学位論文

The Study on the Herring Sperm-Activating Proteins

ニシン精子活性化タンパク質の研究

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The Study on the Herring Sperm-Activating Proteins

Doctoral Thesis

Submitted to The University of Tokyo

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Zoological Institute, Graduate School of Science,
The University of Tokyo

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Abbreviations

ABC,	avidin-biotin-peroxidase complex
AGPC,	acid-guanidinium-thiocyanate-phenol-chloroform
BApNA,	a-N-benzoyl-L-arginine-p-nitroanilida
BSA,	bovine serum albmin
cAMP,	cyclic adenosin 3', 5'-monophosphate
cDNA,	complementary deoxyribonucleic acid
cGMP,	cyclic guanosine 3', 5'-monophosphate
DAB,	3,3'-diaminobenzidine
Da,	dalton
EDTA,	ethylenediaminetetraacetic acid
ELISA,	enzyme-linked immunosorbent assay
ESW,	egg seawater
HAT,	hypoxanthine/aminopterine/thymidine
HSAP,	herring sperm-activating protein
IEF,	isoelectric-focusing
IgG,	immunogloblin G
IPG-IEF,	immobilized-pH-gradient-isoelectric-focusing
kDa,	kilo dalton
mRNA,	messenger ribonucleic acid
OD,	optical density
PBS,	phosphate buffered saline
pI,	isoelecticpoint
PVDF,	polyvinylidene difluoride
SDS-PAGE,	sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis
SMIF,	sperm motility initiation factor
STI,	soybean trypsin inhibitor
STR,	short tandem repeat
TCA,	trichloroaceticacid
UV,	ultraviolet

General introduction

The task of spermatozoa is to deliver the male genetic information to egg. In order to carry out this task, spermatozoa possess the effective motile organelle, flagellum, which confers high motility on spermatozoa so that spermatozoa can approach egg from afar. The regulatory mechanism of flagella motility, namely sperm motility, is one of main subjects of cell biology and it is revealed that several external factors regulate the sperm motility in many species. Changes in external concentrations of Na^+ and H^+ in sea urchin (Christen et al., 1982) and rat (Wong et al., 1981), changes in external concentrations of K^+ and H^+ in salmonid fish (Morisawa and Suzuki, 1980), heavy metal ions in sea urchin (Clapper et al., 1985a), horseshoe crab (Clapper et al., 1985b) and starfish (Fujii et al., 1955; Mizuno, 1956), changes in external osmolality in fresh water cyprinid fish (Morisawa and Suzuki, 1980), marine fish (Oda and Morisawa, 1993), lamprey (Kobayashi W., 1993) and amphibian (Inoda and Morisawa, 1987), changes of environmental temperature in chicken (Wishart and Ashizawa, 1987), external CO_2 content in sea urchin (Johnson et al., 1983) are known to regulate sperm motility. On the other hand, rises in intracellular Ca^{2+} (Morton et al., 1978; Babcock and Pfeiffer, 1987; Tash and Means, 1987; Wishart and Ashizawa, 1987; Cosson et al., 1989; Serres et al., 1991; Boitano and Omoto, 1992; Oda and Morisawa, 1993), rises in intracellular pH (Christen et al., 1982; Lee et al., 1983; Babcock and Pfeiffer, 1987; Oda and Morisawa, 1993) and cyclic AMP-dependent phosphorylation of proteins (Hansbrought and Garbers, 1981a; Ishiguro et al., 1982; Morisawa and Okuno, 1982; Tash and Means, 1982, 1983; Opresko and Brokaw, 1983; Morisawa et al., 1984; Morisawa and Hayashi, 1985; Tash et al., 1986; Brokaw, 1987; Hayashi et al., 1987; Tash et al., 1988; Schoff et al., 1989; Okamura et al., 1990; Jin et al., 1994; Yoshida et al., 1994) are established to be intracellular factors regulating the sperm motility.

Fish spermatozoa are good and unique material for the study on

the mechanism regulating sperm motility (Morisawa, 1985, 1994). Spermatozoa of teleosts lack the acrosome (Jamieson, 1991). This unique feature prevents researchers from mistaking the reactions in the sperm cells those result in acrosome reaction for those result in the regulation of sperm motility. Furthermore, the motility of fish spermatozoa is well regulated by the external ionic concentration and osmolality (Morisawa and Suzuki, 1980), since the spermatozoa, which are completely quiescent in the male reproductive organs, initiate their motility just after they are spawned outside and exposed to the external ionic or osmotic conditions surrounding them (Morisawa and Suzuki, 1980). The motility response of fish spermatozoa to the external conditions is convenient in the study on sperm motility because the sperm motility can be controlled easily by changing in vitro experimental ionic or osmotic conditions. Using these advantages of fish spermatozoa, the mechanisms to regulate their motility is well understood. In salmonid fishes, sperm motility is suppressed by high concentration of K^+ in the seminal plasma and sperm motility is initiated by the decrease in the external K^+ concentration at the spawning. Under the experimental conditions, when the spermatozoa were diluted into K^+-free solution, sperm motility initiation occurs associated with the rises of intracellular Ca^{2+} (Cosson et al., 1989), the activation of adenylate cyclase (Morisawa and Ishida, 1987) and cyclic cAMP-dependent phosphorylation of 15 kDa protein (Morisawa and Okuno, 1982; Morisawa and Hayashi, 1985; Hayashi et al., 1987; Jin et al., 1994). In the marine teleosts, the sperm motility is suppressed by the osmolality isotonic to the Ringer's solution (about 300 mOsm/kg) in the seminal plasma and their motility is initiated in more hypertonic seawater (about 1100 mOsm/kg) at the spawning. Under experimental conditions, the spermatozoa can be kept immotile in solution isotonic to the Ringer's solution and the increase in external osmolality triggers the initiation of sperm motility. The increase in external osmolality initiates the sperm motility through rises of

intracellular Ca^{2+}, intracellular pH (Oda and Morisawa, 1993) and dephosphorylation of proteins (Oda et al., 1991).

In addition to the regulation of sperm motility by the changes of environmental condition at the spawning, it has been known that eggs release the substances which regulate sperm motility. Sperm chemotaxis is a response of motile spermatozoa to the gradient of chemical stimulants released from egg, causing the modulation of the direction of sperm travel so as to approach to the egg. Sperm chemokinesis is a change of swimming velocity of spermatozoa in response to the chemical stimulus from egg (Ralt et al., 1994), which corresponds to the phenomenon called "the activation of sperm motility". The activation of sperm motility by the egg and chemotaxis toward the egg have been reported in Cnidaria (Dan, 1950; Miller, 1966, 1985; Cosson et al., 1984), Mollusca (Miller, 1985), Annelida (Miller, 1985), Arthropoda (Clapper and Brown, 1980), Lophophorata (Miller, 1985), Echinodermata (Ohtake, 1976; Tubb, et al., 1979; Miller, 1985; Ward, et al., 1985; Suzuki, 1990; Nishigaki et al., 1994), Urochordata (Miller, 1975; Yoshida et al., 1993) and Vertebrate; herring (Yanagimachi and Kanoh, 1953; Yanagimachi et al., 1992; Morisawa et al., 1992), salmon (Yoshida and Nomura, 1972; Ohtake, 1988; Ohta and Imada, 1991; Yanagimachi et al., 1992), bitterling (Suzuki 1958, 1961), frog (Campanella, 1975) and human (Gnessi, et al., 1985; Diaz et al., 1990; Ralt et al., 1991, 1994; Zamir et al., 1993). The sperm activation by egg has been reported also in plant kingdom; Phaeophyceae (Kajiwara et al., 1980; Muller and Gassmann, 1985, Brownlee, 1990). Both chemotaxis and chemokinesis, widely distributed in all over the animal and plant kingdoms, are considered to have significant role in fertilization to direct many spermatozoa to the egg.

The study on the mechanism of the sperm activation by egg is a good subject for understanding the intracellular signal transduction system as well as the regulatory mechanism of sperm motility. Sea urchin is the only species of which the molecular mechanism

underlying the sperm activation by egg has been studied in detail. The sperm-activating substances from egg jelly were chemically studied for the first time by Ohtake (1976). The substances from egg jelly were identified to be decapeptides in 1981 and 74 peptides with sperm-activating activity were purified from 17 sea urchin species (Suzuki et al., 1981; Hansbrough and Garbers, 1981b; Garbers et al., 1982; Nomura et al., 1983; Suzuki, 1990) and one of the peptides, resact, is reported to possess the activity of chemoattractant (Ward et al., 1985; Cook et al., 1994). The complementary DNA of the sperm-activating peptides and their receptorgenes have been cloned in 1990 and 1989, respectively (Ramarao et al., 1990; Dangott et al., 1989). The outline of the mechanism of the sperm activation has been clarified; The receptor is associated with a guanylate cyclase and cGMP concentration in sperm cytosol increases in response to the biding of the peptide to the receptor (Shinomura et al., 1986; Bentley et al., 1988). Cyclic GMP rises intracellular pH through the Na^+/H^+ antiportor (Hansbrough and Garbars, 1981b; Lee, 1988; Reynaud et al., 1993) and intracellular Ca^{2+} concentration, causing the activation of sperm respiration (Ohtake, 1976; Repaske and Garbars, 1983; Suzuki and Garbars, 1984; Shackmann and Cook, 1986), the activation of flagellar motility (Ohtake, 1976) and the promotion of acrosome reaction (Yamaguchi et al., 1988, 1989). Since the cDNA of the atrial natriuretic peptides was isolated using a cDNA of guanylate cyclase from sea urchin spermatozoa, cGMP is established to be a common second messenger in spermatozoa and various other cells (Shimomura et al., 1986; Singh et al., 1988; Chinkers et al., 1989). Recently, the sperm-activating substances of starfish, which are different from those of sea urchins, were purified (Nishigaki, et al., 1994), although the molecular mechanism of sperm activation in starfish has not yet known.

The fact that the sperm activation by egg are observed in the various animal species, those may have been diversified in the process

of evolution (Darwin, 1859), suggests that the molecular mechanism of sperm activation by egg is common among the species. According to Kimura and Ohta (1974), molecular evolution is governed by five principles and one of them is that functionally less important molecules or parts of a molecule evolve faster than more important ones. This means that important molecules are tend to be conserved during the process of evolution. When it is possible to apply this principle to the subject discussed here, the mechanism of sperm activation should be conserved in the species because of its requisiteness for fertilization. If this is not true, we must think that the mechanisms of sperm activation by egg were developed independently in each species, even though the activation is fundamental step in fertilization. This opinion urges us to ask how the mechanisms of sperm activation by egg developed in each species. In order to give an answer, the molecular mechanisms of sperm activation by egg is needed to be clarified in many species and to be compared each other. Since the sperm-activating substance has been purified only in the echinoderm, purification and identification of the substances in other species are requisite.

Fish spermatozoa have many advantages in the study on the molecular mechanism of sperm activation by egg, because fish spermatozoa has been a good materials in the study on sperm motility regulation. Sperm-activating substances in fish are suggested in herring (Yanagimachi, 1957b; Yanagimachi et al., 1992; Morisawa et al. 1992) and salmon (Ohtake, 1988; Yanagimachi et al., 1992) and the sperm activation in herring is the most conspicuous phenomenon. The activation of sperm motility by egg in the herring was first observed by Yanagimachi and Kanoh (1953) who reported that the herring spermatozoa are completely immotile in the absence of egg and become motile only in the presence of egg, suggesting that the sperm activation by egg is a prerequisite step for herring fertilization. They also reported that the sperm-activating factor is protein and locates

near the micropylar opening of the egg, causing the attraction of spermatozoa to the micropylar opening (Yanagimachi, 1957b; Yanagimachi et al., 1992). The substance in herring has been partially purified by Morisawa et al. (1992), however, the mechanism regulating the herring spermatozoa remained completely unknown. Furthermore, herring has more advantages; The herring has the spawning season in a year and a large quantity of homogeneous mature egg and spermatozoa are easily obtained in the season. The assay system to measure the activity of purified samples in vitro is easily established, because the herring is one of species with external fertilization. These features are the important advantages when biochemical and physiological studies are performed.

In this study, the sperm-activating proteins were studied to understand the molecular mechanism of sperm activation by egg in herring and to understand the regulation mechanism of sperm flagellar motility. In part 1, 5 herring sperm activating proteins (HSAPs) were purified from the egg seawater, the supernatant of the suspension of ripe unfertilized egg in seawater, using G-50 gel filtration and isoelectric focusing. They have similar molecular weights around 8 kDa and isoelectricpoints of about pH 5. The similarity in molecular weights, isoelectric points, UV absorption spectra and partial amino acid sequences of the HSAPs determined by the Edman degradation method suggested that the HSAPs were isozyme. In part 2, DNA sequence of cDNA of the HSAP gene was estimated using the partial amino acid sequence determined by the Edman degradation method and the 114-mer oligonucleotide probe was synthesized. Using it, each cDNA libraries from an ovary and a liver were screened and clones were isolated. From the DNA sequence of the cloned cDNA of the HSAP, the HSAP was identified to be protein which is resemble to Kazal-type trypsin inhibitors. In part 3, it was demonstrated that the purified HSAPs interact with trypsin and that the trypsin inhibitors, commercially obtained, activated the motility of herring spermatozoa.

The localization of the HSAPs on the outermost layer of egg chorion of egg was also demonstrated by immunohistochemical study using monoclonal and polyclonal antibodies against the HSAP. These results suggested that herring eggs secrete the proteins which are resemble to the trypsin inhibitor to surrounding seawater and activate sperm motility to facilitate fertilization.

Part 1

Purification of Herring Sperm-Activating Proteins

Summary

The spermatozoa of the Pacific herring, Clupea pallasii, diluted in seawater, are quiescent in the absence of egg. They become motile when a ripe-unfertilized egg is present, suggesting that the sperm-activating substance is released from the egg into seawater to facilitate fertilization. Five species of the herring sperm-activating proteins (HSAPs), were purified from the egg seawater (ESW), the supernatant of the egg-suspended seawater, using G-50 gel filtration and two methods of isoelectric focusing, i.e. carrier-ampholyte-gradient-isoelectric-focusing and immobilized-pH-gradient-isoelectric-focusing. All of the 5 proteins activated almost all of the herring spermatozoa at the concentration of 10 ug/ml and the spermatozoa did not activated at the concentration of less than 1 ug/ml. It was suggested that threshold concentration of the proteins to activate the spermatozoa was around 5-10 ug/ml. The purified proteins have slightly different isoelectric point (pI) values (4.8, 4.9, 5.0, 5.1 and 5.4), and similar molecular weight of about 7700 estimated by Tricine SDS-PAGE. The molecular mass of a HSAP (pI=5.1), of which activity was the most conspicuous in the 5 HSAPs, was 8.1 kDa by time-of-flight mass spectrometry and its S value was 1.4 s. UV absorption spectra of the 5 HSAPs were very similar each other, suggesting that the HSAPs are of isoform.

Introduction

The spermatozoa of the Pacific herring, Clupea pallasii, are immotile or swim very slowly when they are put into seawater, while the spermatozoa of most marine teleosts swim vigorously in seawater. Yanagimachi and Kanoh (1953) first observed that the herring spermatozoa are activated when put together with a ripe-unfertilized egg in seawater, especially in the vicinity of the micropylar opening of the egg and reported that the activation of sperm motility by egg is prerequisite for the fertilization in the species. The sperm-activating activity is susceptive to the treatments with acidified Ringer's solution, trypsin and heating, suggesting that the sperm-activating factor is protein (Yanagimachi, 1957a). The factor could not easily removed from the egg chorion by rinse, suggesting that it attaches to the egg chorion firmly (Yanagimachi, 1958; Yanagimachi et al., 1992). Recently, the 105 kDa sperm-activating protein named sperm motility initiating factor (SMIF) was isolated from the egg chorion by the treatment of the chorion with acidified Ringer's solution (Pillai et al., 1992).

On the other hand, Morisawa et al. (1992) found that unfertilized egg activates the spermatozoa surrounding the egg and the supernatant of the egg-suspended seawater (egg seawater; ESW) possesses the ability to activate the herring spermatozoa, suggesting that another factor for sperm activation is released from the egg to the surrounding seawater, although Yanagimachi and his collaborators affirm that the chorion-attached SMIF may not be released to the surroundings (Yanagimachi et al., 1992). The activity of ESW was lost by the treatment with proteolytic enzymes, such as trypsin and pronase E (Morisawa et al., 1992), suggesting its proteinaceous nature. In this part, the herring sperm-activating proteins (HSAPs) contained in the ESW were purified and the characterization of chemical properties of the HSAPs were presented. It was further shown that the origin of the proteins

purified from the ESW is not the ovarian fluid but egg itself.

Materials and Methods

Fish Specimens of the mature Pacific herring, Clupea pallasii, were caught in Mangoku-ura, Matsushima Bay and the waters around Oshika Peninsula in Miyagi Prefecture in January and February, and in Furen-lake in Hokkaido in early April. Fresh female fishes were selected in the fish markets, whose ovulation was completed and eggs were easily extruded from their urogenital pores by gently pressing the abdomens. Purchased fishes were chilled on ice, transported to the laboratories and their ovaries were isolated. Fresh males were also selected in the fish markets and used as fresh as possible, because the spermatozoa lost the ability to respond to the HSAPs within 4-5 hr after the death of fishes, even if fishes were stored on ice.

Preparation of egg-conditioned medium Ripe eggs are so sticky that they adhere each other to form a hard mass when put into seawater. To steep the HSAPs efficiently from the eggs, ovulated ripe unfertilized eggs (600 g) collected from isolated ovaries from 10 females were spread onto the sheet of double-layered nylon nets (23cm x 34cm) to make monolayer of eggs avoiding the disruption of eggs. The nets with eggs were soaked in several trays containing filtered seawater for 45-60 min at ambient temperature of 4-10 ^{o}C and then the nets with eggs were removed. The seawater remained in the trays was pooled and centrifuged (3,000 xg, 10 min, 4 ^{o}C). The supernatant (3 l) obtained was designated as egg seawater (ESW) and used as a starting material for the purification of the HSAPs.

Gel filtration Solid $(NH_4)_2SO_4$ was added to the ESW to a final concentration of 95% to concentrate the sperm-activating activity. After centrifugation (10,000 xg, 10 min, 4 ^{o}C), the precipitate was dissolved in distilled water of 1/10 volume of the original supernatant and then the solution was extensively dialyzed against 0.5 M NaCl

buffered with 0.02 M Tris/HCl at pH 8.2 (Buffer A) using Spectrapor^{R} dialyzing tubes (MWCO=3500, Spectrum Medical Industries, Inc. Los Angeles, CA, USA). A portion (150 ml) of the dialyzate was applied to a column of Sephadex G-50 (Pharmacia, Uppsala, Sweden; 5 x 95 cm) that had been equilibrated with the Buffer A. The column was eluted at a flow rate of 80 ml/hr with the Buffer A and 15 ml of eluted samples were fractionized. Fractions with the sperm-activating activity were pooled, added solid $(NH_4)_2SO_4$ to a final concentration of 95% saturation and then centrifuged (10,000 xg, 10 min, 4°C). The precipitate was dissolved with 100 ml of distilled water and dialyzed extensively against 0.05 M Tris/HCl buffer at pH 8.2.

Carrier-ampholyte-isoelectric-focusing The fraction with the sperm-activating activity through the G-50 gel filtration was successively dialyzed against 0.01 M Tris/HCl buffer at pH 8.2 and a part of the dialyzate (42 ml) was applied to the electrofocusing column with 110 ml capacity. A 0-50 % concentration gradient of glycerol solution was prepared in the column that contained the dialyzate and 1.5 % Ampholine (pH 3-10, Pharmacia, Uppsala, Sweden), according to the method of Vesterberg (1971). Isoelectricfocusing was performed at 600-800 V for 48 hr at 0°C. After electrofocusing, the glycerol solutions and the sample were fractionated using a peristaltic pump preventing perturbation of the pH gradient. The fractions were extensively dialyzed against buffer A to remove glycerol from the fractions and then the sperm-activating activity in each fraction was evaluated as described below.

The active fractions were pooled and applied to the successively carrier-ampholyte-IEF with the pH gradient of pH 4-6. The procedures were the same as described above except that the ampholine of pH 4-6 (Pharmacia, Uppsala, Sweden) was used.

Immobilized-pH-gradient-isoelectric-focusing (IPG-IEF) IPG-IEF was carried out in the pH range of 4.6 - 5.6 according to the

method of Righetti (1990). Immobiline II were purchased from Pharmacia (Uppsala, Sweden). A portion (1 ml) of the dialyzate was applied onto an IPG gel plate (0.5 mm thick) containing 1.0 %(V/V) Ampholine of pH 4-6 (Pharmacia, Uppsala, Sweden) and electrophoresis was carried out at 600 V for 12 hr at 8°C. The gel plate was stained with Coomassie Brilliant Blue R-250. When the HSAPs were recovered from the gel, the gel plate was not stained and each band containing the protein was cut out and the proteins were eluted from the IPG gel using an electroelution instrument (Extraphore™, Pharmacia, Uppsala, Sweden).

Tricine-SDS-polyacrylamide-gel-electrophoresis (Tricine-SDS-PAGE)
Tricine-SDS-PAGE was carried out according to the method of Schägger and Jakow (1987) using 16.5% polyacrylamide gel. After electrophoresis for 4 hr at 15 mA, the gel was stained with Coomassie Brilliant Blue and/or a silver staining kit (2D-silver stain II "Daiichi", Daiichi Pure Chemical, Tokyo, Japan).

UV spectrometry and mass spectrometry UV absorption spectra of the HSAPs were measured using a spectrophotometer(U-best 55, Nihon Bunko, Tokyo, Japan). Time-of-flight mass spectrometry was carried out with a Kompact MALDI III (Shimadzu/Kratos, Tokyo, Japan) equipped with a laser ionization system.

Assay of sperm activating activity Since herring spermatozoa are activated most effectively with the ESW in 300 mM NaCl solution (Morisawa et al., 1992), the sperm-activating activity was assayed in seawater diluted to 60 % with distilled water (60 % seawater). The semen extruded from the urogenital pore of male fish was diluted 1000 times in 60 % seawater (50 ul) on a glass slide to a final concentration of approximately 2.0 $x10^7$ spermatozoa/ml. Then an aliquot (5 ul) of the ESW or of the fraction obtained through each purification step was added to the sperm suspension and the

percentage of motile spermatozoa was evaluated under a microscope. In some experiments, sperm motility was evaluated also in terms of grade 0-5: Grade 5 represents that 80-100 % of spermatozoa were motile, Grade 4; 60-80 % of spermatozoa were motile, Grade 3; 40-60 % of spermatozoa were motile, Grade 2; 20-40 % of spermatozoa were motile, Grade 1; 0-20 % of spermatozoa were motile, grade 0, all spermatozoa were immotile. The microscope (Optiphot, Nikon, Tokyo, Japan) was equipped with a negative phase contrast objective lens (SPlan 20 NH, Olympus, Tokyo, Japan). All experiments were performed at ambient temperature (2-5 ^{o}C), because the herring spermatozoa swam for longer than 1 hr in the presence of the ESW at 4 ^{o}C, while they lost their motility quickly at higher temperature (e.g. within 10 min in spite of the presence of the ESW at 37 ^{o}C).

Results

Origin of HSAPs

Mature herring eggs are covered with viscous fluid which has been presumed to be the ovarian fluid by Yanagimachi et al.(1992). There were possibilities that the origin of the sperm-activating protein in the ESW is the egg itself or the ovarian fluid sticking to the egg surface. When a tip of a micropipette was introduced into a lump of the ovulated eggs, then pulled out and the ovarian fluid sticking to the tip was added to the sperm suspension in a small amount of 60 % seawater, the spermatozoa were not activated, suggesting that the origin of the HSAPs in the ESW is not the ovarian fluid but the egg itself.

Purification of HSAPs

Three liter of the ESW was prepared from 600 g of ripe-unfertilized eggs obtained from 10 females and the HSAPs were purified from the ESW by the procedures shown in Figure 1.1. The ESW was concentrated by ammonium sulfate precipitation and applied onto a Sephadex G-50 column. The column was eluted with the equilibration buffer and fractions with the sperm-activating activity were pooled and concentrated with ammonium sulfate (Fig. 1.2). After dialysis, an aliquot of the concentrated active fraction was applied onto the carrier-ampholyte-IEF in the range of pH 3-10. The fractions with the sperm-activating activity was focused at two pH ranges; the major active fraction around pH 5 and the minor one around pH 6 (Fig. 1.3). When the major fraction focused at pH 5 was applied on the second carrier-ampholyte-IEF in the range of pH 4-6, the activity was separated to 5 peaks, which were focused around pH 5. The activity focused at pH 5.1 was the most conspicuous (Fig. 1.4).

When IPG-IEF was carried out in the pH range of 4.6-5.6, 5 protein bands visualized by Coomassie Brilliant Blue staining were focused (Fig. 1.5) at pI values of 4.8, 4.9, 5.0, 5.1 and 5.4. All of the 5

proteins had the activity to activate almost all the herring spermatozoa with the duration time of longer than 600 sec at the concentration of 10 ug/ml. At the concentration of less than 1 ug/ml, the all HSAPs did not activate the spermatozoa and there seemed to be threshold concentrations of the HSAPs around 5-10 ug/ml in the activation of the spermatozoa (Fig. 1.6). BSA did not activate the spermatozoa at the same or higher concentration. The bands focused at pH 4.9, 5.0 and 5.1 were more clear than the other peaks. About 10 mg of the HSAP (pI=5.1) was obtained from 600 g of ripe-unfertilized eggs.

Properties of HSAPs

Each HSAP migrated as a single band on SDS-PAGE and molecular weights of all of the 5 HSAPs were estimated to be 7700 (Fig. 1.7). The molecular mass of the HSAP (pI 5.1) was determined by time-of-flight mass spectrometry. The spectrum obtained clearly showed that the HSAP (pI 5.1) is a monomeric protein and has the molecular mass of 8.1 kDa (Fig. 1.8). This value is almost the same as that estimated by SDS-PAGE (Fig. 1.7). Its S value was determined to be 1.4 by moving boundary method.

The absorption spectra of the 5 HSAPs were very similar to one another and had common features with a not-sharp peak at 280 nm, a shoulder at 290 nm and a gentle slope around 270 nm (Fig. 1.9).

Discussion

Sperm activation by egg is the phenomenon observed in many animal species. The sperm-activating substance has been purified only in sea urchin (Suzuki et al., 1981) and recent studies have provided the details of the molecular mechanisms of sperm activation in this species (Ward and Kopf, 1993). In several species, purification and identification of chemical structures of sperm-activating substances are trying, for example in ascidian (Yoshida et al., 1993), human (Ralt et al., 1994), starfish (Punnett et al., 1992; Nishigaki et al., 1994) and herring (Pillai et al., 1993). Except for ascidian, the sperm-activating substances are considered to be proteins.

In the fish, the presence of the sperm-activating substance in the vicinity of the micropylar opening was reported not only in herring (Yanagimachi and Kanoh, 1953; Morisawa et al., 1992; Pillai et al., 1993) but also in bitterling (Suzuki, 1958, 1961) and in salmonids (Yanagimachi et al., 1992). In salmonids, the ovarian fluid is known to elongate the duration of sperm motility and it is not clear whether the sperm motility elongation is due to the chemical substance in the fluid or due to the physical properties of the fluid (Yoshida and Nomura, 1972; Ohtake, 1988; Ohta and Imada, 1991; Yanagimachi, et al., 1992). Pillai et al. have reported that the 105 kDa protein in the egg of C. pallasii is a sperm motility initiation factor (SMIF). According to them, the SMIF firmly attaches to the egg chorion so that it can not be released to the surrounding seawater easily, thus spermatozoa need to attach to the egg chorion to be activated. The 105 kDa protein was extracted from the isolated egg chorions by treatment with acidified Ringer's solution and purified by only one step of size exclusion fractionation (Yanagimachi et al., 1992; Pillai et al., 1993). On the other hand, Morisawa et al. (1992) showed that the spermatozoa in the vicinity of egg could be activated without attachment to the egg chorion, suggesting the presence of a water-soluble substance for the sperm activation in herring. Although Pillai and his collaborators

asserted that any sperm-activating activity was not found in the surrounding seawater or Ringer's solution, however, it may be possible that the soluble substance (the HSAPs) has been washed out before the start of the 105 kDa protein purification. The seawater-soluble HSAPs, in contrast to the 105 kDa protein, can be easily released from the eggs just by immersion of eggs in seawater as reported in the previous report (Morisawa et al., 1992). In the preparation of the HSAPs, the eggs were not rinsed before the purification of the HSAPs because the ovarian fluid had no sperm-activating activity.

The sperm-activating proteins in the ESW that were purified by ammonium sulfate precipitation, G-50 gel filtration and IEF, had slight different pI values. However, their ability to activate the herring spermatozoa and their molecular weights analyzed by Tricin-SDS-PAGE were the same extent. The absorption spectra of the HSAPs were very similar to each other. These results suggest the similarity of the 5 HSAPs in the structures and it is likely that these proteins are derived from the HSAP isogenes. To provide the definitive answer to the possibility that the HSAPs were coded by the isogenes, analysis of amino-acid sequences of these HSAPs and cloning of the corresponding genes were performed in part 2.

It was not certain whether the HSAPs and the 105 kDa protein were different proteins or not. There is a possibility that the water-soluble HSAPs are a part of the 105 kDa protein, which is removed by immersion or wash and released into surrounding medium. In order to make clear the relation between the HSAPs and the 105 kDa protein, the immunological studies on the cross reactivity of antibodies against the HSAPs and the 105 kDa protein is worth to be performed. The comparison of amino acid sequences of both the 105 kDa protein and the HSAPs is also necessary. In addition to the HSAPs and the 105 kDa protein, cholestan 3-ol was extracted from the homogenate of herring eggs and reported to be sperm-activating substance, although

its biological relevance in the herring fertilization is uncertain (Koshi and Ogawa, 1951).

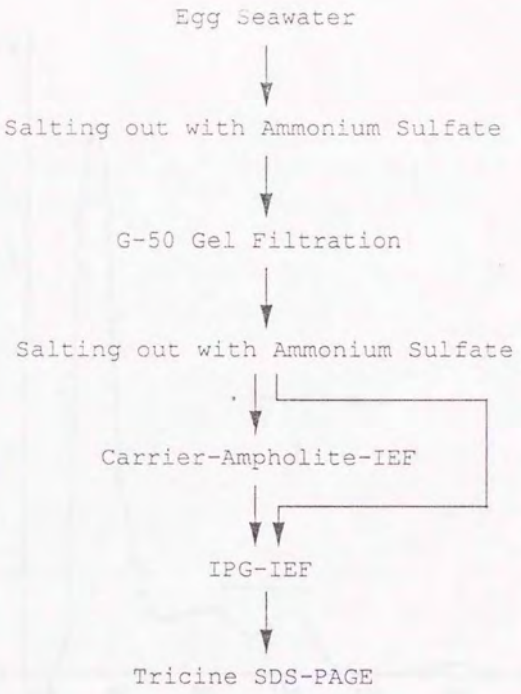


Figure 1.1. Procedures for the purification of the HSAPs from the ESW. The proteins salted out with ammonium sulfate from the ESW were fractionated by G-50 gel filtration. Active fractions were pooled and applied onto IPG-IEF directly or after fractionated by carrier-ampholyte-IEF. Proteins bands focused by IPG-IEF were analyzed by Tricine-SDS-PAGE.

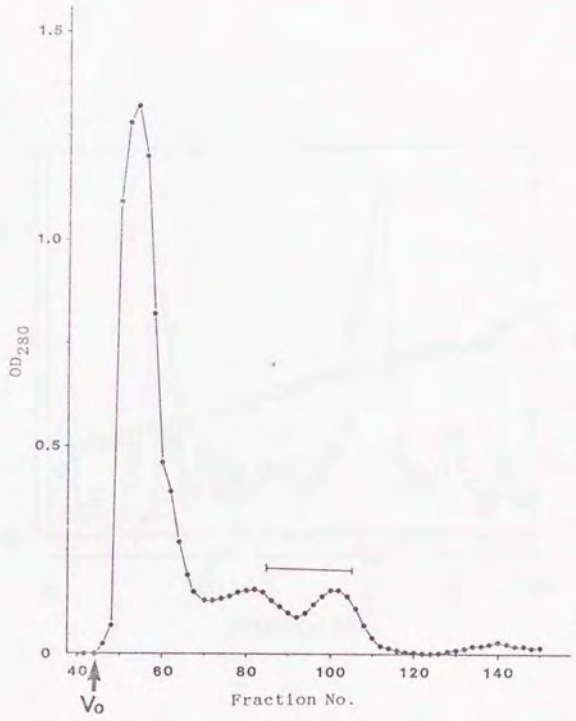


Figure 1.2. Gel filtration of ESW on the Sephadex G-50 column. The ESW was concentrated with $(NH_4)_2SO_4$ and dialyzed against the equilibration buffer; 0.5 M NaCl - 0.02 M Tris/HCl buffer, pH 8.2. A portion (150 ml) of the concentrated ESW was gel filtrated on a column (5 cm x 95 cm) and eluted with the equilibration buffer at a flow rate of 80 ml/hr. Each 15 ml of the eluted sample was collected and each absorbance at 280 nm and the sperm-activating activity were measured. The position of void volume (arrow) are shown. The fractions in which the activity was eluted is marked in the figure.

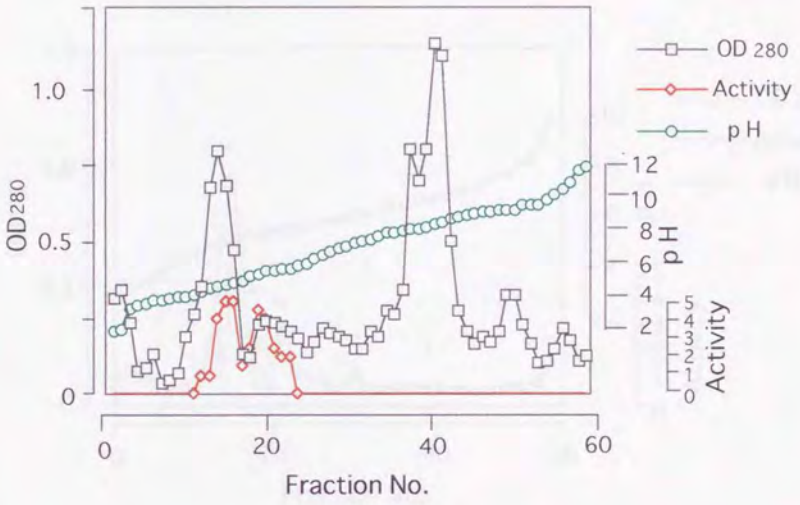


Figure 1.3. Carrier-ampholyte-IEF at the range of pH 3-10. The fractions with the sperm-activating activity were pooled and concentrated with $(NH_4)_2SO_4$, dialyzed extensively against 0.01 M Tris/HCl buffer, pH 8.2 and then applied onto the carrier-ampholyte-IEF at the range of pH 3-10. The activity of each fraction was evaluated in terms of grades as described in the Materials and Methods after removal of glycerol included in the fractions by dialysis. The fractions with the activity were pooled and successively applied onto the second carrier-ampholyte-IEF at the range of pH 4-6 (shown in Fig. 1.4).

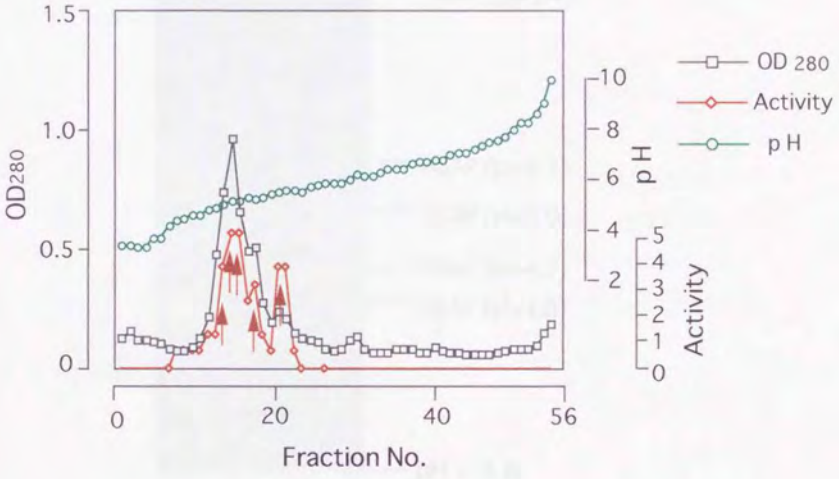


Figure 1.4. Carrier-ampholyte-IEF at the range of pH 4-6. The fractions with the sperm-activating activity through the carrier-ampholyte-IEF at the range of pH 3-10 were pooled and successively applied onto the carrier-ampholyte-IEF at the narrow range of pH 4-6 to separate the peaks of the activity. The peaks of the activity are indicated with red arrows.

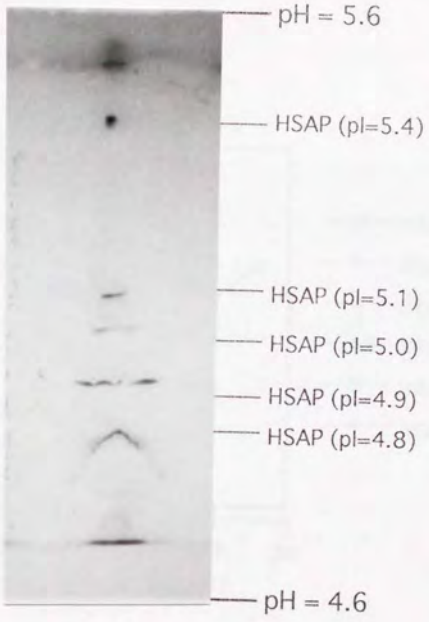


Figure 1.5. Immobilized-pH-gradient-isoelectric-focusing (IPG-IEF) of HSAPs. The aliquot (1 ml) of the active fraction through G-50 gel filtration was directly applied on "Immobiline" IPG-IEF at the pH range of 4.6 - 5.6. After electrophoresis at 600 V for 12 hr at 8 ^{o}C, the gel was stained with Coomassie Brilliant Blue.

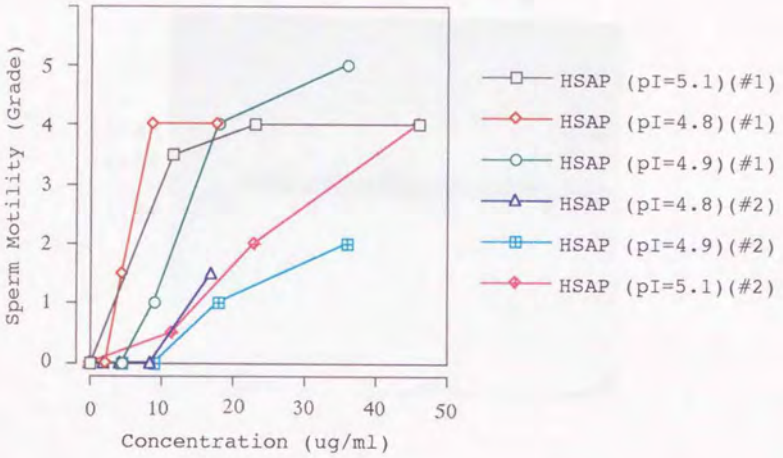


Figure 1.6. Activation of sperm motility by HSAPs. In the presence of the purified HSAPs (pI=4.8, 4.9, 5.1), sperm motility was evaluated in terms of grade 0-5 as described in Materials and Methods. Data from two individual males are shown and numbers of male used (#1 and #2) are shown in parenthesis in the figure. Note that the 3 HSAPs had almost the same specific activities in each individuals, while the specific activities of the 3 HSAPs were different between two individuals.

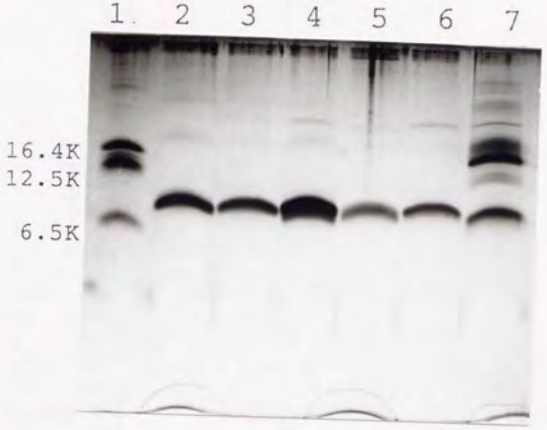


Figure 1.7. Tricine-SDS-polyacrylamide-gel-electrophoresis. Each band of the focused proteins on the IPG gel was eluted and applied on 16.5 % polyacrylamide gel. After electrophoresis,the gel was silver stained. Lane 1, Molecular weight makers; hemoglobin (16.4 kDa), cytochrome C (12.5 kDa) and aprotinin (6.5 kDa), Lane 2, HSAP (pI=5.1), Lane 3, HSAP (pI=5.0), Lane 4, HSAP (pI=4.9), Lane 5, HSAP (pI=5.4), Lane 6, HSAP (pI=4.8), Lane 7, Active fraction eluted through G-50 gel filtration.

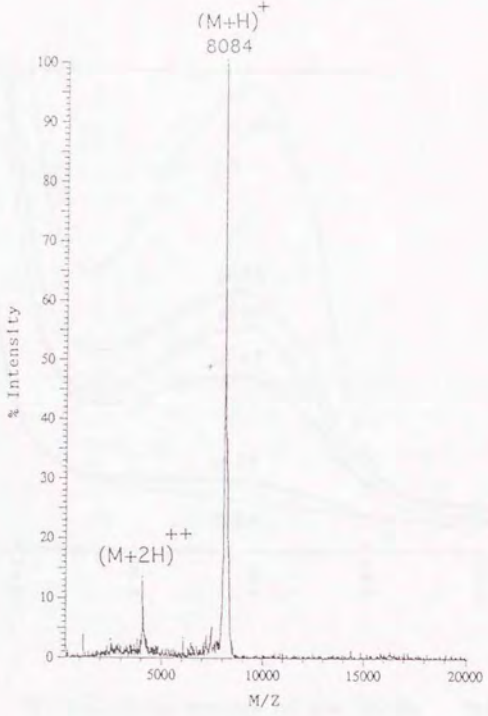


Figure 1.8. Determination of the molecular mass of HSAP (pI=5.1). The HSAP (pI=5.1) was eluted from the IPG gel and its molecular mass (M) was determined by time-of-flight mass spectrometry. Species corresponding to the mono- and di-protonated ($(M+1)^{+}$ and $(M+2)^{++}$, respectively) quasimolecular ions were observed and the position of mono-protonated quasimolecular ion indicates the molecular mass of the protein.

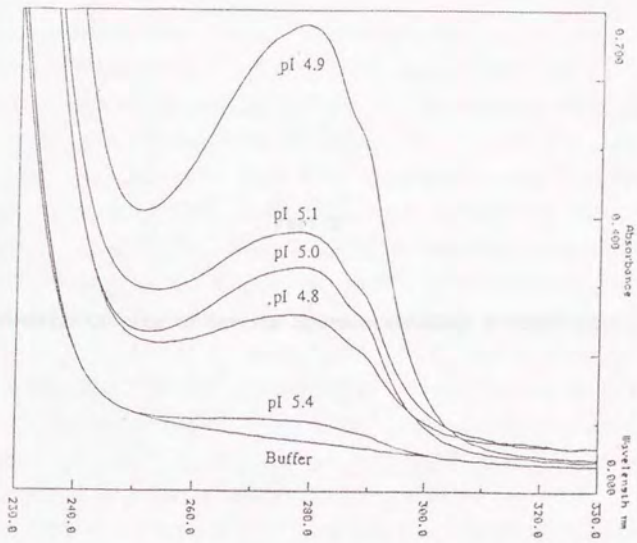


Figure 1.9. UV absorption spectra of the HSAPs. The purified 5 HSAPs were eluted from the IPG gel and those UV absorption spectra were measured. The spectrum of the control sample, which were eluted from the portion of the IPG gel without protein band, are shown (the spectrum with "Buffer" in the figure).

Part 2

Molecular Cloning of Herring Sperm-Activating Protein Gene

Summary

Complementary DNA (cDNA) clones encoding a herring sperm-activating protein (HSAP) were isolated from an ovary cDNA library of the Pacific herring, Clupea pallasii by the following procedures. N-terminal amino acid sequences of three HSAPs (pI=4.9, 5.0 and 5.1) were directly determined by the Edman degradation method to estimate the DNA sequence of HSAP cDNA. The cDNA library was constructed with lambda gt 22A DNA from the purified ovarian mRNAs or liver ones. An oligo-nucleotide probe was chemically synthesized, of which DNA sequence (114 nucleotide long) corresponded to the estimated sequence of the cDNA of the HSAP (pI=5.1). The cDNA libraries were screened with the radio-labeled probe and consequently 16 clones were isolated. Twelve clones from the ovary cDNA library contained the cDNA encoding the HSAP of 73 amino acid residues and the predicted HSAP included the partial amino acid sequence of the HSAP (pI=5.1) determined by Edman degradation method. The clones were classified into 3 species by the sequence of their cDNA inserts and one of the three possessed the initiation codon (ATG). The sizes of cDNAs in all of the clones isolated were about 500 base pair long. The cDNA of one of clones hybridized to three species of ovarian mRNAs; two species with about 500 nucleotide long and one species with about 1000 nucleotide long. It was suggested that the isolated cDNA clones were corresponded to the two smaller species of the mRNAs. The amino acid sequence of the HSAP predicted from the DNA sequence of the cDNA showed a similarity to Kazal-type trypsin inhibitors, such as acrosin inhibitor, pancreatic secretorytrypsin inhibitor and ovomucoid.

Introduction

Sperm activation by egg in the Pacific herring, Clupea pallasii, was first reported by Yanagimachi and Kanoh (1953) as described in the part 1. Yanagimachi (1957) suggested that the herring sperm-activating substance was protein and localized in the vicinity of the micropylar opening. Recently, Morisawa and his collaborators (1992) found that egg seawater (ESW), the supernatant of herring egg-suspension in seawater, contains the water-soluble proteins which activate the motility of herring spermatozoa. In part 1, 5 species of herring sperm-activating proteins (HSAPs) were purified from the egg seawater using G-50 gel filtration and isoelectricfocusing and their molecular weight (about 8 kDa), isoelectricpoints (around pH 5) and S value (1.4 s) were determined. It was also demonstrated that the UV absorption spectra of the 5 HSAPs were very similar each other, suggesting that the HSAPs are of isoform derived from isogenes.

On the contrary, another sperm-activating protein; sperm motility initiating factor (SMIF), was reported to be isolated in C. pallasii (Pillai et al., 1993). The SMIF was a water-insoluble 105 kDa protein and attached firmly to the egg chorion. To clarify the relation between the HSAPs and the 105 kDa protein, it is prerequisite to define each primary structure. The determination of the whole amino acid sequences of HSAPs is also prerequisite to understand the molecular mechanisms of sperm activation by the HSAPs.

The Edman degradation method is a way for determination of the whole amino acid sequences of the proteins by making peptide maps (Edman, 1950a, 1950b, 1956a, 1956b). In this method, the protein is digested by trypsin or other appropriate endoprotease and each fragment obtained is subjected to amino acid sequencing and then the whole amino acid sequence of the protein is estimated by re-constructing the sequences of the fragments (Bartelt et a., 1977; Jonakova, et al., 1992). Cloning of cDNA is another method to determine the whole amino acid sequences of the proteins. The cDNA

corresponding to the protein gene is isolated and cloned from the cDNA library and the amino acid sequence is predicted from the DNA sequence of the cDNA (Moritz et al., 1991; Yamamoto et al., 1985; Sakai, et al., 1989, 1992; Stallings-Mann et al., 1994). The latter method is more advantageous than the former one: Once the cDNA of the HSAPs are cloned, the HSAPs can be prepared in a large scale using expression vector. This is a big advantage in the study on the HSAPs, because the purification of the HSAPs from herring egg is restricted only in the spawning season of herring and the purification in a large scale is a hard work.

In this part, a partial DNA sequence of the HSAPs-cDNA was estimated from the N-terminal amino acid sequence of the HSAP determined directly by the Edman degradation method and an oligonucleotideprobe was synthesized according to it. The ovary and liver cDNA libraries were screened with the probe and cDNA clones encoding the HSAP were isolated. The predicted amino acid sequence of the HSAP showed that the HSAP is a 73 amino acid long protein and has a striking similarity to the members of Kazal-type trypsin inhibitor family.

Materials and Methods

Purification of HSAPs and amino acid sequencing The HSAPs were purified from the egg seawater as described in part 1. The HSAPs focused on IPG gel after IPG-IEF were electroblotted onto PVDF membrane (ProBlott™, Applied Biosystems Inc., Foster City, CA, USA) and dried up. The HSAPs with pIs of 4.9, 5.0 and 5.1, the major HSAP species, were subjected to gas-phase amino acid sequencing with a protein sequencer (Model 473A, Applied Biosystems Inc., Foster City, CA, USA). Analysis of the HSAP (pI=5.0) yielded amino acid sequences up to 20 amino acid residues from N-terminal one, while analysis of the others yielded up to 40 residues. Since the Edman degradation method was employed to sequence the HSAPs in this study, some amino acid residues, such as cysteine, were destroyed due to their susceptibility to the acidic hydrolysis and were undefined (Edman, 1950a, 1950b, 1956a, 1956b). Thus, several amino acid residues were not determined in each sequence of the HSAPs.

Screening of cDNA library and DNA sequencing Female herrings were caught at several times from 2 months before the spawning season to middle of the season, and stored at -30 °C until use. Total cellular RNA was extracted from the ovary and the liver of each female by the "AGPC method" (Chomczynski and Sacchi, 1987). Polyadenylated RNA was isolated from the extracted total RNA with a mRNA purification kit (Pharmacia, Uppsala, Sweden) and used as the template for cDNA synthesis. Lambda gt 22A cDNA libraries were constructed with a SuperScript™ Lambda system (Stratagene, La Jolla, CA, USA) and a Gigapack II packaging extract kit (Stratagene, La Jolla, CS, USA) from ovary polyadenylated RNAs or liver ones.

A part of the cDNA sequence (114 bases) of the HSAP (pI=5.1) was predicted according to the directly determined N-terminal amino acid sequence of the HSAP (pI=5.1) using the codon usage in mammals, since the codon usage in fishes is almost the same as that in mammals

(Wada et al., 1992). In the prediction of the nucleotide sequence, the 5 undefined amino acid residues in the directly determined sequence were judged as cysteine by comparison of amino acid sequences between the HSAPs and Kazal-type trypsin inhibitors (see Results), of which similarity was revealed by the preliminary comparison of their amino acid sequences. According to the DNA sequence at 5'-end region of the estimated cDNA, a single strand oligonucleotide (1-60) for the sense strand and a single strand for the antisense strand (53-114) were synthesized using a DNA synthesizer (Cyclone, Biosearch) and purified with Mono Q (Pharmacia, Uppsala, Sweden) column chromatography. Since their 8 nucleotideds at 3'-ends were designed to be complementary, they could anneal each other when they were combined (see Fig. 2.2). After annealing, DNA polymerase I, Klenow fragment (Amersham, Buckinghamshire, England) was added in the presence of [α-^{32}P] dCTP (Amersham, Buckinghamshire, England) to extend the both strands to 114 nucleotided long and to label them with ^{32}P. Unincorporated [α-^{32}P] dCTP was removed with a spin column (Quick Spin Column G-50, Boehringer-Mannheim, Mannheim) before hybridization.

Almost 5 x 10^5 independent recombinant phage plaques were transferred to nylon filters (Hybond N^+, Amersham, Buckinghamshire, England). Hybridization was carried out at 42 ^{o}C for 24 hr in 6 x SSPE (0.9 M NaCl, 6 mM EDTA, 0.06 M sodium phosphate buffer, pH 7.4) containing 0.5 % sodium dodecyl sulfate (SDS), 100 ug/ml denatured salmon sperm DNA and the ^{32}P-labeled oligonucleotide. Filters were washed at room temperature for 30 min in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5 % SDS and then at 45 ^{o}C for 30 min in 2 x SSC containing 1 % SDS. Sixteen positive clones were isolated and their inserts were subcloned into pBluescript^{TM} (Stratagene, La jolla, CA, USA). cDNAs of all clones were partially sequenced and those of two clones were completely sequenced by the dye-primer cycle sequencing method with a DNA

sequencer (Model 373A, Applied Biosystems Inc., Foster City, CA, USA) and by the dideoxy chain termination method with Sequenase (United states Biochemicals, Cleveland, OH, USA).

Northern blot analysis Total RNA from ovaries and livers were extracted by the "AGPC method" (Chomczynski and Sacchi, 1987) as described above, electrophoresed on a formaldehyde/agarose gel, transferred to nitrocellurose membrane (GeneScreen-plus, DuPont) and fixed to the membrane by baking at 80 °C for 2 hr. The blot was prehybridized for 4 hr followed by hybridization for 24 hr with the ^{32}P-labeled oligonucleotide probe at 42 °C.

Homology search analysis Homology search analysis was performed with the sequence of the clone, O-11, on SWISS-PROT (European Molecular Biology Laboratory), using GENETEX system (GENETEX Ver. 6.0, Software Development Inc, Tokyo, Japan) and DNASIS system (DNASIS, Hitachi Software Engineering Co., Yokohama, Japan).

Results

Amino acid sequences of HSAPs

Three HSAP (pI=4.9, 5.0, 5.1) purified by IPG-IEF were subjected to amino acid sequencing using a protein sequencer and sequences of 30-40 amino acid residues from each N-terminal ends were determined (Fig. 2.1). Sequence analysis demonstrated that the three HSAPs have almost the same N-terminal amino acid sequences, especially those of the pI 4.9 and 5.1 HSAPs were completely identical, suggesting that the HSAPs are of isoform. The homology search analysis with the partial amino acid sequences of the HSAPs revealed that the sequence of the HSAP is strikingly similar to the sequences of Kazal-type trypsin inhibitors (cf. Fig. 2.6) and they had no similarity to those of the sperm-activating peptides in sea urchin or starfish. Because the amino acid sequencing was performed by the standard procedures of the Edman degradation method, some amino acids, such as cysteine, were destroyed and could not be defined (represented as "X" in the Fig. 2.1). All undefined residues in the HSAP sequences corresponded to cysteine in other Kazal-type trypsin inhibitors (cf. Fig. 2.6), so that these undefined amino acid residues were estimated to be cysteines.

Isolation and sequencing of cDNA clones.

Screening of the herring ovary cDNA library (about $1x10^6$ recombinant colonies) with the synthesized oligonucleotide probe yielded 14 positive clones. Twelve clones among the 14 clones contained an cDNA insert encoding 73 amino acid residues, which included the N-terminal amino acid sequence of the HSAP (pI=5.1) determined with a protein sequencer. Twelve clones were classified to 3 species by the size of their cDNA inserts and one of the three possessed the initiation codon (ATG). The cDNA insert is 479 base pairs long, including 31 adenine residues for the poly-(A)+ tail (Fig. 2.3). The size of 479 base pairs was almost the same as that of mRNA (about 500 nucleotide long) determined by Northern blot

analysis (see below). The predicted amino acid sequence included the sequence which was identical to that of the HSAP (pI=5.1) except the 4th proline residue. The upstream region adjacent to the 1st arginine residue of the HSAP showed a typical signal peptides of 20 amino acid residues (von Heijne, 1990) and an initiation codon with the Kozak consensus sequence (CCACCATG) (Kozak, 1984) were pesent. The first terminal codon (TGA) occurred at the position of 317 and a HSAP constructed from 73 amino acid residues was encoded. In the amino acid sequence of the encoded HSAP, the amino acid sequence corresponding to the 38 amino acid residues of the HSAPs was included. The 3'-untranslated region was composed of 162 base pairs that included a consensus signal sequence (AATAAA) for a polyadenylation 18 bases upstream from the poly (A)+ tail. The cDNA predicted a HSAP composed of 73 amino acid residues with a calculated molecular weight of 8.173 kDa and this value was almost the same as those (8 kDa) of the HSAPs determined in part 1. The calculated isoelectric point of 5.13 (Skoog and Wichman, 1986) of the predicted HSAP corresponded to the pI value of the HSAPs (pI=5) (Table 2.1). The good correspondence of the molecular weights and pI values between the purified HSAPs (part 1) and the predicted HSAP suggested the absence of carbohydrate modification of the mature HSAPs. The all of undefined residues which were not defined in the amino acid sequencing by Edman degradation method were cysteine. The amino acid sequence predicted by the cDNA clones did not show any homology to those of the sperm-activating peptides in sea urchin or starfish.

Other clones (0-1, 0-2) coded the same HSAP as that coded by the clone, 0-11 (Fig. 2.4). These cDNA were a little smaller than the clone, 0-11 (Fig. 2.3), because these clones lacked 37 nucleotides from the position 17 to 53 of 0-11. Except this deletion and a point mutation at the position 340 (C →T), the sequence of 0-1 was identical to that of 0-11 (Fig. 2.4). The 3' terminal of the DNA sequence of the clone 0-2 has not determined yet.

Screening of the herring liver cDNA lirary (about $1x10^6$ recombinant colonies) with the synthesized oligonucleotide probe yielded 2 positive clones. They were partially sequenced, but they did not show any homology to the amino acid sequence of the HSAP.

Expression of the HSAPs mRNAs

The distribution of mRNAs for the HSAP was analyzed in ovaries and livers of females by using the clone 0-2, (Fig. 2.5). Two mRNA species of about 500 nucleotides long and one mRNA of 1000 nucleotides long were noted in the ovaries but there was no signal in the liver.

Similarity to Kazal-type trypsin inhibitors

The overall amino acid sequence of the HSAP, predicted from the insert cDNA sequence of the HSAP, was very similar to the sequences of Kazal-type trypsin inhibitors (Fig. 2.6). Between the predicted HSAP and bovine acrosin inhibitor, 44 % residues are common in the middle part of their amino acid sequences (Jonakova et al., 1992). Kazal-type trypsin inhibitors conserve six cysteines, which form three intramolecular disulfide bonds, and asparagine at position of 39 in the boar acrosin inhibitor, which is considered to function as a hinge i the molecules of Kazal-type trypsin inhibitors. These amino acid residues are important to maintain the three dimensional structures of Kazal-type trypsin inhibitor (Bolognesi and Gatti, 1982; Papamokos et al., 1982). The HSAP also possessed the conserved the six cysteines and the arginine in the corresponding positions, suggesting that HSAPs have the very similar three dimensional structure to Kazal-type trypsin inhibitors.

Discussion

The sperm activation by egg is well known in vertebrates, such as fishes including herring (Yanagimachi and Kanoh, 1953; Morisawa et al., 1992), bitterling (Suzuki, 1958, 1961) and salmons (Yoshida and Nomura, 1972; Ohtake, 1988), frog (Campanella, 1975) and mammals including rabbit (Dickman, 1963) and human (Gnessi et al., 1985; Diaz et al., 1990; Ralt et al., 1991, 1994). The herring sperm-activating proteins (HSAPs) were purified from the egg seawater and the characteristics of the proteins were described in part 1. In this part, the primary structure of one of them was determined as the sperm-activating substance first identified in vertebrates. The HSAPs were strikingly homologous to Kazal-type trypsin inhibitors.

Sperm motility initiation factor (SMIF) with molecular weight of 105 kDa was reported to be isolated from the egg chorion of the Pacific herring by Pillai et al. (1993). The 105 kDa protein attaches firmly to the egg chorion so that it can not be easily released to the surrounding seawater (Yanagimachi et al., 1992; Pillai et al., 1993). There was a possibility that the water-soluble HSAPs are the part of the 105 kDa protein and separated from the 105 kDa protein upon the release of egg into seawater at the time of spawning. The Northern blot analysis of mRNAs for the HSAPs demonstrated, however, that the sizes of the mRNAs for the HSAPs were too small to encode the 105 kDa protein, indicating that the HSAPs and the 105 kDa protein are encoded by quite different genes.

The amino acid sequences of the 3 HSAPs determined in this part were almost identical, suggesting that the HSAPs are of isoform. This idea has been already suggested in part 1. Furthermore, the HSAP coded by the isolated clones possessed a sequence of the N-terminal 38 amino acid residues, which was identical to those of the two HSAPs (pI=4.9 and 5.1) except that its 4th amino acid residue was proline instead of glutamine. This change in amino acid residues can occur by one point mutation in the codon encoding 4th glutamine (CAA →CCA or

CAG →CCG). Another HSAP (pI=5.0) contained the 4th proline as the predicted HSAP did, however, its 19th amino acid residue was arginine instead of lysine in the HSAPs (pI=4.9, 5.1 and predicted one). These indicated that the predicted HSAP from the isolated cDNA clones was different HSAP from the 3 HSAPs (pI=4.9, 5.0, 5.1) and suggested the presence of isoforms of the HSAPs encoded by isogenes. Further analysis of the cDNAs of the HSAPs will be necessary to make clear how many species of the HSAPs are present in the herring.

Molecular cloning of the genes encoding the HSAP suggested that HSAPs are the members of Kazal-type trypsin inhibitor family (Kazal et al., 1948; Chechova and Meloun, 1979; Laskowski and Kato, 1980; Meloun et al., 1984; Fink et al., 1990). The seminal plasma of mammals is known to contain several species of Kazal-type trypsin inhibitors called "acrosin inhibitors", since these inhibit a trypsin-like protease, acrosin, located in the sperm acrosome (Fritz, et al., 1974; Zaneveld et al., 1972, 1973; Jonakova et al., 1988). These inhibitors are assumed to protect spermatozoa or the wall of male and female genital tracts from proteolytic digestion by acrosin released from occasionally damaged spermatozoa (Bhattacharyya and Zaneveld, 1978; Moritz et al., 1991). Although, the physiological role of these inhibitors in fertilization is not clear, the blocking of fertilization by these inhibitors (Zaneveld et al., 1970, 1971; Suominen et al., 1973) and the observation that the concentration of these inhibitors in human cervical mucus become the lowest at the ovulatory period during the menstrual cycle (Schumacher and Zaneveld, 1974) have been reported, suggesting the inhibitory effects of acrosin inhibitors in seminal plasma and ovarian fluid on fertilization (Hirschhauser et al., 1971, 1972). Furthermore, the effect of seminal proteinase inhibitors on the sperm motility has been reported in mammals. In porcine, seminal proteinase inhibitors block the inactivation of adenylate cyclase by membrane-bound proteinase and consequently maintaine the bicarbonate activation of adenylate cyclase and sperm motility at high levels (Okamura et al., 1990) and

Lee et al. (1992) reported that an antithrombin III- like protein in follicular fluid stimulates porcine sperm motility. Jeng et al. (1993), however, reported that proteinase inhibitor in the seminal plasma inhibits the porcine sperm motility. The results presented here that the HSAPs, an acrosin inhibitor-like proteins, are responsible in the sperm activation in herring suggests the new physiological role of acrosin inhibitors on fertilization in vertebrates. Further investigation of the effects of HSAPs on sperm motility will be necessary for understanding the role of acrosin inhibitors for fertilization.

	Molecular weight (kDa)	Isoelectric point
Purified HSAP (pI=5.1)	8.1	5.1
Predicted HSAP	8.173	5.13*

Table 2.1. Molecular characteristics of purified HSAP and protein predicted from the DNA sequence of the clone, O-11. *Isoelectric point of the predicted HSAP was calculated by the method of Skoog and Wichman (1986).

```
                              5     10    15    20
HSAP (PI=5.1):    RSVQRIGIDXQGYGSAXTKE
HSAP (PI=5.0):    RSVPRIGIDXQGYGSAXTRE
HSAP (PI=4.9):    RSVQRIGIDXQGYGSAXTKE


                              25    30    35    40
HSAP (PI=5.1):    YRPIXGSDDVTYENEXLFXA
HSAP (PI=5.0):    YRPIXGALXVXY
HSAP (PI=4.9):    YRPIXGSDDVTYEXNXLFXA
```

Figure 2.1. N-terminal partial amino acid sequences of HSAPs. The amino acids are represented by their single-letter code. The purified HSAPs were electroblotted onto PVDF membrane and subjected to gas-phase amino acid sequencing. Since the standard procedure of the Edman degradation method was employed for the amino acid sequencing, some amino acids, such as cysteine, were destroyed and were not defined (represented as X). Note that the sequences of the HSAPs (pI=4.9 and 5.1) are identical and they are very similar to the sequence of another HSAP (pI=5.0).

(a)

ArgSerValGlnArgIleGlyIleAspCysGlnGlyTyrGlySerAlaCysThrLysGlu
CGCUCUGUGCAGCGCAUUGGCAUUGACUGCCAGGGCUAUGGCUCUGCCUGCACCAAGGAG

TyrArgProIleCysGlySerAspAspValThrTyrGluAsnGluCysLeuPhe
UACCGCCCCAUCUGUGGCUCUGAUGAUGUGACCUAUGAGAACGAGUGCCUGUUC

(b)

Probe 1: CGCTCTGTGCAGCGCATTGGCATTGACTGCCAGGGCTATGGCTCTGCCTGCACCAAGGAGT

Probe 2: GAACAGGCACTCGTTCTCATAGGTCACATCATCAGAGCCACAGATGGGGCGGTACTCCTTG

(c)

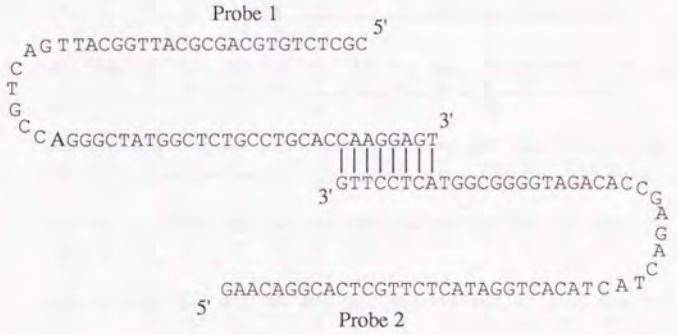


Figure 2.2. Design of probes for cloning of complementary DNA of HSAPs. (a) DNA sequence of HSAP-cDNA was estimated from the amino acid sequence of the HSAP (pI=5.1) according to the codon usage in fish. (b) Two 114-mer probes were synthesized according to the estimated DNA sequence of the HSAPs-cDNA as described in Materials and Methods. (c) The probes were designed to anneal with 8 bases of each 3'-terminals. After they annealed, Klenow's fragment was used to elongate the both chains to 114 bases long.

```
  T CGA CCC ACG CGT CCG GTC CCT TCT GAA GCC ACC ATG AAG  40
                                          M   K
                                             -20

CTG AGC ATT GTG ATC TCC ATC TGC GTT CTA CTT TAC TTC TCT  82
 L   S   I   V   I   S   I   C   V   L   L   Y   F   S
                                  -10

GGT CAC ACT TTG GCC AGG TCA GTC CCG AGG ATC GGG ATT GAT 124
 G   H   T   L   A   R   S   V   P   R   I   G   I   D
                -1  +1

TGT CAA GGC TAC GGT TCT GCT TGC ACT AAG GAG TAC CGC CCT 166
 C   Q   G   Y   G   S   A   C   T   K   E   Y   R   P
+10                                      +20

ATC TGT GGG TCC GAC GAC GTC ACC TAT GAA AAT GAA TGC TTG 208
 I   C   G   S   D   D   V   T   Y   E   N   E   C   L
                        +30

TTC TGC GCT GCC AAA CGA GAA AAT AGA TGG GGG ATT TTG GTC 250
 F   C   A   A   K   R   E   N   R   W   G   I   L   V
       +40                                      +50

GGT CAT CGC GGG GCA TGT ATA GCG TGG GGG GGG ATG GTG GAG 292
 G   H   R   G   A   C   I   A   W   G   G   M   V   E
                                +60

GAG TTG AGG GAG TGG AGC TCC GAC TGA GGT GAA CCC TGA CCC 334
 E   L   R   E   W   S   S   D end
                +70

TGA CCC TGA ACC CTG ATC CTA ACC CTA ACC CTA ACC CTA ACC 376

CTA ACC CCA ACC CTG ACC TGA GAT CAG AGG GAA ATG TCA GTA 418

AAG CAT CAA TAA AGC ATC TGT AAA GCA TAC AAA AAA AAA AAA 460
      ** *** *

AAA AAA AAA AAA AAA AAA A                               479
```

Figure 2.3. DNA sequence of the isolated HSAP-cDNA clone (0-11) and the predicted amino acids sequence of the HSAP. The nucleotide sequence is displayed above the predicted amino acid sequence, which are represented by their single-letter code under the appropriate codon. The signal peptide is underlined with a dotted line. The amino acid sequence corresponding to that of N-terminal amino acid residues of HSAP (pI=5.1), determined by Edman degradation method, is underlined with a solid line. The polyadenylation signal is indicated by asterisks. The boxed sequence indicates the Kozak sequence, the consensus initiation codon for translation.

```
GCG TCC GTC TCC ATC TGC GTT CTA CTT TAC TTC TCT GGT CAC  42
 A   S   V   S   I   C   V   L   L   Y   F   S   G   H
                           -10

ACT TTG GCC AGG TCA GTC CCG AGG ATC GGG ATT GAT TGT CAA  84
 T   L   A   R   S   V   P   R   I   G   I   D   C   Q
        -1  +1                                  +10

GGC TAC GGT TCT GCT TGC ACT AAG GAG TAC CGC CCT ATC TGT 126
 G   Y   G   S   A   C   T   K   E   Y   R   P   I   C
                               +20

GGG TCC GAC GAC GTC ACC TAT GAA AAT GAA TGC TTG TTC TGC 168
 G   S   D   D   V   T   Y   E   N   E   C   L   F   C
                +30

GCT GCC AAA CGA GAA AAT AGA TGG GGG ATT TTG GTC GGT CAT 210
 A   A   K   R   E   N   R   W   G   I   L   V   G   H
+40                                     +50

CGC GGG GCA TGT ATA GCG TGG GGG GGG ATG GTG GAG GAG TTG 252
 R   G   A   C   I   A   W   G   G   M   V   E   E   L
                        +60

AGG GAG TGG AGC TCC GAC TGA GGT GAA CCC TGA CCC TGA CCC 294
 R   E   W   S   S   D end
        +70
TGA ATC CTG ATC CTA ACC CTA ACC CTA ACC CTA ACC CTA ACC 336

CCA ACC CTG ACC TGA GAT CAG AGG GAA ATG TCA GTA AAG CAT 378

CAA TAA AGC ATC TGT AAA GCA TAC AAA AAA AAA AAA AAA A   418
 ** *** *
```

Figure 2.4. DNA sequence of the isolated HSAP-cDNA clone (O-1) and the predicted amino acids sequence of the HSAP. The nucleotide sequence is displayed above the predicted amino acid sequence, which are represented by their single-letter code under the appropriate codon. The region corresponding to signal peptide is underlined with a dotted line. The amino acid sequence corresponding to the sequence of N-terminal amino acid residues of HSAP (pI=5.1), determined by Edman degradation method, is underlined with a solid line. The polyadenylation signal is indicated by asterisks. Note that this clone lacks the Kozak sequence and initiation codon (ATG).

Figure 2.5 Expression of HSAP genes in herring ovary. Total cellular RNA was extracted by the "AGPC method", electrophoresed on formaldehyde/agarose gels and blotted onto nylon membrane. The blot was prehybridized for 4 hr followed by hybridization for 24 hr with the ^{32}P-labeled oligonucleotide probe at 42 ^{o}C. The positions of 28 S and 18 S ribosomal RNA were indicated with arrow heads.

```
                          10          20          30          40
HSAP      :       RSVPRIGIDCQGYGSACTKEYRPICGSDDVTYENECLFCA
BUSI-1    :  EIYFEPDFGFPPDCKVYTEACTREYNPICDSAAKTYSNECTFCN
BUSI-2    :       QGAQVDCAEFKDPKVYCTRESNPHCGSNGETYGNKCAFCK
HUSI-2    :  QFGLFSKYRTPNCSQYRLPGCPRHFNPVCGSDMSTYANECTLCM
BAI       : ARSKKTRKEPDCDVYRSHLFFCTREMDPICGTNGKSYANPCIFCS
BSI       :       TRKQPNCNVYRSHLFFCTRQMDPICGTNGKSYANPCIFCS
BPST-1    :        NILGREAKCTNEVNGCPRIYNPVCGTDGVTYSNECLLCM
HPST-1    :        DSLGREAKCYNELNGCTKIYDPVCGTDGNTYPNECVLCF

                     50          60          70
HSAP      :AKRENRWGILVGHRGACIAWGGMVEELREWSSD
BUSI-1    :EKMNNDADIHFNHFGECEY
BUSI-2    :AVMKSGGKINLKHRGKC
HUSI-2    :KIREGGHNIKIIRNGPC
BAI       :EKLGRNEKFDFGHWGHCREYTSA
BSI       :EKGLRNQKFDFGHWGHCREYTSARS
BPST-1    :ENKERQTPVLIQKSGPC
HPST-1    :ENRKRQTSILIQKSGPC
```

Figure 2.6. Alignment of amino acid sequences of the HSAP and several Kazal-type trypsin inhibitors. The amino acids conserved among four or more species are boldfaced. Amino acid residues are represented by their single-letter code. BUSI: Bovine seminal plasma acrosin inhibitor (Meloun et al., 1983); HUSI: Human seminal plasma acrosin inhibitor (Fink et al., 1990); BAI: Bovine acrosin inhibitor (Jonakova et al.,1992); BPST: Bovine pancreatic secretory trypsin inhibitor (Greene and Bartelt, 1969); HPST: Human pancreatic secretory trypsin inhibitor (Bartelt et al., 1977)

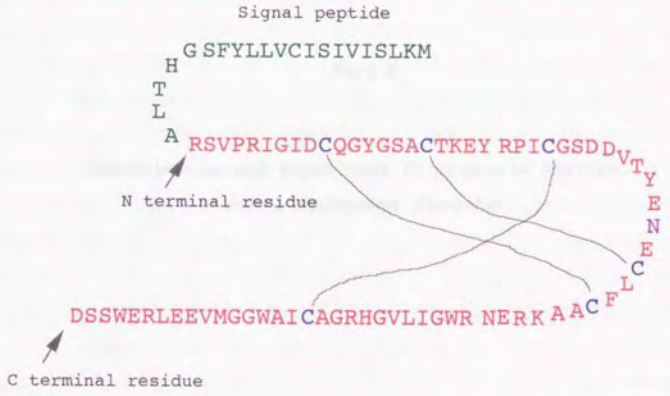


Figure 2.7. Primary structure of HSAP. Amino acids are represented by their single-letter code. Three intermolecular disulfide bonds were drawn imitating those of Kazal-type trypsin inhibitors.

Part 3

Localization and Functional Relevance of Herring Sperm-Activating Proteins

Summary

Molecular cloning of the cDNA encoding herring sperm-activating proteins (HSAPs) defined their primary structures and revealed the similarity between the HSAPs and Kazal-type trypsin inhibitors in part 2. We demonstrated in this part that the purified HSAP (pI=5.1) activated the proteolytic activity of trypsin. Furthermore, it was found that the trypsin inhibitors purchased commercially activated the herring sperm motility at the concentration of 1 mg/ml, although the HSAPs were effective at much lower concentration (10 ug/ml). These results suggest that not only the primary structures but also the three dimensional structures of the HSAPs and the trypsin inhibitors are resemble each other and that the binding of the trypsin inhibitor-like HSAPs from herring egg to the trypsin-like receptor on the sperm cells is the requisite process for triggering the intracellular cascade system for the activation of herring sperm motility.

To make clear the localization of the HSAPs, antisera and monoclonal antibodies were raised against the HSAPs and fixed ripe-unfertilized herring eggs were examined by immunohistochemistry. The immunoreactivity of the antisera and the monoclonal antibodies was detected in the outermost layer of the egg chorion, suggesting that the localization of the HSAPs is not restricted to the vicinity of the micropylar opening where conspicuous activation of the herring sperm was reported. Relationships between the localization of the HSAPs and their function and their origin were discussed.

Introduction

The sperm activation by egg in the Pacific herring, Clupea pallasii, has been reported to be conspicuous around the micropylar opening of the egg (Yanagimachi, 1958; Yanagimachi, et al., 1992) and that the sperm-activating substance is a water-insoluble protein which tightly attaches to the egg chorion in the vicinity of the micropylar opening (Yanagimachi, 1957a; Yanagimachi et al., 1992). Recently, a protein with molecular weight of 105 kDa was isolated from the egg chorion as a sperm motility initiation factor (SMIF) in the Pacific herring (Pillai et al., 1993).

On the other hand, Morisawa et al. (1992) reported that the herring egg releases the sperm-activating proteins (HSAPs) into surrounding seawater (which is soluble to seawater). In this study (part 1), 5 species of the HSAPs were purified from the egg seawater by gel filtration and isoelectricfocusing. The cDNA corresponding to the HSAP gene was isolated from a herring ovarian cDNA library and one of the HSAPs was identified as a proteins with 73 amino acid residues and molecular weight of 8173 Da in part 2. The amino acid sequence of the predicted HSAP was resemble to those of Kazal-type trypsin inhibitor, suggesting that the function of the HSAPs substitutes for those of the trypsin inhibitors. Therefore, questions whether the HSAPs can inhibit trypsin or not and whether the trypsin inhibitors possess the ability to activate the herring spermatozoa or not, will be answered in this part.

It was shown that the HSAPs increased the proteolytic activity of trypsin, suggesting the interaction of the HSAPs and trypsin. It was also found that the some trypsin inhibitors possessed the ability to activate the herring sperm motility, suggesting the similarity of three dimensional structures of the HSAPs and the trypsin inhibitors. Furthermore, the localization of the HSAPs on the herring mature egg was studied using the monoclonal and the polyclonal antibodies raised against the HSAPs to make clear whether the HSAPs were localized

around the micropylar opening or not.

Materials and Methods

Purification of HSAPs The HSAPs were purified as described in part 1. The egg seawater (ESW) were prepared and the contained proteins were precipitated with ammonium sulfate and then fractionated with Sephadex G-50 gel filtration after dialysis. The active fractions were pooled and directly applied to immobilized-pH-gradient-isoelectric-focusing (IPG-IEF). For the preparation of monoclonal antibodies, the HSAP (pI=5.1) purified with IPG-IEF (see Fig. 1.5) was used as the immunogen. In the preparation of antisera against the HSAPs, the fraction focused around pH 5 in the carrier-ampholyte-isoelectric-focusing (IEF) in the range of pH 3-10 (see Fig. 1.3) was pooled, dialyzed and provided.

Assay of sperm motility

Males of the Pacific herring, Clupea pallasii, were purchased in the fish markets as described in part 1. Fresh male fishes were selected whose semen was easily extruded from their urogenital pores when their abdomens were gently pressed, because only the spermatozoa from such males responded to the sperm-activating activity of the ESW or the purified HSAPs. Their testes were isolated as soon as possible and chilled on ice until use.

Sperm motility was evaluated as described in part 1 with a slight modification as described below. The semen extruded from the isolated testis was diluted 100 times within an isotonic solution of 150 mM NaCl buffered with 20 mM HEPES/NaOH at pH 8.2, and then incubated for 30-60 min at 2-4 ^{o}C. One ul of the sperm suspension was further diluted into 50 ul of 60 % seawater containing the trypsin inhibitors or the HSAPs. The percentage of motile spermatozoa was evaluated under a microscope. All experiments were performed at ambient temperature (2-5 ^{o}C) and never performed at higher temperatures.

Measurement of proteolytic activity of trypsin Proteolytic activity of trypsin was measured according to the method of Said (1991) with a slight modification. Trypsin (20 ug/ml) was incubated with Soybean trypsin inhibitor (type 1-S, #9003, Sigma, St Louis, MO, USA) or the HSAP (pI=5.1) purified by IPG-IEF in 100 mM Tris/HCl buffer (7.5) containing 3 mg/ml Casein for 20 min at 30 ^{o}C. Then, equal volume of 10 %(W/V) trichloroacetic acid (TCA) was added, centrifuged for 5 min and UV absorption at 280 nm of the supernatant was measured.

Preparation of antiserum The HSAPs fraction (200 ul) obtained by carrier-ampholyte-IEF (pH 3-10) was diluted with 1 ml of PBS (13.7 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4) and emulsified in 1.5 ml Freund's complete adjuvant (Yatoron, Tokyo, Japan). Antisera against the HSAPs were prepared according to the standard method (Harlow and Lane, 1988). For the first immunization, the HSAP sample was directly injected into both left and right subpopliteral-lymph nodes. In the following immunizations, the emulsified HSAP sample was subcutaneously injected at several dorsal sites on the footpads of a rabbit every 2 weeks for 2 months. Three days after the final immunization, the rabbit was bled from the heart and the serum was separated and stored at -80 ^{o}C. Its titer to react with the HSAP were tested by the ELISA as described later.

Immunization The HSAP (pI=5.1) (10 ug) purified by IPG-IEF was blotted on nitrocellulose membrane and the membrane was homogenized by sonication in PBS buffer (1 ml). Then the homogenate was emulsified with Freund's complete adjuvant (Yatoron, Tokyo, Japan) with a ultrasonic sonicator and injected intraperitoneally into each of 6 male BALB/c mice (8 weeks old). Each mouse received 10 ug of the purified HSAP (pI=5.1) emulsified with Freund's incomplete adjuvant every 10 days for 3-4 times and sacrificed three days later of the last injection.

Cell fusion and screening of hybridoma The mouse was killed and its spleen cells were fused with the mouse myeloma NS-1 cells at a ratio of 10:1 by the standard procedures (Koehler and Milstein, 1976). Polyethylene glycol 4000 was used as the fusing agent and the cells were plated in 96-well tissue culture dishes in hypoxanthine /aminopterin/thymidine selection medium (HAT medium). The hybridoma cells were screened for the production of specific antibodies against the purified HSAP (pI=5.1). The supernatant of each well were tested for their activity by enzyme-linked immunosorbent assay (ELISA). Antibody-producing clones were obtained by the limiting-dilution method and several clones were injected into the pristane (Sigma, St. Louis, MO, USA)-treated mice for the production of ascites. The cloned hybridoma were frozen and stored at -80 ^{o}C.

ELISA ELISA was performed with the purified native HSAP (pI=5.1). The antibodies generated by the hybridoma cells were spotted on the plates and the intensity of the color developed by the protocol of the ABC-anti-mouse-IgG (H+L) kit (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) was quantified with an ELISA reader. The amount of immunoglobulin contained in the culture medium was determined by ELISA using purified normal mouse IgG as standards.

Preparation of tissue Ripe unfertilized herring eggs were collected from the urogenital pores of females by gentle pressing the abdomens. Masses containing about 10 eggs were fixed in Bouin's solution, dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin and cut in thickness of 3 um.

Immunohistochemistry Immunohistochemical detection of the HSAP was carried out with the ABC-anti-mouse-IgG (H+L) Kit on the paraffin sections. The sections were incubated with the primary antibodies (diluted 1:5000 with 50 mM PBS (4.3 mM Na_2HPO_4, 0.625 mM

NaH_2PO_4, 7.75 mM NaCl, pH 7.3) containing 0.05 % Triton X-100 and 1 % normal horse serum) or the diluted ascites containing about 0.12 ug/ml IgG for over night at 4°C, rinsed with 50 mM PBS and incubated with biotinylated anti-mouse or anti-rabbit IgG for 1 hr at room temperature. Next, the sections were rinsed with 50 mM PBS and incubated with the ABC-kit for 1 hr at room temperature. After the several PBS rinses, the sections were reacted with 0.05 % 3,3'-diaminobenzidine (DAB) - 0.01 % H_2O_2 - 50 mM Tris/HCl (pH 7.6) for 15 min. The sections were rinsed with 50 mM PBS to terminate the reaction and examined with a microscope.

Trypsin inhibitors Three inhibitors from soybean (type 1-S, #T9003, type 2-S, #T9128, and Bowman-Birk type, #T9777), two inhibitors from chicken egg white (ovomucoid, type 3-O, #T2011, and ovoinhibitor, type 4-O, #T1886) and one inhibitor from bovine pancreas (Kunitz type, type 1-P, #T0256) were purchased from Sigma (St. Louis, MO, USA).

Results

Interaction of HSAP and trypsin

The similarity of the amino acid sequences between the HSAP and Kazal-trypsin inhibitors raised the question whether the HSAPs inhibit trypsin or not. However, when the HSAP (pI=5.1) and trypsin were co-incubated, the trypsin activity to digest casein increased (Fig. 3.1). In the presence of the HSAP at the concentration of 25 ug/ml trypsin was activated for 1.5 times of that in the absence of the HSAPs, while the same concentration of soybean trypsin inhibitor (type 1-S, #T9003, Sigma) inhibited trypsin activity. These results suggested that the HSAP interacts with trypsin and acts as the activator rather than the inhibitor.

Activation of sperm motility by trypsin inhibitors

Seven trypsin inhibitors including three inhibitors from soybean (type 1-S, #T9003, type 2-S, #T9128, and Bowman-Birk type, #T9777), two inhibitors from chicken egg white (ovomucoid, type 3-O, #T2011, and ovoinhibitor, type 4-O, #T1886) and one inhibitor from bovine pancreas (Kunitz type, type 1-P, #T0256) were commercially purchased and their ability to activate the motility of the herring spermatozoa were examined. Among them, 3 inhibitors, soybean trypsin inhibitor (type 2-S, #T9128), Bowman-Birk type trypsin inhibitor from soybean (#T9777) and Kunitz-type trypsin inhibitor from beef pancreas (#T0256), activated the herring sperm motility at the concentration of 1 mg/ml (table-3.1), while the purified HSAP (pI=5.1) are effective at the much lower concentration (10 ug/ml) (see part 1). The other 4 inhibitors did not activate the sperm motility at the same concentration of 1 mg/ml. This activation would be due to the specific activity of trypsin inhibitors, because BSA and IgG did not activate the sperm motility at the same or higher concentrations (1-10 mg/ml).

Localization of HSAPs

Although there are the reports suggesting that sperm-activating proteins are localized in the vicinity of the micropylar opening (Yanagimachi, 1958; Yanagimachi et al., 1992), the immunoreactivity to the antiserum to the HSAPs distributed in the outermost layer of the egg chorion (Fig. 3.2). The egg chorion of herring is constructed from three layers and only the outermost layer was immunoreacted to both of the antiserum and the monoclonal antibodies, while the inner two layers did not show any immunoreactivity (Fig. 3.2). The cytosol and the yolk droplets were not reacted in most eggs, however, in some eggs some staining was seen in the cytosol and the vesicles beneath the plasma membrane (Fig. 3.2c, 3.4). Non-immunized rabbit serum did not stain the outermost layer of the egg chorion nor the other parts of the egg (Fig. 3.3). Removal of mucin by the treatment with neuraminidase did not affect the staining of the outermost layer (Fig. 3.4). Moreover, pre-incubation of the antibodies with the excess amount of the HSAP (pI=5.1) before the staining decreased the stain in the outermost layer of the egg chorion (data not shown).

Twenty-five clones whose IgG reacted to the HSAP (pI=5.1) on ELISA were obtained. When two kinds of monoclonal antibodies (No. 27, 31) were used, the immunoreactivity to those antibodies was also seen in the outermost layer of the egg chorion (Fig. 3.5). These results suggests that the immunoreactivity in the outermost layer of the egg chorion represents the localization of HSAP (pI=5.1). In the vicinity of the micropylar opening, there was no particular immunoreactivity with either the antiserum or the monoclonal antibodies against the HSAP (pI=5.1) (Fig. 3.6).

Discussion

Due to the similarity of the amino acid sequences between the herring sperm-activating proteins (HSAPs) and Kazal-type trypsin inhibitors, it was likely that the HSAPs might have the ability to inhibit trypsin. The HSAP, however, activated trypsin when it was co-incubated with trypsin. Some substrates for trypsin are known to have various effects on different types of proteinases: BApNA (a-N-benzoyl-L-arginine-p-nitroanilide), a well known trypsin-specific substrate, inhibits the activity of the Streptomyces proteinases (Nakata et al., 1972), but activates bovine trypsin (Nakata et al., 1970). While porcine trypsin is not activated nor inhibited by BApNA (Nakata et al., 1973). The effect of the HSAP on the trypsin activity is not unexpected one. It is possible that the HSAP activates trypsin as BApNA activates bovine trypsin. This result suggests that the HSAPs actually interact with trypsin.

Three of seven tested trypsin inhibitors as well as the HSAP activated the motility of the herring spermatozoa, suggesting that the HSAPs and the trypsin inhibitors possess a common three dimensional structure, or some parts of their structures are resemble. The sperm activation by the trypsin inhibitors also suggested the possibility that the HSAP-receptor on the herring spermatozoa is a trypsin-like molecule. If the receptor for the HSAPs is trypsin or its analogue, the presence of a unique intercellular and intracellular signal transduction systems will be suggested; Binding of the trypsin-like receptor and the trypsin inhibitor-like ligand induces the activation of sperm motility. Recently, much evidence has accumulated suggesting the significant roles of protease for the regulation of cell functions. For example, some protease inhibitors are reported to act as endothelial growth factors in human hepatoma cells (McKeehan et al., 1994).

It were reported that the sperm activation occurs only around the micropylar opening of the herring egg and that the structure of the

egg chorion around the micropylar opening is different from other parts of the egg chorion with special staining patterns by some dyes (Yanagimachi, 1958; Yanagimachi et al., 1992). Based on these observations, it seems possible that the sperm-activating factor is localized in the vicinity of the micropylar opening. However, the immunohistochemical study with the antiserum and the monoclonal antibodies against the HSAPs showed here that the HSAPs mainly localized at the outermost layer of the egg chorion surrounding the surface of the egg. There was no particular immunoreactive region in the vicinity of the micropylar opening. The sperm activation, however, is reported to occur in the vicinity of the micropylar opening (Yanagimachi, 1958, Yanagimachi et al., 1992) and it is possible that the HSAPs are released into the surrounding seawater only from the vicinity of the micropylar opening by unknown mechanisms. On the other hand, there is other observation that the sperm activation is not limited around the micropylar opening but also all over the surface of egg (Morisawa et al., 1992).

There are possibilities that sperm-activating/attracting factors other than HSAPs which guide the swimming spermatozoa to the micropylar opening is localized in the vicinity of the micropylar opening. The special staining of the micropylar opening by some dyes (Yanagimachi, 1958, Yanagimachi et al., 1992) suggests the localization of some substances in the micropylar region. Recently, the 105 kDa protein was proposed to be such factor, although its chemical properties and localization in the egg are not reported (Pillai et al., 1993). Since the mRNA of the HSAPs were too small to code the 105 kDa protein as demonstrated in part 2, it is reasonable to say that the HSAPs are different proteins from the 105 kDa protein. There is a possibility that the HSAPs and the 105 kDa protein co-operate to activate the herring spermatozoa; The HSAPs released from the egg activate the immotile spermatozoa around the egg and then the 105 kDa protein guides the swimming spermatozoa to the micropylar opening

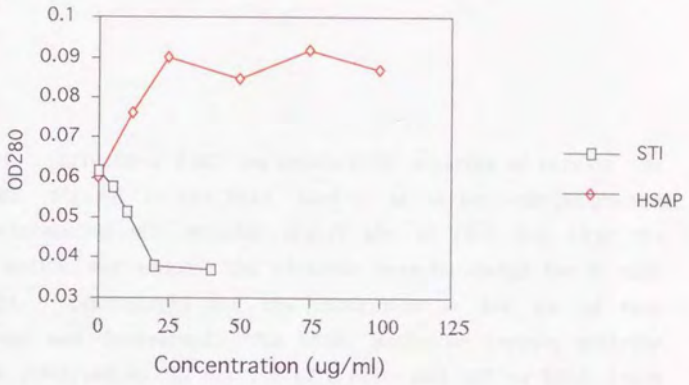
when the activated spermatozoa reach the micropylar opening. It is also possible that the egg of the Japanese herring species possess the HSAPs and the American herring species possess the 105 kDa protein to activate the spermatozoa, since the recent study on isozyme analysis revealed that the Japanese herring species and the American herring species are too different to be classified in the same species (Kobayashi,T., 1993).

In Medaka, Olyzias latipes, the components of egg chorion are synthesized in liver, then transported to ovary by the blood flow and constructed into the egg chorion (Yamagami et al., 1992). On the other hand, major egg chorion proteins are originates within the follicle in the pipefish, Syngnathus scovelli (Begovac and Wallace, 1989) and many morphological studies suggest that the egg chorion is formed by the oocyte in several fishes (Anderson, 1967; Wourms, 1976; Tesoriero, 1977). The components of zona pellucida of mammalian egg, ZP-1,2,3, are synthesized by egg itself (Wolgemuth et al., 1984). As shown in part 2, the amino acid sequence of HSAP predicted from the cDNA nucleotide sequence had a signal peptide, suggesting that the HSAPs were secretory proteins. The immunohisto-chemical studies showed that the vesicles which were located beneath the plasma membrane and looked like secretion granules were immunoreactive to the antiserum against the HSAPs in some eggs (Fig. 3.2C, 3.4). Furthermore, HSAP genes were transcripted only in ovaries as shown in part 2. These suggest that the HSAPs are synthesized by egg itself and secreted into the surroundings in contrast to the case in Olyzias latipes. In situ hybridization analysis is necessary to determine what cell subpopulations synthesize the HSAPs.

Tryosin inhibitors	Activation	Effective concentration
Inhibitor from Soybean; type 1-S #T9003	+	1 mg/ml <
Inhibitor from Soybean; type 2-S, #T9128	-	
Inhibitor from Soybean; Bowman-Birk type, #T9777	+	1 mg/ml <
Inhibitor from chicken white; ovomucoid, type 3-O, #T2011	-	
Inhibitor from chicken white; ovoinhibitor, type 4-O, #T1886	-	
Inhibitor from bovine pancreas; Kunitz type, type 1-P, #T0256	+	1 mg/ml <
HSAP (pI=5.1)	+	10 ug/ml <
BSA, Frction V	-	
IgG, not-specific to HSAPs	-	

Table 3.1 Effects of trypsin inhibitors on the herring sperm motility. All trypsin inhibitors and BSA (Fraction V) were from Sigma (St. Louis, MO, USA). HSAP (pI=5.1) was purified from IPG-IEF gel.

a


0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
OD280
0
25
50
75
100
125
Concentration (ug/ml)
STI
HSAP

b

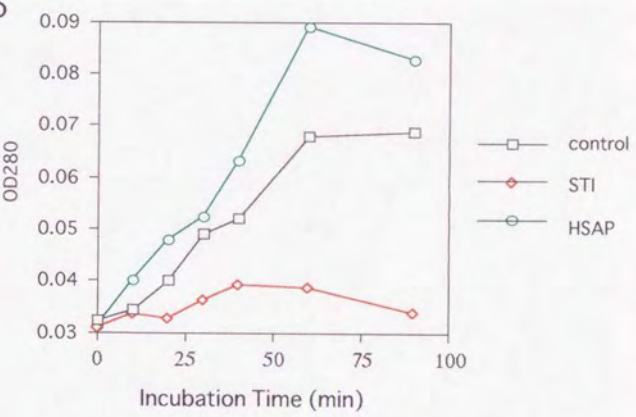
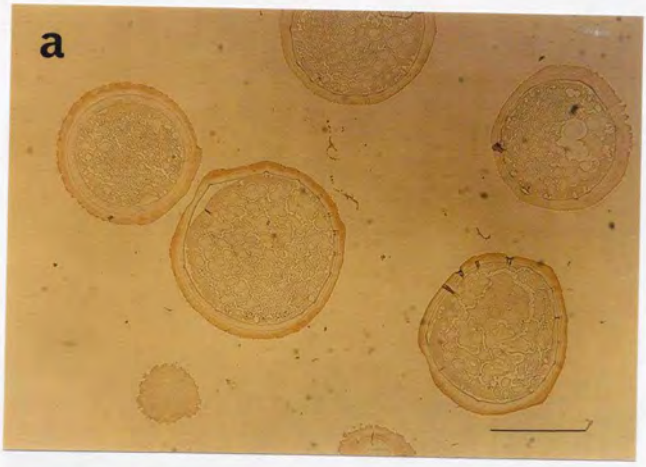
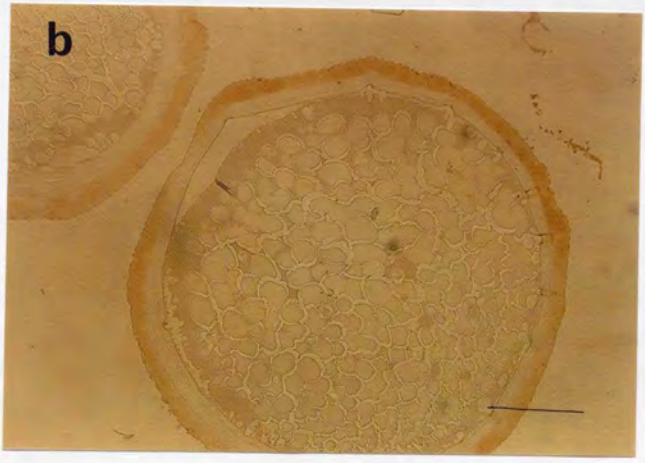

0.09
0.08
0.07
0.06
0.05
0.04
0.03
OD280
0
25
50
75
100
Incubation Time (min)
control
STI
HSAP

Figure 3.1. Effects of HSAP on proteolytic activity of trypsin. a) STI (#9003, Sigama) or the HSAP (pI=5.1) at various concentrations were pre-incubated with trypsin for 15 min at 30°C and then the reaction medium was added. The mixtures were incubated for 60 min, added TCA, centrifuged and the absorption at 280 nm of each supernatant was determined. The HSAP activated trypsin activity, while STI inhibited it. b) Mixture of trypsin and STI or HSAP (each 40 ug/ml) and trypsin alone were further incubated with the reaction medium for the time indicated. After the reaction, TCA was added, centrifuged and the absorption at 280 nm were determined.


a


b

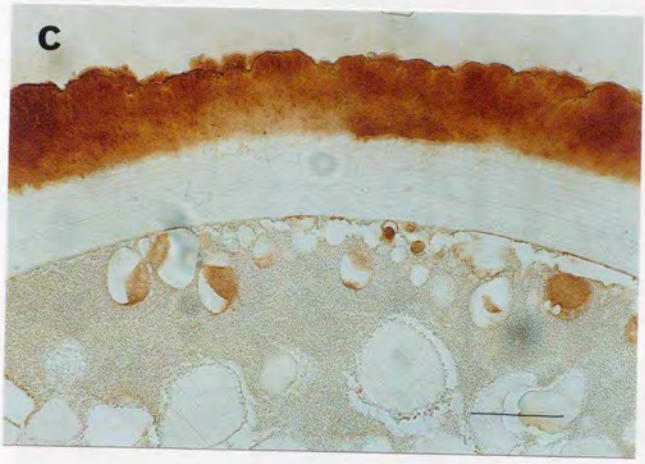


Figure 3.2. Photomicrographs of sections of herring ripe-unfertilized eggs stained immunohistochemically with the antiserum against the HSAPs. Figure 3.2.c shows high magnification of section of the egg chorion. Scale bar = 500 um (a), 200 um (b), 50 um (c).

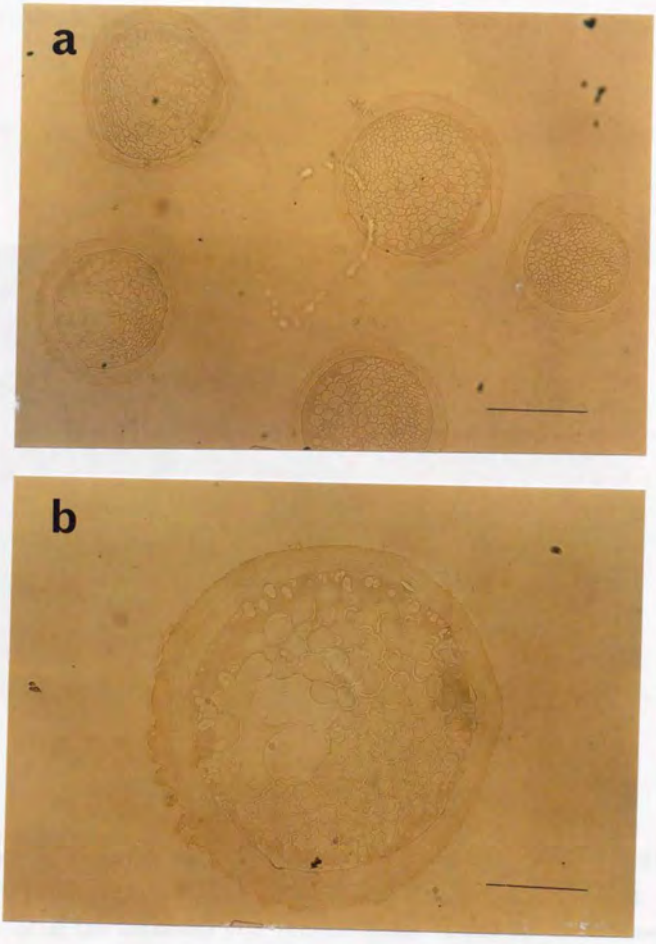


Figure 3.3. Photomicrographs of sections of herring ripe-unfertilized eggs stained immunohistochemically with the serum from a not-immunized rabbit. Scale bar = 500 um (a), 200 um (b).

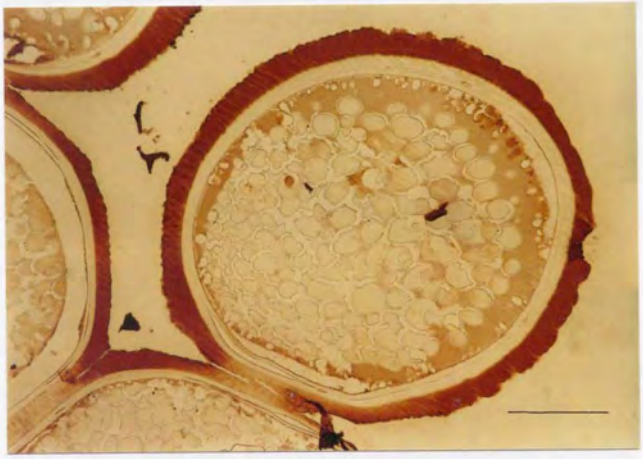


Figure 3.4. Photomicrographs of sections of herring ripe-unfertilized eggs stained immunohistochemically with the antiserum against the HSAPs after the treatment with neuraminidase. Scale bar = 200 um.

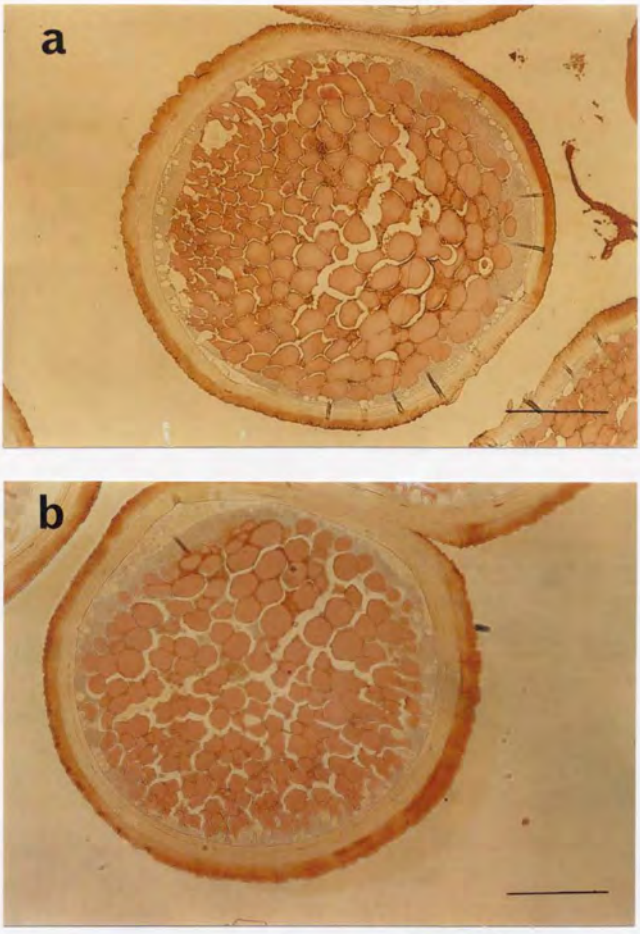


Figure 3.5. Photomicrographs of sections of herring ripe-unfertilized egg stained immunohistochemically with the monoclonal antibodies against the HSAP (pI=5.1). The sections were stained with monoclonal antibody, No. 27 (a) and No. 31 (b). Scale bar = 200 um.

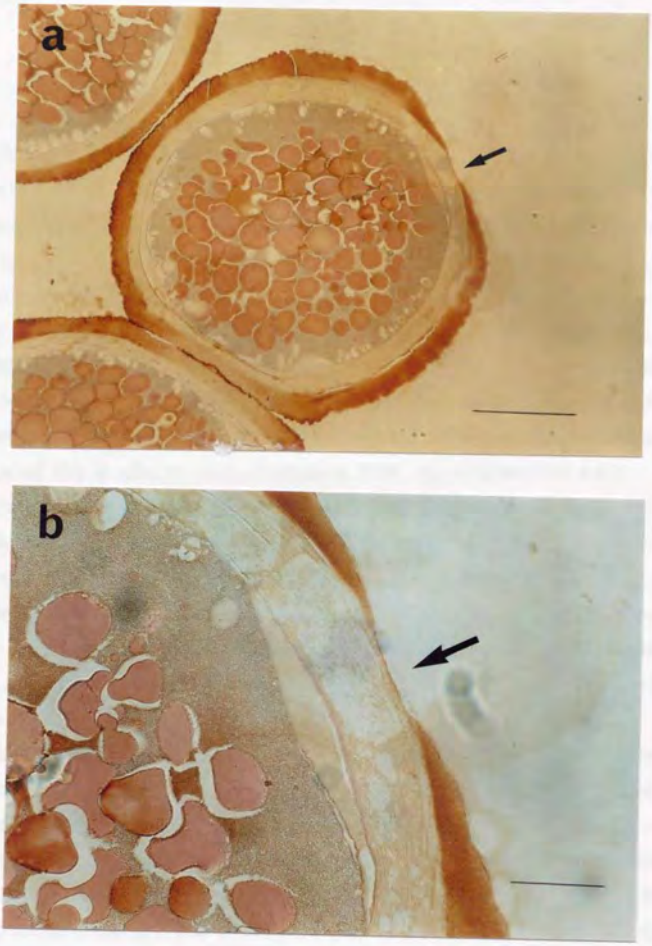


Figure 3.6. Localization of the immunoreactivity to HSAPs in the vicinity of the micropylar opening. The region of the micropylar opening is indicated with arrows. The micropylar canal is not present in this section. The section was stained with the monoclonal antibody, No. 27. Figure 3.6.b shows high magnification of micropylar opening region (indicated with an arrow in Figure 3.6.a). Scale bars = 200 um (a), 50 um (b).

General Conclusion

The spermatozoa of the Pacific herring, Clupea pallasii, are immotile upon dilution into seawater and they become active when contact with the proteinaceous substance(s) secreted from the egg. In part 1, 5 acidic proteins with different isoelectricpoints (4.8, 4.9, 5.0, 5.1 and 5.4) were purified from the egg-conditioned medium, egg seawater, using G-50 gel filtration and isoelectric focusing. The proteins were named the herring sperm-activating proteins (HSAPs). All the HSAPs had the ability to activate the motility of the herring spermatozoa at the concentration higher than 5-10 ug/ml. Molecular masses of the 5 HSAPs were estimated 7700 by Tricine-SDS-PAGE and molecular weight of one of the 5 HSAP (pI=5.1) was determined to be 8.1 kDa by mass spectrometry. Its S value was determined to be 1.4 by sedimentation analysis. UV absorption spectra of the HSAPs were very similar one another and the sequences of 20 amino acid residues from their N-terminal ends, analyzed by the Edman degradation method, were almost identical (part 2). These strongly suggest that the 5 HSAPs are of isoform.

In part 2, complementary DNA (cDNA) of the HSAPs was cloned to determine the whole amino acid sequences of the HSAPs. A 114-mer oligonucleotide probe for cDNA encoding the HSAP (pI=5.1) was chemically synthesized according to the amino acid sequence directly determined by the Edman degradation method. A cDNA library constructed from herring ovarian mRNAs was screened using the probe and 12 clones were obtained. One clone contained 479 base pairs long cDNA insert including an open reading frame of 356 base pairs long. The cDNA insert encoded a HSAP with 73 amino acid residues which N-terminal 38 amino acid residues were identical to those of the purified HSAP (pI=5.1). The consensus sequence reported by Kozak and the initiation codon of ATG were found in the cDNA. Another clone contained the 418 base pairs long insert, which also included the sequence of 38 amino acid residues of the HSAP (pI=5.1) and this clone

did not contain the initiation codon. Two mRNAs with sizes of about 500 nucleotide long and one mRNA with 1000 nucleotide long were found in ovaries but not in livers and the cDNA cloned in this study seems to correspond to two smaller mRNAs. Signal peptides were present in both clones, suggesting that the HSAPs were secretory proteins. This is in good accordance with the findings that the HSAPs is released into the surroundings when put into seawater.

The amino acid sequence of the HSAP determined by molecular cloning of HSAPs cDNA was closely resemble to the sequences of Kazal-type trypsin inhibitors, such as acrosin inhibitor, pancreatic secretory trypsin inhibitor and ovomucoid. The homology between the amino acid sequence of the HSAP and the bovine acrosin inhibitor was about 44 %. The HSAP possessed 6 cysteine residues which are conserved among all of the Kazal-type trypsin inhibitors and considered to be important to their three dimensional structures. The 34th residue, asparagine, in the bovine acrosin inhibitor is the other kind of amino acid residue which is also important to the three dimensional structure of the Kazal-type trypsin inhibitors and it was also conserved in the HSAP.

The similarity of amino acid sequences of the HSAP and the Kazal-type trypsin inhibitors predicted the interaction of the HSAPs and trypsin. In part 3, it was shown that trypsin inhibitors, such as the soybean trypsin inhibitor, a Bowman-Birk type trypsin inhibitor and a Kunitz-type trypsin inhibitor from beef pancreas activated the herring sperm motility. The HSAP activated the proteolytic activity of trypsin. These results suggest that the receptor for the HSAPs are present in the herring spermatozoa and it can interact with the trypsin inhibitors as well as with the HSAPs. Namely, it seems possible that the receptor for the HSAPs is a trypsin-like molecule.

Since the first observation of the sperm activation by egg in the herring (Yanagimachi and Kanoh, 1953), it has been believed that the sperm-activating factor localized in the vicinity of the micropylar

opening. In contrast to this belief, it was revealed in part 3 that the sperm-activating proteins distribute all over the egg surface. There is a possibility that another protein, such as the SMIF (Pillai et al., 1993) is present and attracts the spermatozoa in the vicinity of the micropylar opening. Another possibility is that the HSAPs are released into surroundings only from the vicinity of the micropylar opening by unknown mechanism. Further examination on the activation of the spermatozoa around the egg will be required.

Perspectives

Homology between the HSAPs and acrosin inhibitors

Purification of the sperm-activating proteins (HSAPs) and the amino acid sequences were performed in the study of this doctoral thesis and it was revealed that the HSAPs are ones of the members of the Kazal-type trypsin inhibitor family and resemble to acrosin inhibitor, pancreatic secretory trypsin inhibitor, ovomucoid, ovoinhibitor, etc. Among the Kazal-type trypsin inhibitors, the acrosin inhibitors are known to inhibit a trypsin-like proteinase, acrosin, contained in the acrosome of mammalian spermatozoa and it is considered that they play an important role in the acrosome reaction in the mammals fertilization. The acrosin inhibitors are contained in a large quantity in both the seminal plasma and the acrosome of mammalian spermatozoa (Zaneveld et al., 1972, 1973; Fritzs et al., 1974), suggesting that they protect the spermatozoa or the male and the female genital tracts from proteolytic digestion by acrosin, which are occasionally released from damaged spermatozoa (Bhattacharyya and Zaneveld, 1978). On the other hand, it is no doubt that they have more significant roles in fertilization; It is well known that the seminal plasma contains many species and a large quantity of the acrosin inhibitors (Hirschhauser et al., 1971, 1972; Zaneveld et al., 1972; Schumacher and Zaneveld, 1974; Meloun et al., 1984; Moritz et al., 1991; Jonakova et al., 1992; Stallings-Mann et al., 1994) and that the inhibitors prevent the in vitro fertilization in mammals (Zaneveld et al., 1970, 1971; Suominen et al., 1973). Recently, Jeng et al. (1993) showed that protease inhibitors suppress the sperm motility in porcine. An acceleratory effect of the seminal proteinase inhibitor on the porcine sperm motility has been suggested (Okamura et al., 1990; Lee, et al., 1992). These findings and the result concerning the activation of the herring sperm motility by the trypsin inhibitor-like HSAPs suggest a new possible function of the acrosin inhibitors on the regulation of sperm motility in the fertilization.

Origin of HSAPs

The other Kazal-type trypsin inhibitors, ovomucoid (Rhodes et al., 1960; Catterall et al., 1979; Nordstrom et al., 1979) and ovoinhibitor (Matsushima, 1958), are contained in the avian egg white and a possible function of those are considered to be protection of the egg from the attack by the bacterial or the fungal proteinases (Matsushima, 1958). The localization of the HSAPs covering all the surface of the egg presented in part 3 suggests that the intrinsic role of the HSAPs in the herring egg was the protection of the egg from attack by the exogenous proteinases. In this opinion, the HSAPs were never invested with the ability to activate the herring spermatozoa intrinsically. The herring spermatozoa are produced in a large quantity (about 5×10^{11} spermatozoa/male) and a large amount of mutations may possibly occur in the process of spermatogenesis, so that it seems possible to consider that the herring spermatozoa acquired the reception system for the HSAPs by which the HSAPs activate the herring spermatozoa.

Mechanism of sperm activation by the HSAPs

The significant roles of proteases for cell functions are well understood recently; The proteases are considered to be not scavengers in cells but the important component of the intercellular and the intracellular signal transduction systems. For example, proteosome plays essential roles in several cell functions (review by Ciechanover, 1994), including cell cycle regulation (Glotzer et al., 1991; Kawahara et al., 1992) and sperm motility regulation (Inaba et al., 1992, 1993). Tumor associated protease inhibitors are reported to act as growth factors in human hepatoma cells (McKeehan et al., 1994), suggesting that protease inhibitors can act as a ligand. This evidence and the fact that the HSAPs and the other protease inhibitors could activate the herring sperm motility suggest the presence of the intercellular signaling system triggered by the interaction between

protease inhibitor and protease, which is not known in cell biology. To answer the question, what signal transduction system mediate the sperm activation by the HSAPs, it is prerequisite to purify and characterize the HSAPs-receptor. ^{125}I- labeled HSAP will be prepared by labelling the HSAP synthesized at a great deal using expression vectors recombinated the cDNA of the HSAP and will be used to purify the HSAPs-receptor. The monoclonal and the polyclonal antibodies will be also used. When the receptor will be purified and the cDNA encoding the receptor will be cloned, the primary structure of cytosolic domain of the HSAP-receptor will be defined and the mechanism how the HSAPs activate the herring sperm motility will be understood. The characterization of the HSAPs-receptor will give us a new insight not only into the sperm motility regulation but also into the signal transduction systems in cells.

The mechanism of evolution

Kimura and Ohta (1974) have proposed 5 principles governing molecular evolution. One of them is that functionally less important molecules or parts of molecule evolve faster than more important ones. Although they did not assured us that the important molecules do not evolve, it means that the more important molecule tends to be conserved during the process of evolution. The sperm activation by egg is a fundamental and prerequisite step in the fertilization and observed over the various animal species as described in the part of General Introduction. When it is possible to apply the opinion by Kimura and Ohta to the subject discussed here, these facts suggest that the sperm-activation factor and the mechanisms of sperm activation might be conserved over the species. However, the sperm-activating proteins in herring (presented in this study), sea urchin (Nomura et al., 1981) and starfish (Nishigaki et al., 1994) are different protein molecules and it was indicated that the molecular mechanisms of the sperm activation is not conserved over the species but may developed independently in each species. The development of different

mode of fertilization, such as generation of a new substance for the sperm activation or a new mating behavior of parents, can lead homogenous animals to be divided into different groups; The modes of fertilization are common within a group and different between groups and the gametes of each group do not fertilize each other, consequently there is no exchange of genetic information between the two groups and the two groups become two different species. The mechanisms, by which the sperm-activating proteins was developed in the species which was the ancestor of Clupea pallasii, produced a new mode of fertilization in the ancestor species and evolved the descendant species, C. pallasii, with the sperm activation by egg. It can be one of the mechanisms to produce a new species, namely one of the mechanisms of evolution in the opinion proposed here.

Herrings in the North Pacific

Taxonomists have classified the herring, which is widely distributed all over the North Pacific Ocean from the yellow sea to the west coast of Canada, into one species named Clupea pallasii. The present study and the other study, however, showed the presence of completely different two sperm-activating factors, 8.1 kDa HSAPs and 105 kDa SMIF, in the Japanese (presented in this study) and the Canadian (Pillai et al., 1993) herrings, respectively. Recently, the analysis of isozyme polymorphism has reported that the herring around Japan and Canada should be classified into different species (Kobayashi,T., 1993). If it is the case, it is very reasonable to consider that the sperm-activating systems in the Japanese herring and the Canadian herring are different. As shown in part 2, the cDNA of the 8.1 kDa HSAP contains the short tandem repeat (STR) in its non-coding region near the 3'-end. STR is a portion where 4-8 nucleotide sequence is tandem repeated for 10-50 times, whose polymorphism is utilized for the identification of individuals in the field of ethology, forensic science and population genetics (Litt and Luty, 1989; Weber et al., 1989; Edmans et al., 1991). Since it is very

likely that the polymorphism in the STR of the HSAP-cDNA is present, it will be able to be used to evaluate the genetic distributions of the C. pallasii groups in the North Pacific Ocean. Consequently it will give us an answer to the question whether the Japanese and the canadian herring is different species or not.

The substances for the sperm-activation are tried to be purified and to be characterize in many animal species, such as ascidian (Yoshida et al., 1992) and human (Ralt et al., 1994). In sea urchin (Nomura et al., 1981), starfish (Nishigaki et al., 1994) and herring (presented in this study), the substances have been already characterized and the clarification of the molecular mechanisms of sperm activation are in progress. When the mechanisms of sperm activation in the each species are clarified and the comparison of the mechanisms will become possible, the roots of the molecular mechanism of sperm activation by egg will be able to be discussed in the course of evolution.

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References

1. Anderson,E. (1967) The formation of the primary envelope during oocytedifferentiation in teleosts.J. Cell Biol. 35, 193-212.

2. Babcock,D.F. and Pfeiffer,D.R. (1987) Independent elevation of cytosolic [Ca^{2+}] and pH of mammalian sperm by volatage-dependent and pH-dependent machanisms. J. Biol. Chem. 262, 15041-15047.

3. Bartelt,D.C., Shapanka,R. and Greene,L.J. (1977) The primary structure of the human pancreatic secretory trypsin inhibitor. Arch. Biochem. Biophys. 179, 189-199.

4. Begovac,P.C. and WallaceR.A. (1989) Major vitelline envelope proteins in pipefish oocytes originate within the follicleand are associated with the Z3 layer. J. Exp. Zool. 251, 56-73.

5. Bentley,J.K., Khatra,A.S. and Garbers,D.L. (1988) Receptor-mediated activation of detergant-solubilized guanylate cyclase. Biol. Reprod. 39, 639-647.

6. Bhattacharyya,A.S. and Zaneveld,L.J.D. (1978) Release of acrosin and acrosin inhibitor from human spermatozoa. Fertil. Steril. 30, 70-78.

7. Boitano,S. and Omoto,C.K. (1992) Trout sperm swimming patterms and role of intracellular Ca^{2+}. Cell Motil. Cytoskel. 21, 74-82.

8. Bolognesi,M. and Gatti,G. (1982) Three-dimensional structure of the complex between pancreatic secretory trypsin inhibitor (Kazal type) and trypsinogen at 1.8 A resolution.J. Mol. Biol. 162, 839-868.

9. Brokaw,C.J. (1987) Regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation.J. Cell Biochem. 35, 175-184.

10. Brownlee,C. (1990) Intracellular signalling during fertilization and polarisation in Fucoid algae. NATO ASI series, H45, 579-590.

11. Campanella,C. (1975) The site of spermatozoon entrance in the unfertilized egg of Discoglossus pictus (Anura): An electron microscope study. Biol. Reprod. 12, 439-447.

12. Catterall,J.F., Stein,J.P., Lai,E.C., Woo,S.L.C., Dugaiczyk,A., Mace,M.L., Means,A.R. and O'Malley,B.W. (1979) The chick ovomucoid gene contains at least six intervening sequences. Nature 278, 323-327.

13. Cechova,D. and Meloun,B. (1979) Differences in the evolution of seminal plasma acrosine inhibitors and pancreatic secretory trypsin inhibitors. Hoppe-Seyler's Z. Physiol. Chem. Bd.360, 1497-1500.

14. Chinkers,M., Garbers,D.L., Chang,M.S., Lowe,D.G., Chin,H. Goeddel,D.V. and Schulz,S. (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor.Nature 338, 78-83.

15. Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

16. Christen,R., Schackmann,R.W. and Shapiro,B.M. (1982) Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, Strongyloentrotus purpuratus. J. Biol. Chem. 257, 14881-14890.

17. Ciechanover,A. (1994) The ubiquitin-proteasome proteolyticpathway. Cell 79, 13-21.

18. Clapper,D.L. and Brown,G.G. (1980) Sperm motility in the horseshoe crab, Limulus polyphemus. Dev. Biol. 76, 341-349.

19. Clapper,D.L., Davis,J.A. Lamothe,P.J., Patton,C. and Epel.D. (1985a) Involvement of Zinc in the regulation of pH_i, Motility , and acrosome reaction in sea urchin sperm. J. Cell Biol. 100, 1817-1824.

20. Clapper,D.L., Lamothe,D.J., Davis,J.A. and Epel,D. (1985b) Sperm motility in the horseshoe crab. V: Zinc removal mediates chelator initiation of motility. J. Exp. Zool. 236, 83-91.

21. Cosson,M.P., Billard,R. and Letellier,L. (1989) Rise of internal Ca^{2+} accompanies the initiation of trout sperm motility. Cell Motil. Cytoskel. 14, 424-434.

22. Cosson,M.P., Carre,D. and Cosson,J. (1984) Sperm chemotaxis in siphonophores. J. Cell Sci. 68, 163-181.

23. Cook,S.P., Brokaw,C.J., Muller,C.H. and Babcock,D.F. (1994) Sperm chemotaxis: Egg peptides control cytosolic calcium to regulate flagellar responses. Dev. Biol. 165, 10-19.

24. Dan,J.C. (1950) Fertilization in the medusan, Spirocodon saltatrix. Biol. Bull. 99, 412-415.

25. Dangott,L.J., Jordan,J.E., Bellet,R.A. and Garbers,D.L. (1989) Cloning of the mRNA for the protein that crosslinks to the egg peptide speract. Proc. Natl. Acad. Sci. USA, 86, 2128-2132.

26. Darwin,C. (1859) On the Origin of Species. Murray, London.

27. Diaz,C.V., Perez,M.A.D., Ortega,F.V., Krivitzky,S.K. and Ambe,A.K. (1990) Evidence that human follicular fluid contains a chemoattractant for spermatozoa. Fertil. Steril. 54, 1180-1182.

28. Dickman,z. (1963) Chemotaxis of rabbit spermatozoa. J. Exp. Biol.

40, 1-5.

29. Edman,P. (1950a) Preparation of phenyl thiohydantoins from some natural amino acids. Acta Chem. Scand. 4, 277-282.

30. Edman,P. (1950b) Method for dertermination of the amino acid sequence in peptides. Acta Chem. Scand. 4, 283-293.

31. Edman,P. (1956a) On the mechanism of the phenyl isothiocyanate degradation of peptides. Acta Chem. Scand. 10, 761-768.

32. Edman,P. (1956b) Mechanism of the phenyl isothiocyanate degradation of peptides. Nature 177, 667-668.

33. Edwards,A.I., Civitello,A., Hammond,H.A. and Caskey,C.T. (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am. J. Hum. Genet. 49, 746-756.

34. Fink,E., H-Fink,C., and Eulitz,M. (1990) Amino acid sequence elucidation of human acrosin-trypsin inhibitor (HUSI-2) reveals that Kazal-type proteinase inhibitors are structurally related to b-subunits of glycoproteinhormones. FEBS lett. 270, 222-224.

35. Fritzs,H., Schleuning,W.D., Schiessler,H., Schill,W.B. and Winkler,G. (1974) Boar, bull and human sperm acrosin - isolation, properties and biological aspects. In "Proteases and Biological Control" Reich/Rifkin/Shaw eds. Cold Spring Harbor Conferences on Cell Proliferation. Vol.2. pp715-735.

36. Fujii,T., Utida,S., Mizuno,T. and Nanao,S. (1955) Effects of amino acids and some chelating substances on the motility and the oxygen uptake of starfish spermatozoa. J. Fac. Sci. Univ. Tokyo IV. 7, 335-345.

37. Garbers,D.L., Watkins,H.D., Hansbrough,J.R., Smith,A. and Misono,K. (1982) The amino acid sequence and chemical synthesis of speract and of speract analogues. J. Biol. Chem. 257, 2734-2737.

38. Glotzer,M., Murray,A.W. and Kirschner,M.W. (1991) Cyclin is degraded by the ubiquitin pathway. Nature 349, 132-138.

39. Gnessi,L., Ruff,M.P., Fraioli,F. and Pert,C.B. (1985) Demonstration of receptor-mediated chemotaxis by human spermatozoa. Exp. Cell Res. 161, 219-230.

40. Greene,L.J. and Bartelt,D.C. (1969) The structure of the bovine pancreatic secretory trypsin inhibitor-Kazal's inhibitor. J. Biol. Chem. 244, 2646-2657.

41. Hansbrough,J.R. and Garbers,D.L. (1981a) Speract: Purification and characterization of a peptide associated with eggs that activates spermatozoa. J. Biol. Chem. 256, 1447-1452.

42. Hansbrough,J.R. and Garbers,D.L. (1981b) Sodium-dependent activation of sea urchin spermatozoa by speract and monensin. J. Biol. Chem. 256, 2235-2241.

43. Harlow,E. and Lane,D. (1988) Antibodies: A laboratory manual. Cold Spring Harbor Laboratory.

44. Hayashi,H., Yamamoto,K., Yonekawa,H. and Morisawa,M. (1987) Involvement of tryrosine protein kinase in the initiation of flagellar movement in rainbow trout spermatozoa. J. Biol. Chem. 262, 16692-16698.

45. Hirschhauser,C., Baudner,S. and Daume,E. (1972) Immunologic identification of human seminal plasma protease inhibitor in cervical

mucus. Fertil. Steril. 23, 630-632.

46. Hirschhauser,C., Kionke,M., Daume,E. and Buchholz.R. (1971) Trypsin inhibitors in the human female genital tract. Acta Endocrinol. 68, 413-416.

47. Inaba,K., Akazome,Y. and Morisawa,M. (1992) Two high molecular mass proteases from sea urchin sperm. Biochem. biophys. Res. Commun. 182, 667-674.

48. Inaba,K., Akazome,Y. and Morisawa,M. (1993) Purification of proteasomes from salmonid fish sperm and their localization along sperm flagella. J. Cell Sci. 104, 907-915.

49. Inoda,T. and Morisawa,M. (1987) Effect of osmolality of the initiation of sperm motility in Xenopus laevis. Comp. Biophys. Biochem. 88A, 539-542.

50. Ishiguro,K., Murofushi,H. and Sakai,H. (1982) Evidence that cAMP-dependent protein kinase and a protein factor are involved in reactivation of Triton X-100 models of sea urchin and starfish spermatozoa. J. Cell Biol. 92, 777-782.

51. Jamieson,B.G.M. (1991) Fish Evolution and Systematics: Evidence from spermatozoa. Cambridge University Press, Cambridge.

52. Jeng,H., Liu,K-M. and Chang,W-C. (1993) Purification and characterization of reversible sperm motility inhibitors from porcine seminal plasma. Biochem. Biophys. Res. Commun. 191, 435-440.

53. Jin,Z.X., Inaba,K., Manaka,K., Morisawa,M. and Hayashi,H. (1994) Monoclonal antibodies against the protein complex that contains the flagellar movement-initiating phosphoprotein of Oncorhynchus keta. J.

Biochem. 115, 885-890.

54. Johnson,C.H., Clapper,D.L., Winkler,M.M., Lee,H.C. and Epel,D. (1983) A volatile inhibitor immobilizes sea urchin sperm in semen by depressing the intracellular pH. Dev. Biol. 98, 493-501.

55. Jonakova,V., Calvete,J.J., Mann,K., Shafer, W., Schmid,E.R., and T-Petersen,E. (1992) The complete primary structure of three isoforms of a boar sperm-associated acrosin inhibitor. FEBS lett. 297, 147-150.

56. Jonakova,V., Cechova,D., Meloun,B. and Veselsky,L. (1988) An acrosin inhibitor from boar seminal vesicle fluid immunologically related to the trypsin-kallikrein inhibitor (Kunitz). Biol. Chem. Hoppe-Seyler 369, 43-49.

57. Kajiwara,T., Kodama,K. and Hatanaka,A. (1980) Attractants of male-gamate from a marine brown alga Sargassum horneri. Bull. Japan. Soc. Sci. Fish. 46(5), 555-557.

58. Kawahara,H., Sawada,H. and Yokosawa,H. (1992) The 26 S proteasome is activated at two points in the ascidian cell cycle. FEBS lett. 310, 119-122.

59. Kazal,L.A., Spicer,D.S. and Brahinsky,R.A. (1948) Isolation of a crystalline trypsin inhibitor-anticoagulant protein from pancreas. J. Am. Chem. Soc. 70, 3034-3040.

60. Kimura,M. and Ohta,T. (1974) On some principles governing molecular evolution. Proc. Nat. Acas. Sci. USA 71, 2848-2852.

61. Kobayashi,T. (1993) Iden (Tokyo) 47(6), 53-58.

62. Kobayashi,W. (1993) Effects of osmolality on the motility of sperm

from the lamprey, Lampetra japonica. Zool. Sci. 10, 281-285.

63. Kohler,G. and Milstein,C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

64. Koshi,M. and Ogawa,Y. (1951) Studies of fertilization. I. Separation and chemical formula of activation and chemotaxis substance of sperm from the Clupea pallasii eggs. Med. Biol. Japan 18, 184-187.

65. Kozak, M. (1984) Compliation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nuc. Acids Res. 12, 857-872.

66. Laskowski, M, Jr. and Kato, I. (1980) Protein inhibitors of proteinases. Ann. Rev. Biochem. 49, 593-626.

67. Lee,H.C. (1988) Internal GTP stimulates the speract receptor mediated voltage changes in sea urchin spermatozoa membrane vesicles. Dev. Biol. 126, 91-97.

68. Lee,H.C., Johnson,C. and Epel,D. (1983) Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. Dev. Biol. 95, 31-45.

69. Lee,S-L., Kuo,Y-M., Kao,C-C., Hong,C-Y. and Wei,Y-H. (1992) Purification of a sperm motility stimulator from porcine follicular fluid. Comp. Biochem. Physiol. 101B, 591-594.

70. Litt,M. and Luty,J. (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. 44, 397-401.

71. Matsushima,K. (1958) An undescribed trypsin inhibitor in egg white.

Science 127, 1178-1179.

72. McKeehan,W., Sakagami,Y., Hoshi,H. and McKeehan,A. (1986) Two apparent human endothelial cell growth factors from human hepatoma cells are tumor-associated proteinase inhibitors. J. Biol. Chem. 261, 5378-5383.

73. Meloun,B., Jonakova,V., and Cechova,D. (1983) Coll. Czech. Chem. Commun. 48, 2558-2568.

74. Meloun,B., Cechova,D., and Jonakova,V. (1984) Homologies in the structures of bull seminal plasma acrosin inhibitors and comparison with other homologous proteinase inhibitors of the Kazal-type. Hoppe-Seyler's Z. Physiol. Chem. 364, 1665-1670.

75. Miller,R.L. (1966) Chemotaxis during fertilization in the hydroid Campanularia. J. Exp. Zool. 162, 23-44.

76. Miller,R.L. (1975) Chemotaxis of the spermatozoa of Ciona intestinalis. Nature 254, 244-245.

77. Miller,R.L., (1985) Sperm chemo-orientation in the metazoa. In Biology of Fertilization. (Eds. C.B.Metz and A.Monroy) vol. 2, pp. 275-337. Academic Press, New York.

78. Mizuno,T. (1956) Relationship between zinc and sperm motility in some marine forms. J. Fac. Sci. Univ. Tokyo 7, 477-487.

79. Morisawa,M. (1985) Initiation mechanism of sperm motility at spawning in teleosts.Zool. Sci. 2, 605-615.

80. Morisawa,M. (1994) Cell signaling mechanism for sperm motility. Zool. Sci. 11, 647-662.

81. Morisawa,M. and Hayashi,H. (1985) Phosphorylation of a 15 K axonemal protein is the trigger initiating trout sperm motility. Biomed. Res. 6, 181-184.

82. Morisawa,M. and Ishida,K. (1987) Short-term changes in levels of cyclic AMP, adenylate cyclase, and phosphodiesterase during the initiation of sperm motility in rainbow trout. J. Exp. Zool. 242, 199-204.

83. Morisawa,M., Morisawa,S. and de Santis,R. (1984) Initiation of sperm motility in Ciona intestinalis by calcium and cyclic AMP. Zool. Sci. 1, 237-244.

84. Morisawa,M. and Okuno,M. (1982) Cyclic AMP induces maturation of trout sperm axoneme to initiate motility. Nature 295, 703-704.

85. Morisawa,M. and Suzuki,K. (1980) Osmolality and potassium ion: Their roles in initiation of sperm motility in teleosts. Science 210, 1145-1147.

86. Morisawa,M., Tanimoto, S. and Ohtake,H. (1992). Characterization and partial purification of sperm-activating substance from eggs of the herring, Clupea palasii. J. Exp. Zool., 264, 225-230.

87. Moritz,A., Lilja,H. and Fink,E. (1991) Molecular cloning and sequence analysis of the cDNA encoding the human acrosin-trypsin inhibitor (HUSI-II). FEBS lett. 278, 127-130.

88. Morton,B.E., Sagadraca,R. and Fraser,C. (1978) Sperm motility within the mammalian epididymis: Species variation and correlation with free calcium levels in epididymal plasma. Fertil. Steril. 29, 695-698.

89. Muller,D.G. and Gassmann,G. (1985) Sexual reproduction and the

role of sperm attractants in monoecious species of the brown algae order Fucales (Fucus, Hesperophycus, Pelvetia, and Pelvetiopsis). J. Plant Physiol. 118, 401-408.

90. Nakata,H. and Ishii,S. (1970) Substrate activation in the trypsin-catalyzed hydrolysis of benzoyl-L-arginine p-nitroanilide. Biochem. Biophys. Res. Commun. 41, 393-400.

91. Nakata,H., Yoshida,N., Narahashi,Y. and Ishii,S. (1972a) Substrate activation and substrate inhibition of trypsin-like enzymes from three strains of Streptomyces species. J. Biochem., 71, 1085-1088.

92. Nakata,H. and Ishii,S. (1972b) Substrate activation of trypsin and acetyltrypsin caused by a-N-benzoyl-L-arginine p-nitroanilide. J. Biochem. 72, 281-290.

93. Nishigaki,T., Chiba,K., Yoshida,M., Morisawa,M. and Hoshi,M. (1994) Structure-activity relationship of starfish sperm-activating peptides. Zool. Sci. 11, 77.

94. Nomura,K., Suzuki,N., Ohtake,H., and Isaka,S. (1983) Structure and action of sperm activating peptides from the egg jelly of a sea urchin, Anthocidaris crassispina. Biochem. Biophys. Res. Comm. 117, 147-153.

95. Nordstrom,J.L, Roop,D.R.,Tsai,M-J. and O'Malley,B.W. (1979) Identification of potential ovomucoid mRNA precursors in chick oviduct nuclei. Nature 278, 328-331.

96. Okamura,N., Onoe,s., Kawakura,K., Tajima,Y. and Sugita,Y. (1990) Effects of a membrane-bound bicarbonate-sensitive adenylate cyclase in porcine sperm plasma membranes. Biochim. Biophys. Acta, 1035, 83-89.

97. Oda,S. and Morisawa.M. (1993) Rises of intracellular Ca^{2+} and pH mediate the initiation of sperm motility by hyperosmolality in marine teleosts.Cell Motil. Cytoskel. 25, 171-178.

98. Oda,S., Morisawa,M. and Hayashi,H. (1992) Dephosphorylation of protein participates in osmolality-dependent initiation of sperm motility in marine teleosts.Zool. Sci. 8, 1059.

99. Ohta,H. and Imada,K. (1992) Effects of viscosity of coelomic fluid on sperm motility in masu salmon, Oncorhynchus masou. Suisan Zoshoku 40, 177-182.

100. Ohtake,H. (1976) Respiratory behavior of sea-urchin spermatozoa. I. Effect of pH and egg water on the respiratory rate. J. Exp. Zool. 198, 313-322.

101. Ohtake,H. (1988) Kaiyo Kagaku (Tokyo), 20, 170-175.

102. Operesko,L.K. and Brokaw,C.J. (1983) cAMP-dependent phosphorylation associated with activation of motility of Ciona sperm flagella. Gamete Res. 8, 201-218.

103. Papamokos,E., Weber,E., Bode,W. and Huber,R. (1982) Crystallographic refinement of Japanese quail ovomucoid, a Kazal-type inhibitor, and model building studies of complexes with serine proteases.J. Mol. biol. 158, 515-537.

104. Pillai,M.C., Shields,T.S., Yanagimachi,R. and Cherr,G.N. (1993) Isolation and partial characterization of the sperm motility initiation factor from eggs of the Pacific herring, Clupea pallasii. J. Exp. Zool., 265, 336-342.

105. Punnett,T.P., Miller,R.L. and Yoo,B.H. (1992) Partial purification

and some chemical properties of the sperm chemoattractant from the forcipulate starfish, Pycnopodia helianthoides (Brandt, 1835). J. Exp. Zool. 262, 87-96.

106. Ralt,D., Coldenberg,M., Fetterolf,P., Thompson,D., Dor,J., Mashiach,S., Garbers,D.L. and Eisenbach,M. (1991) Sperm attraction to a follicular factor(s) correlates with human egg fertilizability. Proc. Natl. Acad. Sci. 88, 2840-2844.

107. Ralt,D., Manor,M., C-Dayag,A., T-Kaspa,I, B-Shlomo,I, Makler,A., Yuli,I., Dor,J., Blumberg,S., Mashiach,S. and Eisenbach,M. (1994) Chemotaxis and chemokinesis of human spermatozoa to follicular factors. Biol. Reprod. 50, 774-785.

108. Ramarao,C.S., Burks,D.J. and Garbers,D.L. (1990) A single mRNA encodes multiple copies of the egg peptide speract. Biochemistry 29, 3383-3388.

109. Repaske,D.R. and Garbers,D.L. (1983) A hydrogen ion flux mediates stimulation of respiratory activity by speract in sea urchin spermatozoa. J. Biol. Chem. 258, 6025-6029.

110. Reynaud,E., de La Torre, L.D., Zapata,O., Lievano,A. and Darszon,A. (1993) Ionic bases of the membrane potential and intracellular pH changes induced by speract in swollen sea urchin sperm. FEBS lett. 329, 210-214.

111. Rhodes,M.B., bennett,N. and Feeney,R.E. (1960) The trypsin and chymotrypsin inhibitors from avian egg whites. J. Biol. Chem. 235, 1686-1693.

112. Righetti,P.G., (1990) Immobilized pH Gradients: Theory and Methodology. Elsevier, Amsterdam.

113. Saido,T. (1991) Experimental Medcine (Tokyo), 9, 177-181.

114. Sakai,K., Hirai,M., Minoshima,S., Kudoh,J. and Shimizu,N. (1989) Isolation of cDNAs encoding a substrate for protein kinase C: Nucleotide sequence and chromosomal mapping of the gene for a human 80K protein. Genomics 5, 309-315.

115. Sakai,K., Hirai,M., Kudoh,J., Minoshima,S. and Shimizu,N. (1992) Molecular cloning and chromosomal mapping of a cDNA encoding human 80K-L protein: Major substrate for protein kinase C. Genomics 14, 175-178.

116. Shackmann,R.W. and Chock,P.B. (1986) Alteration of intracellular [Ca^{2+}] in sea urchin sperm by the egg petide speract. J. Biol. Chem. 261, 8719-8728.

117. Schägger,H. and G.von Jagow, (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem., 166. 368-379.

118. Schoff,P.K., Cheetham,J. and Lardy,H.A. (1989) Adenylate kinase activity in ejaculated bovine sperm flagella. J. Biol. Chem. 264, 6086-6091.

119. Serres,C., Feneux,D. and Berthon,B. (1991) Decrease of internal free calcium and human sperm movement. Cell Motil. Cytoskel. 18, 228-240.

120. Shimomura,H., Dangott,L.J. and Garbers,D.L. (1986) Covalent coupling of a resact analogue to guanylate cyclase. J. Biol. Chem. 261, 15778-15782.

121. Schumacher,G.F.B. and Zaneveld,L.J.D. (1974) Proteinase inhibitors in human cervical mucus and their in vitro interactions with human

acrosin. In Bayer-Symposium V "Proteinase inhibitors", pp178-186.

122. Singh,S., Lowe,D.G., Thorpe,D.S., Rodriguez,H., Kuang,W,J., Dangott,L.J., Chinkers,M., Goeddel,D.V. and Garbers,D.L. (1988) Membrane guanylate cyclase is a cell-surface receptor with homology to protein kinases. Nature 334, 708-712.

123. Skoog,B. and Wichman,A. (1986) Trends in Analytical Chemistry 5, 82.

124. Stallings-Mann,M.L., Burke,M.G., Trout,W.E. and Roberts,R.M. (1994) Purification, characterization, and cDNA cloning of a Kunitz-type proteinase inhibitor secreted by the porcine uterus. J. Biol. Chem. 269, 24090-24094.

125. Suominen,J., Kaufman,M.H. and Setchell,B.P. (1973) Prevention of fertilization in vitro by an acrosin inhibitor from rete testis fluid of the ram. J. Reprod. Fertil. 34, 385-388.

126. Suzuki,N. (1990) Structure and function of sea urchin egg jelly molecules. Zool. Sci. 7, 355-370.

127. Suzuki,N. and Garbers,D.L. (1984) Stimulation of sperm respiration rates by speract and resact at alkaline extracellular pH. Biol. Reprod. 30, 1167-1174.

128. Suzuki,N., Nomura,K., Ohtake,H. and Isaka,S. (1981) Purification and the primary structure of sperm-activating peptides from the jelly coat of sea urchin eggs. Biochem. Biophys. Res. Comm. 99, 1238-1244.

129. Suzuki,R. (1958) Sperm activation and aggregation during fertilization in some fish. I. Behavior of sperm around the micropyle. Embryologia, 4, 93-102.

130. Suzuki,R. (1961) Sperm activation and aggregation during fertilization in some fish. VI. The origin of the sperm-stimulating factor. Annot. Zool. Jpn., 34, 24-39.

131. Tash,J.S. and Means,A.R. (1982) Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol. Reprod. 26, 745-763.

132. Tash,J.S. and Means,A.R. (1983) Cyclic adenisine 3', 5' monophosphate, calcium and protein phosphorylation in flagellar motility. Biol. Reprod. 28, 75-104.

133. Tash,J.S. and Means,A.R. (1987) Ca^{2+} regulation of sperm axonemal motility. Methods in Enzymol. 139, 808-823.

134. Tash,J.S., Hidaka,H. and Means,A.R. (1986) Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for sperm flagellar motility. J. Cell Biol. 103, 649-655.

135. Tash,J.S., Krinks, M., Patel.J., Means,R.L., Klee,C.B. and Means,A.R. (1988) Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm. J. Cell Biol. 106, 1625-1633.

136. Tesoriero,J.V. (1977) Formation of the chorion (zona pellucida) in the teleost, Oryzias latipes I. Morphology of early oogenesis. J. Ultrastruct. Res. 59, 282-291.

137. Tubb,D.J., Kopf,G.S. and Garbers,D.L. (1979) Starfish and horseshoe crab egg factors cause elevations of cyclic nucleotide concentrations in spermatozoa from starfish and horseshoe crabs. J. Reprod. Fetil. 56, 539-542.

138. Vesterberg,O. (1971) Isoelectric focusing of proteins. Methods in Enzymol. 22, 389-412.

139. von Heijne,G. (1990) The signal peptide. J. Memb. Biol. 115, 195-201.

140. Wada,K., Wada,Y., Ishibashi,F. Gojobori,T., and Ikemura,T. (1992) Codon usage tabulated from the GenBank genetic sequence data. Nuc. Acid Res. 20, 2111-2118.

141. Ward,G.E., Brokaw,C.J., Garbers,D.L. and Vacquier,V.D. (1985) Chemotaxis of Arbacia punctulata spermatozoa to resact, a peptide from the egg jelly layer. J. Cell Biol. 101, 2324-2329.

142. Ward,C. and Kopf,G.S. (1993) Molecular events mediating sperm activation. Dev. Biol. 158, 9-34.

143. Weber,J.L. and May,P.E. (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. 44, 388-396.

144. Wishart,G.J. and Ashizawa,K. (1987) Regulation of the motility of fowl spermatozoa by calcium and cAMP. J. Reprod. Fertil. 80, 607-611.

145. Wolgemuth,D.J., Celenza,J., Bundman,D.S. and Dunbar,B.S. (1984) Formation of the rabbit zona pellucida and its relationship to ovarian follicular development. Dev. Biol. 106, 1-14.

146. Wong,P.Y.D., Lee,W.M. and Tsang,A.T.F. (1981) The effects of extracellular sodium on acid release and motility initiation in rat caudal epididymal spermatozoa in vitro. Exp. Cell Res. 131, 97-104.

147. Wourms,J.P. (1976) Annual fish oogenesis 1. Differentiation of the mature oocyte and formation of the primary envelope. Dev. Biol. 50,

338-354.

148. Yamagami,K., Hamazaki,T.S., Yasumasu,S., Masuda,K. and Iuchi,I. (1992) Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. Int. Rev. Cytol. 136, 51-92.

149. Yamaguchi,M., Niwa,T., Kurita,M. Suzuki,N. (1988) The participation of speract in the acrosome reaction of Hemicentrotus pulcherrimus. Develop. Growth Differ., 30, 159-167.

150. Yamaguchi,M., Kurita,M. and Suzuki,N. (1989) Induction of the acrosome reaction of Hemicentrotus pulcherrimus spermatozoa by the egg jelly molecules, fucose-rich glycoconjugate and sperm-activating peptide I. Develop. Growth Differ., 31, 233-239.

151. Yamamoto,T., Nakamura,Y., Nishide,T., Emi,M., Ogawa,M., Mori,T., and Matsubara,K. (1985) Molecular cloning and nucleotide sequence of human pancreatic secretory trypsin inhibitor (PSTI) cDNA. Biochem. Biophys. Res. Commun. 132, 605-612.

152. Yanagimachi,R. (1957a) Some properties of sperm-activating factor in the micropyle area of the herring egg. Annot. Zool. Jpn. 30, 114-119.

153. Yanagimachi,R. (1957b) Studies of fertilization in Clupea pallasii 3. Manner of sperm entrance into the egg. Zool. Mag. (Tokyo) 66, 226-233.

154. Yanagimachi,R. (1958) Studies of fertilization in Clupea pallasii. 7. On th specific affinity of the micropyle area of the mature egg to some vital dyes. Zool. Mag. (Tokyo) 67, 310-314.

155. Yanagimachi,R., Cherr,G.N., Pillai,M.C. and Baldwin,J.D. (1992) Factors controlling sperm entry into the micropyles of salmonid and

herring eggs. Develop. Growth and Differ. 34, 447-461.

156. Yanagimachi,R. and Kanoh,Y. (1953) Manner of sperm entry in herring egg, with special reference to the role of calcium ions in fertilization. J. Fac. Sci. Hokkaido Univ. (Ser. 6), 11, 487-498.

157. Yoshida,M., Inaba, K. and Morisawa, M. (1992) Sperm chemotaxis during the process of fertilization in the acidians, Ciona savignyi and Ciona intestinalis. Dev. Biol., 157, 497-506.

158. Yoshida,M., Inaba,K., Ishida,K. and Morisawa.M. (1994) Calcium and cyclic AMP mediate sperm activation, but Ca^{2+} alone contributes sperm chemotaxis in the ascidian, Ciona savignyi. Develop. Growth Differ. 36, 589-595.

159. Yoshida,T. and Nomura,M. (1972) A substance enhancing sperm motility in the ovarian fluid of rainbow trout. Bull. Jpn. Soc. Sci. Fish., 38, 1073.

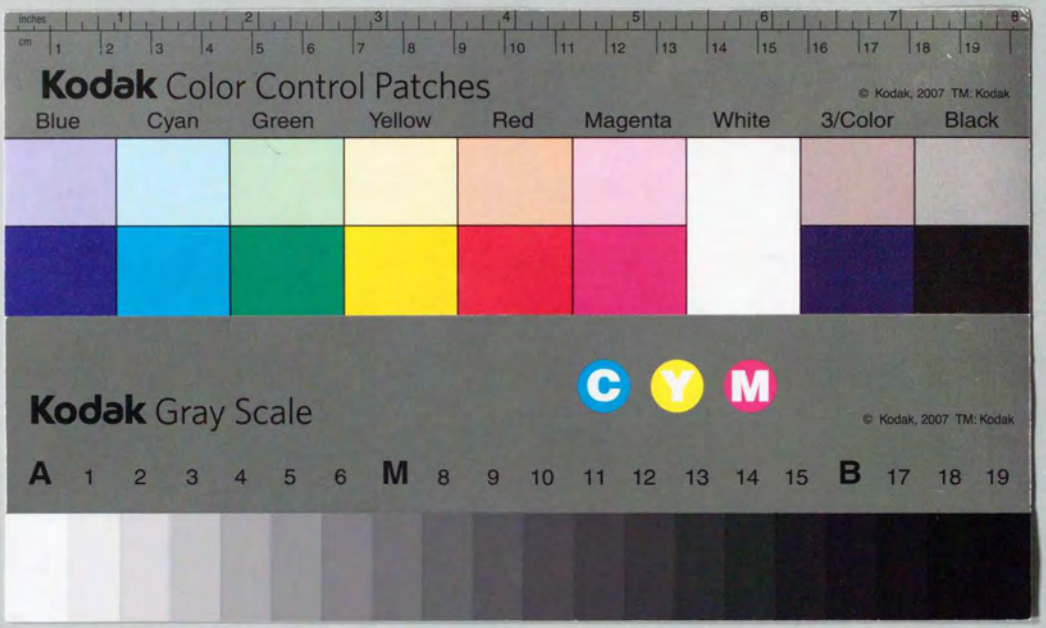
160. Zamir,N., Kreitman,R.R., Manor,M., Makler,A., Blumberg,S., Ralt,D. and Eisenbach,M. (1993) Atrial natriuretic peptide attracts human spermatozoa in vitro. Biochem. Biophys. Res. Commun. 197, 116-122.

161. Zaneveld,L.J.D., Dragoje,B.M. and Schumacher,G.F.B. (1972) Acrosomal proteinase and proteinase inhibitor of human spermatozoa. Science 177, 702-703.

162. Zaneveld,L.J.D., Robertson,R.T., Kessler,M. and Williams,W.L. (1971) Inhibition of fertilization in vitro by pancreatic seminal plasma trypsin inhibitors. J. Reprod. Fertil. 25, 387-392.

163. Zaneveld,L.J.D., Robertson,R.T. and Williams,W.L. (1970) Synthetic enzyme inhibitors as antifertility agents. FEBS lett. 11, 345-347.

164. Zaneveld,L.J.D., Schumacher,G.F.B., Fritz,H., Fink,E. and Jaumann,E. (1973) Interaction of human sperm acrosomal proteinase with human seminal plasma proteinase inhibitors. J. Reprod. Fertil. 32, 525-529.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