Selective Inhibition of Exoplasmic Membrane Fusion in Echinoderm Gametes with Jaspisin, a Novel Antihatching Substance Isolated from a Marine Sponge*

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A specific inhibitor of fertilization of the sea urchin Hemicentrotus pulcherrimus was isolated from the extract of the marine sponge, Jaspis species. Chemical and spectral data of the purified substance, which was designated jaspisin, showed that it is a novel substance with the structure of sodium (E)-5,6-dihydroxyxysteryl sulfate. Jaspisin at 15 μg/ml inhibited exoplasmic fusion of the plasma membrane of acrosome-reacted sperm with the plasma membrane of the egg; it did not affect either the acrosome reaction in sperm or the egg cortical reaction, both of which involve endoplasmic membrane fusion events. When a fertilized egg was cultured in jaspisin, the embryo developed through the mesenchymal blastula stage. However, it was unable to hatch from the fertilization envelope, and spiculogenesis, in which cell-cell fusion of primary mesenchyme cells is involved, was prevented. Jaspisin at 8.6 μg/ml inhibited half the activity of hatching enzyme, a kind of Zn²⁺-dependent metalloendoproteinases. Because Zn²⁺-activated metalloendoproteinases are suggested to be involved in both sperm-egg fusion and fusion of primary mesenchyme cells (Lennarz, W. J., and Strittmatter, W. J. (1991) Biochim. Biophys. Acta 1071, 149–158), one of the possible explanations of the jaspisin effects is that the sulfate inhibits these cellular events through blockage of Zn²⁺-activated metalloendoproteinases that are involved in membrane fusion processes.

The ability to undergo membrane fusion constitutes one of the most central properties of cell membranes. Membrane fusion events have crucial roles in endocytosis, secretion, cell division, phagocytosis, and numerous other cellular activities (1–3). A distinction is made between fusion events that involve an initial contact between cytosolic membrane surfaces (endoplasmic fusion) and those that involve an initial contact between externally oriented membrane surfaces (exoplasmic fusion) (3). Endoplasmic fusion reactions occur during exocytosis and cell division, whereas exoplasmic fusion reactions take place during cell-cell fusion and viral membrane-cell membrane fusion. There are few, if any, chemicals that inhibit exoplasmic fusion events without affecting endoplasmic fusion events. To study the molecular basis of exoplasmic cell fusion events, we tried to find natural endoplasmic-fusion inhibitors by using echinoderm gametes as a bioassay system.

Fertilization of echinoderm gametes involves at least three membrane fusion events (4). The first membrane fusion event is the acrosome reaction, which involves Ca²⁺-dependent endoplasmic fusion of the membrane of the acrosome vesicle with the sperm plasma membrane (5–8). In the second fusion event, which occurs after the sperm undergoes the acrosome reaction and binds to an egg receptor, the sperm plasma membrane fuses with the plasma membrane of the egg (8–11). This sperm-egg fusion involves an exoplasmic fusion event. The third fusion event, which involves an endoplasmic fusion reaction, is an exocytotic event involving the cortical granule membrane and the egg plasma membrane (12–14).

We searched for animal extracts capable of preventing exoplasmic fusion of gametes of the sea urchin Hemicentrotus pulcherrimus without affecting the sperm acrosome reaction and the egg cortical reaction. We found that an extract prepared from the marine sponge, Jaspis species, exhibited such activity. The purification of the active substance was subsequently carried out based on its specific inhibition against sperm-egg fusion. The purified substance was a novel sulfate, which we designated as jaspisin (Fig. 1). We studied its effects on the fusion events during fertilization of H. pulcherrimus and the starfish Asterina pectinifera. The effects of jaspisin on early embryonic development of fertilized sea urchin and starfish eggs were also investigated. Jaspisin at concentrations of 15–100 μg/ml affected neither cell division nor cell differentiation; however, it prevented hatching of sea urchin and starfish embryos at the late blastula stage and blocked spiculogenesis of the sea urchin embryo at the pluteus stage. Jaspisin showed specific inhibition against H. pulcherrimus hatching enzyme, a kind of Zn²⁺-dependent metalloendoproteinase. The results provide strong, albeit indirect, evidence that metalloendoproteinases are involved in the exoplasmic fusion of gametes.

MATERIALS AND METHODS

Organisms—The sea urchin, H. pulcherrimus, and the starfish, A. pectinifera, were collected from various areas of Japan at their breeding season and were kept in artificial seawater (ASW) in laboratory aquariums. The sponge, Jaspis species, were collected from the coast off Nichinan-Oshima, Miyazaki Prefecture, Japan, and kept frozen at –20°C until use.

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Chemicals—Diaion HP-20, Sephadex LH-20, Kieselgel 60F$_{254}$ plates, and Inertfll for HPLC were obtained from Mitsubishi Kasei (Tokyo), Pharmacia Fine Chemicals (Uppsala), E. Merck (Darmstadt) and GL Sciences (Tokyo), respectively. Hoechst 33342 and 1-methyladenine were obtained from Calbiochem and Sigma, respectively. ASW was obtained from Jamarin Laboratory (Osaka).

Assay for Inhibition of Fertilization—All of the experiments were carried out at 20 °C unless otherwise stated. Sea urchin gametes were collected from adults by pouring 0.5 M KCl into opened body cavities to induce spawning. Starfish gametes were induced to shed by the addition of 150 mg/ml 1-methyladenine (15) to isolated gonadal fragments. Eggs were exposed to a known concentration of a test sample in a dish. Sperm were added after incubation of eggs in test samples for 15 min, except where noted. Eggs were examined at 5 min after insemination for elevation of the fertilization envelope.

Purification of the Inhibitor—An inhibitor of fertilization was purified from the methanol extract of the sponge body guided by using sea urchin gametes in a bioassay. The sponge body was cut into pieces and immersed overnight in 3,000 mg wet weight of methanol. The extract was separated by filtration, and the residue was reextracted with 1,000 ml of methanol. The extracts were combined and condensed in vacuo to afford an oily residue. This was dissolved in 200 ml of methanol, and 1,800 ml of ethyl acetate was added to the solution. The precipitate was collected by centrifugation (1,000 x g, 10 min) and dissolved in 100 ml of distilled water. The solution was applied to a Diaion HP-20 column (4.0 x 16 cm), which was washed with 1,000 ml of distilled water. The active material was eluted with 600 ml of 50% aqueous methanol. The solvents of the active fraction were evaporated to afford an oily residue, which was dissolved in 18 ml of distilled water. Nine 2-ml aliquots were applied separately to a Sephadex LH-20 column (1.4 x 46 cm), which was eluted with distilled water. The active fractions eluted at 3.0 times the column volume; they were combined and concentrated in vacuo. The concentrate was a purified oily substance, designated substance 1.

Methylation Experiments—Substance 1 (0.3 mg) was dissolved in 0.1 ml of methanol, and the solution was poured into 30 ml of diethyl ether containing excess diazomethane. The solution stood on ice for 6 h in the dark. Evaporation of the solvent gave an oily residue (0.3 mg), designated substance 2.

Thin Layer Chromatography—The purity of substances 1 and 2 was examined by TLC using Kieselgel 60F$_{254}$ plates and chloroform-methanol-water (10:5:1, v/v) as the solvent system. After development, the plates were dried and sprayed with saturated aqueous AgNO$_3$ and 0.4% (v/v) 2,4-dinitrophenylhydrazine in 2 N HCl to locate phenols and carboxyl compounds, respectively.

Mass Spectrometry—FABMS and HRFABMS were carried out on a JEOL JMX-DX303 spectrometer in a negative mode using a glycerol matrix.

Nuclear Magnetic Resonance Analyses—NMR spectra were recorded on a JEOL GSX-500 spectrometer operating in the Fourier transform mode. The measurements of $^{1}$H and $^{13}$C were performed at 500 and 125 MHz, respectively. Substances 1 and 2 were dissolved in D$_2$O and CD$_{3}$OD, respectively, and NMR spectra were measured using tetramethylsilane dissolved in CDCl$_3$ as the external standard. Chemical shifts of all other signals were expressed in ppm downfield of tetramethylsilane. $^{1}$H normal NMR, $^{1}$H COSY, $^{1}$C-H COSY, HMBC, NOESY and NOEDS were recorded at 27 °C. Data were analyzed according to Rahman (16).

Other Spectrophotometric Analyses—Substances 1 and 2 were dissolved in acidified water (pH 4.5) and methanol, respectively, and their UV and visible light absorption spectra were recorded on a Shimadzu UV-160A spectrophotometer. The IR spectrum was recorded as films in CaF$_2$ on a JASCO FT/IR 5300 spectrometer. Data were analyzed according to Silverstein et al. (17).

Assay for the Sperm Acrosome Reaction—The acrosome reaction of sea urchin sperm was induced by egg jelly as described by Decker et al. (18). The sperm was concentrated or treated with various concentrations of substance 1 for 5 min, followed by the addition of egg jelly. Twenty seconds after the addition of egg jelly, the sperm were fixed in 80% ASW containing 1% glutaraldehyde, 1% OsO$_4$, 0.1 mm sodium cacodylate (pH 7.0), and 0.5 mg/ml ruthenium red. Specimen were viewed with a Hitachi H-300 electron microscope.

Assay for Sperm-Egg Fusion—Fusion of the plasma membrane of a sea urchin egg with the sperm was examined as described by Hinkley et al. (19). Unfertilized eggs, which were dejellied by brief exposure to acidified ASW (pH 5.0), were treated with 10 µg/ml Hoechst 33342 for 30 min, washed five times with ASW, and then either untreated or treated with various concentrations of substance 1 for 5 min. They were fertilized and, 30 s later, fixed in ASW containing five drops/ml of 5.6% formalin, and observed with a fluorescence microscope. Excitatory light was provided by a 390-nm band pass interference filter and emitted light through a 490-nm band pass filter. Cortices were isolated on glass surfaces at 4 °C from unfertilized eggs, and cortical vesicle discharge was induced by the method described by Sasaki (20). The medium used for isolation of the cortices was composed of 300 mm glycine, 210 mm potassium glutamate, 50 mm NaCl, 10 mm MgCl$_2$, 10 mm EDTA, and 10 mm PIPES, and its pH was adjusted to 6.7 with KOH.

Assay for Inhibition of Embryonic Development—Insemination was carried out with a slight excess of sperm. Fertilized eggs were washed several times with ASW Only batches with more than 98% fertilization were used. Embryos were cultured in the presence or absence of various concentrations of substance 1 starting from 5 to 10 min after insemination. They were grown at 1,000 eggs/ml without stirring in plastic Petri dishes. In some experiments, the effect of substance 1 was tested on sea urchin blastulae whose fertilization envelope was removed by treatment, for 8 h beginning 10 min after fertilization, with the minimum effective concentration of the bathing supernatant (21, 22). The demembranated blastulae were washed several times with ASW and kept in ASW or ASW containing 30 µg/ml substance 1 until controls reached the pluteus stage.

Hatching Enzyme Activity—Medium of hatched sea urchin blastulae was prepared as described by Nomura et al. (22). The enzyme preparation was the fraction obtained by chromatography using reactive red 120-agarose (22). The activity of hatching enzyme was monitored as the activity that dissolved the fertilization envelope of methanol-fixed, two- to four-cell stage embryos. Incubation was carried out at 20 °C for 4 h in a reaction mixture containing 20 mm Tris-HCl (pH 8.2), 0.5 mm NaCl, and 10 mm MgCl$_2$, fixed 100-150 embryos, and a minimum effective concentration of the enzyme preparation sufficient to "hatch" all of the fixed embryo.

RESULTS

Purification of a Substance That Has an Inhibitory Activity on Sperm-Egg Fusion—Substance with gamete fusion inhibitory activity was purified by the method described under "Materials and Methods." From 1 kg, wet weight, of the specimen of the marine sponge, Jaspis species, 92 mg of substance 1 was isolated.

To confirm that the inhibition of sperm-egg fusion of H. pulcherrimus is due to this substance itself and not to other minor components contaminating the fraction, the purified inhibitor was chromatographed on a HPLC system with an ODS Inertsil column (0.46 x 25 cm) using water as the eluent. A single UV (210 nm)-absorbing peak, which was associated with the inhibitory activity, was detected at 2.2 times the column volume. Furthermore, when the purified material was subjected to TLC using a silica gel plate (Kieselgel 60F$_{254}$), which was developed with chloroform-methanol-water (10:5:1, v/v), a single UV-absorbing band was detected at $R_0$. 0.25. When the material extracted from the band with chloroform-methanol-water (10:5:1, v/v) was subjected to bioassay after removal of the solvent, it showed specific inhibition of sperm-egg fusion as described below. These results indicated that the inhibition of gamete fusion is due to the purified substance.

Structural Studies on the Purified Inhibitor—The HRFABMS spectrum of substance 1 revealed the presence of (M-Na)$^+$ ion at m/z 230.9968, showing that the molecular formula is C$_{9}$H$_{13}$OSNa (calculated value for the (M-Na)$^+$ ion being 230.9963). The negative FABMS spectrum of substance 1 (Fig. 2) exhibited an ion peak at m/z 97, indicating the presence of a HSO$_3$ unit in the molecule. Substance 1 showed positive reactions on the AgNO$_3$ test but did not react with 2,4-dinitrophenol.
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**Fig. 2. Negative FABMS (glycerol matrix) of substance 1.**

Hyalidrazine, showing that substance 1 contains a phenolic group but lacks carbonyl groups. The IR spectrum of substance 1 suggested the presence of hydroxyl groups (3,370 cm⁻¹), a vinyl group (1,657 cm⁻¹), benzene ring (1,607, 1,528, and 1,501 cm⁻¹), and a sulfate unit (1,242 cm⁻¹). The UV spectrum of substance 1 (Fig. 3) showed absorption maxima at 211 nm (ε 19,600), 258 nm (ε 10,300), and 298 nm (ε 3,400), suggesting that substance 1 is a styryl derivative. ¹H NMR spectrum of substance 1 (Table I) exhibited the presence of three aromatic protons and two vinyl protons. The spin coupling constant for two aromatic protons with signals at δ 6.67 (H-7) and 6.66 (H-8) was 8.3 Hz, suggesting that they were located vicinal to each other. The signals of the remaining aromatic proton at δ 6.75 (H-4) and the proton at δ 6.66 (H-8) coupled with a coupling constant of 1.8 Hz, suggesting that these protons orient in the meta position of the benzene ring. The ¹³C NMR spectrum of substance 1 (Table I) exhibited the presence of three hydrogen-bearing aromatic carbon atoms at δ 112.1 (C-4), 116.1 (C-7), and 118.9 (C-8); two oxygen-bearing aromatic carbon atoms at δ 143.8 (C-5) and 143.3 (C-6); and one aromatic carbon atom at δ 126.3 (C-3) attached to a vinyl group; a vinyl carbon atom at δ 116.9 (C-2) attached to a benzene ring; and an oxygen-bearing vinyl carbon atom at δ 136.4 (C-1). ¹H-¹H COSY and ¹³C-¹H COSY spectra of substance 1 clearly showed the attachment of the five unexchangeable protons to five carbon atoms (Table I). The attachment of the vinyl group to the benzene ring was determined on the basis of NOEDS and HMBC spectra. When the vinyl proton (H-1) at δ 6.09 was irradiated, 8 and 7% NOEs were observed for the aromatic protons H-4 (δ 6.75) and H-8 (δ 6.66), respectively. These observations suggested that the vinyl group is located at C-3. This assignment was supported by the HMBC correlations of the vinyl proton H-1 (δ 6.90) to C-2 (δ 116.9) and C-3 (δ 126.3), and those of the other vinyl proton H-2 (δ 6.15) to C-1 (δ 136.4), C-4 (δ 113.1), and C-8 (δ 118.9). The geometry of the vinyl group was assigned to be E based on a large coupling constant (J = 12.4 Hz) for the two vinyl protons H-1 and H-2. The geometry was also confirmed by NOE experiments. No NOE was observed between H-1 and H-2. However, irradiation of the vinyl proton H-1 caused 17 and 18% NOEs on H-4 and H-8, respectively. Because substance 1 did not react with 2,4-dinitrophenylhydrazine, the attachment of a hydroxyl group to C-1 was unlikely. The carbon signals of C-1, C-5, and C-6 shifted downfield (0.01, 0.20, and 0.18 ppm, respectively) after changing the solvent from D₂O to H₂O. These deuterium-induced shifts indicated that the sulfate group is located at C-1, and two hydroxyl groups are located at C-5 and C-6. To confirm this, substance 1 was subjected to methylation reaction using diazomethane to give a single product, substance 2, which showed an Rₜ value of 0.75 on TLC under the conditions specified under "Materials and Methods." The negative FABMS of substance 2 showed the (M–Na)⁺ ion at m/z 259, suggesting that two hydroxyl groups were methylated. ¹H NMR spectrum of substance 2 measured at 27 °C in D₂O and CD₃OD, respectively, recorded in the literature, we named it "jaspisin." Carbon

<table>
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<th>Substance</th>
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<th>δc (mult., δhH)</th>
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<td>136.4 (J = 12.4 Hz)</td>
<td>7.21 (d, J = 12.4 Hz)</td>
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<td>2</td>
<td>116.9</td>
<td>6.15 (d, J = 12.4 Hz)</td>
<td>6.18 (d, J = 12.4 Hz)</td>
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<tr>
<td>3</td>
<td>126.3</td>
<td>6.75 (d, J = 1.8 Hz)</td>
<td>6.93 (d, J = 1.8 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>113.1</td>
<td>6.75 (d, J = 1.8 Hz)</td>
<td>6.93 (d, J = 1.8 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>143.8</td>
<td>6.67 (d, J = 1.8 Hz)</td>
<td>6.85 (d, J = 1.8 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>143.3</td>
<td>6.66 (dd, J = 1.8 Hz)</td>
<td>3.83 (s)</td>
</tr>
<tr>
<td>7</td>
<td>116.1</td>
<td>6.67 (d, J = 1.8 Hz)</td>
<td>6.86 (d, J = 1.8 Hz)</td>
</tr>
<tr>
<td>8</td>
<td>115.9</td>
<td>6.66 (dd, J = 1.8 Hz)</td>
<td>3.81 (s)</td>
</tr>
</tbody>
</table>

* Chemical shift of carbon atoms.
* Chemical shift of protons.
* Multiplicities of signals.
* Spin coupling constant between proton signals.
* Symbol s denotes singlet, d doublet, and dd double doublets.

**Fig. 3. UV spectrum (H₂O, pH 4.5) of substance 1.**

**TABLE I**

Chemical shifts and spin coupling constants for substances 1 and 2 measured at 27 °C in D₂O and CD₃OD, respectively

<table>
<thead>
<tr>
<th>Carbon</th>
<th>δc</th>
<th>δc (mult., δhH)</th>
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* Chemical shift of carbon atoms.
* Chemical shift of protons.
* Multiplicities of signals.
* Spin coupling constant between proton signals.
* Symbol s denotes singlet, d doublet, and dd double doublets.

Effects of Jaspisin on Membrane Fusion Events during Fertilization—When unfertilized H. pulcherrimus eggs were treated with jaspisin at concentrations of 15–100 µg/ml (approximately 60–400 µM) for 15 min and subsequently inseminated, sperm attached to the vitelline coat surrounding each egg but did not induce the fertilization envelope (Fig. 4). This result suggested that although the sperm acrosome reaction took place, some later processes of fertilization were blocked by jaspisin. To confirm that jaspisin does not affect exocytosis of the acrosome vesicle, sperm were incubated in 100 µg/ml jaspisin for 5 min, then the solution containing egg jelly was added to induce the sperm acrosome reaction. Twenty seconds later, the sperm were fixed as described under "Materials and Methods." Electron microscopic examination of the sperm head revealed that the acrosome reaction took place normally (data not available).
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FIG. 4. Inhibition of fertilization of sea urchin by jaspisin. Unfertilized eggs were incubated in the presence (panel A) and absence (panel B) of 30 μg/ml jaspisin for 10 min before insemination. The photographs were taken 5 min after insemination. Bar indicates 20 μm.

shown). Fertilization of A. pectinifera was also prevented by jaspisin at concentrations of 15–100 μg/ml.

By using the method of Hinkley et al. (19), we determined whether or not jaspisin inhibits fusion of the acrosome-reacted, sea urchin sperm with the plasma membrane of the sea urchin egg. In this assay, eggs were loaded with the DNA-staining vital dye Hoechst 33342. When sperm fuse with eggs that have taken up this dye, their nuclei become brightly fluorescent. In the control egg, one bright fluorescent sperm nucleus was visible, whereas in the egg that had been treated with 100 μg/ml jaspisin for 5 min, there were no fluorescent sperm nuclei, although several sperm were attached to the egg (Fig. 5). These data indicated that jaspisin prevents sperm-egg fusion.

We next examined the effect of jaspisin on exocytosis of the cortical granule of the sea urchin egg. “Lawns” of intact cortical granules that were bound to the egg’s plasma membrane-vitelline layer complex were prepared as described by Vacquier (13). These isolated cortical granule lawns were incubated with or without 50 μg/ml jaspisin for 15 min. We added CaCl₂ to give a final concentration of 1.5 mM in the medium, which was the minimum effective concentration to induce the cortical reaction. The jaspisin-treated cortices and control cortices discharged all of the cortical granules (data not shown). These data showed that gamete fusion, but not other membrane fusion events during the fertilization processes, was susceptible to jaspisin.

Effects of Jaspisin on Early Embryonic Development—We examined the effect of jaspisin on early embryonic development of H. pulcherrimus. When fertilized eggs were cultured at 20 °C in the presence of 15 μg/ml jaspisin from fertilization, they segregated completely to form two blastomeres of equal size. The embryos blastulated normally after passing through a rapid cleavage period and formed cilia on schedule. The ciliated blastula, however, were unable to hatch (Fig. 6). Primary mesenchyme cells migrated into the blastocoel, and the unhatched embryo gastrulated normally. When starfish embryos were cultured in the presence of 15 μg/ml jaspisin from fertilization, similar unhatched gastrulae formed. Mesenchyme cells formed at the midgastrula stage as in a control embryo (data not shown).

Hatching is the escape of the blastula from its fertilization
Inhibition of Echinoderm Gamete Fusion by Jaspisin

Recently, we found that halenaquinol sulfate, a hydroquinone sulfate obtained from the sponge Xestospongia sp. (27–29), prevented cell membrane fusion events of echinoderm gametes and hatching of embryos but affected neither cleavage, blastulation, nor gastrulation during embryogenesis (30). That halenaquinol sulfate did not affect the capability of H. pulcherrimus hatchling enzyme to dissolve the fertilization envelope of fixed embryos led us to conclude that the sulfate inhibits hatching through blockage of hatching enzyme secretion. However, halenaquinol sulfate is unstable in aqueous media and converted to halenaquinol and its oxidized product, halenaquinone (27), both of which do not affect hatching. Therefore, our conclusion that halenaquinol sulfate does not affect the dissolution of the fertilization envelope catalyzed by hatching enzyme was questionable. Since our trials to stabilize the substance were unsuccessful, we searched for more stable substances capable of inhibiting fertilization and hatching among extracts of marine sponges. From an extract of Jaspis species, we isolated a substance that specifically inhibits fertilization and hatching without affecting cell division and cell differentiation during early embryonic development. Structural analysis showed that this inhibitor is a novel sulfate, which we designated as jaspisin. In contrast to halenaquinol sulfate, jaspisin did not affect two exocytotic secretory events during fertilization, i.e., the sperm acrosome reaction and the egg cortical reaction. Therefore, it is unlikely that jaspisin inhibits exocytotic secretion of hatching enzyme from blastomeres. In this study, we demonstrated that jaspisin directly inhibits the activity of hatching enzyme to dissolve the fertilization envelope.

Hatching enzyme is a type of metalloendoproteinase which requires Zn$^{2+}$ for catalytic activity (25, 31). The metal chelator, 1,10-phenanthroline, inhibits the activity of H. pulcherrimus hatchling enzyme (22). 1,10-Phenanthroline also inhibits the sperm-egg fusion event during fertilization; this inhibition can be reversed by Zn$^{2+}$, which reconstitutes the process of fertilization (32, 33). Since jaspisin exists as an anionic form in ASW, it is rather difficult for the ion to diffuse through the plasma membrane. This may explain why endoplasmic membrane fusion events and cytokinesis are immune to the action of jaspisin. On the other hand, 1,10-phenanthroline is a hydrophobic molecule and readily enters the cell (34). Therefore, the endoplasmic fusion event during cytokinesis is expected to be blocked by 1,10-phenanthroline. In fact, the chelator inhibited

DISCUSSION

envelope by the action of a “hatching enzyme,” a Zn$^{2+}$-activated metalloendoproteinase secreted from the blastomeres (21–25). Hatching enzyme purified from medium of hatched sea urchin blastulae “hatched” methanol-fixed, two- to four-cell stage embryos; it dissolved their fertilization envelopes (21). The hatching enzyme activity was inhibited by jaspisin: under the conditions specified under “Materials and Methods,” jaspisin inhibited hatching by 50% at a concentration of 8.6 µg/ml when diluted hatched blastula medium was used as the enzyme preparation (Fig. 7). The same degree of inhibition was observed using a purified enzyme preparation.

In contrast to the starfish embryo, skeleton formation occurs in the sea urchin embryo. We looked for a jaspisin effect on spiculogenesis using specially prepared H. pulcherrimus embryos. Starting from 10 min after fertilization, we removed the fertilization envelope by treating with the hatched blastula medium (24) for 8 h. When jaspisin was added to a final concentration of 30 µg/ml in a culture of the denuded blastulae, after several washings with ASW, they gastrulated normally. The primary mesenchyme cells migrated within the blastocoel as they do in the untreated embryo (26). However, no spicules were formed even at 48 h after fertilization, although the embryos continued to do swim and appeared viable (Fig. 8). At this time, untreated controls had developed complete triradial skeletons.

![Fig. 7. Effect of jaspisin on the activity of hatching enzyme in the hatched blastula medium of sea urchin embryos. The enzyme activity was expressed as the percent of fixed two- to four-cell stage embryos whose fertilization envelopes were removed under the conditions described under “Materials and Methods.”](image)

**FIG. 7.** Effect of jaspisin on the activity of hatching enzyme in the hatched blastula medium of sea urchin embryos. The enzyme activity was expressed as the percent of fixed two- to four-cell stage embryos whose fertilization envelopes were removed under the conditions described under “Materials and Methods.”

![Fig. 8. Effect of jaspisin on spiculogenesis of sea urchin embryos. Embryos whose fertilization envelope was removed by the addition of the hatched blastula medium were washed in ASW and then incubated in the presence (panel A) and absence (panel B) of 30 µg/ml jaspisin for 40 h from 8 h after fertilization. Bar indicates 20 µm.](image)

**FIG. 8.** Effect of jaspisin on spiculogenesis of sea urchin embryos. Embryos whose fertilization envelope was removed by the addition of the hatched blastula medium were washed in ASW and then incubated in the presence (panel A) and absence (panel B) of 30 µg/ml jaspisin for 40 h from 8 h after fertilization. Bar indicates 20 µm.

S. Ikekami, H. Kobayashi, and Y. Myoyoishi, unpublished data.
the first cleavage of fertilized *H. pulcherrimus* eggs at concentrations approximately comparable to the minimum effective concentration to block fertilization. The sperm acrosome reaction is also inhibited by 1,10-phenanthroline (6). These studies implicate the involvement of metalloendoproteinases not only in exoplasmic membrane fusion events but also in endoplasmic membrane fusion events.

After removal of the embryonic *H. pulcherrimus* fertilization envelope, embryos treated with jaspin in ASW did not develop spicules (Fig. 8). The spicule is formed as a result of deposition of a glycoprotein matrix and mineral within the syncytial cable in a syncytium derived from cell-cell fusion of primary mesenchyme cells (35). The spiculogenesis of *Strongylocentrotus purpuratus* embryos is also prevented by 1,10-phenanthroline (34). Furthermore, carbenzoxy-Gly-Gly-NH$_2$, a dipeptide metalloendoproteinase substrate, also inhibits spicule formation in the embryo. On the other hand, addition of the dipeptide carbenzoxy-Gly-Gly-NH$_2$, which is not a metalloendoproteinase substrate, has no effect on spiculogenesis of the embryos (34). These observations suggest that a Zn$^{2+}$-dependent metalloendoproteinase is involved in the fusion of primary mesenchyme cells via filodipodium, which is a prerequisite to skeleton formation in sea urchin embryos. Lepage *et al.* (31) isolated a cDNA clone coding for a sea urchin embryonic protein, which was designated as BP10. BP10 contains a catalytic domain with an active center typical of a Zn$^{2+}$-activated metalloendoproteinase. BP10 is constructed with the same domains as the human bone mor-

References