al., 1980; Kleinschmidt et al., 1985]. It was further demonstrated that *Xenopus* nucleoplasmin lacks species specificity, since it is able to disassemble human sperm chromatin in vitro [Itoh et al., 1993]. In addition, nucleoplasmin-like molecules have been identified in *Drosophila* embryos, *Spissula solidissima* oocytes and in extracts from the mussel *Mytilus californianus* [Kawasaki et al., 1994; Herlands and Maul., 1994; Rice at al., 1995]. In contrast to the well-documented mediation of nucleoplasmin in sperm chromatin remodeling, the mechanisms responsible for the disappearance of sperm nuclear proteins after their disassembly from sperm chromatin remain essentially obscure.

During the past 5 years, evidence has been accumulating to support the idea that male pronucleus remodeling in sea urchin embryos

Contract grant sponsor: Universidad de Concepción; Contract grant numbers: FONDECYT 1.93.063.1 and 97.31.74-1.2.

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Received 2 May 1997; Accepted 18 June 1997

involves the proteolytic cleavage of proteins associated with the sperm chromatin. Cameron and Poccia [1994] found by using protease inhibitors that non-histone chromosomal degradation is required for male pronucleus formation. In addition, we have previously reported that the complete set of sperm histones were no longer present in zygotes at 30 min postinsemination (p.i.) [Imschenetzky et al., 1991a]. It was suggested that this disappearance could be due to selective proteolysis activated concomitantly with other fertilization-related events. It was also demonstrated that the lost sperm-specific histones (SpH) are replaced by maternal cleavage-stage (CS) histone variants that are forming the chromatin of unfertilized eggs and the initial two cleavage divisions [Green and Poccia, 1985; Imschenetzky et al., 1991a]. These changes in chromatin composition result in differences in chromatin organization that may be followed by micrococcal nuclease digestion studies. Sperm nucleosomes are packed by sperm histone variants (SpH) interacting with 220- to 240-bp DNA fragments [Savic et al., 1981]. Unfertilized egg nucleosomes are organized by CS histone variants that protect 126 bp of DNA [Imschenetzky et al., 1989]. At an intermediate stage of male pronucleus remodeling, the chromatin is formed by nucleoprotein particles containing a subset of SpH, as well as maternally derived CS histone variants [Imschenetzky et al., 1996a]. At final stages of male pronucleus decondensation, the organization of chromatin is similar to the female pronucleus, exhibiting an overall histone composition similar to that of unfertilized eggs and a shortened DNA repeat length, as compared with sperm nucleosomes. Such chromatin organization persists during the initial cleavage divisions [Imschenetzky et. al., 1996a]. Based on these lines of evidence, we have decided to investigate the presence of a proteolytic activity that degrades the five sperm histones in sea urchins shortly after fertilization.

This report describes a novel 60-kDa cysteine protease that was found as a proenzyme in unfertilized eggs and was activated shortly after fertilization. At pH 8.0, the condition found postinsemination of sea urchin eggs [Nishioka and McGwin, 1980; Whitaker and Steinhardt, 1985], this enzyme specifically degrades the five sperm-specific histones (SpH), while the maternally derived CS histone variants remain intact. Consistently, the inhibition of this enzyme blocks the normal degradation of the five SpH that occurs during male pronucleus remodeling.

MATERIALS AND METHODS

Gametes and Zygotes

Sea urchins *Tetrapygus niger* were collected from the bay of Concepcion and maintained at room temperature in an aquarium containing natural seawater under constant aeration. Unfertilized eggs, sperms, and zygotes were obtained as described previously [Imschenetzky et al., 1984, 1986, 1991a].

Isolation of Sperm and CS Histone Variants

SpH and native CS histone variants were isolated from sperm, eggs, and zygotes as described previously [Imschenetzky et al., 1984, 1986]. SpH1 was isolated by exclusion chromatography on Bio Gel P-60, as described by von Holt et al. [1989]. Proteins were analyzed by electrophoresis in one-dimensional 18% (w/v) polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), as described by Laemmli [1970].

Radioactive Labeling of Proteins In Vitro

SpH, SpH1, CS and casein were labeled with ^{14}C-formaldehyde essentially according to the method of Rice and Means [1971]; 0.3 mg of the proteins to be labeled was dissolved in 86 µl of 100 mM Hepes buffer pH 7.5. To this solution, 4.4 µl of ^{14}C-formaldehyde (53 mCi/mmol) and 100 µl of 200 mM sodium cyanoborohydride were added. The mixture was incubated for 2 h at room temperature and dialyzed overnight to remove low-molecular-weight components. The specific activities for the proteins were (methyl-^{14}C)SpH 80,000 cpm/µg, (methyl-^{14}C)SpH1 85,000 cpm/µg, (methyl-^{14}C)CS 65,000 cpm/µg, (methyl-^{14}C)casein 75,000 cpm/µg.

Protease Assay

The extracts were incubated for 2 h at 37°C in 0.05 M phosphate buffer pH 7.5 containing 1 mM DTT, 1 mM EDTA and 0.1 % Triton X-100 to allow proteolysis. Protease activity was determined by measuring the hydrolysis of (methyl-^{14}C)SpH, (methyl-^{14}C)SpH1, (methyl-^{14}C)CS, or (methyl-^{14}C)casein into acid-soluble peptides as described by DeMartino [1981]. Units of activity were expressed as cpm released per min per microgram of protein at 37°C (cpm/min µg pro-

tein at 37°C). The products resistant to the digestion were analyzed by electrophoresis in SDS-PAGE and the radioactivity associated to each electrophoretic band was detected by fluorography as described by Chamberlain [1979].

Activation of the Proenzyme

The proenzyme was activated by preincubation with pepsin as described by Lipps et al. [1996]. Briefly, extracts were preincubated at 37°C for 30 min in the presence of 0.1 mg/ml of pepsin dissolved in 20 mM EDTA, 0.5 M sodium formate pH 3.2, and then assayed for protease activity as described above.

Subcellular Fractionation and Assay of Acid Phosphatase as a Lysosomal Marker Enzyme

Whole homogenates in a buffer 10 mM sodium phosphate pH 6.0 containing 0.4 M NaCl, 2 mM EDTA, and 0.2% Triton X-100 (PSETB) from unfertilized eggs or zygotes harvested 5 min p.i. were used to obtain the chromatin, lysosomal and cytoplasm enriched fractions. The chromatin was isolated from the 3,000*g* pellet and the resulting supernatant was centrifuged at 20,000*g* for 30 min to obtain a pellet enriched in lysosomes and a supernatant corresponding to the cytoplasm. The activity of acid phosphatase, chosen as lysosome marker, was measured in each one of the subcellular fractions with p-nitrophenylphosphate as substrate, as described by Bergmeyer [1974]. One unit of enzyme hydrolyzes 1 µmole of p-nitrophenylphosphate/min at 25°C and pH 5.0.

Chromatin Isolation and Enzyme Purification

The unfertilized eggs and zygotes obtained 5 min p.i. were homogenized in PSETB. The homogenate was filtered through a 40-µm-pore-size plankton network and centrifuged at 3,000*g* for 10 min. The pellet corresponding to the crude chromatin fraction was subsequently resuspended in PSETB and centrifuged at 3,000*g* for 10 min. This step was repeated three times; the resulting chromatin pellet was loaded on 35-ml sucrose gradient (10–40% w/v) containing 10 mM sodium phosphate pH 6.0. The gradients were centrifuged in a Beckman SW 28 rotor for 21.5 h at 28,000 rpm and 4°C; 2.0-ml fractions were collected, and the protein concentration was measured as described by Bradford [1976]. Protease activity was measured in each fraction, using SpH1 as substrate.

The fractions containing protease activity were concentrated and then subjected to gel filtration through a column (2.0 × 80 cm) of Sephadex G-100 equilibrated with 10 mM sodium phosphate buffer pH 6.0. This column was then eluted with the same buffer, 2-ml fractions were collected, and the protein was monitored by measuring the absorbance at 230 nm. Protease activity was determined for each second fraction, using (methyl-^{14}C)SpH1 as substrate. The products of proteolysis were then analyzed by SDS-PAGE and fluorography.

SDS-PAGE Zymography

SDS gels containing 12% (w/v) acrylamide were polymerized in the presence of 0.1% SpH1 before standard SDS-PAGE was carried out. The samples were diluted 1 : 1 in SDS sample buffer and boiled 2 min prior to loading onto gels. After electrophoresis the gel was incubated in 2.5% Triton X-100 for 1.5 h to remove SDS and restore proteolytic activity, rinsed twice in distilled water, and incubated for 24 h at 37°C in 0.05 M buffer phosphate pH 7.5 containing 1 mM DTT, 1 mM EDTA and 0.1% Triton X-100 to allow proteolysis. The gels were stained in a 0.1% solution of Coomassie blue in methanol–acetic acid–water (400 : 70 : 530) overnight and destained in the same solvent. Proteases were visualized as clear bands in a blue background wherever digestion of the copolymerized SpH1 occurred [Vafa and Nishioka, 1995].

SpH Western Immunoblot Analysis

After SDS-PAGE of proteins, the gels was transferred to nitrocellulose membranes and analyzed by Western immunoblots. The procedures followed to produce antibodies against sperm histones and for the electrophoretic transfer, blotting and immunodetection were described previously [Imschenetzky et al., 1991a, 1996a]. One extra lane containing whole SpH or alternatively the native CS histone variants was included in each SDS-PAGE as positive and negative controls, respectively.

RESULTS

Detection of Proteolytic Activity in Sea Urchin Zygotes

The presence of proteolytic activity capable of degrading SpH was investigated in sea urchin sperm, unfertilized eggs, and zygotes that were harvested 5 min p.i. Protein extracts were pre-

pared from gametes and zygotes and their nuclear, cytoplasmic, and lysosomal-peroxisomal fractions were assayed by measuring proteolytic degradation of whole ^{14}-labeled sperm histones (^{14}C-SpH) or ^{14}C-labeled-sperm histone H1 (^{14}C-SpH1). As shown in Figure 1, by using as substrates either ^{14}C-SpH (Fig. 1A) or ^{14}C-SpH1 (Fig. 1B), a proteolytic activity was found that was associated with the nuclear fraction of zygotes harvested 5 min p.i. This activity was undetectable in the cytoplasm or lysosomal-peroxisomal fractions of these zygotes, nor was it present in any of the fractions obtained from either sperm or unfertilized eggs.

To discard the possibility of contamination of the nuclear preparations with lysosomes, the activity of acid phosphatase, used as a lysosome marker enzyme, was determined in each fraction. Figure 2 shows that acid phosphatase activity was almost undetectable in the nuclear preparations, confirming that these extracts were free of lysosome contamination.

To explore the possibility that the proteolytic activity detected in zygotes could be derived from a proenzyme present in unfertilized eggs, the subcellular fractions were preincubated with pepsin in order to activate the putative protease zymogen. The proteolytic activity was then measured in each fraction as determined in materials and methods. The results obtained were compared with those derived from the activated subcellular fractions from zygotes, as well as with those obtained from zygotes and unfertilized eggs before their preincubation with pepsin (Fig. 3). As shown in Figure 3, a proenzyme capable of degrading ^{14}C-SpH1 after activation was found in the nuclear extracts from unfertilized eggs. The activity present in zygotes was increased by the zymogen activation treatment, indicating that the nucleus from zygotes still contained a significant amount of proenzyme. It is also shown that preincubation with pepsin did not produce the activation of any SpH degrading protease in lysosomal-peroxisomal or cytoplasmic extracts, in either unfertilized eggs or zygotes.

In summary, these results demonstrated the presence of a proteolytic activity that degrades sperm histones in the nuclear fraction of sea urchin zygotes. A similar activity was found in unfertilized eggs that was activated by treatment with pepsin, therefore it is present in an inactive form.

Partial Purification and Characterization of the SpH Protease

To characterize the enzyme responsible for the proteolytic activity detected in sea urchin zygotes, the chromatin from zygotes harvested 5 min p.i. was isolated and subjected to a sucrose gradient fractionation (Fig. 4A). The active fractions from the gradient (fractions 11-

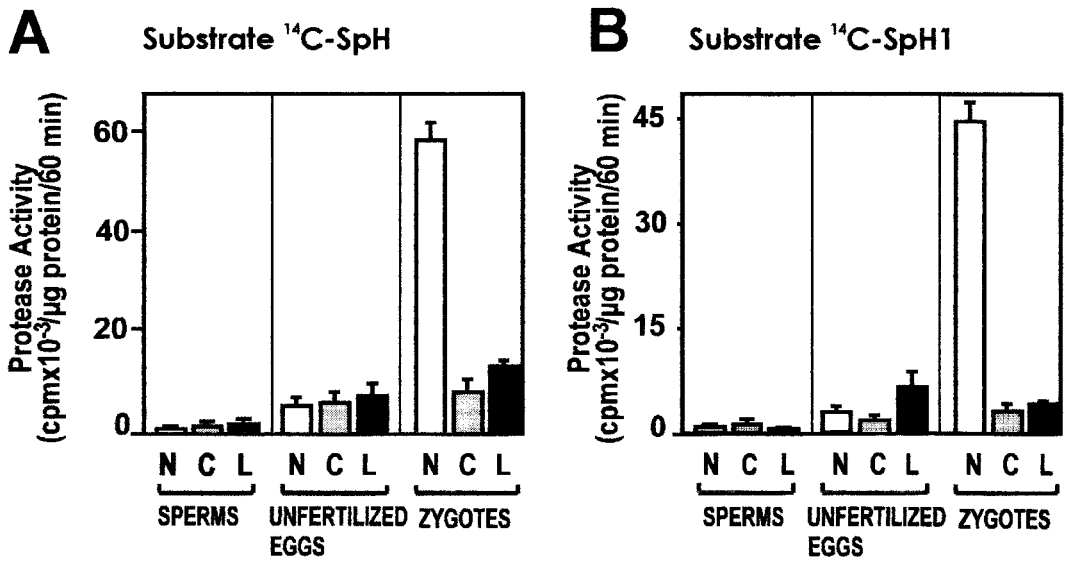

Fig. 1. Detection and subcellular distribution of a proteolytic activity that degrades sperm-specific histones. Protein extracts were prepared from the nuclear (N), cytoplasmic (C), and lysosomal-peroxisomal (L) fractions of sea urchin sperm, unfertilized eggs, and zygotes harvested 5 min postinsemination (p.i.). Protease activity was measured by using labeled substrates: **(A)** total sperm histones (^{14}C-SpH), and **(B)** sperm-specific histone H1 (^{14}C-SpH1).

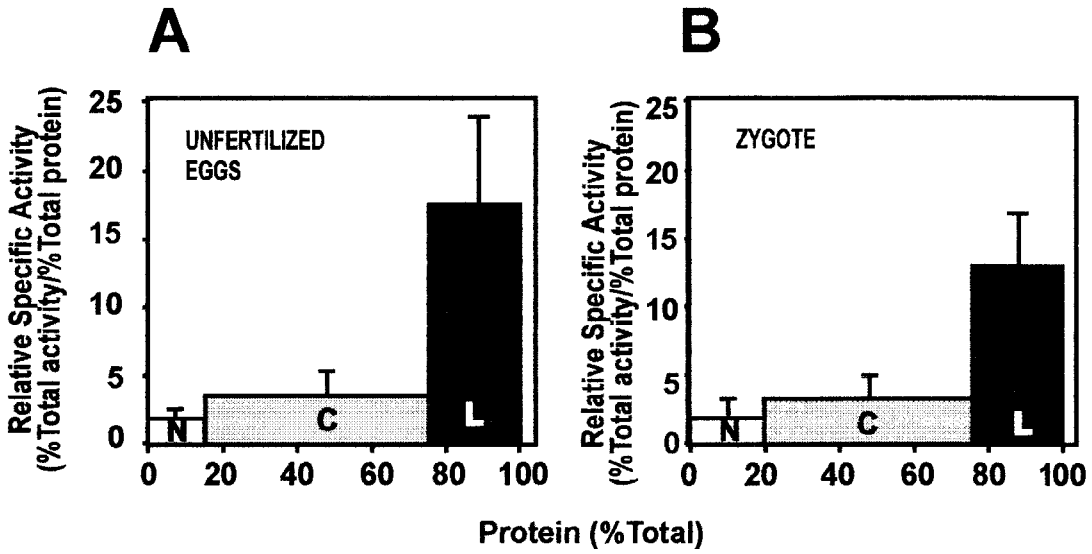

Fig. 2. Intracellular distribution of acid phosphatase, used as a lysosomal marker enzyme. Subcellular fractions were obtained by differential centrifugation of total homogenates from unfertilized eggs or zygotes as described under Methods. The activity of acid phosphatase was then determined in each fraction. N, nuclei; C, cytoplasm; L, lysosomes-peroxisomes. Distribution of the phosphatase activity in fractions obtained from **(A)** Unfertilized eggs, and **(B)** zygotes.

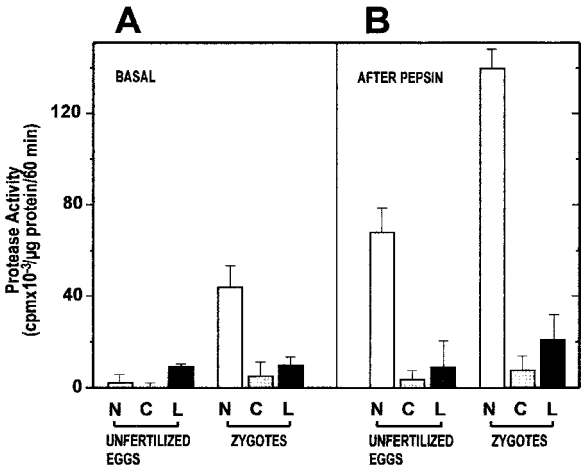

Fig. 3. Activation of a SpH protease zymogen by pepsin. Protein extracts from the nuclear (N), cytoplasmic (C), and lysosomal-peroxisomal (L) fractions obtained from sea urchin unfertilized eggs and zygotes were preincubated with 100 mg/ml of pepsin for 30 min at pH 3.2 and 37°C. Subsequently, the presence of a proteolytic activity that can cleave sperm-specific histone H1 (^{14}C-SpH1) was measured as described under Materials and Methods. **A:** Basal proteolytic activity measured before treatment with pepsin. **B:** Proteolytic activity determined after the protease zymogen activation with pepsin.

15) as determined by proteolytic degradation of ^{14}C-SpH1 (Fig. 4B), were pooled and further purified by chromatography on a Sephadex G-100 column (Fig. 5A). ^{14}C-SpH1 was used as substrate in all steps of this enzyme purification. The ^{14}C-SpH1 proteolytic degradation was

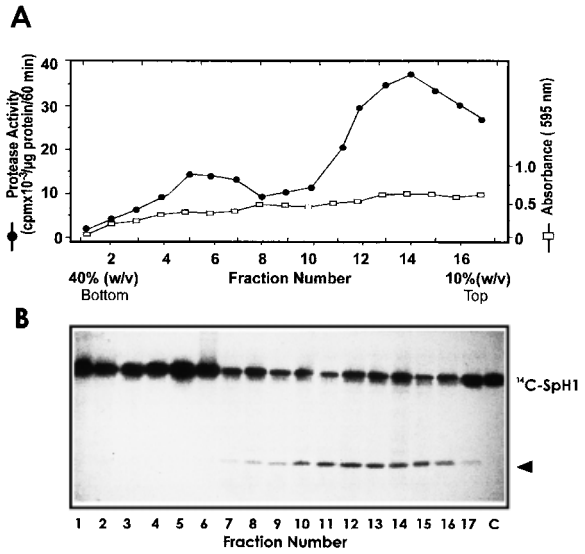

Fig. 4. Purification of the SpH-protease by sucrose gradient centrifugation. Chromatin isolated from sea urchin zygotes was loaded on a 10–40% (w/v) sucrose gradient and centrifuged for 21.5 h at 141,000 g at 4°C. Fractions of 2 ml were collected, and the presence of proteolytic activity was determined in each fraction by using ^{14}C SpH1 as substrate. **A:** Elution profile of the sucrose gradient. (□) Protein concentration pattern, determined as described by Bradford. (●) **B:** Proteolytic activity pattern using ^{14}C-SpH1 as substrate. The products of ^{14}C-SpH1 degradation were analyzed by fluorography in SDS-PAGE. The electrophoretic migration of ^{14}C-SpH1 and the fraction numbers are indicated. Arrowhead, position of ^{14}C-SpH1 degradation products.

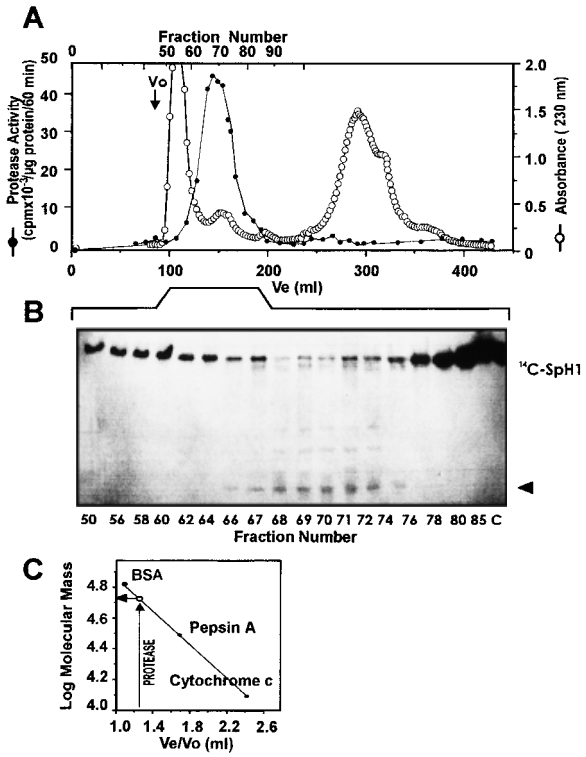

Fig. 5. Protease purification by chromatography on a Sephadex G-100 column. The pool (fractions 11–15) containing the proteolytic activity from the sucrose gradient was concentrated, loaded onto a Sephadex G-100 column, and eluted as described under Methods. **A:** Elution profile with the fraction numbers and elution volumes indicated. Protein concentration (o) was followed by measuring the absorbance at 230 nm. Proteolytic activity (●) was determined in each second fraction with ^{14}C-SpH1 as substrate. The products of ^{14}C-SpH degradation derived from the proteolytic activity present in fractions 50–85 were further analyzed by SDS-PAGE and fluorography. **B:** Fluorography of the ^{14}C-SpH1 degradation products obtained with proteins from fractions 50–85, the electrophoretic migration of ^{14}C-SpH1 is indicated. **C:** Apparent molecular mass of the SpH-protease in its active form, as calculated from its elution volume from the Sephadex G-100 column. Phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55 kDa), ovoalbumin (42.7 kDa), aldolase (40 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa) were used as molecular mass markers.

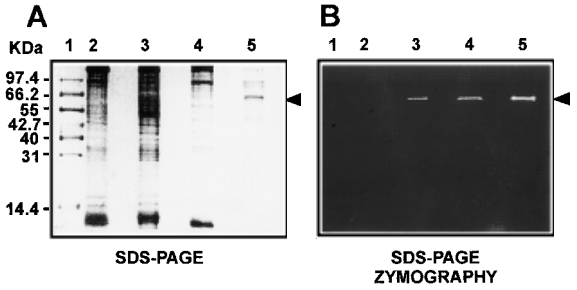

Fig. 6. SDS-PAGE zymogram of SpH-protease partial purification; 50–100 µg of proteins present in the extracts derived from the different purification steps was electrophoresed in a SDS-PAGE gel and in a zymogram gel containing 0.1% SpH1 as described in methods. **Lane 1**, size markers as described in Figure 5; **lane 2**, total cell homogenates; **lane 3**, nuclear fraction; **lane 4**, pool of fractions 11–15 of the sucrose gradient; and **lane 5**, pool of fractions 67–75 of the Sephadex G-100 chromatography. **A:** SDS-PAGE gel stained with Coomassie blue. **B:** The zymogram gel was washed in 2.5% Triton X-100 for 30 min at room temperature and then stained with Coomassie blue. The position of the protease is observed in the gel as a clear band against a dark background.

TABLE I. Purification of the SpH-Protease

Fraction	Specific activity (mU/mg)	Yield (%)	Purification (-fold)
Homogenate	0.62	100	1
Chromatin	26.63	18.7	42
Sucrose gradient	57.49	11.7	93
Sephadex G-100	152.17	3.2	314

further confirmed by SDS-PAGE analysis of the products that were detected by fluorography (Figs. 4B and 5B, respectively). An approximate molecular mass of 60 kDa for the protease in its active form was determined from the elution on the Sephadex G-100 column. Phosphorylase b (97.4 kDa), bovine serum albumin (BSA) (66.2 kDa), glutamate dehydrogenase (55 kDa), ovoalbumin (42.7 kDa), aldolase (40 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa) were used as molecular mass markers (Fig. 5C). This value was further confirmed by visualization of protease activity in a zymogram gel (Fig. 6B, cf. lane 5 in SDS-PAGE and zymogram). The protease corresponds to the major 60-kDa protein derived from the Sephadex G-100 chromatography. The purification results are summarized in Table I.

As shown in Table II, the SpH-protease requires for its activity the presence of thiol reducing agents, such as dithiothreitol (DTT) or 2-mercaptoethanol. Consistently, the enzyme was found to be sensitive to the sulfhydryl blocking compounds: iodoacetamide and p-hydroxy mercuribenzoate. Furthermore, the SpH-protease was sensitive to 1 mM each of the cysteine-type protease inhibitors leupeptin, cystatin and (2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3 methylbutane ethyl ester (E-64 d), whereas it was not affected by the serine type-protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and pepstatin (Fig. 7). Taken together, this evidence defines this enzyme as a SpH-cysteine protease.

TABLE II. Thiol Group Requirement of the SpH-Protease

Activators	Activity (cpm $\times 10^{-3}$/μg protein/60 min)	Relative activity (% control)
Basal activity	10.4 ± 0.2	100
Dithiothreitol (DTT), 1 mM	36 ± 2.5	346
Dithiothreitol (DTT), 5 mM	45 ± 3.0	433
β-Mercaptoethanol, 1 mM	45.2 ± 1.5	435
β-Mercaptoethanol, 5 mM	46 ± 2.5	442

Inhibitor	Activity (cpm $\times 10^{-3}$/μg protein/60 min)	Inhibition (%)
Basal activity	10.4 ± 0.2	—
Iodoacetamide, 1 mM	2.9 ± 0.2	72
Iodoacetamide, 5 mM	0.3 ± 0.1	98
p-Hydroxymercuribenzoate, 1 mM	1.0 ± 0.2	90
p-Hydroxymercuribenzoate, 5 mM	0 ± 0	100

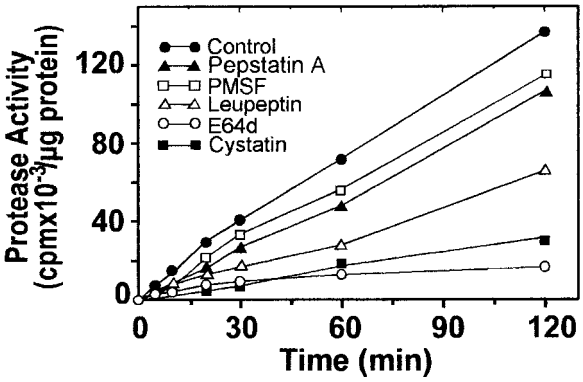

Fig. 7. Effect of protease inhibitors. The protease activity was measured in the presence of 1 mM of each of the inhibitors, using labeled-sperm-histones as substrates as described under Materials and Methods. (■) cystatin, (○) E64, (△) leupeptin, (▲) pepstatin, and (□) PMSF.

The SpH thiol-protease was found to be active between pH 7.0 and 8.0, exhibiting an optimum at pH 7.5-8.0. Within this range of pH, the protease was selective for the degradation of ^{14}4-SpH and ^{14}4-SpH1, with no proteolytic activity detectable against ^{14}C-casein (Fig. 8). As reported previously, following fertilization of sea urchins there is an increase of the intracellular pH which determines egg metabolic activation (Nishioka and Mc Gwin, 1980; Whitaker and Steinhardt, 1985]. Consequently, under the conditions that follow fertilization, the SpH-cysteine protease described in this report would exhibit its full activity in vivo.

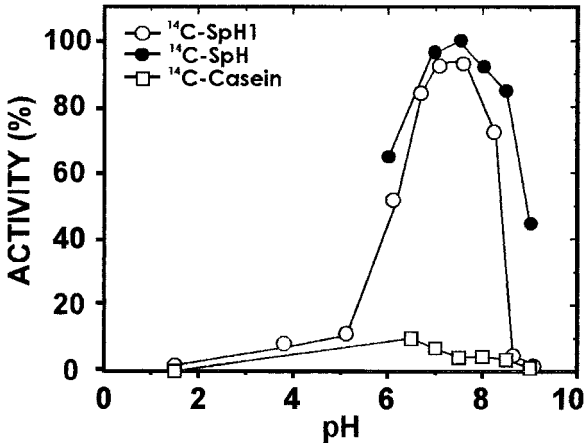

Fig. 8. SpH-protease pH dependency and substrate selectivity of the SpH-protease The effect of pH on the proteolytic activity was determined as described under Methods, with the following labeled substrates: (●) total sperm-histones (^{14}C-SpH), (○) sperm histone H1 (^{14}C-SpH1), and (□)^{14}C-casein.

Assessment of the Role of the SpH-Protease in Male Pronucleus Chromatin Remodeling In Vitro and In Vivo

It is well established that the selective loss of the sperm-specific histones that occurs after fertilization is accompanied by their replacement by CS histone variants derived from maternal stores (Poccia, 1986; Imschenetzky et al., 1996a]. Such selective loss was previously suggested to be due to degradation of sperm histones (Imschenetzky et al., 1991a]. This mechanism would require a protease recognition of the SpH, leaving the maternally derived CS histone variants intact. Following this rationale, we had analyzed in vitro the activity of the SpH-protease against either the whole complement of ^{14}C-SpH or the native ^{14}C-CS histone variants as substrates. The products of digestion were characterized by analysis in SDS-PAGE followed by fluorography. As shown in Figure 9, this cysteine protease exhibited substrate selectivity toward sperm histones, since the maternal CS variants were not degraded. The five ^{14}C-SpH: SpH1, SpH2A, SpH2B, SpH3, and SpH4 were degraded after incubation with the protease (Fig. 10 A,B). Under similar experimental conditions, none of the ^{14}C-CS variants was significantly degraded (Fig. 10C). These

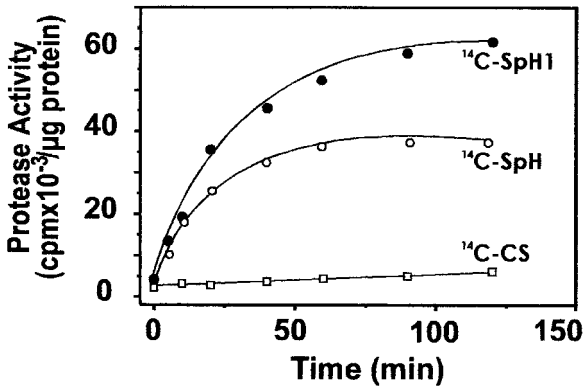

Fig. 9. Substrate selectivity of the SpH-protease. The time course activity of SpH-protease activity was compared using as substrates: (□) CS histone variants (^{14}C-CS), (○) total sperm histones (^{14}C-SpH) and (●) sperm histone H1 (^{14}C-SpH1).

results are consistent with our initial hypothesis.

As described in Figure 7, the activity of this SpH-cysteine-protease can be strongly inhibited by E-64 d. Based on this information, we incubated sea urchin zygotes from 3 min p.i. until the end of the first cleavage division (90 min p.i.) with E-64 d in order to inhibit the cysteine protease in vivo. The presence of SpH on the chromatin of zygotes treated with E-64 d was then determined and compared with normal zygotes. For this purpose, the complete set of histones present in the chromatin of zygotes was isolated and analyzed by Western blot revealed with antibodies that specifically recognize the SpH (Imschenetzky et al., 1991a, 1996a]. The results obtained are presented in Figure 11. As shown, in sea urchin embryos incubated with E-64 d from 3 to 90 min p.i., the whole complement of SpH were not degraded (Fig. 11, lane 2), as seen in the control embryos (Fig. 11, lane 3).

DISCUSSION

A cysteine protease bound to chromatin of zygotes harvested shortly after fertilization has been identified that was able to perform a complete and selective proteolysis of labeled sperm histones. We have demonstrated that this enzyme is present in an inactive state in unfertilized eggs and is activated in zygotes harvested 5 min p.i. This enzyme is a 60-kDa protein that selectively degrades sperm histones in vitro within a very narrow range of pH 7.0–8.0. By contrast, native CS histone variants remained intact after treatment with the protease. The substrate selectivity shown by the cysteine-protease described in this report strongly suggests its role in the degradation of SpH, which in turn determines male pronucleus chromatin remodeling. This selectivity may be explained by the extensive poly(ADP-ribosylation) of CS histone variants in unfertilized eggs and zygotes (Imschenetzky et al., 1991b, 1993, 1996b]. We speculate that the high concentration of negative charges provided by the ADP-ribose moieties bound to the CS histone variants may protect these proteins from proteolysis by this enzyme. ADP-ribose polymers have been shown to function as protease inhibitor in other systems (Inagaki et al., 1980].

The evidence obtained from the experiments in vitro on the role of the SpH cysteine protease during male pronucleus remodeling was further substantiated by the experiments in vivo. Indeed, Figure 11 shows that the proteolysis of the complete set of sperm-specific histones was prevented by incubating the zygotes in E-64 d, a specific inhibitor cysteine proteases. These results are not due to undesirable side effects of E-64 d on the early events occurring after fertilization, since both male pronuclei swelling and amphimixis occurred in a similar manner in E-64-d treated embryos and control embryos (data not shown).

Taken together, these results established a very good correlation between the characteristics of this enzyme found in vitro and its possible role in vivo. An additional line of evidence which correlates the properties of this enzyme determined in vitro with its potential function in male pronucleus remodeling is provided by the pH modulation of its activity. The enzyme degrades sperm histones in a range of pH that extends from pH 7.0 to pH 8.0. This is exactly the pH range found in vivo in sea urchin zygotes following fertilization (Nishioka and Mc Gwin, 1980; Whitaker and Steinhardt, 1985].

The biochemical characteristics of the SpH cysteine protease described in this report are consistent with its role in the progression through the different steps that are involved in male pronucleus remodeling described by Cothreen and Poccia (1993]. Three sequential steps were defined according to male pronucleus morphology: an initial conical condensed state (state I), an ovoid intermediate state (state II), and a fully decondensed spherical male pronucleus (state III). As reported, the

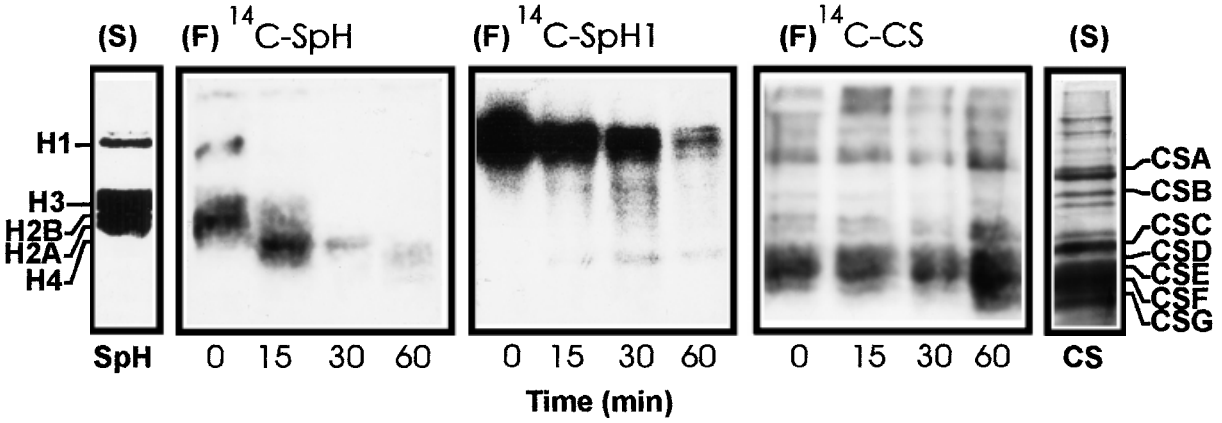

Fig. 10. The SpH-protease does not degrade the maternally derived cleavage-stage (CS) histone variants. The SpH-protease was incubated for the times indicated with the following labeled substrates: total sperm histones (^{14}C- SpH), sperm histone H1 (^{14}C-SpH1), and CS histone variants (^{14}C-CS). The products obtained after each incubation were analyzed by SDS-PAGE followed by fluorography (F). Slots containing the Coomassie blue-stained (S) native histone variants from sperms (SpH, left) and from unfertilized eggs (CS, right) are included for comparison of their electrophoretic migration.

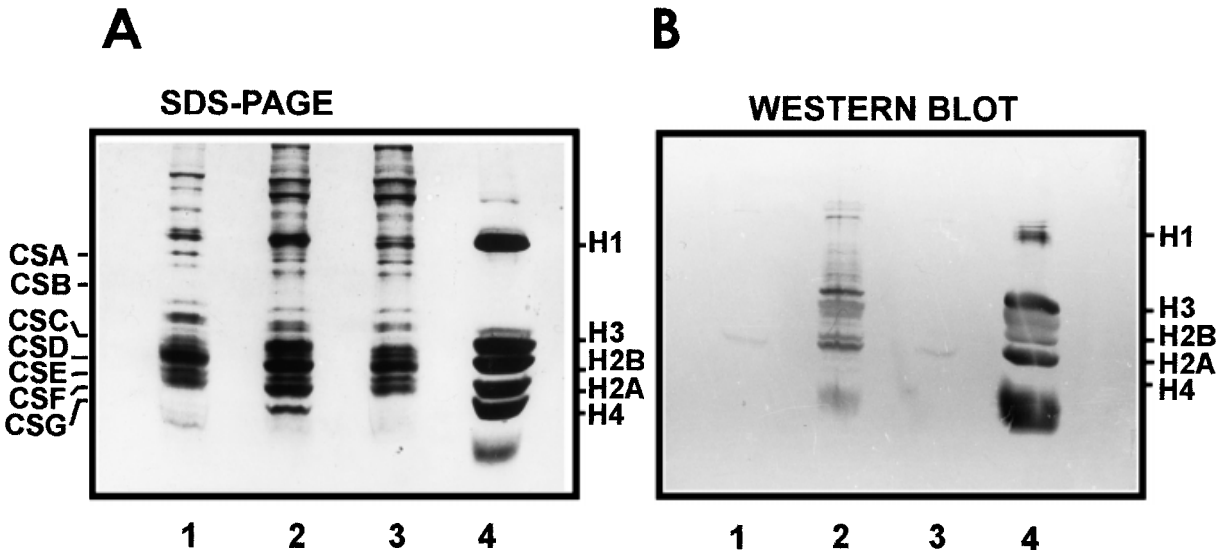

Fig. 11. Inhibition of the SpH-protease by E-64 d in vivo. Sea urchin zygotes were incubated with E-64 d between 3 and 90 min p.i. At 90 min p.i., whole histones were isolated from the E-64-day-treated zygotes, as well as from control zygotes that were incubated in seawater during this period. The impact of E-64 days treatment on sperm histone degradation was determined by Western blots revealed with polyclonal antibodies specific against sea urchin sperm histones. **A:** Coomassie blue-stained SDS-PAGE. **B:** Corresponding Western blot. Each slot of the gel was charged with 100 µg of histones. Slot 1 contains CS histone variants, slot 2 contains whole histones isolated from E-64-day-treated zygotes harvested 90 min p.i., slot 3 contains whole histones isolated from control zygotes harvested 90 min p.i., and slot 4 contains whole sperm histones.

transformation from state I to state II requires protein phosphorylation by factors that are not present in oocytes before maturation and may be blocked by protein kinases inhibitors such as staurosporine (Cothreen and Poccia, 1993; Cameron and Poccia, 1994]. Alternatively, this step may be inhibited by N-ethylmaleimide, a compound that blocks sulfhydryl groups (Luttmer and Longo, 1987]. This requirement for thiol groups may be correlated with the participation of the cysteine protease in the first step of male pronucleus formation. Progression from state II to state III is promoted by the rise of intracellular pH that occurs at fertilization (Carron and Longo, 1980; Cothreen and Poccia, 1993; Cameron and Poccia, 1994]. In this context, it may be hypothesized that the activity of the SpH cysteine protease can also be modulated

by this rise in intracellular pH, thus participating in the progression from state II to state III of male pronucleus remodeling. In addition, the presence of the cysteine protease in an inactive state in unfertilized eggs and its activation post fertilization, is in agreement with the independence of male pronucleus formation on new protein synthesis after fertilization (Poccia, 1986; Imschenetzky et al., 1991a].

The participation in male pronucleus remodeling of a 28-kDa protease that specifically degrades the SPKK motif in histones has been previously postulated by Susuki et al. (1990]. In common with the cysteine protease described in this report, the SPKK-protease was sensitive to leupeptin, suggesting that this SPKK-protease is also a cysteine type protease. However no other formal evidence was provided to enable the unequivocal classification of the enzyme as a cysteine protease. Undoubtedly the SPKK-protease is distinct from the cysteine protease described in this report, both in its apparent molecular mass and in its restricted specificity. As reported by Susuki et al. [1990], the SPKK protease promotes a limited proteolysis only of sperm histones H1 and H2B. It seems obvious that although the participation of the SPKK-protease in sperm histone degradation during male pronucleus formation can not be dismissed, its role appears more limited than that expected for the cysteine protease described in this report.

The molecular weight and strict substrate selectivity of the SpH cysteine protease clearly define it as unique and distinct from all other proteases described thus far in sea urchin gametes (Taniguchi et al., 1986; Okada and Yakota, 1990; Inaba et al., 1992; Inaba and Morisawa, 1995] or in embryos (Grainger and Winkler, 1989; Le Page and Gache, 1990; Quigley et al., 1993; Vafa and Nishioka, 1995]. Moreover, the cysteine protease described in this report also differs from cysteine proteases that have been described as associated with the nucleus in regenerating rat liver (Tsurugi and Ogata, 1980], embryonic carcinoma (Scholtz et al., 1996] or in other systems (Alnemri, 1997]. The SpH cysteine protease certainly also differs from aspartic or serine-type neutral and alkaline proteases described as nuclear proteases in other biological systems (Chong et al., 1974; Hagiwara et al., 1981; Dyson and Walker, 1984; Tsurugi and Ogata, 1986; Motizuki et al., 1988; Tòkes and Clawson, 1989].

Although the detailed mechanism responsible for male pronucleus formation is not yet fully understood, in batracians it was previously reported that nucleoplasmin and a yet undefined cytoplasmic factor participate in this event (Philpott et al., 1991; Philpott and Leno, 1992]. Nucleoplasmin is essential for the assembly of nucleosomes because it promotes the addition of the H2A–H2B dimers to the histone H3–H4 tetramer. The H3–H4 tetramer has being reported to be primarily organized by proteins N1/N2 (Dilworth et al., 1987]. In sea urchin zygotes, sperm histone disappearance appears to occur in a non coincidental manner during male pronucleus remodeling. Consistently the SpH (H3–H4) tetramer is removed, followed by the release of the SpH (H2A–H2B) dimers (Imschenetzky et al., 1991a]. Such stepwise replacement results in an intermediate chromatin composed of hybrid nucleoprotein particles containing both SpH (H2A–H2B) and maternal type CS histone components (Imschenetzky et al., 1996a]. The substrate selectivity of the SpH cysteine protease described in this report is consistent with the specific degradation of sperm-histones, while leaving the CS histone variants as intact proteins. It is however puzzling that at an intermediate stage of male pronuclei remodeling the SpH (H3-H4) tetramer is no longer associated with chromosomal nucleoprotein particles, whereas SpH dimers (H2A–H2B) are still remaining. This stepwise mechanism of SpH release can not be explained only by the action of SpH-protease. In this context, it seems predictable that the SpH cysteine protease is not the unique molecule responsible for sperm chromatin remodeling. As shown, the SpH-protease is unable to discriminate between SpH (H3–H4) tetramers and SpH (H2A–H2B) dimers. In this context, it is tempting to speculate the participation of factors such as nucleoplasmin and proteins N1/N2 that in combination with the SpH cysteine protease may lead to a successful male pronucleus remodeling in sea urchins. Nucleoplasmin-type molecules or N1/N2 type factors have not yet been identified in sea urchins and further work is thus required.

ACKNOWLEDGMENTS

We are very grateful to Dr. Linda Fothergill-Gilmore from the University of Edinburgh, U.K.,

for critical reading and for correcting the English of the manuscript. Supported by grants FONDECYT 1.93.063.1 and 97.31.74-1.2 from Universidad de Concepción, Chile.

REFERENCES

Ausio J (1995): In Jamieson BGM, Ausio J, Justine JL (eds): "Advances in Spermatozoal Taxonomy and Phylogeny." Mémoires de Musée National d'Histoire Naturelle, Paris, pp. 447–462.

Alnemri E (1997): Mammalian cell death proteases: A family of highly conserved aspartate specific cysteine proteases. J Cell Biochem 64:33–42.

Bergmeyer M (1974): Reagents for enzymatic analysis. In Hans Ulrich Bergmeyer (eds): Methods of Enzymatic Analysis. Vol. 1. Academic Press, Inc, pp 495–496.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Cameron LA, Poccia DL (1994): In vitro development of the sea urchin male pronucleus. Dev Biol 162:568–578.

Carron CP, Longo FJ (1980): Relation of intracellular pH and pronuclear development in the sea urchin, *Arbacia punctulata*. Dev Biol 79:478–487.

Chamberlain JP (1979): Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor sodium salicylate. Anal Biochem 98:132–135.

Chong M, Garward W, Bonner J (1974): Purification and properties of a neutral protease from rat liver chromatin. Biochem 13:5128–5134.

Cothren CC, Poccia DL (1993): Two steps required for male pronucleus formation in the sea urchin egg. Exp Cell Res 205:126–133.

Das NK, Micou-Eastwood J, Alfert M (1975): Cytochemical studies on the protamine-type protein transition in sperm nuclei after fertilization and early embryonic histones of *Urechis caupo*. Dev Biol 43:333–339.

De Martino G (1981): Calcium dependent proteolytic activity in rat liver. Identification of two proteases with different calcium requirements. Arch Biochem Biophys 211: 253–257.

Dilworth SM, Black SJ, Laskey RA (1987): Two complexes that contain histones are required for nucleosome assembly in vitro: Role of nucleoplasmin and N1 in *Xenopus* egg extracts. Cell 51:1009–1018.

Dyson M, Walker J (1984): Chromatin associated protease from calf thymus. Int J Peptide Protein Res 24:201–207.

Earnshaw W, Honda B, Laskey R (1980): Assembly of nucleosomes: The reaction involving *Xenopus laevis* nucleoplasmin. Cell 21:373–383.

Ecklund PS, Levine L (1975): Mouse sperm basic nuclear protein electrophoretic characterization and fate after fertilizaton. J Cell Biol 66:251–262.

Grainger J, Winkler M (1989): The sea urchin multicatalytic protease: Purification, biochemical analysis, subcellular distribution and relationship to snRNPs. J Cell Biol 109:675–683.

Green GR, Poccia D (1985): Phosphorylation of sea urchin sperm H1 and H2B histones precedes chromatin decondensation and H1 exchange during male pronuclear formation. Dev Biol 108:235–245.

Hagiwara H, Miyazaki K, Matuo Y, Yamashita J, Horio T (1981): Purification and characterization of alkaline protease and neutral protease from chromatin of rats. Biochim Biophys Acta 660:73–82.

Herlands L, Maul G (1994): Characterization of a major nucleoplasmin-like germinal vesicle protein which is rapidly phosphorylated before germinal vesicle breakdown in *Spisula solidissima*. Dev Biol 161:530–537.

Imschenetzky M, Oliver MI, Gutierrez S, Morin V, Garrido C, Bustos A, Puchi M (1996a): Hybrid nucleoprotein particles containing a subset of male and female histone variants form during male pronucleus formation in sea urchins. J Cell Biochem 63:385–394.

Imschenetzky M, Morin V, Carvajal N, Montecino M, Puchi M (1996b): Decreased heterogeneity of CS histone variants after hydrolysis of the ADP-ribose moiety. J Cell Biochem 61:109–117.

Imschenetzky M, Montecino M, Puchi M (1993): Temporally different poly(adenosine diphosphate-ribosylation) signals are required for DNA replication and cell division in early embryos of sea urchins. J Cell Biochem 51:198–205.

Imschenetzky M, Puchi M, Pimentel C, Bustos A, Gonzales M (1991a): Immunobiochemical evidence for the loss of sperm-specific histones during male pronucleus formation in monospermic zygotes of sea urchins. J Cell Biochem 47:1–10.

Imschenetzky M, Montecino M, Puchi M (1991b): Poly(ADP-ribosylation) of atypical CS histone variants is required for the progression of S phase in early embryos of sea urchins. J Cell Biochem 46:234–241.

Imschenetzky M, Puchi M, Gutierrez S, Massone R (1989): Analysis of supranucleosome particles from unfertilized eggs of sea urchin. Exp Cell Res 182:436–444.

Imschenetzky M, Puchi M, Massone R (1986): Characterization of cleavage stage chromosomal proteins from zygotes of the sea urchin *Tetrapygus niger*. Comp Biochem Physiol 84B:23–27.

Imschenetzky M, Puchi M, Oyarce A, Massone R, Inostroza D (1984): A comparative study of the histones isolated from sperm of the sea urchin *Tetrapygus niger*. Comp Biochem Physiol 78B:393–399.

Inaba K, Morisawa M (1995): Identification of a 10 S trypsin-like protease that cross-reacts with anti-proteasome antibody in sea urchin egg jelly. Arch Biochem Biophys 319: 177–184.

Inaba K, Akazome Y, Morisawa M (1992): Two high molecular mass proteases from sea urchin sperm. Biochem Biophys Res Commun 182:667–674.

Inagaki T, Miura K, Murachi T (1980): Identification of a protease inhibitor from rat peritoneal macrophages as poly(ADP-ribose). J Biol Chem 255:7746–7750.

Itoh T, Ohsumi K, Katagiri CH (1993): Remodeling of human sperm chromatin mediated by nucleoplasmin from amphibian eggs. Dev Growth Diff 35:59–66.

Katagiri C, Ohsumi K (1994): Remodeling of sperm chromatin induced in egg extracts of amphibians. Int J Dev Biol 38:209–216.

KawasakiA K, Philpott A, Avilion A, Berrios M, Fisher PA (1994): Chromatin decondensation in *Drosophila* embryo extracts. J Biol Chem 269:10169–10176.

Kleinschmidt J, Fortkamp E, Krohne G, Zentgraf H, Franke W (1985): Co-existence of two different types of soluble histone complexes in nuclei of *Xenopus laevis* oocytes. J Biol Chem 260:1166–1176.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Laskey R, Honda B, Mills A, Finsch J (1978): Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature 275:416–420.

Le Page T, Gache G (1990): Early expression of a collagenase-like hatching enzyme gene in the sea urchin embryo. EMBO J 9:3003–3012.

Lipps G, Fullkrug R, Beck E (1996): Cathepsin B of *Schistosoma mansoni*. Purification and activation of the recombinant proenzyme secreted by *Saccharomyces cerevisiae*. J Biol Chem 271:1717–1725.

Luttmer SJ, Longo FJ (1987): Rates of male pronuclear enlargement in sea urchin zygotes. J Exp Zool 243:289–298.

Motizuki M, Kohno H, Tsurugi K (1988): A study on the identities of the three species of chromatin-associated proteinases in a mutant of *Saccharomyces cerevisiae* which lacks four major vacuolar proteinases. J Biochem 104:192–195.

Nishioka D, Mc Gwin N (1980): Relationships between the release of acid, the cortical reaction and the increase of protein synthesis in sea urchin eggs. J Exp Zool 212:215–223.

Okada Y, Yakota Y (1990): Purification and properties of cathepsin B from sea urchin eggs. Comp Biochem Physiol 96B:381–386.

Philpott A, Leno GH (1992): Nucleoplasmin remodels sperm chromatin in *Xenopus* egg extracts. Cell 69:759–767.

Philpott A, Leno GH, Laskey RA (1991): Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin. Cell 65:569–578.

Poccia D (1986): Remodeling of nucleoproteins during gametogenesis, fertilization and early development. Int Rev Cytol 105:1–65.

Quigley J, Braithwaite S, Armstrong P (1993): Matrix metalloproteases of the developing sea urchin embryo. Differentiation 54:19–23.

Rice RH, Means GE (1971): Radioactive labeling of protein in vitro. J Biol Chem 246:831–832.

Rice P, Garduño R, Itoh T, Katagiri CH, Ausio J (1995): Nucleoplasmin-mediated decondensation of *Mytilus* sperm chromatin. Identification and partial characterization of a nucleoplasmin-like protein with sperm-nuclei decondensing activity in *Mytilus californianus*. Biochem 34:7563–7568.

Savic A, Richman P, Williamson P, Poccia D (1981): Alterations in chromatin structure during early sea urchin embryogenesis. Proc Natl Acad Sci USA 78:3706–3710.

Scholtz B, Lamb K, Rosfford E, Kingsley M, Rizzino A (1996): Appearance of nuclear protease activity after embryonal carcinoma cell undergo differentiation. Dev Biol 173:420–427.

Suzuki M, Sugiura M, Ebashi S (1990): Sea urchin protease specific to the SPKK motif in histone. J Biochem 108:347–355.

Tanigushi Y, Akasaka K, Shraya T, Shimada H (1986): A Zn^{2+}-dependent and histone H1-specific protease in sea urchin sperm. J Biochem 99:931–938.

Tokes Z, Clawson G (1989): Proteolytic activity associated with the nuclear scaffold. The effect of self-digestion on lamins. J Biol Chem 264:15059–15065.

Tsurugi K, Ogata K (1980): Presence of a thiol protease in regenerating rat liver nuclei. Partial purification and some properties. Eur J Biochem 109:9–15.

Tsurugi K, Ogata K (1986): Effects of DNA and urea on the specificity for H1 histone of the neutral protease B partially purified from rat liver chromatin. J Biochem 99:237–241.

Vafa O, Nishioka D (1995): Developmentally regulated protease expression during sea urchin embryogenesis. Mol Repr Dev 40:36–47.

Von Holt C, Brandt WF, Greyling HJ, Lindsay GG, Rodrigues J, Schwager S, Sewell BT (1989): Isolation and characterization of histones. Methods Enzymol 170:431–523.

Whitaker MJ, Steinhardt RA (1985): Ionic signaling in the sea urchin egg at fertilization. In Metz CB, Monroy A (eds): "Biology of Fertilization." Vol 3. San Diego: Academic Press, pp 167–221.