主論文

Studies on the functions of sperm autoantigens in the Nile tilapia, *Oreochromis niloticus*

（ナイルテラピア精子自己抗原の機能に関する研究）

Kazuhiko Mochida

1995

①

Studies on the functions of sperm autoantigens in the Nile tilapia,

<u>Oreochromis</u> <u>niloticus</u>

Kazuhiko Mochida

Division of Biology and Aquaculture

Graduate School of Fisheries Science

Hokkaido University

1995

CONTENTS

I. Introduction

In our country, after the reduction of pelagic fishery became unavoidable owing to the establishment of the economic sea zone, the necessity of the development of coastal fishery have been emphasized, and the culture for useful fish have been expanded in the coastal zone. However, in many case, since the insurance of parent fish depend on natural resources, the management of culture is not always stable because of its difficulty in obtainment seeding stable. Accordingly, it is imoportant for the stable management of culture to rear parent fish and artificial produce seeding in security. For the purpose, it is necessary to clarify the physiological mechanism on reproduction and to develop the mean of the artificial control of the reproduction.

In culture of tilapia which is one of useful fish for fishery, the importance of the induction of infertility is pointed out by Balarin (1979). Hence, it is one of the most important subjects in the artificial control of reproduction to establish the technique of producing infertile fish mainly to prevent the overpopulation effect on body growth of objective fish. In the present study, in order to accumulate basical informations for the purpose of the artificial control of male reproduction, especially in sperm fertility, attempts were made at characterization of several sperm proteins in a teleost.

Several membrane proteins were expressed on the surface of the germ cell in the late stages of spermiogenesis in some teleosts (Parmentier et al., 1984; Lou and Takahashi, 1991). Limited data indicate that testicular autoimmunity can be artificially induced in several teleosts such as Atlantic salmon, <u>Salmo salar</u> (Laird et al., 1978), rainbow trout, <u>Oncorhynchus mykiss</u> (Secombes et al., 1985a) and Nile tilapia, <u>Oreochromis niloticus</u> (Lou and Takahashi, 1987) and that only spermatozoa are attacked by its own-immune system during the autoimmune response. In the Nile tilapia, the blood-testis barrier was formed by adjacent Sertoli cells that encircle the germ-cell cysts containing spermatids at middle or late phases of chromatin condensation (Lou and Takahashi, 1989a). These results provide indirect evidence that several membrane proteins which are expressed on the germ cell surface during spermiogenesis are autoantigens. This assumption suggests that sperm surface autoantigens are sperm-specific antigens and thought to have sperm-specific functions. In mammals, several membrane proteins are expressed on the surface of germ cell during late spermatogenesis and play important roles in fertilization, i.e., sperm-zona binding (Yanagimachi et al., 1981; Naz et al., 1983; Primakoff et al., 1985; Saling and Lakoski, 1985) and sperm-egg plasma membrane binding (Saling et al., 1985; Primakoff et al., 1987).

The sperm plasma membrane fraction of the Nile tilapia was separated by sonication and analyzed with immunoblotting with the antisperm autoantibody (Lou and Takahashi, 1991). This autoantibody was produced in the serum of fish with artificially induced testicular autoimmunity, and at least six different peptides were identified as autoantigens (Lou et al., 1989; Lou and Takahashi, 1991). However, no study dealing with the functions of sperm autoantigens in teleost fish have been conducted. In the present study, therefore, first, monoclonal antibodies to these sperm autoantigens were produced as a step towards determining their function (chapter III).

Specific activation of sperm motility and attraction of spermatozoa to the egg in fertilization process have been studied in a wide variety of animals, e.g. Siphonophores (Carré et al., 1981; Cosson et al.,1984), Arthropoda (Clapper and Brown, 1980), Echinodermata (Miller, 1985; Ward et al., 1985), Prochordata (Yoshida et al., 1993) and vertebrate

(Villanueva-Diaz et al., 1990; Ralt et al., 1991, 1994). Especially in the sea urchin, these phenomena were extensively studied at a molecular level (Garbers, 1989; Ward and Kopf, 1993). Also in teleost species, activation and prolongation of sperm motility have been reported (Yanagimachi, 1957; Suzuki, 1958; Yoshida and Nomura, 1972; Ohta et al., 1989; Yanagimachi et al., 1992), however, the molecualr substrates involved in this phenomena have not yet been well clarified both in spermatozoa and egg. Recently, in mammals, the olfactory receptor genes showed to express in the male germ line (Parmentier et al., 1992), further the antiserum, which were generated against the deduced amino acid sequenece of the expressed olfactory receptor in dog testis, detected the gene product in late stage of spermatids and on the tail midpiece of mature spermatozoa (Vanderhaeghen et al., 1993). These findings lead to the hypothesis that members of the olfactory receptor family on spermatozoa are implicated in reception of chemoattractant from the egg. In the chapter IV, several experiments were carried out in view of the association with the sperm autoantigens and the specific activation of sperm motility during fertilization.

In mammals, testicular spermatozoa which completed spermiogenesis have no fertilizing ability; the spermatozoa acquire the ability during their transit through the epididymis (Orgebin-Crist et al., 1975). Therefore, seminal plasma is thought to contain various kind of molecules which are responsible for sperm functional maturation and maintenance of fertilizing ability until spermiation. Many studies have conducted the epididymal secretory protein in several mammalian species such as rat (Wagner and Kistler, 1987; Moore et al., 1990), mouse (Flickinger et al., 1988; Vreeburg et al., 1990; Araki et al., 1992; O'Brien et al., 1993), porcine (Okamura et al., 1992) and human (Thaler et al., 1990). In addition, it has been appeared the functions of the seminal plasma protein which are involved in sperm maintenance (Reddy and Bhargava 1979; Scheit et al., 1979; Papp et al., 1994) capacity for motility (Acott and Hoskins, 1981; Acott et al.,1983) and fertility (Cuasnicu et al., 1984; Audhya et al., 1987; Killian et al., 1993).

Spermatozoa which acquired full capacity for motility in epididymis are actually kept immotile until they are either ejaculated or diluted from the seminal plasma. The protein factors are also responsible for this motility quiescence in some mammalian species such as rat (Turner and Giles,1982; Usselman and Cone, 1983) and bovine (Baas et al., 1983; Carr and Acott, 1984). Especially, the sperm motility inhibiting factor reported in rat is a high molecular weight glycoprotein "immobilin" which is thought to keep spermatozoa immotile mechanically simply by creating a high viscoelastic environment (Usselman and Cone, 1983). Also in teleosts, spermatozoa which even acquire the potential for motility are kept immotile in the seminal plasma, and the motility inhibiting factor appeared to be potassium ion in salmonid fish and to be osmolarity in cyprinid fish (Morisawa and Suzuki, 1980; Morisawa et al., 1983). In contrary, our preliminary result shows that, in the Nile tilapia Oreochromis niloticus, the motility inhibiting factor is neither the potassium ion nor osmolarity. These results suggest that there are some kinds of sperm motility quiescence factors in teleosts. In the chapter V, several experiments were carried out in view of the association with the sperm autoantigens and the mechanism of immobilization of tilapia spermatozoa in the seminal plasma.

II. Acknowledgements

Sincere gratitude is first due to Professor Kohei Yamauchi, Faculty of Fisheries, Hokkaido University, for his invaluable guidance and encouragement throghout this study. I am greatly obliged also to Professor Fumio Yamazaki and Lecturer Shinji Adachi, Faculty of Fisheries, Hokkaido University, for their important suggestions and kind reading of the manuscript.

I also would like to take this opportunity to thank the following people for their help and co-operation: Assistant Professor Takeshi Miura and my colleagues at the laboratory of Fresh-Water Fish Culture, Faculty of Fisheries, Hokkaido University, for their encouragement and valuable help. Especially, Mrs. Hideaki Kudo and Toshitaka Ikeuchi, and Misses. Ikumi Nakamura and Tomoko Kondo for their direct assistance.

Associate Professors Akihiko Hara and Hiroshi Ueda, and Professor Emeritus Hiroya Takahash of Faculty of Fisheries, and Professor Norio Suzuki of Faculty of Science, Hokkaido University, Associate Professors Ya-huan Lou of Virginia University, and Akihiro Takemura of Ryukyu Univeristy, and Assistant professor Kiyoshi Soyano of Nagasaki University, and Dr. Yasunori Koya of Hokkaido National Fisheries Research Institute, for their interesting discussion and helpful advice.

The Wakamono Seisan Katsudo Center and Mr. Kazuo Maekawa for providing fish sample. A library clerk, Mrs. Nanbu Makiko of Hokkaido University for her odering many literatures for me.

I would like to thank the NIHON IKUEIKAI and the Japan Society for the Promotion of Science for the scholarship grant extended to me.

III. Production of monoclonal antibodies to sperm autoantigens

Several proteins expressed on the germ cells in the late stage of spermiogenesis, so-called autoantigens, think to be sperm specific functions. However, no studies have dealed with the functions of sperm autoantigens in teleosts. Production of monoclonal antibody is one of useful way to investigate the function of the specific ligand. In this chapter, attempts are made at purification of the autoantigens for the immunization by affinity chromatography using tilapia anti-sperm autoantibody from the fish experimentally induced testicular autoimmunity by the injection with allogeneic sperm in adjuvant and at production of monoclonal antibodies raised against the obtained sperm autoantigens as a step towards determining their functions.

Materials and Methods

1) Purification of sperm autoantigens

Sperm cells of the Nile tilapia were collected either by sacrificing the males and macerating the testis or by stripping milt of spermiating fish. Testis from freshly killed animals were minced thoroughly in the artificial seminal plasma (ASP; 125mM NaCl, 12mM KCl, 0.8mM $CaCl_2$ and 0.55mM $MgCl_2$ buffered with 10mM Hepes pH7.8). The resulting tissue suspension was passed through a 425μm of stainless steel mesh followed by a 250μm of mesh to remove larger mass of tissue. The final tissue suspension was then centrifuged at 1,000g for 15 min and resuspended in a small volume of the ASP. The suspension was layered on a discontinuous Nycodenz (Nycomed, Oslo, Norway) gradient (9.2% and 18%, w/v) dissolved in 5mM Hepes (pH7.4) containing 3mM KCl, and centrifuged at 1,500g for 30 min. The second and third layers out of four layers contained the sperm cells were further collected and centrifuged at 1,000g for 15 min. Resultant sperm pellets were washed twice with ASP to remove Nycodenz, yielding sperm for solubilization. The second means of collecting sperm consisted of freshly stripped milt centrifuged at 1,000g for 30 min to produce a sperm pellet.

The resultant sperm cells obtained by macerating the testis and by stripping were pooled and resuspended at about 1×10^{10} cells/ml in solubilization buffer (SB;10mM Tris-HCl pH 7.8, 0.15M NaCl, 30mM n-octyl-ß-D-thioglucopyranoside (OTG), 1mM PMSF and 2mM EDTA 2Na), or in SB without OTG as control. These suspensions were frequently stirred for 15 - 20 min and then centrifuged at 100,000g for 1 hr. These solubilization processes were done at 4°C. The resultant supernatant of solubilized sperm called solubilized sperm protein fraction (SSP), the residual sperm pellet (RSP), and non-treated sperm pellet (NSP), sperm cells which were not solubilized, were collected for electrophoretic and Western blotting analysis. About 2ml of SSP was first applied to a 2ml pre-column of Sepharose 4B to remove materials that would bind non-specifically to Sepharose. The eluate was applied to a 5ml affinity column previously prepared with cyanogen-bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and tilapia antisperm autoantibody obtained by the method described previously (Lou et al., 1989). The SSP was loaded onto the column at a flow rate of 3ml/hr, and the column was washed at 10ml/hr with SB containing 10 mM OTG instead of 30 mM OTG. After the column was washed, the sperm autoantigen fraction was eluted with 8M urea containing 10mM OTG. This affinity chromatography was done at 15°C.

The sperm pellet was assayed for protein by the Lowry method following homogenization with a teflon homogenizer in ASP. The SSP and the autoantigen fraction were assayed using a Bio-rad protein assay kit (Bio-rad, South Richmond, CA, USA). In both assays, bovine serum albumin (BSA; Sigma, St Louis, MO) was used as the standard.

2) Electrophoresis

Samples were pretreated with 3% sodium dodecyl sulfate and 10% mercaptoethanol at 100°C for 2 min and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel (Laemmli, 1970). The separated proteins were stained with either silver (silver staining kit, Wako) or 0.1% Coomassie Brilliant Blue in 0.5% ethanol 5% acetic acid solution (CBB). Before SDS-PAGE, the eluted autoantigen fraction was concentrated by adding trichloroacetic acid (TCA) at a final concentration of 10%, and incubating for 1 hr, and then centrifuging at 1,000g for 1 hr, all at 4°C. The resultant pellet was washed with cold-acetone and centrifuged at 10,000g for 15 min to remove the TCA. This step was repeated twice, and the autoantigen pellet was air-dried. Protein molecular weight markers (low MW set) were obtained from Pharmacia.

3) Production of monoclonal antibody

The air-dried autoantigen fraction was dissolved in 0.9% NaCl, or the autoantigen of 80kDa which was excised from polyacrylamide gel and stained with Coomassie Brilliant Blue was homogenized with 0.9% NaCl and used as antigen for immunization. Two 6-week-old female BALB/c mice were injected intraperitoneally with these antigens (10-20μg protein/mouse/injection). For immunization on Day 1 and Day 14, these antigens were emulsified in an equal volume of Freund's complete adjuvant and injected intraperitoneally into each mouse. On Day 28, each mouse received only the antigens. The mice were sacrificed on Day 31.

The spleen cells of the injected mice were fused with the mouse myeloma P3-X63-Ag8-U1. Culture supernatants were initially analyzed for binding to SSP in an enzyme linked immunosorbent assay (ELISA) as a first screening. About 10μg of SSP were added to a 96-well ELISA plate (Flow laboratories, VA, USA) and incubated overnight at 4°C. The SSP coated plate were then incubated with 1% BSA solution for 30 min at room temperature to block the non-specific protein binding sites. One hundred μl of culture media were added to each well, and the plates were then incubated overnight at 4°C. Finally, the plates were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Bio-rad, dilution 1:1000) for 3 hr at room temperature. After extensive washing with 0.01M phosphate bufferd saline pH7.2 (PBS) containing 0.05% tween 20, the peroxidase activity was colored by adding 100μl of o-phenylenediamine (600μg/ml in citrate-phosphate buffer pH5.0) containing 0.02% H_2O_2, and the reaction was stopped by adding 100μl of 1N H_2SO_4. Positive wells were optically evaluated. The positive wells were further selected by both Western blotting analysis of the autoantigen fraction and immunohistochemical analysis of tilapia testis. The positive hybridomas were recloned by limiting dilution analysis to establish a permanent cell line.

4) Western blotting

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) with a Bio-rad semidry trans-blot SD (Towbin et al., 1979). The following procedures were done at room temperature. Before immunostaining, the membrane was treated for 30 min with 20mM Tris-HCl buffer (pH7.5), 500mM NaCl (TBS), containing 5% skim milk to block the non-specific protein binding sites. For immunoblotting using antisperm autoantibody, the membrane was then incubated with antisperm autoantibody overnight following with rabbit anti-tilapia IgM antiserum prepared by the method described by Lou et al. (1989). Finally the membrane was incubated with HRP conjugated goat anti-rabbit IgG (Bio-rad, dilution 1:1000) for 3 hr. For immunoblotting using monoclonal antibody, the PVDF membrane blotted with the autoantigen fraction was incubated with culture media overnight. Then, the membrane was incubated with HRP conjugated goat anti-mouse IgG (Bio-rad, dilution 1:1000) for 3 hr. After washing with TBS, the PVDF membranes reacted with HRP conjugated antibody were incubated with 0.06% 4-chloro-1-naphtol in TBS containing 0.06% H_2O_2 (30% solution) to visualize the peroxidase reaction products.

5) Immunohistochemistry

Testes from freshly killed maturing male tilapia were fixed in Bouin's fluid for 3 hr at room temperature and washed at least 3 times with PBS for 2 days at 4°C. The testes were then embedded in paraffin (m.p. 54°C). Five μm sections of the tissue were deparaffinized, immersed in 0.3% H_2O_2 in methanol for 40 min to block endogenous peroxidase activity, and then treated with 10% normal rabbit serum in PBS for 1 hr at room temperature to avoid non-specific binding of antibodies. Sections were incubated with the culture media for 6 hr at room temperature, and then, after washing with PBS, incubated with a solution of biotinylated rabbit anti-mouse IgG at 1:400 (Dako, Glostrup, Denmark) for 3 hr at room temperature. Finally, the streptavidin and biotinylated HRP complex (Dako) was applied. After 30 min incubation, the sections were washed with 50mM Tris-HCl (pH7.6) (TB), and incubated with 0.01% diaminobenzidine-0.03% H_2O_2 in TB. The immuno-stained sections and the contiguous sections stained with hematoxylin-eosin were observed with a Zeiss Axiophot microscope. Staging of spermiogenesis followed the criteria proposed by Lou and Takahashi (1989b).

Freshly ejaculated sperm washed once in PBS were placed on slides precoated with 0.01% poly-L-lysine and incubated for 2 hr. The slides were incubated with 10% normal goat serum for 1 hr to block non-specific binding of antibodies. Then, each culture medium was added on the slides and incubated for 3 hr; finally, the slides were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Sigma) at a dilution of 1:100 for 3 hr in a dark, humid chamber. Excess antibody was removed by washing the slides with PBS. The slides were observed under a Zeiss Axiophot microspcope equipped with epifluorescence.

RESULTS

1) Autoantigen purification

The amount of protein obtained in the SSP was approximately 4mg/10^{10}sperm, and this represented about 12% of the total protein of the sperm. The affinity-purified autoantigen fraction was about 4% of the total protein in the SSP.

Electrophoretic patterns and Western blotting analysis by antisperm autoantibody of the NSP, RSP, SSP and the purified autoantigen fraction are shown in figure 1. In the NSP, several peptides which showed positive reactions with autoantibody were observed in the region of 70-120kDa, but in the RSP only a weak staining by autoantibody was found in 90kDa band. In the SSP, many polypeptide bands were recognized on the gel with silver staining, and autoantibody recognized mainly 90-120kDa bands and minor bands of 60kDa and 40kDa. In the autoantigen fraction, mainly two polypeptide bands of 80kDa and 27kDa were observed on silver staining gel. The electrophoretic pattern of the autoantigen fraction was the same

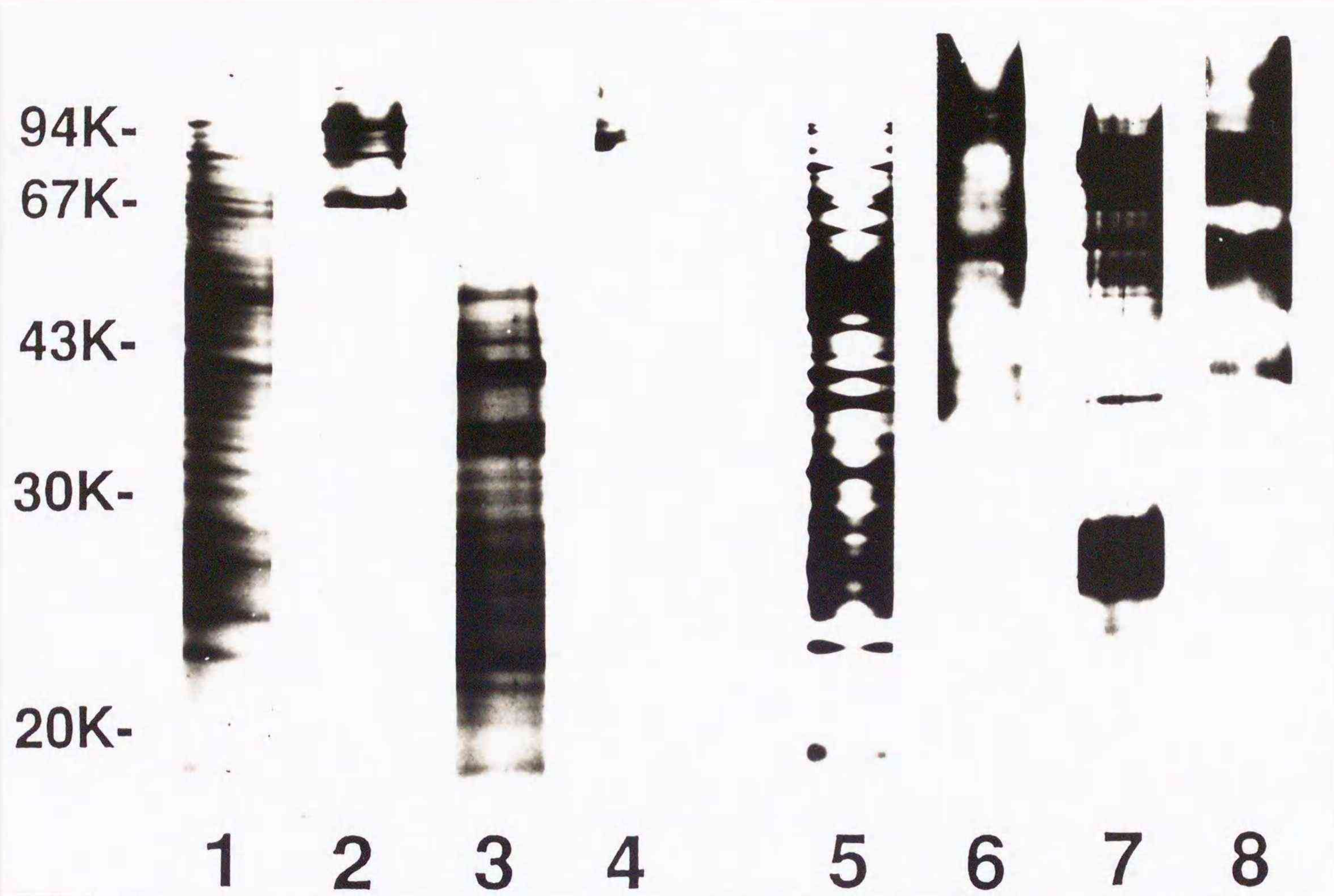


Fig. 1 Electrophoretic pattern and Western blot analysis by antisperm autoantibody of non-treated sperm pellets (1 and 2), residual sperm pellets after solubilization (3 and 4), solubilized sperm protein (5 and 6) and the autoantigen fraction (7 and 8). Ten μg of protein applied to lanes 5- 8. Odd numbered lanes show each fraction exaqmined on SDS-PAGE with Coomassie Brilliant Blue staining (1 and 3) and silver staining (5 and 7). Even numbered lanes show each fraction analyzed by Western blotting with antisperm autoantibody. Positions of molecular weight markers are indicated on the left side of the figure.

as that under the non-reduced condition. The autoantibody intensely recognized 80kDa polypeptide band. Faint staining with autoantibody was observed on 40-70kDa bands. Smear-type staining by autoantibody was also recognized around 100kDa. Some bands were not apparent on the silver staining gel. The 27kDa peptide in the purified autoantigens was not stained by autoantibody.

2) Establishment of monoclonal antibodies to sperm autoantigens

From spleen cells of mice immunized with the air-dried autoantigen fraction or the 80kDa band excised from polyacrylamide gel - myeloma fusions about 400 culture supernatants were assayed and 70 positive supernatants were found by ELISA that bind to the SSP fraction. Four of those were positive after subsequent screenings. Immunoblotting analysis of autoantigens by culture media of these clones were shown in figure 2. Monoclonal antibodies were named Testicular Antigen of Tilapia (TAT)-10, 20, 21 and 30, respectively. TAT-10 reacted with a 27kDa polypeptide as a single clear band. However, the 27kDa band which was stained by silver staining was of the smear type. TAT-20 reacted with 80kDa + 30-40kDa peptides, while TAT-21 reacted with only a 80kDa peptide. TAT-30 reacted with the 120kDa peptide, a peptide barely in evidence on gel with silver staining.

Immunohistochemical analysis of tilapia testis by the culture media of four hybridomas were shown in figure 3. The antigen recognized by TAT-10 was localized in type A and early B spermatogonia, especially in their cytoplasm, and the cytoplasm of early type B spermatogonia was stained stronger than that of type A spermatogonia. Spermatids at later stage of spermiogenesis and spermatozoa were also strongly stained by TAT-10. The antigens recognized by TAT-20, 21 occurred at the same locations in the testis, i.e., spermatids at later stages of spermiogenesis and

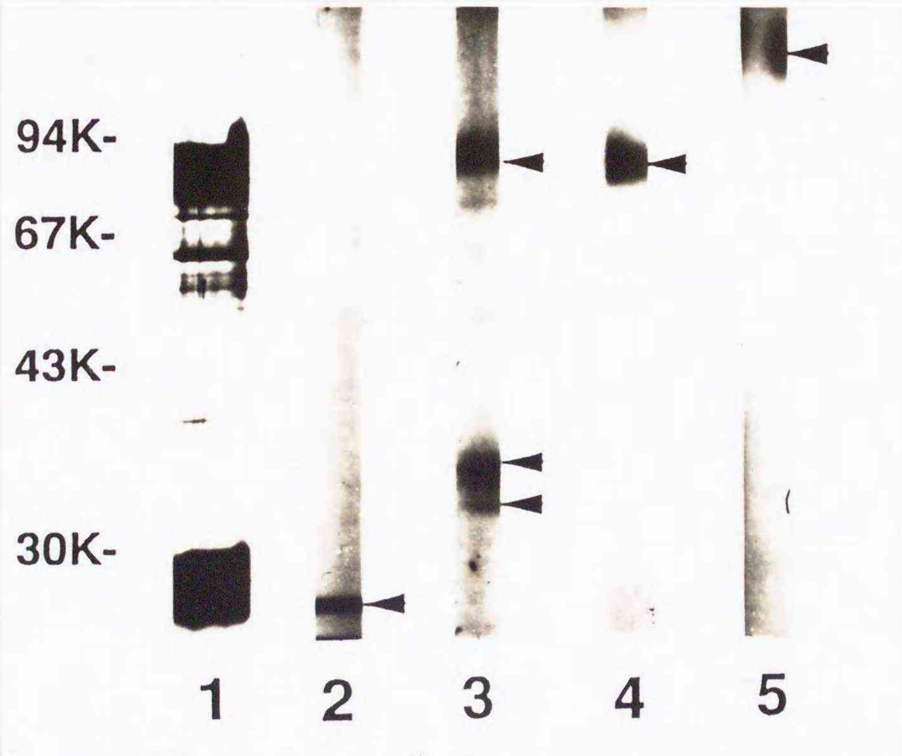


Fig. 2 Immunoblotting of the autoantigen fraction by the monoclonal antibodies. Lane 1, autoantigen fraction with silver staining; lane 2, TAT-10; lane 3, TAT-20; lane 4, TAT-21; lane 5, TAT-30. Arrowheads show the peptides reacted with the monoclonal antibodies. Positions of molecular weight markers are indicated on the left side of the figure.

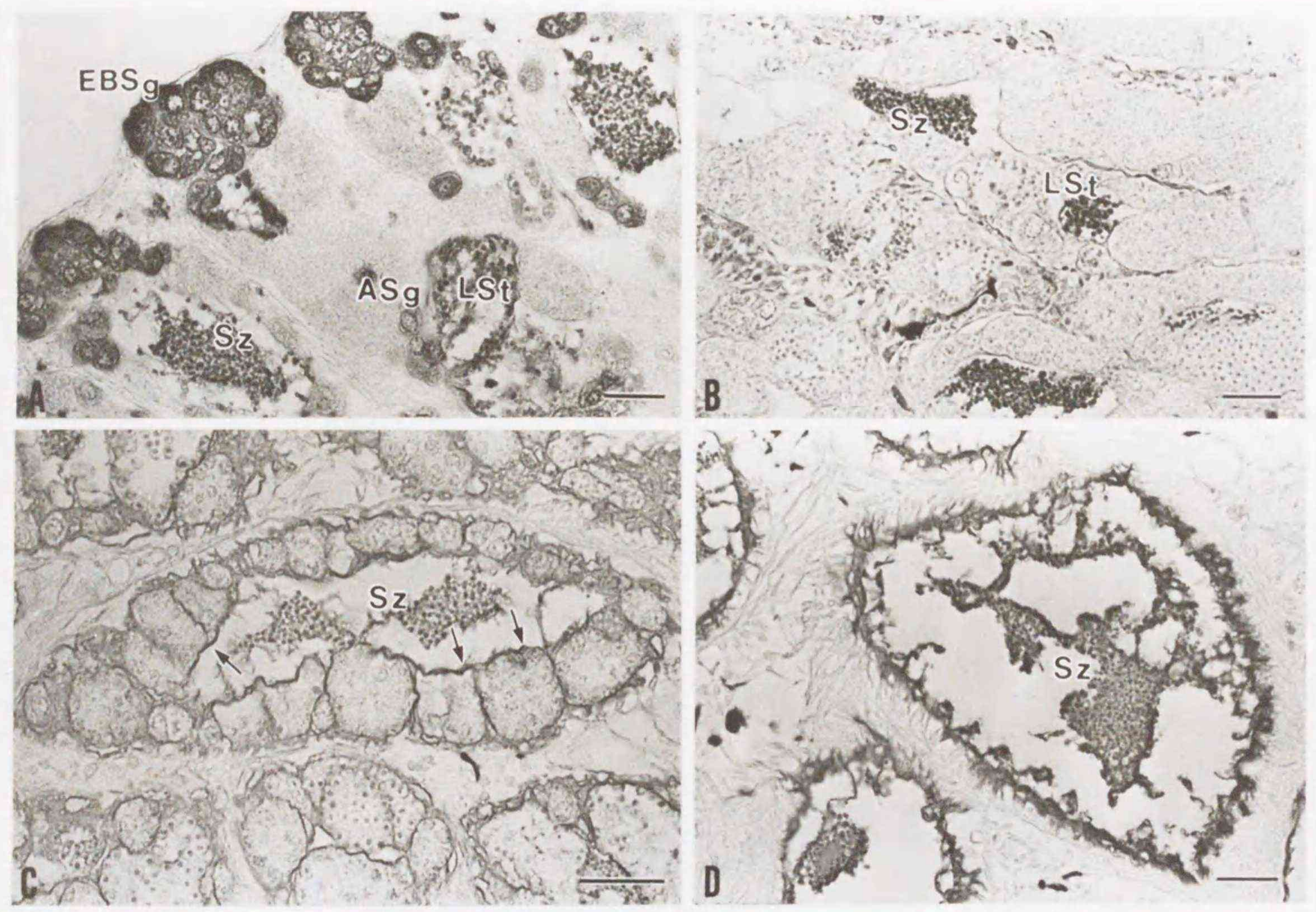


Fig. 3 Localization of antigen recognized by the monoclonal antibodies on tilapia testis. (A), TAT-10; (B), TAT-20 (the same as TAT-21); (C) and (D), TAT-30. Note that the antigen found in the cytoplasm of Sertoli cells primarily nest to the lumen (arrows in c). ASg, type A spermatogonia; EBSg, early type B spermatogonia; LSt, spermatid at the late stage of spermiogenesis; Sz, spermatozoa. Bars, 20μm.

spermatozoa. TAT-30 antigen localized in the whole cytoplasm of Sertoli cells, epithelial cells of seminal ducts, and spermatozoa in both seminal lobule lumen and seminal ducts on tilapia testis. The cytoplasm of Sertoli cells facing the lobule lumen tended to be stained by TAT-30 stronger than those facing the interstitium. All spermatogenic cells in cysts were not stained.

The localization of the antigens on the surface of intact tilapia spermatozoa reacting to the culture media of four hybridomas were determined by an indirect immunofluorescence method (Fig. 4). Apparent fluorescence staining by TAT-10 was found in the midpiece of spermatozoa. Intense fluorescence staining with TAT-30 was observed over the sperm head. Weaker staining was observed on head of spermatozoa when TAT-20 and 21 were reacted (data not shown). No antibodies that bound to the tail of spermatozoa were observed.

Immunohistochemical characteristics of monoclonal antibodies to testicular autoantigens of the Nile tilapia are summarized in Table 1.

DISCUSSION

In this study, sperm autoantigens were purified with antisperm autoantibody affinity chromatography from the solubilized sperm protein and produced monoclonal antibodies to sperm autoantigens of the Nile tilapia. These should prove useful for investigation of the functions of the antigens.

In the previous study, nitrogen cavitation was primarily used in analysis of mammalian sperm plasma membrane components (Peterson et al., 1980; Mack et al., 1986) while in the masu salmon, <u>Oncorhynchus masou</u>, sonication was used to separate the sperm plasma membrane fraction (Lou et al., 1990). These methods, however, are unsuitable to

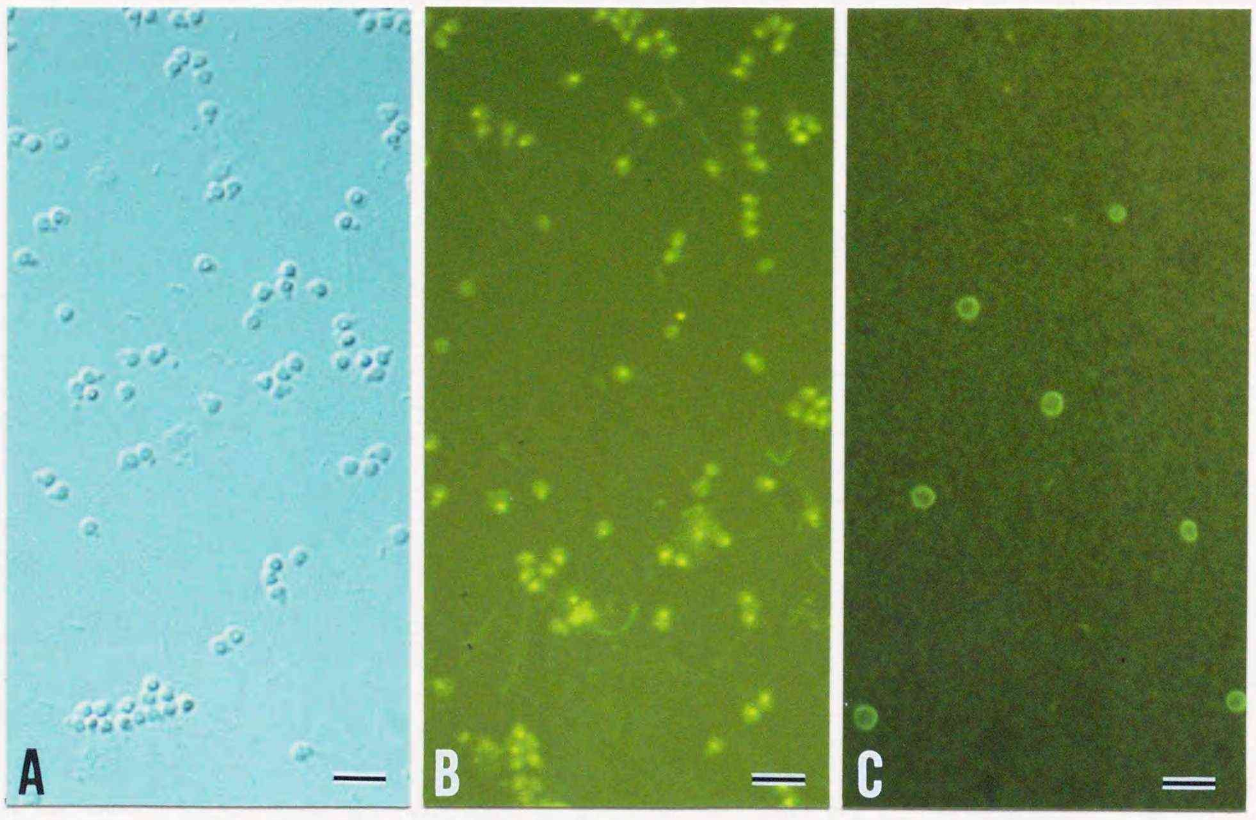


Fig. 4 Localization of antigens reacting to TAT-10 and TAT-30. (A), differential interference image of tilapia spermatozoa. (B), immunofluorescent staining patterns of spermatozoa by TAT-10 and (C), TAT-30. Bars, 5μm.

Table 1. Characteristics of monoclonal antibodies specific to sperm autoantigens

Monoclonal antibodies	Testis with Bouin's fixative					Autoantigens	
	A and early B spermatogonia	late B spermatogonia and spermatocyte	spermatids	sperm	sperm duct and Sertoli cells	localizations on intact sperm surface	molecular weights (reduced)
TAT-10	+	–	+	+	–	midpiece	27kDa
TAT-20	–	–	+	+	–	head	80, 30 - 40kDa
TAT-21	–	–	+	+	–	head	80kDa
TAT-30	–	–	–	+	+	head	120kDa

purify high yields of autoantigens for immunization. Primakoff et al. (1988a) purified sperm-specific proteins by applying the detergent octylglucoside-extracted sperm membrane protein to specific-antibody affinity chromatography. In this study, I used this procedure to purify autoantigens of tilapia sperm where OTG was used for solubilization instead of octylglucoside. The capacity of OTG to solubilize membrane proteins of Escherichia coli is equivalent to that of octylglucoside (Tsuchiya and Saito, 1984).

Electrophoretic and Western blotting analysis of ultracentrifuged sperm pellet confirmed that almost all of protein that reacted positively with autoantibody were solubilized. Also, electrophoretic patterns of the autoantigen fraction showed that the main polypeptides of 80kDa and 27kDa were highly purified, indicating that the main, but not all, autoantigens of tilapia sperm could be purified by applying the SSP to tilapia antisperm autoantibody affinity chromatography. In the affinity purified autoantigen fraction, the main 80kDa peptide, several peptides of 40-70kDa and the one around 100kDa were recognized by autoantibodies, and were identified as autoantigens. Lou and Takahashi (1991) demonstrated the existence of autoantigens of the Nile tilapia sperm at around 80kDa and 100kDa, and also reported that several peptides at relatively low molecular weight around 30kDa were also identified as autoantigens. Autoantigens of low molecular weight were not detected, perhaps because a different method of preparation of the sperm membrane protein fraction was used for electrophoresis. The autoantigen of about 100kDa was faintly observed with silver staining but was clearly observed with autoantibody staining. Similarly, in SSP this polypeptide was almost not apparent with silver staining but was stained with autoantibody. These observation suggested that this peptide had strong autoimmunogenic epitope. The 27kDa peptide of autoantigen fraction was highly purified with autoantibody affinity chromatography, but was not reacted with autoantibody at all. The treatment of SDS was likely to inactivate the immunogenic activity against autoantibody.

Lou and Takahashi (1991) observed that Nile tilapia spermatids at the late stage of spermiogenesis and spermatozoa on testis sections were immunohistochemicaly stainable with rabbit antisperm antibody suggesting that almost all of sperm surface antigen began to emerge on spermatids at the final stage of spermiogenesis as autoantigens. The electrophoretic pattern and Western blot analysis by autoantibody of SSP demonstrated all of solubilized sperm protein were not autoantigens. In RSP, the complete disappearance of plasma membrane and expansion of chromatin were observed by transmission electron microscope indicating that SSP was contaminated by proteins in the cytoplasm and the chromatin of spermatozoa. The 80kDa and 27kDa peptides were highly purified with autoantibody affinity chromatography, suggesting that these peptides are the main autoantigens on the sperm's surface and may play important roles in sperm-specific functions, e.g. fertilization.

I was able to produce monoclonal antibodies which specifically react with testicular autoantigens. The autoantigen of 27kDa had a smear type of staining pattern when tested on silver staining gel, but TAT-10 recognized a sharp polypeptide band of 27kDa. It is possible that the 27kDa autoantigen contained several peptides, and TAT-10 recognized one of these peptides. The TAT-10 antigen was present not only in the later stages of spermatids and spermatozoa, but also in A and early B type spermatogonia as well, although previous studies showed that autoantigens appear on the surface of spermatids only at the late stage of spermiogenesis (Lou and Takahashi, 1989b, 1991). These peptides which were expressed on both spermatogonia and spermatozoa might belong to the same protein family, but the peptide expressed on spermatozoa might contain autoantigenic epitope. This may be elucidated by investigating whether or not the antigens recognized by TAT-10 expressed on both spermatogonia and spermatozoa are the same molecule. It was recently showed that the antigen recognized by TAT-10 was also localized in olfactory epithelial cells and olfactory nerves as well as testes. Parmentier et al. (1992) reported that the mammalian olfactory receptor gene family was expressed on germ cells and suggested that the receptor was involved in chemotaxis during fertilization. Noteworthy perhaps is the fact that the TAT-10 detected antigen localized in midpiece of spermatozoa where mitochondria were closely aggregated, suggesting that this antigen may play a role in sperm motility.

The antigens recognized by TAT-20 and 21 were localized on spermatids late in spermiogenesis and on spermatozoa. The immunohistochemical observation showed that the autoantigen of 80kDa recognized by TAT-20 and 21 belonged to a sperm membrane type of antigen. This type of autoantigen was first identified in rabbit on the surface of pachytene spermatocytes, and finally distributed over the acrosomal area of spermatozoa (O'Rand and Romrell, 1981). In addition, the monoclonal antibodies to these antigens inhibit the penetration by spermatozoa of the zona pellucida of the egg in vitro (O'Rand et al., 1984). These monoclonal antibodies also cross-react with human ejaculated spermatozoa, and inhibit human spermatozoa from penetrating the zona-free hamster oocyte in vitro (O'Rand and Irons, 1984). These results suggest that the molecules which play an important role in fertilization had common epitopes across animal species. TAT-20 also reacts with spermatids and spermatozoa of Japanese eel, <u>Anguilla japonica</u>. The epitope recognized with TAT-20 may be conserved in some teleost species, and have critical sperm-specific functions.

The TAT-30 antigen was localized on Sertoli cells, epithelial cells of seminal ducts, and spermatozoa, primarily on the head. I recently showed that the Nile tilapia had the 120kDa peptide as one of the main proteins in seminal plasma which had cross-react with TAT-30. This result strongly suggests that the peptide is of the sperm coating antigen type where the 120kDa autoantigen was synthesized in Sertoli cells and epithelial cells of seminal ducts and then secreted into the seminal plasma to attach sperm heads. Although the 120kDa peptide was identified as one of the autoantigens in the present study, it was noteworthy that a functional barrier apparently does not exist between Sertoli cells and the interstitium (Lou and Takahashi, 1989a), and that Sertoli cells were not attacked by immune-competant cells, such as macrophages during the autoimmune response (Lou and Takahashi, 1987). Here I found that the cytoplasm of Sertoli cells facing the lobule lumen tended to stain more strongly by TAT-30 than those facing the interstitium. Danahey et al. (1986) showed a selective unidirectional sorting of Sertoli cell secretory proteins. Ueda et al. (1988) also reported that Sertoli cells cultured in a polarized fashion secreted some proteins unidirectionally in rat. Sertoli cells may secrete the 120kDa peptide only into seminal lobule lumina, and the peptide may thus be unavailable to the animal's immune system. In mammals, many studies have considered the properties of sperm coating antigens secreted from epididymis (Wong and Tsang, 1982; Brooks and Tiver, 1983; Rankin et al., 1989) which are essential for spermatozoa to acquire fertilizing ability (Orgebin-Crist and Fournier-Delpech, 1982). The antibodies to these epididymal glycoproteins blocked fertilizing ability in rat (Cuasnicu et al., 1984). In addition, some of these glycoproteins were known as androgen-regulated proteins (Holland and Orgebin-Crist, 1988; Moore et al., 1990). Thus, in the Nile tilapia, it is interesting for analysis of the sperm maturation process to determine if the secretion of the 120kDa peptide is under endocrine control.

Sperm surface antigens that play important roles in fertilization were found on sperm head (Kallajoki et al., 1986; Hardy et al., 1988). Considering the molecules detected by TAT-30 or TAT-20 and 21, they were all localized in the head of sperm. Further the molecule recognized by TAT-20 and 21 was the main component of sperm surface autoantigen. It should consequently be important for the investigation of the function of these autoantigens to assay the fertility of sperm treated with these monoclonal antibodies.

Primary conclusion is that the monoclonal antibodies to sperm surface autoantigens that we developed may have utility for the analysis of the fertilization process from initiation of sperm motility to sperm-egg membrane binding. The possible functions of the autoantigens detected by these monoclonal antibodies are now being analyzed in our laboratory.

IV. sperm autoantigen and specific activation of sperm motility by ovarian fluid

In the previous chapter, the production of four monoclonal antibodies to the autoantigens on spermatozoa was reported in order to investigate the functions of these antigens. One of these monoclonal antibodies, the Testicular Antigen of Tilapia (TAT-10) recognized the 27kDa antigen localized in spermatids in late spermiogenesis and in the midpiece of spermatozoa, although the antigens also localized in type A and early B spermatogonia. This distribution pattern on spermatozoa suggests the possibility that the antigen involved in the regulation of sperm motility. In this chapter, two major experiments were carried out in an attempt to speculate the physiological significance of TAT-10 antigen on spermatozoa in the regulation of sperm motility. First, whether the ovarian fluid stimulate sperm motility in the Nile tilapia, Oreochromis niloticus, and then TAT-10 treatment affect sperm motility regulated by the ovarian fluid were examined. Second, tissue specificity of TAT-10 antigen was analyzed, especially, it was attempted to demonstrate the localization of the TAT-10 antigen in olfactory epithelium.

MATERIALS AND METHODS

1) Animal

Nile tilapia, Oreochromis niloticus, of both sexes used in the present study were maintained in indoor concrete ponds on the campus of the Faculty of Fisheries, Hokkaido University, at 25-30°C under natural light conditions, and fed on a commercial diet 2 to 3 times a day.

2) Preparations of spermatozoa, ovarian fluid and somatic tissues

Spermatozoa were freshly obtained by stripping the abdomen of three male Nile tilapia, and the each milt were diluted in the artificial seminal plasma (ASP: 125mM NaCl, 12mM KCl, 0.8mM $CaCl_2$ and 0.55mM $MgCl_2$ buffered with 10mM Hepes, pH7.8), the Ca^{2+} free ASP and the Ca^{2+} free ASP containing 0.5mM EGTA. And then, these diluted milts were centrifuged at 1000g for 15 min at 4°C and the supernatants were removed. After repeating this step twice, the final resultant sperm cells were resuspended at about 2-3x10^7 cells/ml in the same solutions.

Ovarian fluid was obtained by passing ovulated eggs stripped from female fish through a 425μm of stainless steel mesh. The ovarian fluid from 5 individuals were pooled and used in the present experiments. The ovarian fluid was centrifuged at 10000g for 15 min at 4°C to remove insoluble materials. Final supernatant was stored at -80°C until use.

The fish were sacrificed and immediately, their various organs (olfactory rosette, whole brain, heart, intestine, kidney, liver, gill muscle, spleen, ovary and testis) were removed. Blood was collected from caudal vein with 2.5ml syringe (Terumo, Tokyo Japan). Each tissue except blood was washed quickly in 50mM Tris-HCl pH8.0, 1mM PMSF, 2mM EDTA 2Na and was homogenized in about 1.5 volumes of the same solution. These tissue suspensions and the serum were stored at -40°C until use for Western blot analysis.

3) Sperm motility assay

Diluent buffer (DB) was made up with 10mM Tris-HCl pH7.4, 1% bovine serum albumin, containing required concentrations of Ca^{2+}. All treatments were made at room temperature. Estimats of motility of the spermatozoa were made as follows. Ten μl of sperm suspension was diluted with 200μl of the diluent buffer with or without the ovarian fluid.

After gentle stirring, 10μl of the mixture was placed in a haemocytometer slide, the coverslip put over and the droplet placed in the field of view of the microscope. The percentages of moving spermatozoa in several constant minutes after dilution were calculated by counting the number of spermatozoa with blurred outlines in several photomicrographs of 4 different fields at 100x magnification with 0.5sec exposure.

4) Antibody treatment

Monoclonal antibodies from culture media for the hybridomas established in the previous chapter were purified by affinity chromatography. The media were centrifuged at 1000g for 10 min to eliminate cellular and particulate debris, and then were dialyze against 1.5M glycine pH8.9 containing 0.3M NaCl overnight at 4°C. After the dialysis, the media were applied to Protein A-Sepharose CL-4B equilibrated with above mentioned buffer and the antibodies peaks were eluted with 0.1M citric acid buffer pH4.0. Immediately after the elutions, the eluted antibodies were dialyzed against the ASP overnight at 4°C and then stored at -20°C before use.

Sperm suspensions in ASP adjusted to 2-3x10^7 cells/ml were incubated with equal volumes of the purified monoclonal antibodies solutions (100μg of the antibody/ml ASP) for 2 hr at room temperature.

5) Gel filtration

Gel filtration of ovarian fluid was performed using Sephadex G-25 (1.5x16 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the DB containing 0.2mM $CaCl_2$ and eluted with the same buffer at a flow rate of 12ml/h. Aprotinin (M_r 6500), a synthetic peptide (13 residues, M_r 1400) and tryptophane (M_r 204) were used as standard. Five hundred μl of the ovarian fluid was loaded and the eluate fractions were collected at 1 ml/tube and each fraction was examined for the sperm motility assay.

6) Electrophoresis

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was preformed as previously described (Laemmli, 1970). Samples containing 10μg protein, as determined by Lowry's method, were pretreated with 3% SDS and 10% mercaptoethanol at 100°C for 2 min, and applied to 15% polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue, or silver (silver staining kit, Wako). Protein molecular weight markers (low M_r set) were obtained from Pharmacia.

7) Western blot analysis

The separated protein samples on gels without staining were then transferred to polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, USA) by the method described previously (Towbin et al., 1979). Before immunostaining, the membrane was incubated 5% skim milk in Tris-buffered saline (TBS, 20mM Tris-HCl pH7.5, containing 500mM NaCl) to block the non-specific protein binding sites. The membrane was then incubated with culture supernatant of TAT-10 obtained before overnight, followed by treatment with horse-radish peroxidase (HRP) conjugated goat anti-mouse IgG (Bio-rad, South Richmond, CA, USA) at a dilution 1:1000 in TBS for 3 hr at room temperature. After washing with TBS, 4-chloro-1-naphthol solution (60mg in 100ml TBS containing 0.01% H_2O_2) was applied to visualize the peroxidase reaction products.

8) Immunohistochemistry

Tissues were fixed with Bouin's solution for 4 to 5 hr at room temperature. After washing with 0.01M phosphate buffered saline pH7.2 (PBS) for 2 days (more than three changes) at 4°C. The tissues were embedded in paraffin (m.p. 54°C). Five micron-thick sections of the tissues were first rinsed in 0.3% hydrogen peroxidase in methanol for 40 min at eliminate endogenous peroxidase activity. After washing with PBS, the sections were treated with 10% normal rabbit serum in PBS to avoid non-specific binding of antibodies, and then treated with the culture supernatant of the TAT-10 hybridoma for 3 hr at room temperature. The sections were further incubated with biotinylated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) at a dilution 1: 500 for 1 hr, and then incubated with streptABC complex HRP (Dako) for 30 min at room temperature. After washing with TB (50mM Tris-HCl pH7.6), 3.3'-diaminobenzidine in TB containing 0.03% H_2O_2 was applied to visualize the localization of the antigen recognized by TAT-10. In order to confirm specificity, other culture supernatant of TAT series hybridomas or PBS was substituted for TAT-10 culture supernatant.

For immunoelectron microscopic observations, tissues were rapidly freezed in liquid nitrogen, and then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer pH7.4. After being throughly washed with 0.1M phosphate buffer pH7.4 containing 10% sucrose, the specimens were dehydrated through a graded ethanol series and embedded in LR-White (London Resin Co. Hampshire, England). Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome with diamond knife. The sections were first incubated with PBS containing 1% normal goat serum (Funakoshi, Tokyo, Japan) to block non-specific protein binding site, and then incubated with the culture supernatant of the TAT-10 hybridoma for 1 hr at room temperature. The sections were further incubated with 10nm-gold conjugated goat anti-mouse IgG+IgM (Bio Cell, Cardiff, UK) at a dilution 1:200 for 1 hr. The sections were viewed with a Hitachi H-7000 electron microscope after staining with uranyl acetate. In the present study, stages of spermiogenesis was determined according to the criteria proposed by Lou and Takahashi (1989b).

9) Statistical analysis

In the present study, results were expressed as means±SE. The significance of differences between various experimental and control groups was analyzed by means of the paired Student's *t*-test.

RESULTS

1) Effect of ovarian fluid on sperm motility

Sperm suspensions in Ca^{2+}-free ASP and Ca^{2+}-free ASP containing 0.5mM EGTA were dissolved in the DB, or the DB containing 1mM EGTA with or without 1/100 volume of the ovarian fluid. The ratio of motile spermatozoa were measured 1, 2, 4 and 8 min after dilution. As can be seen in figure 5, at any time after the dilution, there were no significant difference between the ratio of the motile spermatozoa in the diluent buffer with the ovarian fluid and those without the ovarian fluid. Both groups of spermatozoa could not have motile for 8 min, and especially all of spermatozoa suspended in the Ca^{2+}-free ASP containing 0.5mM EGTA ceased to move only around 2 to 3 min after dilution. Also the sperm suspensions in Ca^{2+}-free ASP dissolved in the DB containing 0.2mM Ca^{2+} with or without 1/100 volume of the ovarian fluid. Motile spermatozoa in the DB containing the ovarian fluid was significantly higher than those without the ovarian fluid 4 and 8 min after the dilution.

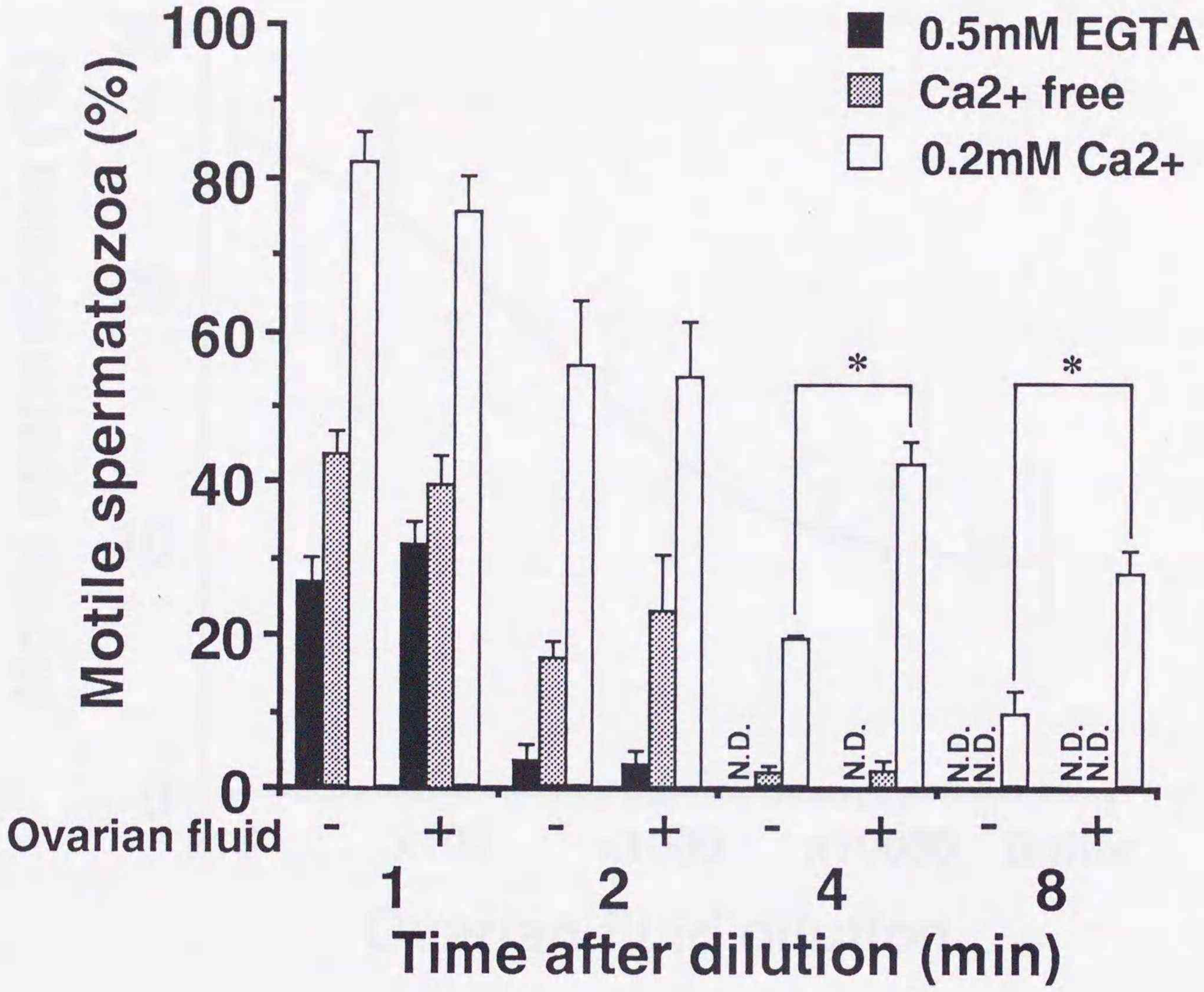


Fig. 5 The ratio of motile spermatozoa of the Nile tilapia 1, 2, 4 and 8 min after dilution with the DB with (+) or without (-) 1/100 volume of the ovarian fluid. Dark shaded column, spermatozoa dissolved in Ca2+-free ASP containing 0.5mM EGTA was suspended in the DB containing 1mM EGTA. Light shaded column, spermatozoa dissolved in Ca2+-free ASP was suspended in the DB. Open column, spermatozoa dissolved in ASP was suspended in the DB containing 0.2mM Ca2+. N.D., motile spermatozoa was not detected. Asterisks represent significant difference from the value without the ovarian fluid ($p < 0.01$).

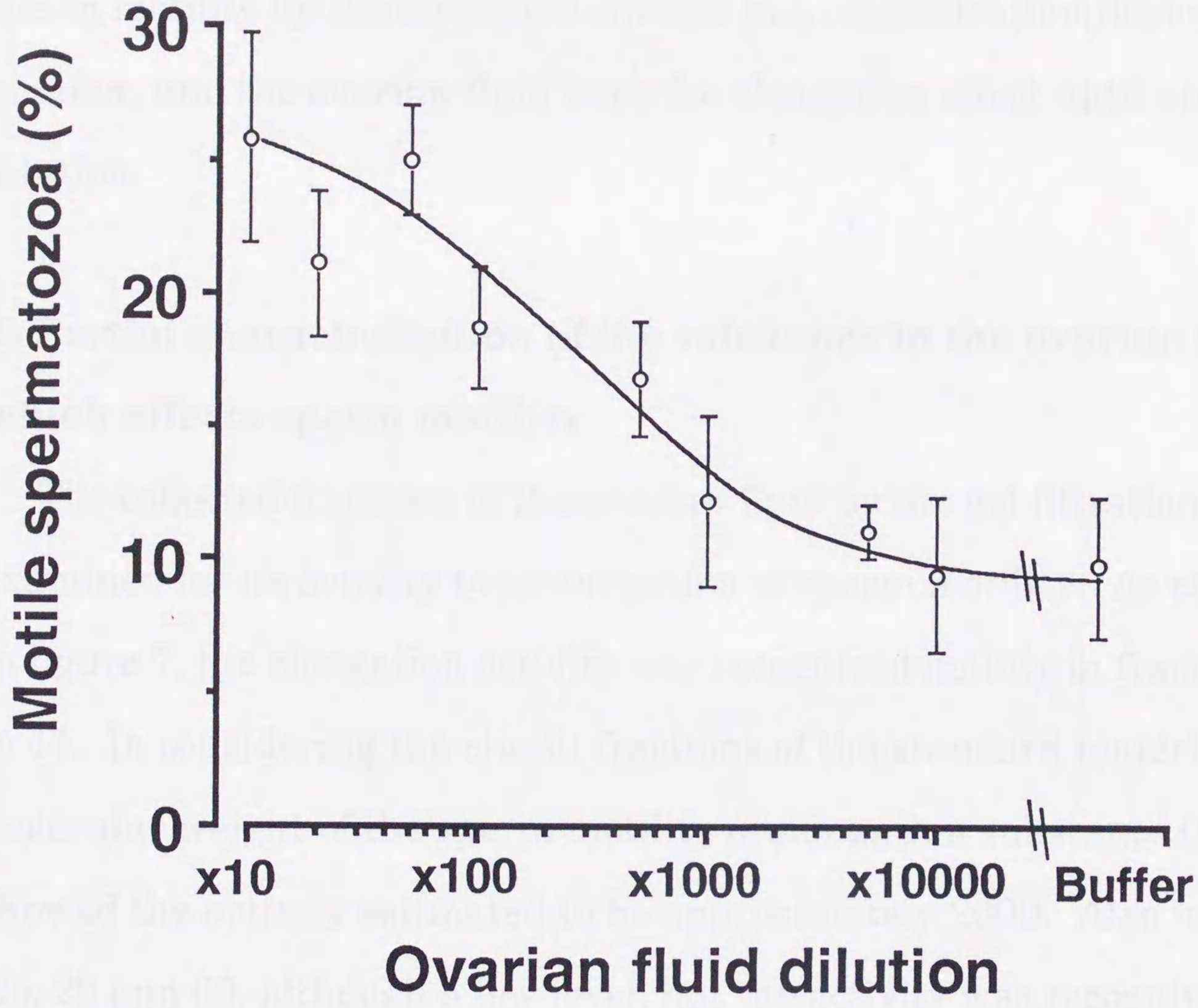


Fig. 6 Concentration-dependency of the prolongation effect of the ovarian fluid on the sperm motility of the Nile tilapia. Control, the DB containing 0.2mM Ca^{2+} without the ovarian fluid.

Effect of the ovaian fluid concentrations on sperm motility was shown in figure 6. Sperm suspension in ASP was dissolved in the DB containing 0.2mM $CaCl_2$ with various concentrations of the ovarian fluid, and motile spermatozoa were measured 4 min after dilution. The prolongation of sperm motility by the ovarian fluid was in a concentration-dependent manner, and the ovarian fluid kept the elongation effect until about 500x dilution.

2) Partial characterization of the substance in the ovarian fluid which affects sperm motility

The collected fractions of the ovarian fluid by the gel filtration were each examined for its activity to prolongation of sperm motility. As can be seen in figure 7, the elongation activity was recognized mainly in fraction No. 40 to 44. In considering the eluant fractions of the standard materials, the molecular weight of the sperm motility prolongation substance (SMPS) showed the activity estimated to be approximately 2000. Also in fraction No. 20 and 60, although a low level, but the activity was recognized.

The ovarian fluid was incubated in boiling water for 5 min. After the incubation, whether the ovarian fluid maintained the above observed activity was analyzed. The result was shown graphically in figure. 8. The ovarian fluid maintained the activity which elongated sperm motility even after the boiling for 5 min.

3) Effect of the TAT-10 on sperm motility prolongation

The TAT-10 and TAT-21 as control antibody treated sperm suspensions and non-treated sperm suspension were dissolved in the pooled fractions (No.40-44) of the ovarian fluid by the gel filtration containing 1% BSA, or the DB containing 0.2mM Ca^{2+}. The TAT-10 at the concentrations 0.5μg to 50μg significantly inhibited prolongation effect of sperm motility by the

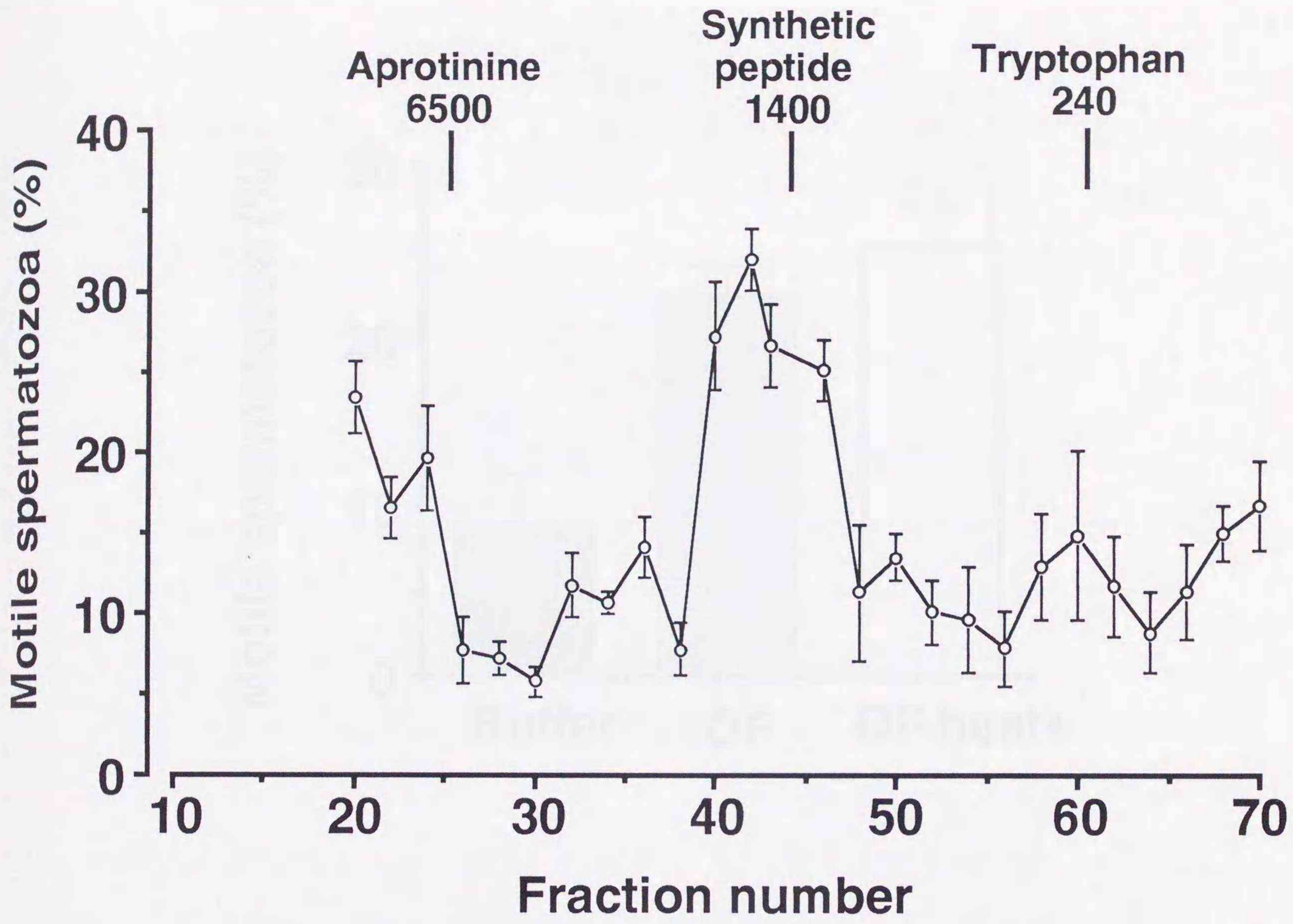


Fig. 7 Sperm motility prolongation activity in each fraction of the ovarian fluid of the Nile tilapia separated by the gel filtration with Sephadex G-25 (1.5x16cm) equilibrated with the DB containing 0.2mM Ca^{2+}. Bars show that the positions of these materials are eluted. Numbers beneath the names of the materials represent the molecular weight of the materials.

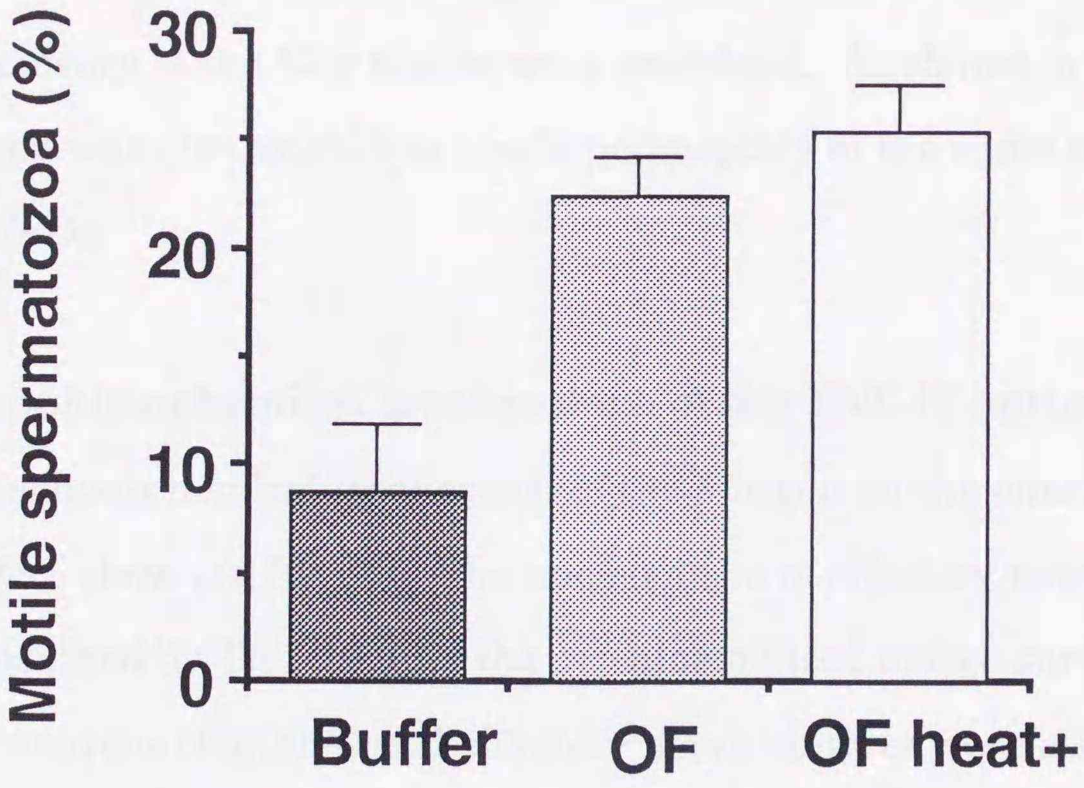


Fig. 8 Heat stability of the sperm motility prolongation activity of the ovarian fluid of the Nile tilapia. Sperm solutions were suspended in the diluent containing 0.2mM Ca2+, the diluent containing 0.2mM Ca2+ with 1/100 volume of the ovarian fluid (OF) and the diluent containing 0.2mM Ca2+ with 1/100 volume of the ovarian fluid incubated at 100°C for 5 min (OF heat+). Motile spermatozoa was estimated 4 min after the dilution.

SMPS, although the TAT-21 did not inhibited the effect of the SMPS. There were no visible effect of both the TAT-10 and the TAT-21 treatment on percent motility (Fig. 9).

4) Tissue specificity of the TAT-10 antigen

The localization of TAT-10 recognized antigen in testis, olfactory organ, brain and ovary of the Nile tilapia were examined. As shown in figure 10, the antigen was also existed as single polypeptide of the same molecular weight 27kDa.

5) Immunohistochemical localizations of the TAT-10 antigen

Immunohistochemical localization of the antigen on the olfactory system were shown in Fig. 11. The nucleoplasm of olfactory neurons were strongly stained by TAT-10, and the cytoplasm faced to free surface of olfactory neurons (Fig.11A) and olfactory nerve bundles were also stained by TAT-10. On the sagital section of the brain, fibres of olfactory bulb were especially strongly stained by TAT-10 (Fig. 11B). In the ovary, TAT-10 antigen first emerged in the cytoplasm of oogonia. The cytoplasm of perinucleolus stage oocyte had especially strong immunoreactivity to TAT-10 (Fig. 12A). In the late perinucleolus stage oocyte, the nucleoplasm was also stained weakly by TAT-10, and only nucleoplasm was stained by TAT-10 in the early yolk vesicle stage oocyte (Fig. 12B). No immunoreactivity to TAT-10 was observed in vitellogenic oocyte (Fig. 12C). Immunoelectron microscopic study on testis showed that especially the cytoplasm of spermatid at initial and middle phase of chromatin condensation was strongly stained in patches, and also the TAT-10 antigen localized on the plasma membrane of these spermatids (Fig. 13).

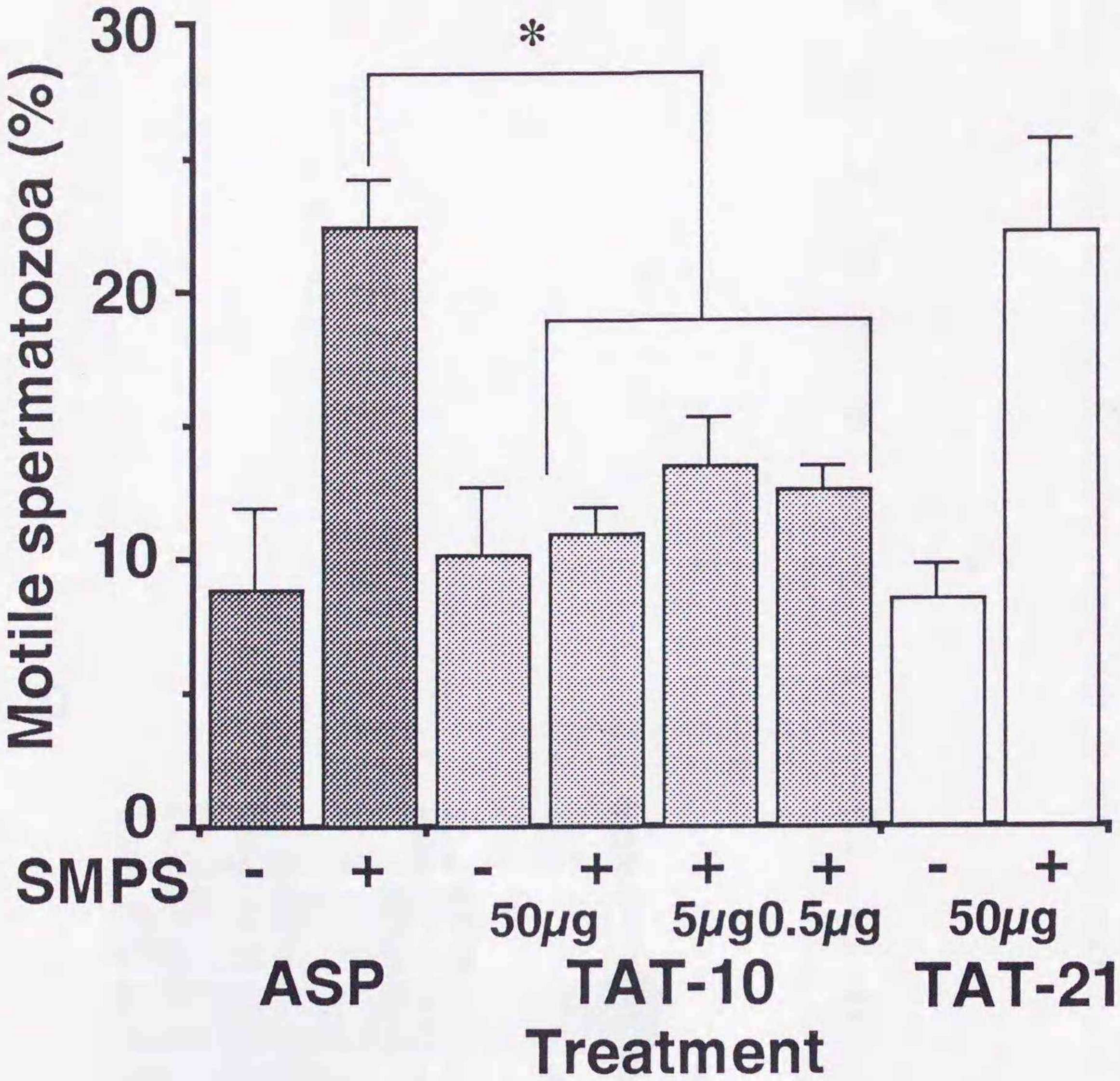


Fig. 9 Effect of antibody treatment on sperm motility prolongation by the SMPS of the Nile tilapia. Spermatozoa-antibody solutions (conc. μg/5x10^6 cells) were dissolved in the diluent containing 0.2mM Ca^{2+} with (+) or without (-) the SMPS fractionated by the gel filtration of the ovarian fluid with the Sephadex G-25, and motile spermatozoa were measured 4 min after dilution. Dark shaded column, ASP as control treatment group. Light shaded column, the TAT-10 treatment group. Open column, the TAT-21 treatment group. Asterisk represents significant difference from the ASP (+) (p< 0.05).

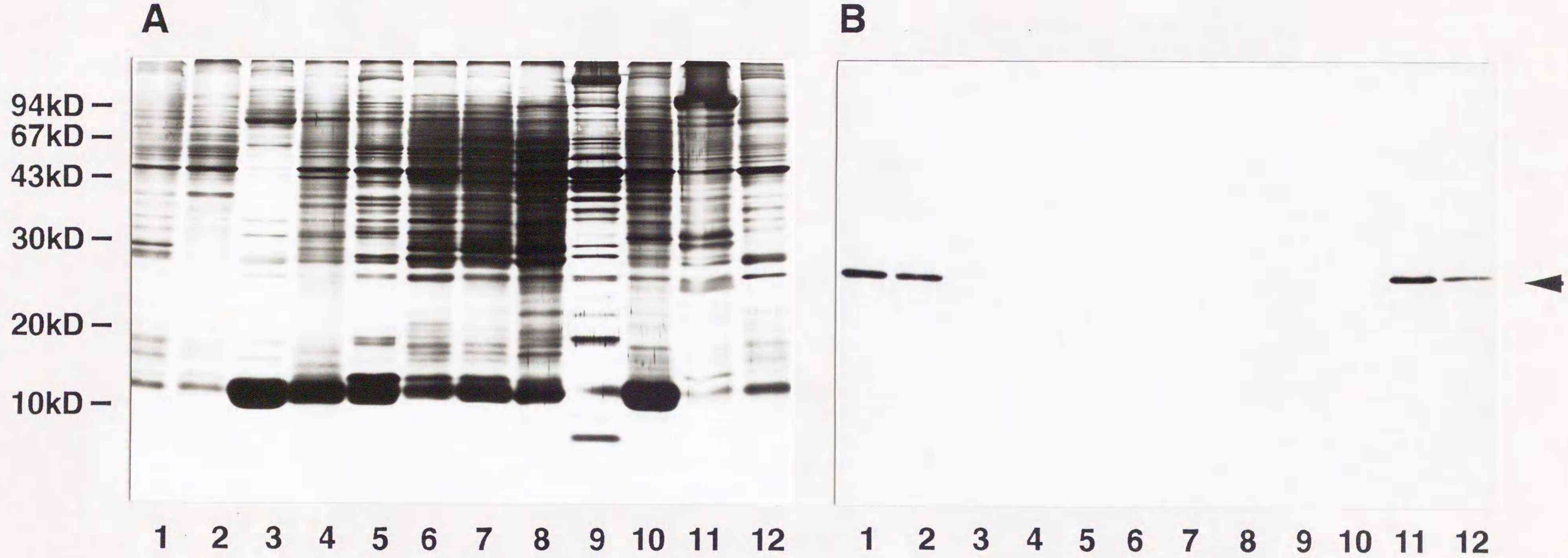


Fig. 10 Detection of the TAT-10 antigen in various tissues of the Nile tilapia by Western blotting using TAT-10. A, electrophoretic pattern of the homogenates of the various tissues in 15% SDS-PAGE with silver staining. Size markers are shown in kD. B, Western blotting of the tissue homogenates by the TAT-10. Ten microgram of protein was applied to each lane. 1, olfactory rosette; 2, brain; 3, blood; 4, gill; 5 heart; 6, intestine; 7, kidney; 8, liver; 9, muscle; 10, spleen; 11, ovary; 12, testis. Arrow shows the position of the TAT-10 antigen.

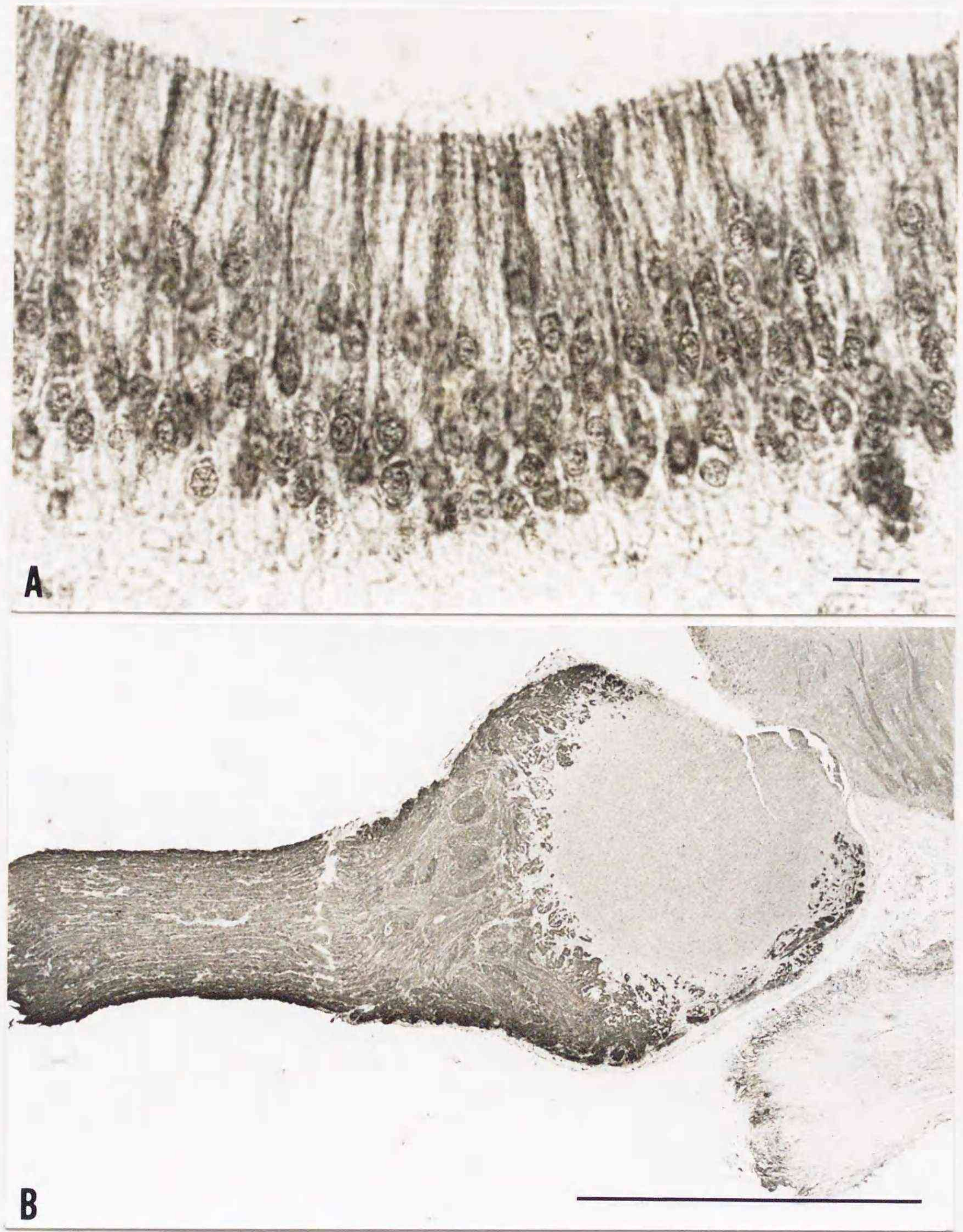


Fig. 11 Localization of the TAT-10 antigen in olfactory epithelium (A) and olfactory bulb (B) of the Nile tilapia. Note that the antigen found mainly in the nucleoplasm and also in cytoplasm of olfactry epithelial cells (A). Bars, 100μm.

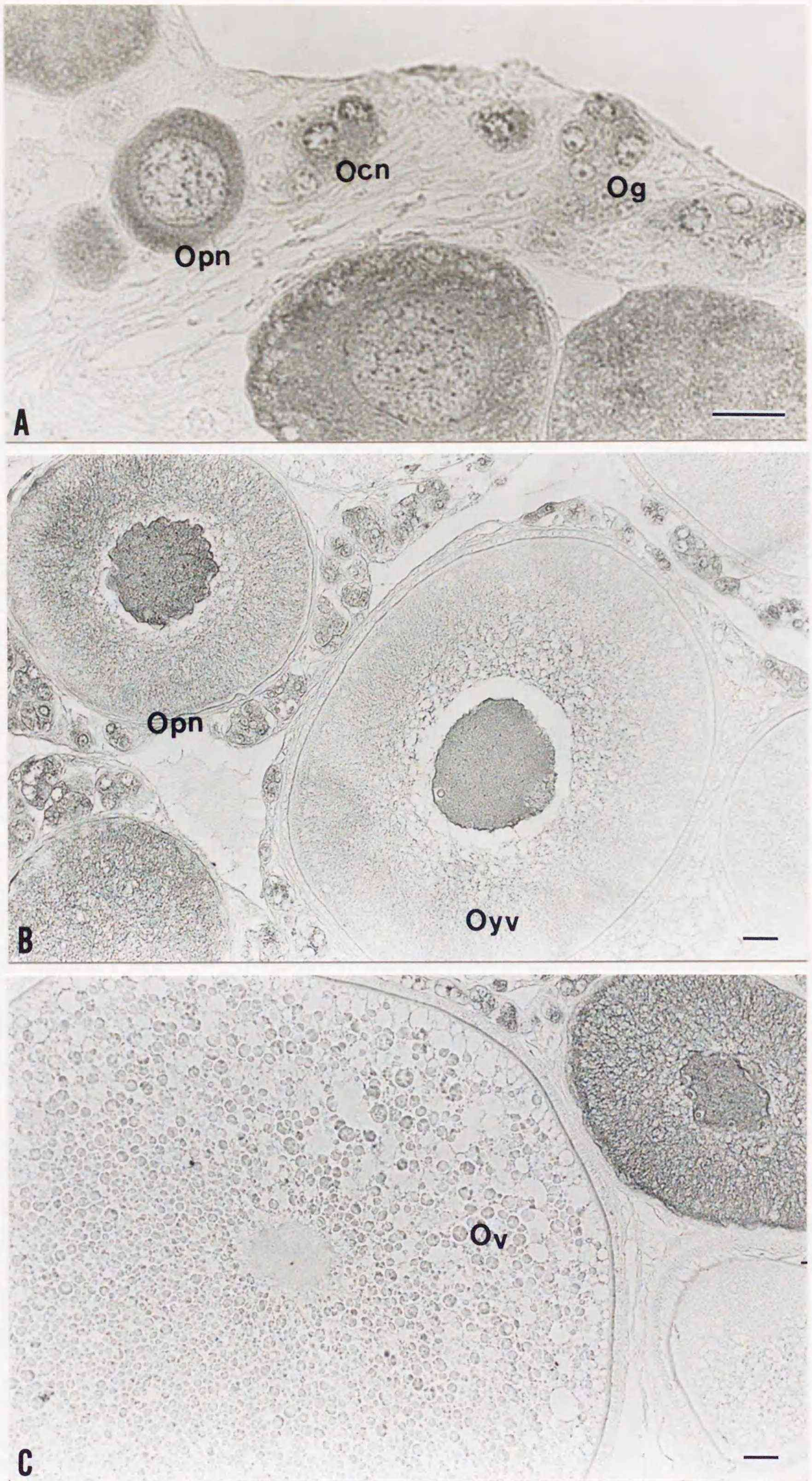


Fig. 12 Localization of the TAT-10 antigen in ovary of the Nile tilapia. Og, oogonia; Ocn, oocyte at chromatin nucleolus stage; Opn, oocyte at perinucleolus stage; Oyv, oocyte at yolk vesicle stage; Ov, vitellogenic oocyte. Bars, 100μm.

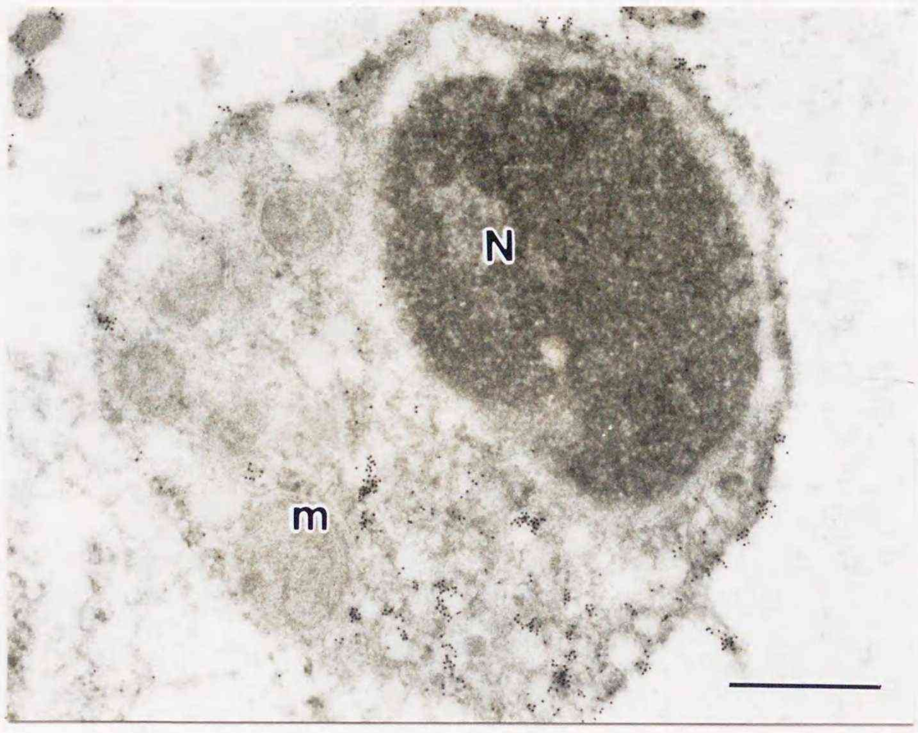


Fig. 13 Electron micrograph of a spermatid at the initial phase of chromatin condensation in the testis of the Nile tilapia reacted with the TAT-10. Gold particles are predominetly seen in the electron-dense structures in the cytoplasm and plasma membrane of the cell. N, nucleus. m, mitochondria. Bar, 0.5μm.

DISCUSSION

The first aim of this study was to determine whether the ovarian fluid would express the sperm motility activating effect. In teleosts, two typical types of sperm motility activation around eggs during fertilization have been reported. One is shown in herring, Clupea palasii and bitterling, Acheilognathus lanceolata; sperm motility is activated by the substance which exists in the egg chorion around micropyle (Yanagimachi, 1957; Suzuki, 1958, 1959). The other is that sperm motility is prolonged by the substance existed in ovaran fluid in rainbow trout, Oncorhynchus mykiss and masu salmon, Oncorhynchus masou (Yoshida and Nomura, 1972; Ohta et al., 1989), and that the increment of velocity is also observed in the masu salmon (Ohta et al., 1989). In the present study, I especially paid attention to sperm motility prolongation by ovarian fluid, and demonstrated that the ovarian fluid had the prolongation effect on sperm motility in the diluent buffer 0.2mM of Ca^{2+} was added. In the environmental water, the concentration of Ca^{2+} is approximately 0.2mM, and it is plausible that the ovarian fluid reveals the prolongation effect efficiently at a concentration of 0.2mM of calcium ion. Further, the ovarian fluid maintained the prolongation effect even in a dilution 1:500. This may be one of strategy to fertilize eggs more efficiently in external fertilization.

In the present study, sperm motility could be observed even in the Ca^{2+}-free diluent buffer. In contrast, when the Ca^{2+}-free ASP and the diluent buffer were chelated by EGTA, the sperm motility decreased further. These results suggest that preservation of sperm motility requires a few millimolar of extracellular Ca^{2+}. Additionally, the expression of its activity of the SMPS required a few millimolar of Ca^{2+}. Therefore, it is likely that the SMPS in the ovarian fluid is a promotor which directly or indirectly intake extracellular Ca^{2+} especially necessary for regulating flagellar motility. In sea urchin, the sperm activating peptide from egg jelly layer regulates Ca^{2+} entry of spermatozoa (Schackmann and Chock, 1986).

The calcium ion is known to play important roles in various kind of behavior of spermatozoa, e.g. acrosome reaction of sea urchin (Kopf and Garbers, 1980), chemotaxis for egg of sea urchin (Brokaw and Nagayama, 1985) and flagellar motility both of sea urchin and some mammalian species (Tash et al., 1988). In flagella of spermatozoa, dynein ATPase think to be phosphorylated by the calmodulin-dependent protein phosphatase (Tash et al., 1988), although the regulation of sperm motility by the calmodulin-dependent protein kinase may be one of possible mechanisms. In some case, calcium exerts its influences on cellular events by binding to calmodulin, and the calmodulin also has been detected in the spermatozoa of several mammalian species (Jones et al., 1980; Feinberg et al., 1981; Moore and Dedman, 1984). The moility of human spermatozoa is inhibited by calmodulin antagonists such as trifluoperazine and calmidazolium, and the treatment of these antagonists induced a concomitant rise in Ca^{2+} and a decline in cAMP internal spermatozoa (Aitken et al., 1988). On the other hand, although cAMP-dependent phosphorylation also suggests to be involved in the control of axonemal function in sea urchin (Murofushi et al., 1986). In salmonid fish (Morisawa and Hayashi, 1985), similarly, the stimulation of adenylate cyclase by extracellular Ca^{2+} is also observed in spermatozoa (Hyne and Garbers, 1979; Morisawa and Ishida, 1987). In considering the above, it is plausible that the Ca^{2+} as one messenger, which enter into spermatozoa by the stimulation of the SMPS regulates motility prolongation via calmodulin-dependent and/or cAMP dependent protein kinase in the Nile tilapia.

Studies on the gel filtration of the ovarian fluid showed that the M_r of the SMPS was approximately 2000. Furthermore, this substance was heat stable. In addition to this fraction, other two fractions which showed minor prolongation effect were also detected. The substance eluted around void volume may be a quite different molecule which also has sperm motiliy prolongation activity or the SMPS originally coupled with a carrier protein. It is likely, therefore, that it may be separated from the carrier protein by means of the dilution. The fraction eluted near around tryptophan which affect sperm motility is thought to containe mainly a various kind of electrolytes. Although at least Ca^{2+} played some role in its motility in the spermatozoa of the Nile tilapia, preliminary result showed that also Na^+ has an activation effect on sperm motility. In the sea urchin spermatozoa, motility and respiration can be activated by the Na^+: H^+ exchange upon addition of Na^+ to sperm in Na^+-free sea water (Christen et al., 1982; Lee et al., 1983; Bibring et al., 1984). As a result of the Na^+: H^+ exchange, intracellular pH increases followed by the activation of flagellar dynein ATPase (Tombes and Shapiro, 1985). Potassium in sea urchin and sodium bicarbonate in several mammalian species also stimulate their sperm motility (Mita and Yasumasu, 1984; Okamura et al., 1985). Similarly, in the Nile tilapia, these electrolytes may regulate sperm motility. In the sea urchin, sperm activation substances detected in the egg jelly layer are several peptides composed of approximately 10 residues of amino acid (Hansbrough and Garbers, 1981; Suzuki et al.,1981, 1984, 1987, 1988a,b; Yoshino et al., 1990, 1992). This substance has been purifying in order to analyze the structure of this molecule in our laboratory.

TAT-10 treatment significantly inhibited the prolongation of sperm motility by the SMPS. The antibody treatment did not affect sperm motility in the diluent buffer without the SMPS. These results suggest that the TAT-10 antigen on spermatozoa plays an important role in some part of the signal transduction by which the SMPS expressed its activity. Also, immunoelectron microscopic study showed that the TAT-10 antigen localized on the plasma membrane of midpiece region of spermatozoa, suggesting to be a membrane receptor of the SMPS. The preliminary result revealed that partial amino acid sequence of the TAT-10 antigen purified from solubilized sperm protein resembles several G protein-coupled receptors. Sperm motility prolongation by the ovarian fluid may be expressed through the G protein related signal transduction in the Nile tilapia. In the sea urchin, two types of receptors for sperm activating peptides have been identified; one is a protein at M_r 77000 and its predicted amino acid sequence resembles that of the low density lipoprotein receptor (Dangott and Garbers, 1984), and the other receptor is identified as a member of the family of guanylyl cyclases (Shimomura et al., 1986; Garbers, 1992). Although sperm motility activating molecule was recently purified in Pacific herring (Pillai et al., 1993), sperm receptors for the molecules containing those for motility prolongation substances have not yet been identified in teleosts. Cloning and sequence of gene encoding the TAT-10 antigen in spermatozoa, which is putative receptor for the SMPS, are now being in our laboratory.

The second aim of this study was to explore whether the TAT-10 antigen expressed in somatic tissues other than testis, especially in olfactory organ. It is the noteworthy hypothesis supposed in the mammalian studies (Parmentier et al., 1992; Vanderhaeghen et al., 1993) that olfactory receptor gene family expressed in sperm cells may be involved in sperm motility regulation or chemotaxis, although there is no direct evidence that the olfactory receptor which expressed in spermatozoa involved in sperm migration during fertilization. Also in a teleost, catfish <u>Ictalurus punctatus</u>, as well as mammalian species, olfactory receptor genes are expressed in the olfactory epithelium (Ngai et al., 1993a, b). Western blot analysis using the TAT-10 revealed that the TAT-10 antigen was also present in olfactory rossette, brain and ovary other than testis as the same M_r polypeptides. However, immunostaining on olfactory organ demonstrated that the TAT-10 antigens distributed not only in the olfactory neurons, but also in olfactory nerve bundles and in the olfactory bulb. In rat olfactory epithelium, the antibody against one of putative olfactory receptor proteins specifically recognized cilia of a small subset of olfactory neuron, suggesting that the olfactory receptor is localized selectively in the cilia (Koshimoto et al., 1992). Although the TAT-10 antigen expressed in the olfactory organ may not be a olfactory receptor, it is possible that the TAT-10 antigen play an important role in neuro-signal transduction being implicated in odorant recognition.

The TAT-10 antigens localized in A and early B spermatogonia and also in previtellogenic oocyte, suggesting to be implicated in primary growth of germ cells. In ovary, the TAT-10 antigen in oocyte changed their distribution pattern in cytoplasm to nucleus during primary growth phase, and localized mainly in nucleus as well as in the olfactory neurons. The preliminary result in the present study showed that the TAT-10 antigens were detected mainly in cytosolic and microsome fractions by differential centrifugation in these tissues. In these tissues, the TAT-10 antigens which are synthesized in cytoplasm may express their specific functions in the nucleus. Further study is needed to clarifiy the significance of the distribution pattern. Present immunoelectron microscopic study indicated that the TAT-10 antigen was actively synthesized in the cytoplasm of spermatids at the initial phase of chromatin condensation during spermiognesis. It is uncertain whether all of the TAT-10 antigens expressed in spermatids during spermiogenesis are implicated in chemoreception for the SMPS. Some of the antiges may play an important role in sperm maturation. It is known that final maturation inducing steroids as pheromonal hormones induce ovulation and increase of milt volume by the mediation of the olfactory receptor systems in some teleosts (Stacey and Sorensen, 1991), therefore, the TAT-10 antigen may also be involved in final maturation. Now, it has been analyzed whether the TAT-10 antigen is detected in mature oocyte. The present investigation on the localization also indicates the possibility that the TAT-10 antigens in these three tissues, which have several functions belong to the same gene family, indicating several intracellular distribution patterns.

In conclusion, I have demonstrated in the present study that, in the Nile tilapia as well as several other teleosts, the ovarian fluid contained the sperm motility prolongation substance (SMPS), which was a M_r 2000 and heat stable. The SMPS required at least a few millimolar of external Ca^{2+} in order to express their activity. Because of antagonist to the SMPS, the TAT-10 antigen on spermatozoa suggests to be implicated in a signal transduction of motility prolongation by the SMPS. Western blot analysis revealed that the TAT-10 antigen also localized in the olfactory organ and ovary as well as testis. However, immunocytochemical study suggests the TAT-10 antigen in olfactory organ was not olfactory receptor. According to the localization of the antigen in the ovary, testis and olfactory organ, it seems likely that the TAT-10 antigen plays an important role in primary growth and final maturation of germ cells. Additional research should focus on the real function(s) of the TAT-10 antigen(s) in each tissues by determining the corresponding ligand(s), and on the clarification of signal transduction cascade(s) in the physiological phenomena.

V. Sperm autoantigen and a sperm motility inhibiting factor in seminal plasma

In this chapter, the mechanism of immobilization of tilapia spermatozoa in the seminal plasma will be addressed. One of the monoclonal antibodies established in the previous chapter, the testicular antigen of tilapia (TAT)-30, recognized electrophoretically the 120kDa antigen under reduced condition, which localized on head of spermatozoa. In addition, the antigen was the main protein component of the seminal plasma. Furthermore, the previous result revealed that the intact TAT-30 antigen in the seminal plasma was a high molecular weight glycoprotein, suggesting the possibility that the TAT-30 antigen is the inhibiting factor of sperm motility in the seminal plasma like as the "immobilin" (Usselman and Cone, 1983). Therefore, first, it was investigated whether the TAT-30 antigen in the seminal plasma had sperm motility inhibiting effect. Succeedingly, partial characterization especially of sugar residue, tissue specificity and the localization in testis of the TAT-30 antigen were analyzed.

MATERIALS AND METHODS

1) Animal

Male Nile tilapia, Oreochromis niloticus used in the present study were maintained in indoor concrete ponds on the campus of the Faculty of Fisheries of Hokkaido University, at 25-30°C under natural light conditon, and fed on a commercial diet 2 to 3 times a day.

2) Preparations of milt, seminal plasma and somatic tissues

Milt freshly stripped from three male Nile tilapia were pooled, and mixed with equal volume of the artificial seminal plasma (ASP: 125mM NaCl, 12mM KCl, 0.8mM $CaCl_2$ and 0.55mM $MgCl_2$ buffered with 10mM Hepes, pH7.8). Sperm suspension obtained was used in the sperm motility assay.

Seminal plasma for its protein components purification was collected by the following method as described in the previous chapter. Namely, testes from freshly sacrificed males were macerated in the ASP. The testis suspension was passed through a stainless steel mesh and final testis suspension was centrifuged at 8000g for 15 min at 4°C. The resultant supernatant was collected as the seminal plasma. For electrophoresis, milt stripped from the males was centrifuged at 8000g for 15 min at 4°C. The resultant supernatant was collected as the seminal plasma. These collected seminal plasma were stored at -80°C until use.

The fishes were sacificed and immediately, their various organs (whole brain, heart, intestine, kidney, liver, gill, muscle, spleen, ovary and testis) were removed. Blood were collected from caudal vein with shiringe (Terumo, Tokyo, Japan). Each tissue except blood was washed quickly in 50mM Tris-HCl pH8.0, 1mM PMSF, 2mM EDTA 2Na and was homogenized in about 1.5 volumes of the same solution. These tissue suspensions and the blood were assayed for protein by the Lowry method using bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) as the standard and stored at -40°C until use for Western blot analysis.

3) Sperm motility assay

Diluent buffers were made up with 10mM Tris-HCl pH7.4, 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), containing required concentrations of the seminal plasma protein. All measurement were made at room temperature. Sperm motility were estimated as follows. Two μl of the sperm suspension was dissolved in 200μl of the diluent buffer containing required concentrations of seminal plasma protein. Then, 10μl of the mixture was placed in a haemocytometer slide. The percentage of moving spermatozoa 1.5 min after the dilution was calculated by counting the number of spermatozoa with blurred outlines in several photomicrographs of 4 different fields of 100x magnification with 0.5 sec exposure.

4) Antibody treatment

Monoclonal antibodies from culture media for the hybridomas of TAT-30 and TAT-21 as control established in the former study were purified by affinity chromatography. The media were centrifuged at 1000g for 10 min to eliminate cellular and particulate debris, and were dialyzed against 1.5M glycine pH8.9 containing 0.3M NaCl overnight at 4°C. After the dialysis, the media were applied to Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with above mentioned buffer and the antibodies peaks were eluted with 0.1M citric acid buffer pH4.0. Immediately after the elutions, the eluted antibodies were dialyzed against the diluent buffer without seminal plasma protein overnight at 4°C and stored at -20°C until use. The antibody solutions were assayed using Bio-Rad protein assay kit (Bio-Rad, South Richmond, CA) using bovine IgG (Sigma) as standard.

The diluent buffer with seminal plasma protein was incubated with equal volume of the purified monoclonal antibody solution (100μl of the antibody/ml) overnight at 4°C and used in the sperm motility assay.

5) Gel filtration

For partial purification of proteins in the seminal plasma, gel filtration was carried out by Sepharose 6B (Pharmacia) (2.5x80 cm) at a flow rate of 12ml/h equilibrated with 20mM Tris-HCl pH8.0 containing 2% NaCl and NaN_3. The eluate fraction was collected in 4ml each. For molecular weight determination of the TAT-30 antigen in the seminal plasma, gel filtration was performed using Superose 6 with Pharmacia FPLC system equilibrated with 20mM Tris-HCl pH8.0, 2% NaCl with the same buffer at a flow rate of 0.5ml/min, and the eluted fractions were collected at 1ml/tube and each fractions was examined for the detection of TAT-30 antigen by dot blot analysis. Gel filtration calibration kit for molecular weight determination of high molecular weight proteins (Pharmacia) were used as standards.

6) Immunoprecipitation Procudure

Ten-20μg of the TAT-30 or control antibody was added to 50μl of Protein A-Sepharose CL 4B (Pharmacia) and mixed with tilapia seminal plasma. The reaction mixture was incubated first at room temeprature for 3 hr and then 4°C overnight. The immune complexes were obtained by centrifugation (10000g for 2min), the supernatants were discarded, and the pellets were washed three times with 0.01M phosphate buffered saline pH7.2 (PBS). The washed gels were incubated in 8M urea for 1hr at room temperature and then centrifuged at 10000g for 2 min. The resultant supernatant was subjected to SDS-PAGE.

7) Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% and 12.5% acrylamide was done in a Tris buffer system (Laemmli, 1970). For SDS-PAGE, samples were pretreated with 3% SDS and 10%

mercaptoethanol at 100°C for 2 min. The gels were stained with Coomassie Brilliant Blue or silver (silver staining kit, Wako). Protein molecular weight markers (low Mr set) were obtained from Pharmacia.

8) Blotting

The separated protein samples on gels without staining were then transferred to polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, USA) by the method described previously (Towbin et al., 1979). Before immunostaining, these membranes were incubated with 5% skim milk in Tris-buffered saline (TBS, 20mM Tris-HCl pH7.5 containing 500mM NaCl) to block non-specific protein binding sites. For immunostaining, the membrane was then incubated with culture supernatant of TAT-30 obtained before overnight, followed by treatment with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Bio-rad, South Richmond, CA, USA) at a dilution 1:1000 in TBS for 3 hr at room temperature. For lectin staining, the membranes were incubated with several biotinyl lectins (Biotinyl lectin set containing ConA, Conavalia ensiformis agglutinin; DBA, Dolichos biflorus agglutinin; LCA, Lens culinaris agglutinin; PNA, Arachis hypogaea agglutinin; RCA_{120}, Ricinus communis agglutinin; SBA, Glycine max agglutinin; UEA-I, Ulex europaeus-I agglutinin; WGA, Triticum vulgaris agglutinin, Wako, Tokyo, Japan) at 20μg/ml concentration overnight, followed by treatment with the streptavidin and biotinylated HRP complex (Dako, Glostrup, Denmark) for 1 hr. After washing above treated membranes with TBS, 4-chloro-1-naphthol solution (60mg in 100ml TBS containing 0.01% H_2O_2) was applied to visualize the peroxidase reaction products.

Eluted fractions of the seminal plasma by the gel filtration were dotted on nitrocellulose membrane (Bio-rad), and the membrane was stained with TAT-30 by the method described above.

9) Histochemistry

Testes from freshly killed maturing male tilapia were fixed in Bouin's fluid for 3 hr at room temperature for immunostaining, or in 10% formalin in 0.1M phosphate buffer pH7.2 overnight at 4°C for lectin staining. After fixation, these testes were washed at least three times with PBS for two days at 4°C, and then embedded in paraffin (m.p. 54°C). Five micrometers sections of the testes were deparaffinized and immersed in 0.3% H_2O_2 in methanol for 40 min to block endogenous peroxidase activity. For immunostaining, the sections were treated with 10% normal goat serum in PBS for 1 hr at room temperature to avoid non-specific binding of antibodies. Sections were incubated with the culture media of the TAT-30 for 6 hr at room temperature, and then, after washing with PBS, incubated with a solution of biotinylated rabbit anti-mouse IgG at 1:400 (Dako) for 3 hr at room temperature. For lectin staining, the sections were treated wih 1% BSA-PBS for 1 hr at room temperature for blocking, and then incubated with previously described biotinyl lectins at 2μg/ml for 6 hr at room temperature. Finally, the sections for both immunostaining and lectin staining were incubated with the streptavidin and biotinylated HRP complex (Dako). After 30 min incubation, the sections were washed with 50mM Tris-HCl pH7.6 (TB), and incubated with 0.01% diaminobenzidine-0.03% H_2O_2 in TB. These sections and the contiguous sections stained with hematoxylin-eosin were observed with a Zeiss Axiophot microscope.

For immunoelectron microscopic observations, tissues were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer pH7.4 (PB) for 3hr at room temperature. After being throughly washed with the PB containing 10% sucrose, the specimens were dehydrated through a graded ethanol series and embedded in LR-White (London Resin Co. Hampshire, England). Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome with diamond knife. The sections were first incubated with PBS containing 1% BSA (Sigma) to block non-specific protein binding site, and then incubated with the culture supernatant of the TAT-30 hybridoma for 3 hr at room temperature. The sections were further incubated with 10nm-gold conjugated goat anti-mouse IgG+IgM (Bio Cell, Cardiff, UK) at a dilution 1: 100 for 1hr. The sections were viewed with a Hitachi H-7000 electron microscope after staining with uranyl acetate.

10) Statistical analysis

In the present study, results were expressed as mean±SE, and differences between groups were compared by paired Student's t -test.

RESULT

1) Partial purification of protein components in the seminal plasma

The seminal plasma was salted out by 50% saturated ammonium sulfate, and centrifuged at 10000g for 15min. Resultant precipitate dissolved in PBS was extensively dialyzed against 20mM Tris-HCl pH8.0 containing 2% NaCl and 0.1% NaN_3 overnight at 4°C. After the dialysis, insoluble material was removed by centrifugation at 10000g for 10 min and the supernatant was fractionated by means of Sepharose 6B gel filtration. The chromatogram of the gel filtration of the seminal plasma and the TAT-30 antigen-rich fractions by the dot bot analysis were shown in figure 14. Fraction No.35-45 were pooled as the seminal plasma protein (SPP) and used in the sperm motility inhibition assay after dialysis against 10mM Tris-HCl pH7.4 containing 1% BSA. Several protein components of the seminal plasma were concentrated in the SPP (Fig. 14, inset).

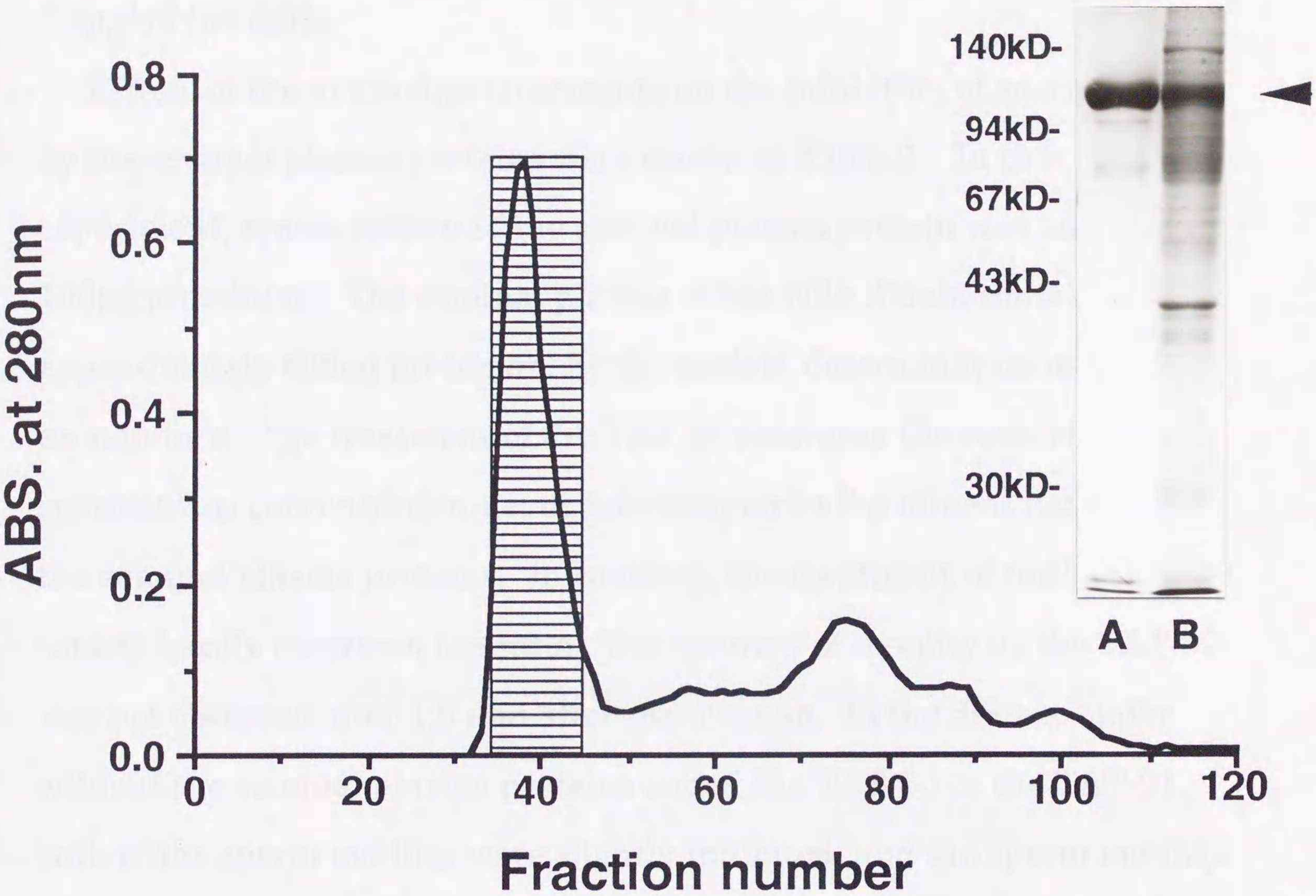


Fig. 14 Sepharose 6B column chromatography (2.5x80cm) of the TAT-30 positive fraction obtained by the salting out of the seminal plasma of the Nile tilapia. Shaded square shows the SPP (TAT-30 antigen-rich) fraction. Inset: electrophoretic patterns of the seminal plasma (A) and the SPP fraction (B) on 10% SDS-PAGE. Arrow shows the TAT-30 antigen. Positions of molecular weight markers are indicated on the left of the figure.

2) Inhibition of sperm motility by the seminal plasma protein

The seminal plasma proteins inhibited sperm motility with concentration-dependent fashion, and the inhibitory effect reached plateau at approximately 100μg/ml (Fig. 15). The ratio of motile spermatozoa was already significantly suppressed by adding the seminal plasma proteins 0.1μg/ml ($p < 0.05$).

Effects of the antibodies treatments on the inhibition of sperm motility by the seminal plasma proteins were shown in Table 2. In this experiment, concentration of the seminal plasma protein was adjusted at 100μg protein/ml. The seminal plasma of the Nile tilapia contained approximately 400μg protein/ml by the protein determination using BSA as standard. The treatment of the TAT-30 recovered the ratio of motile spermatozoa concentration-dependent manner in the diluent buffer with the seminal plasma proteins. In contrary, the treatment of the TAT-21 as control hardly recovered the ratio. The recovery of motility by the TAT-30 was not observed after 1.5 min after the dilution. In the diluent buffer without the seminal plasma proteins added the TAT-30 or the TAT-21, both of the sperm motility were slightly inhibited, and the sperm motility added the TAT-30 in the diluent buffer tend to be lower than that added the TAT-21 in the diluent buffer.

3) Partial characterization of the sperm motility inhibiting factor (SMIF)

The seminal plasma was centrifuged at 1000g for 10 min to remove insoluble material, and the supernatant was loaded onto the Superose 6 gel filtration. Each eluted fraction was assayed for detection of the TAT-30 antigen by dot blot analysis in order to determine the molecular weight of the SMIF, comparing with the eluted fractions of standard materials.

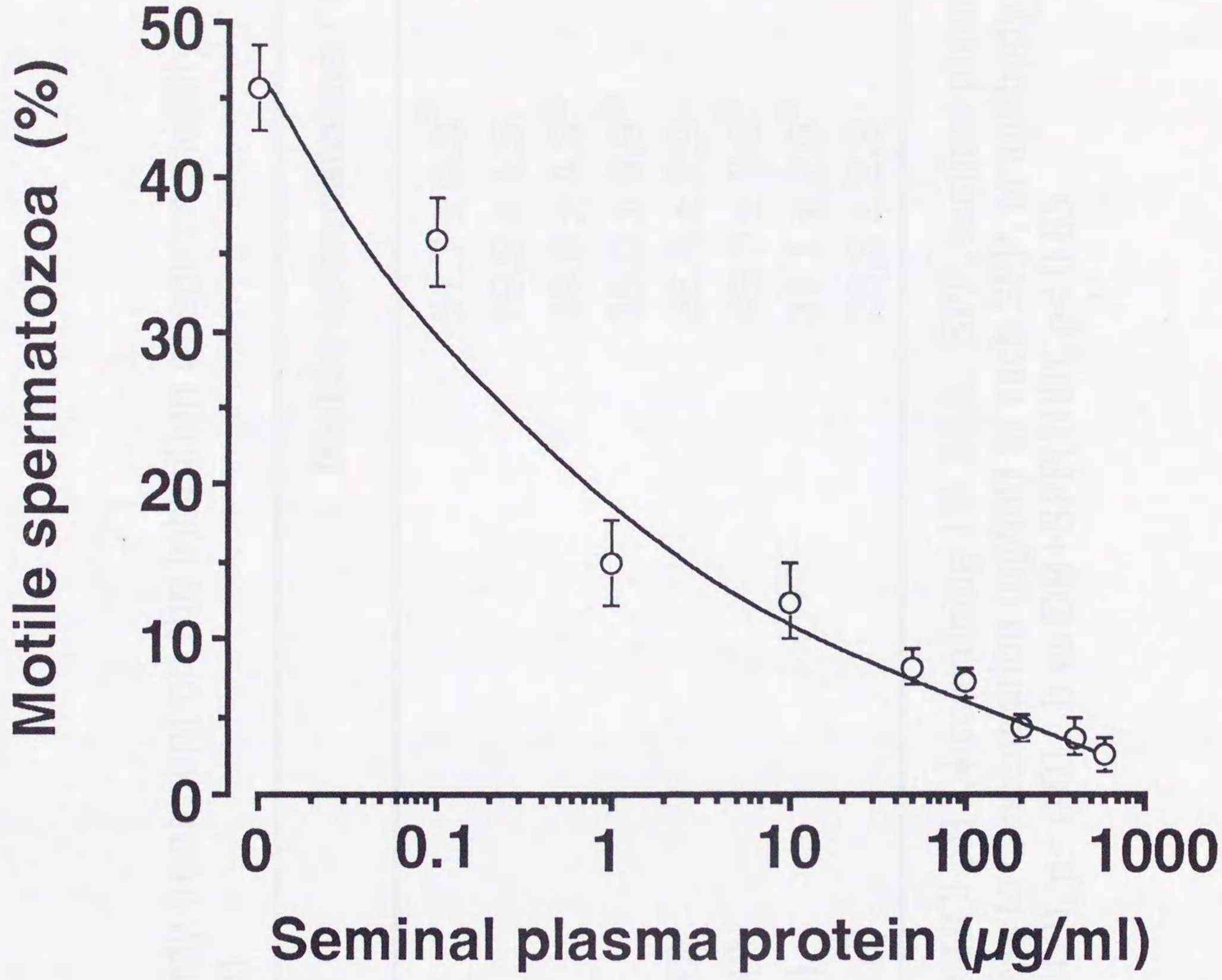


Fig. 15 Concentration-dependency of the SPP on sperm motility inhibiton of the Nile tilapia

Table 2. Effects of the antibody treatment on the inhibition of sperm motility by the seminal plasma protein

Diluent	Motile spermatozoa (%)
DB	51.7 ± 4.3^{a}
DB + SPP (100)	19.9 ± 1.5
DB + TAT-21 (50)	39.6 ± 4.3^{b}
DB + TAT-30 (50)	35.0 ± 3.9^{b}
DB + SPP (100) + TAT-21 (50)	26.4 ± 2.9
DB + SPP (100) + TAT-30 (50)	49.0 ± 3.9^{a}
DB + SPP (100) + TAT-30 (5)	31.1 ± 2.0^{a}
DB + SPP (100) + TAT-30 (0.5)	23.3 ± 2.8

DB, the diluent buffer 10mM Tris-HCl pH7.4 containing 1% BSA. SPP, seminal plasma protein. Parenthetical number refers to concentration (μg/ml) of each SPP, or antibody in the diluent buffer. a vs DB+SPP(100), $p< 0.01$. b vs DB+SPP(100), $p< 0.05$

The SMIF was eluted in the same position as the blue dextran, and was estimated approximately M_r 2,000,000 (Fig. 16A, B).

The electrophoretic pattern and Western blotting with the TAT-30 and the lectins were shown in figure 17. The seminal plasma contained mainly 120kDa and minor 78kDa, 43kDa, 38kDa and 27kDa polypeptides. The 120kDa protein was only recognized by the TAT-30, however, the minor polypeptides other than 120kDa were not reacted with the TAT-30. The lectin binding characteristics of the seminal plasma showed that LCA recognized mainly the 120kDa polypeptide and 38kDa polypeptides. The 38kDa polypeptide was also stained by the UEA-I (data not shown). These blots by the lectins incubated in the presence of the competing sugar failed to produce bands.

The immunoprecipitate was carried out SDS-PAGE in 12.5% acrylamide with silver staining (Fig.18). The electric pattern of the immunoprecipitate was subjected to densitometric analysis. Peak areas of the 120kDa protein, the 27kDa protein and the 18kDa protein were measured and ratio of 1: 0.24: 0.1 between the peaks were given. Taking the molecular weight of these proteins of the SMIF into account, the molar ratio of these polypeptides was calculated to be 1: 1.1 : 0.7.

4) The TAT-30 antigen localization

Tissue specificity of the TAT-30 antigen was shown in figure 19. The antigen was observed only in testis as a single protein of 120kDa.

Immunohistochemical localization of the antigen on the testicular tissue were shown in figure 20. The TAT-30 antigen localized in the whole cytoplasm of Sertoli cells, epithelial cells of seminal ducts, and spermatozoa in both seminal lobule lumen and seminal ducts of tilapia testis. All spermatognic cells in cysts were not stained by TAT-30. The

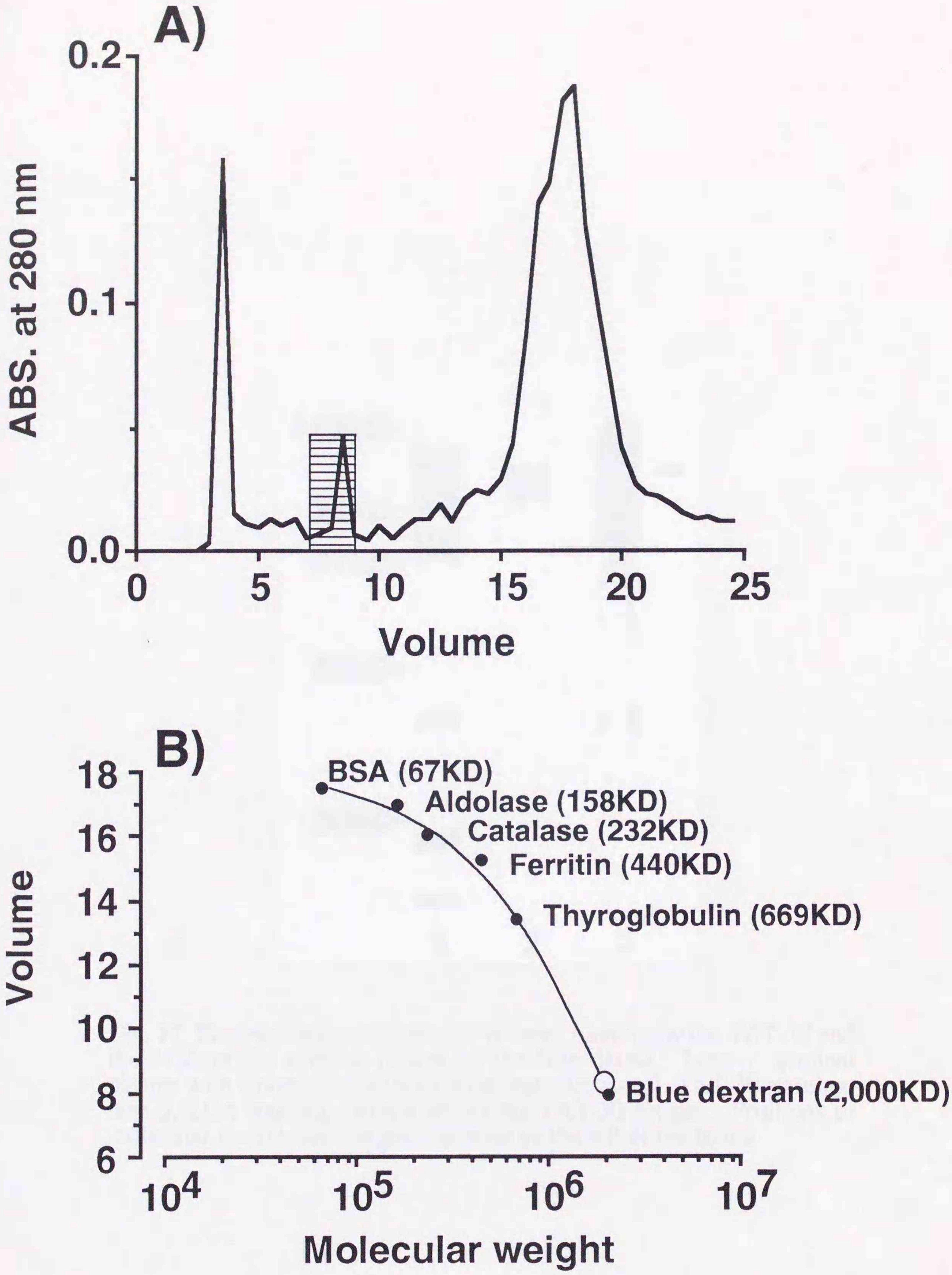


Fig. 16 A) Superose 6 column chromatography of the seminal plasma of the Nile tilapia. Shaded square shows the TAT-30 antigen-rich fraction. B) Molecular weight estimation of the SMIF. Closed circles are standards and open circle is the SMIF.

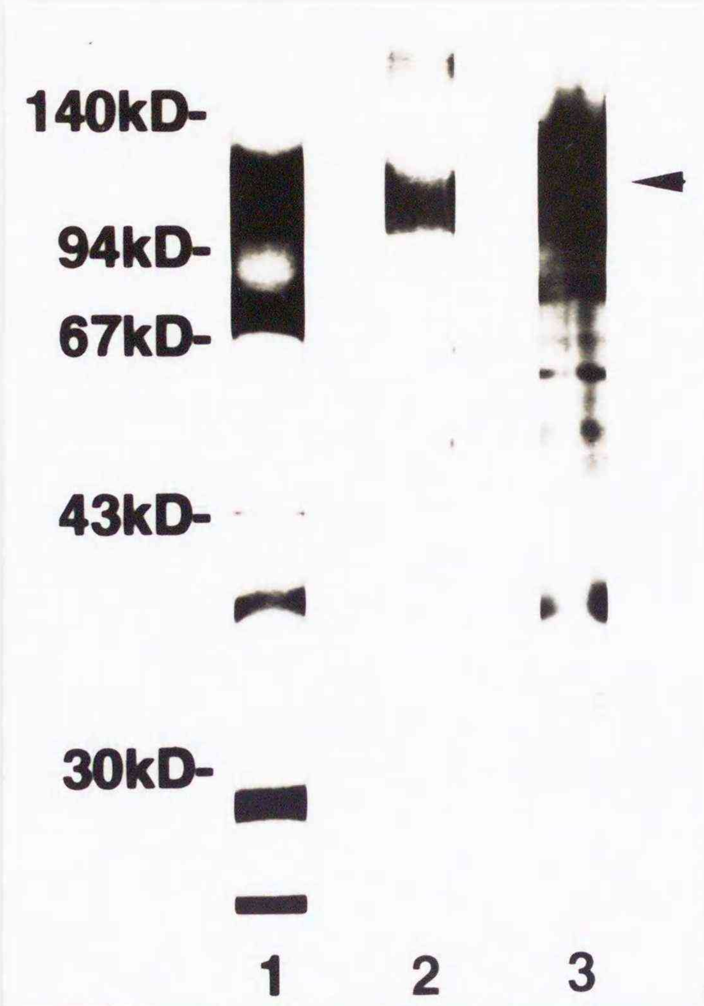


Fig. 17 Electrophoretic pattern and Western blotting by the TAT-30 and the LCA of the seminal plasma of the Nile tilapia. Lane 1, seminal plasma with Coomassie Brilliant Blue staining; lane 2, TAT-30 staining; lane 3, LCA staining. Arrow shows the TAT-30 antigen. Positions of molecular weight markers are indicated on the left of the figure.

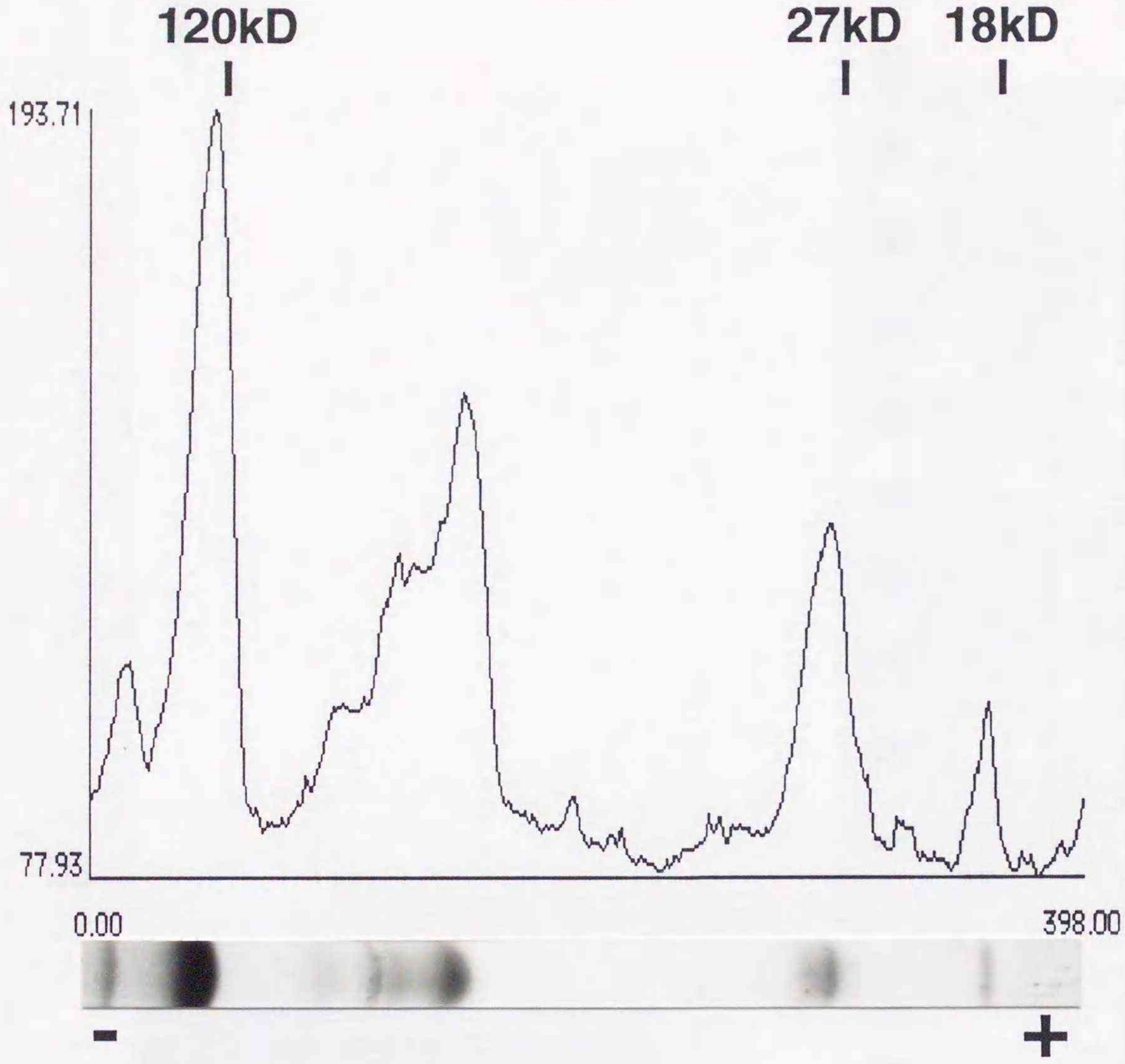


Fig. 18 Densitmetric analysis of 12.5% SDS-PAGE of the reduced SMIF of the Nile tilapia. Bars show the positions of the components of the SMIF.

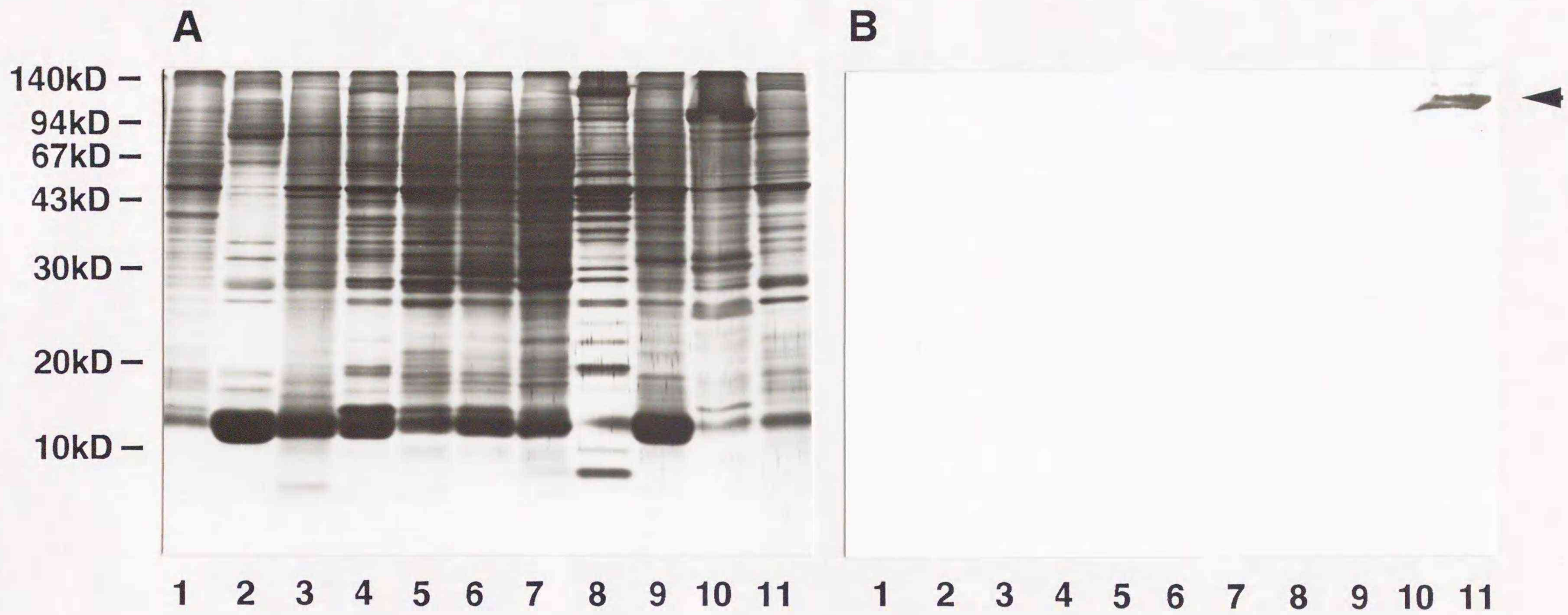


Fig. 19 Detection of the TAT-30 antigen in various tisseus of the Nile tilapia by Western blotting using the TAT-30. A, electrophoretic pattern of the homogenates of the various tissues in 15% SDS-PAGE. Size markers are shown in kD. B, Western blotting of the tissue homogenates by the TAT-30. Ten microgram of protein was applied to each lane. 1, whole brain; 2, blood; 3, gill; 4, heart; 5, intestine; 6, kidney; 7, liver; 8, muscle; 9, spleen; 10, ovary; 11, testis. Arrow shows the position of the TAT-30 antigen.

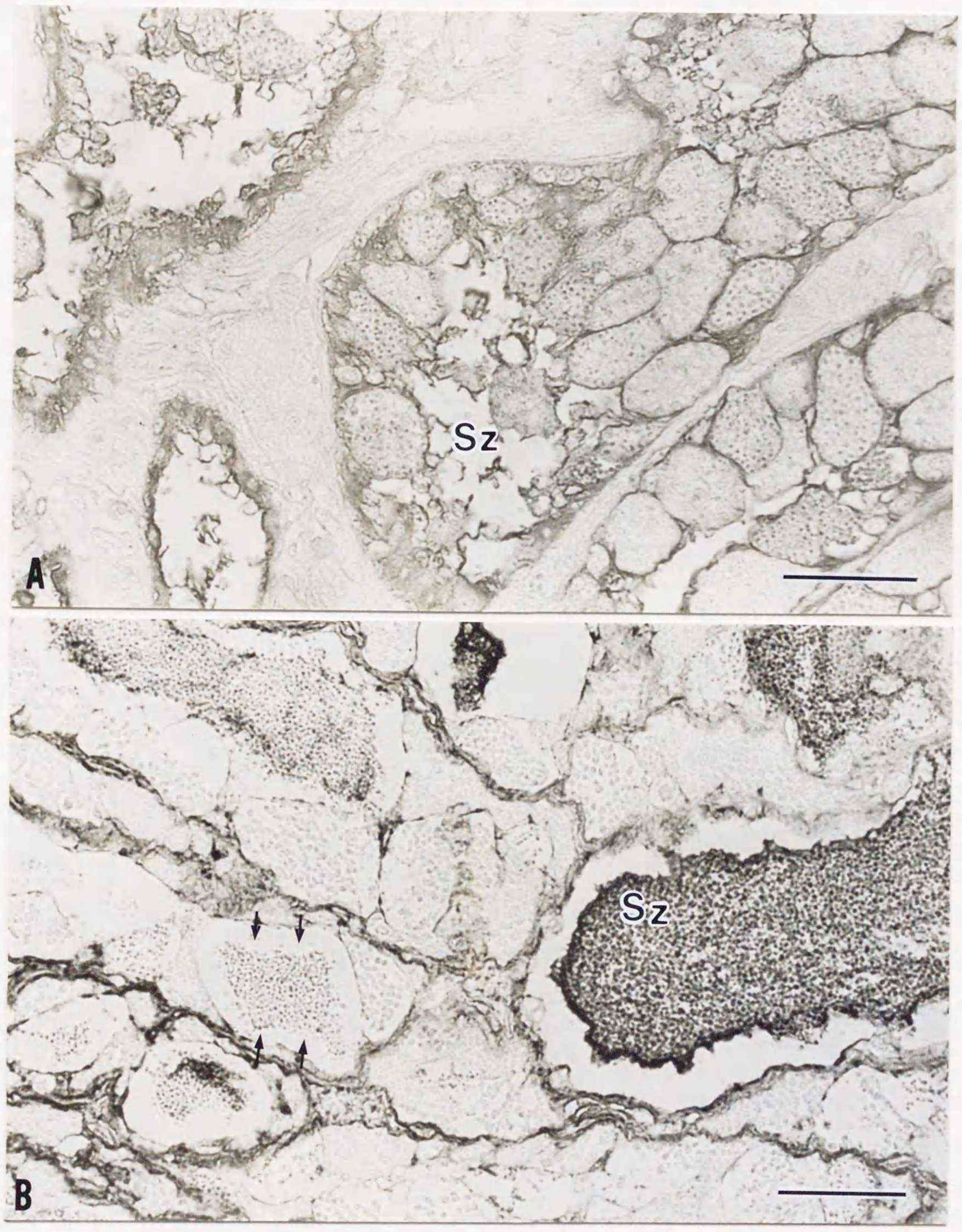


Fig. 20 Localization of the SMIF and sugar residues recognized by the TAT-30 and the LCA, respectively on tilapia testis. Note that some of spermatozoa in the seminal lobule are not recognized by the LCA (arrows in B). A, TAT-30 staining; B, LCA staining. Sz, spermatozoa. Bars, 20μm.

LCA reacted with spermatozoa in seminal ducts and some of spermatozoa in the seminal lobule, and also reacted with interstitial cells, epithelial cells of seminal ducts and some part of Sertoli cells.

Electron microscopic observation indicated an intense immunogold labelling in the cytoplasm facing seminal lobule lumen and in the lumen in association with spermatozoa (Fig. 21A). In the cytoplasm of Sertoli cells, gold particles were also evident in the rough endoplasmic reticulum and in lysosomes (Fig. 21B). Electron lucent vesicles were observed in the cytoplasm of Sertoli cells, but gold particles were not found in the vesicles. In the cytoplasm of epithelial cells of sperm ducts, the staining pattern was similar but not as intense.

DISCUSSION

The present result demonstrated that the SPP of the Nile tilapia contained one of the sperm motility inhibiting factor (SMIF). Similarly, the TAT-30 treatment significantly decreased the inhibitory effect of the SMIF on the sperm motility in concentration-dependent manner revealed that the SMIF was the TAT-30 antigen. However, sperm motility was not completely inhibited even when the SPP added at higher concentration than that of the seminal plasma. Spermatozoa may be inhibited its motility by the TAT-30 antigen cooperated with other factors, such as several kind of electrolytes. The preliminary result showed that spermatozoa were kept immotile in the ASP used in the present study, but the rapid decrease of sperm motility in the ASP was observed comparing to that in the milt. The physiological significance of the motility immobilization by these factors above mentioned in testis seems to be caused preservation of the sperm motility activity.

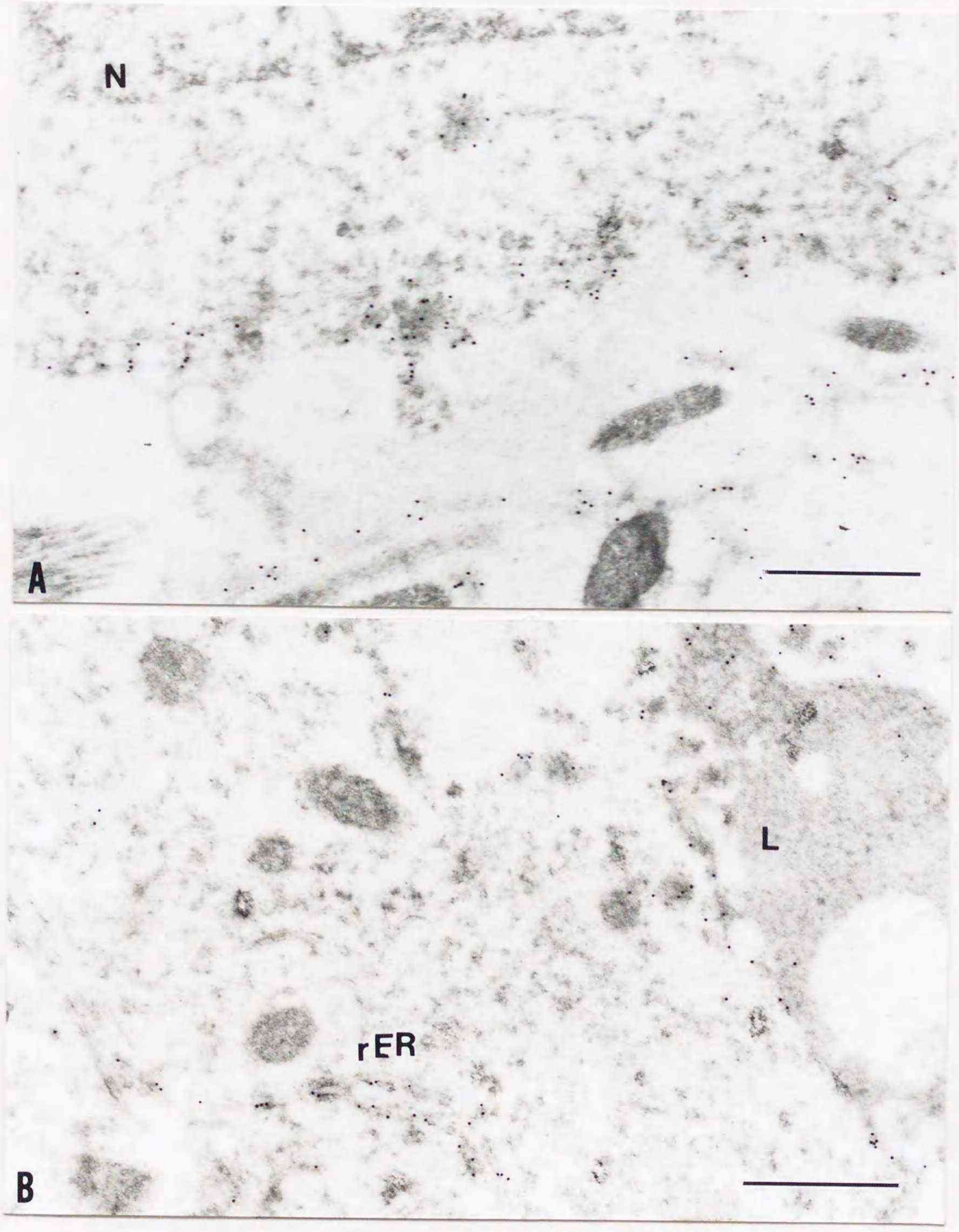


Fig. 21 Electron micrographs of the apical (A) and perinuclear (B) regions of Sertoli cells immunolabeled with the TAT-30. N, nucleus; L, Lysosome; rER, rough endoplasmic reticulum. Bars, 0.5μm.

With respect to the mechanism of the inhibition of motility by the SPP, the inhibition seems to be mechanical immobilization, but not to be caused, at least, by the high osmolarity, because sperm motility recovers only adding the TAT-30 in the DB+SPP. Incidentally, the preliminary result showed that the pH values and osmolarities of the DB+SPP (100μg/ml) and the seminal plasma of the Nile tilapia were 240mOsm and 320mOsm, respectively. Moreover, almost all of spermatozoa were motile in the mannitol solution at 240mOsm. In addition, about 60% of spermatozoa are motile in the mannitol solution at 240mOsm in Oreochromis mossambicus (Harvey and Kelly, 1984). In mammals, there are many reports indicating that epididymal secretory proteins bind to spermatozoa, e.g. rat (Wong and Tsang, 1982; Brooks and Tiver, 1983; Hermo et al., 1994), buffalo (Bergamo et al., 1992) and mouse (Rankin et al., 1992). Since the TAT-30 antigen localized even in the washed spermatozoa with saline as described in the chapter III, the antigen seems likely to belong to this type of antigens, and to bind spermatozoa via integrin like molecule which is receptor for extracellular matrix protein (Hynes, 1987), although there is no direct evidence that the secretory proteins bind to spermatozoa via integrins even in mammals. Based on above informations, the motility of the spermatozoa may be inhibited by the mutual binding of the TAT-30 antigens both in the seminal plasma and on spermatozoa, and the TAT-30 may recognize the epitope which exists in the mutual binding domain of the TAT-30 antigen. Assumption that the sperm motility inhibition is caused by this mechanism explain possibly that sperm motility in the DB + TAT-30 or TAT-21 is lower than that in the DB + antibodies + SPP. It is possible that the TAT-30 mediate between the TAT-30 antigen on spermatozoa and in the milt. As a result, it may cause the inhibition of sperm motility in the DB + TAT-30. However, almost all of the TAT-30 are trapped by the TAT-30 antigen in the milt in the DB + SPP + TAT-30, and the inhibition of sperm motility is not observed. Also in the DB + TAT-21, sperm motility was inhibited comparing to that in the DB, although the value tended to be higher than that in the DB + TAT-30. The TAT-21 may non-specifically mediate between the TAT-30 antigen on spermatozoa and in the milt because of its having a low affinity to the TAT-30 antigen. The immobilin, which is sperm motility inhibiting factor in rat seminal plasma, is not observed to bind spermatozoa. The mechanism of immobilization of spermatozoa by the SMIF may be different from those by the immobilin.

Since sperm motility is prominently inhibited even in the DB adding quitely low concentration (0.1μg/ml) of the SPP, the SMIF seems to bind mutually at very high affinity. In natural fertilization process, it is unlikely that the TAT-30 antigens are dissociated only due to dilution. Concentrations of several kind of ions and pH change may affect the binding and dissociation of the SMIF. Interestingly, the quiescence factor observed in bovine caudal epididymal fluid expresses its effect strong pH-dependent manner (Acott and Charr, 1984). Further studies are needed to clarify the dissociation mechanism between the SMIF molecules of the Nile tilapia.

The calibration of the TAT-30 positive fraction by the gel filtration indicated that M_r of the SMIF was approximately 2,000,000. Furthermore, the immunoprecipitation analysis revealed that the SMIF contained the 120kDa referred to the TAT-30 antigen, 27kDa and 18kDa polypeptides. Probably, the whole SMIF is composed of these three proteins at molar ratio 1: 1: 1. Additionally, Western blot analysis by the lectins showed that the 120kDa polypeptide has sugar residues such as α-Mannose, α-Glucose and N-Acetyl-D-glucosamine. The molecules of sperm motility inhibiting factors observed in epididymal fluid of several mammalian species other than rat is not yet clarified. The immobilin observed in rat epididymal fluid is a high molecular weight glycoprotein more than 1,000,000 dalton, which has sugar residues such as galactose, N-acetylglucosamine and N-acetylgalactosamine (Usselman et al., 1985). The characteristics of sugar residues of epididymal fluid glycoproteins are analyzed in mouse by Rankin et al. (1989). However, the functions of these sugars are not yet clarified. Recent study shows that, in mouse, β-1,4-galactosyltransferase on sperm head mediates fertilization by binding oligosaccharide residues on glycoprotein of zona pellucida, and sugar residues play important roles in the complementary recognition of the molecules both on spermatozoa and on egg (Miller et al., 1992). The sugar residues on the SMIF may be involved in recognition between the molecules composed of the SMIF, and the SMIF and other molecules on spermatozoa. The TAT-30 recognized only one polypeptide at 120kD in testis, suggesting the SMIF specifically expressed on testis. This tissue spcificity of the TAT-30 antigen may be related with the TAT-30 antigen is one of testicular autoantigen.

Immunohistochemical study using the TAT-30 showed that the SMIF localized on the cytoplasm of Sertoli cells, epithelial cells of sperm duct and spermatozoa in seminal lobule lumen and in sperm duct. Also, immunoelectron microscopic study showed that the gold particles were especially evident in rough endoplasmic reticulum in cytoplasm facing the lobule lumen in the Sertoli cells. These observations suggest that the SMIF are actively synthesized by the Sertoli cells to secrete to the lumen, and also that the SMIF synthesized in Sertoli cells are more actively than epithelial cells of sperm ducts. In salmonid fish, testis has specified main sperm duct, and the testicular spermatozoa are immotile, but acquire a potential for motility during their passage through the sperm duct (Miura et al., 1992). However, in the Nile tilapia, testis does not have specified sperm duct, and also, almost all of testicular spermatozoa have a potential for motility. Accordingly, it is likely that Sertoli cells actively synthesize the SMIF in order to immobilize the spermatozoa. In rat, the immobilin are synthesized and secreted actively in the caput epididymis (Ruiz-Bravo, 1988; Hermo et al., 1992), where spermatozoa first acquire the potential for motility (Orgebin-Crist et al., 1975). The gold particles were also predominantly seen in lysosomes. It is likely that acid phosphatase-positive dense secondary lysosomes are involved in endocytosis. In the rat epididymis, several proteins including the immobilin seem to be endocytosed, because of its localizations on endocytotic structures such as secondary lysosomes (Hermo et al., 1991, 1992). Although it is not clear whether the lysosomes which the SMIF were detected in the present study are secondary lysosomes, Sertoli cells may endocytose the excess and degenerated SMIF.

In the present observations, all of spermatozoa in both seminal lobule lumen and sperm ducts were recognized by the TAT-30. However, the LCA recognized all of spermatozoa in the sperm ducts, but not all of spermatozoa in the seminal lobule lumen. Therefore, the spermatozoa which are not reacted with the LCA may not acquire the potential for motility. This hypothesis leads that Sertoli cells secrete the SMIF precursor to the seminal lobule lumen where spermatozoa which do not acquire a potential for motility exist, and become to secrete the completed SMIF which have sugar residues to the seminal lobule lumen where spermatozoa which aquire a potential for motility exist. As a result, the SMIF become to bind mutually by the help of complemental recognition between the sugar residues and the lectin-like ligand on both spermatozoa and the SMIF as mentioned in the prvious paragraph. In addition, the LCA also recognized the interstitium except for some other testicular somatic tissues such as some part of Sertoli cells and epithelial cells of sperm duct . In the interstitium, the LCA seems likely to recognize specific sugar residue on the molecules quite different from the SMIF.

In conclusion, in the Nile tilapia same as some mammalian species, seminal plasma contained the sperm motility inhibiting factor (SMIF). The SMIF was a high molecular weight glycoprotein at M_r approximately 2,000,000, composing of 120kDa as TAT-30 antigen, 27kDa and 18kDa polypeptides at molar ratio 1: 1: 1. The TAT-30 antigen had sugar residues which had affinity for the LCA. Immunohistochemical studies showed that the SMIF were actively synthesized to secrete by the Sertoli cells. The SMIF were endocytosed by the Sertoli cells. Additionally in the epithelial cells of the sperm ducts, these cellular activity was slightly observed. Although all of the spermatozoa in the seminal loblule lumen and sperm ducts were recognized by the TAT-30, some of spermatozoa in the seminal lobule lumen were not recognized by the LCA, suggesting the Sertoli cells also secrete SMIF precursor.

VI. General consideration

The present study clarified that the functions of two sperm autoantigens of the Nile tilapia recognized by the monoclonal antibodies newly established. As a result, one of the autoantigens is the TAT-10 antigen which is involved in the sperm motility prolongation by the factor of the ovarian fluid during fertilization. The other is the TAT-30 antigen as a sperm motility inhibiting factor in the seminal plasma. However, the functions of the 80kDa antigen recognized by both TAT-20 and TAT-21 are remained to be elucidate in the future study.

Also in the Nile tilapia, it appears that the sperm motility prolongation factor exist in the ovarian fluid. Up to date, sperm motility activating substances from egg chorion, or ovarian fluid have been detected in some teleosts such as herring, bitterling, rainbow trout and masu salmon (Yanagimachi, 1957; Suzuki, 1958, 1959; Yoshida and Nomura, 1972; Ohta et al., 1989). However, the structure of these factors have not yet been clarified in any teleost species. In the pacific herring, the sperm motility activating factor is a 105kDa glycoprotein which showed affinity for ConA (Pillai et al., 1993), while in the Nile tilapia, the prolongation substance suggests to be a approximately 2kDa peptide which is heat-stable.

In fish culture, there are some cases that the amount of milt is too small, and motility is not enough artificially to fertilize eggs efficiently. Therefore, sperm motility activating factors may contribute to solve these problems. The sperm activating peptides from egg jelly layer of sea urchins are known to express their activity species-specific manner; the peptides only activate sperm from the same species, but do not affect sperm from different orders (Ward and Kopf, 1993). It is important, therefore, for fish culture to clarify the structure of the sperm motility prolongation substance of the Nile tilapia and to investigate also whether the substance affect motility of spermatozoa in other fish species. In order to analyze the biological and physiological characteristics of the sperm motility prolongation substance, a large amount of peptides are required. However, recent recombinant protein technology assure the amount of protein and peptide. Therefore, gene technology is likely to be applicated in the future study.

In addition, in order to regulate sperm motility artificially, it is also important to clarify the mechanism that how sperm motility prolongation substance affect sperm motility and where the TAT-10 antigen on spermatozoa is implicated. In any animal species which have been studied, the molecular cascade of the sperm motility activation by the sperm motility activating factor have not yet been clear. Since the Nile tilapia have long spawning season through a year and are easily reproduced in culture pond. Accordingly, Nile tilapia as a experimental materials will give a excellent advantage to clarify the mechanism of sperm motility activation.

It is noteworthy that the TAT-10 antigen existed in various tissues such as olfactory organ, ovary and also A and early B spermatogonia in testis other than late stage of spermatids and spermatozoa. These localization especially in male germ cells and olfactory organ suggests that the TAT-10 antigen are implicated in the final maturation. The final maturation inducing steroid $17\alpha,20\beta$-dihydroxy-4-pregnene-3-one (DHP) is suggested to be also involved in the proliferation of spermatogonia in rainbow trout (Depeche and Sire, 1982). As described in chapter IV, the olfactory organ plays an important role in the reception of the DHP as a pheromone which induce the final maturation (Stacy and Sorensen, 1991). The TAT-10 antigen, at least, in male organs, may be a DHP receptor. However, since the DHP receptor has not yet been identified in any fish, the characteristics of cellular localization and tissue specificity of a DHP receptor is not clarified. The localization of the TAT-10 antigen in ovary suggests that the antigen is implicated in the primary growth phase of oocyte. Hence, the antigen may play an important role in the preparation of vitellogenesis; induction of vitellogenin receptor. Until now, in any vertebrates, few study have dealed with the hormonal regulation and the molecular mechanism of primary growth of germ cells both in ovary and testis. The analysis of the functions of the TAT-10 antigens in the previtellogenic oocyte and A and early B spermatogonia probably contribute to clarify the above mentioned subjects.

The TAT-30 antigen showed to be one of components of the sperm motility inhibiting factor (SMIF) in the seminal plasma. In the present study, mutual binding of the SMIF both in the seminal plasma and on spermatozoa suggests to be one of reasons of sperm immobilization in the seminal plasma, because the monoclonal antibodies against the TAT-30 antigen which was a major component of the SMIF prevented the sperm immobilization of spermatozoa. Especially in culture of the Nile tilapia, one of the most important subjects is to establish the technique of producing infertile fish in order to prevent the overpopulation effect on growth of object fish. Further analyze the mechanism of the sperm immobilization by the SMIF helps for prevention of the overpopulation in culture pond.

In mammals, it has been shown that infertility can be induced efficiently by immunizing animals with sperm antigens (Primakoff et al., 1988b). One of the factors involved in the induction of infertility was thought to be agglutination and immobilization of spermatozoa by anti-sperm autoantibodies occuring in the blood and subsequently entering into seminiferous tubules (Menge et al., 1982; Bronson et al., 1982). Several attempts have been made to obtain infertile fish by experimentally inducing testicular autoimmunity in Atlantic salmon Salmo salar (Laird et al., 1978, 1980), rainbow trout Oncorhynchus mykiss (Secombes et al., 1985a, b) and the Nile tilapia (Lou and Takahashi, 1987, 1988). In these fishes, following immunization with testis mateiral, macrophages enter into the lumen of seminal lobules to phagocytize spermatozoa, resulting in the formation of giant cell granulomas in the lobule lumen. In affected testes, however, spermatogenesis proceeds normally along the wall of the lobules, indicating applying this method to successful induction of infertility in fish. In considering the immunological approaches to control fertility of fish, the TAT-30 antigen is also an interesting object. As demonstrated in the previous chapter, the TAT-30 antigen is existed in spermatozoa and is one of testis-specific autoantigen. The more intense testicular autoimmunity, which induce the infertility due to azoospermia, is possibly induced by the immunization with the concentrated TAT-30 antigen than those induced by the immunization with testis homogenate. In addition, Secombes et al (1986) showed that monoclonal antibodies to sperm of carp and rainbow trout could kill spermatozoa by complement-mediated cytotoxicity and presented new immunological method for the induction of infertility. They described the possibility that lysis of germ cells could be induced by the injection with monoclonal antibodies specific to germ cells and guinea pig serum as a source of complement. However, it remains many problems to be solved for application of this method to aquaculture. Since the TAT-10 reacts also with A and early B spermatogonia, the TAT-10 may be useful for the induction of degeneration of testis by this method.

The summary of the present study and possible functions of rest sperm autoantigens are illustrated in figure 22. The present study demonstrated that the TAT-10 antigen and the TAT-30 antigen are implicated in sperm motility prolongation caused by the molecule in the ovarian fluid and the

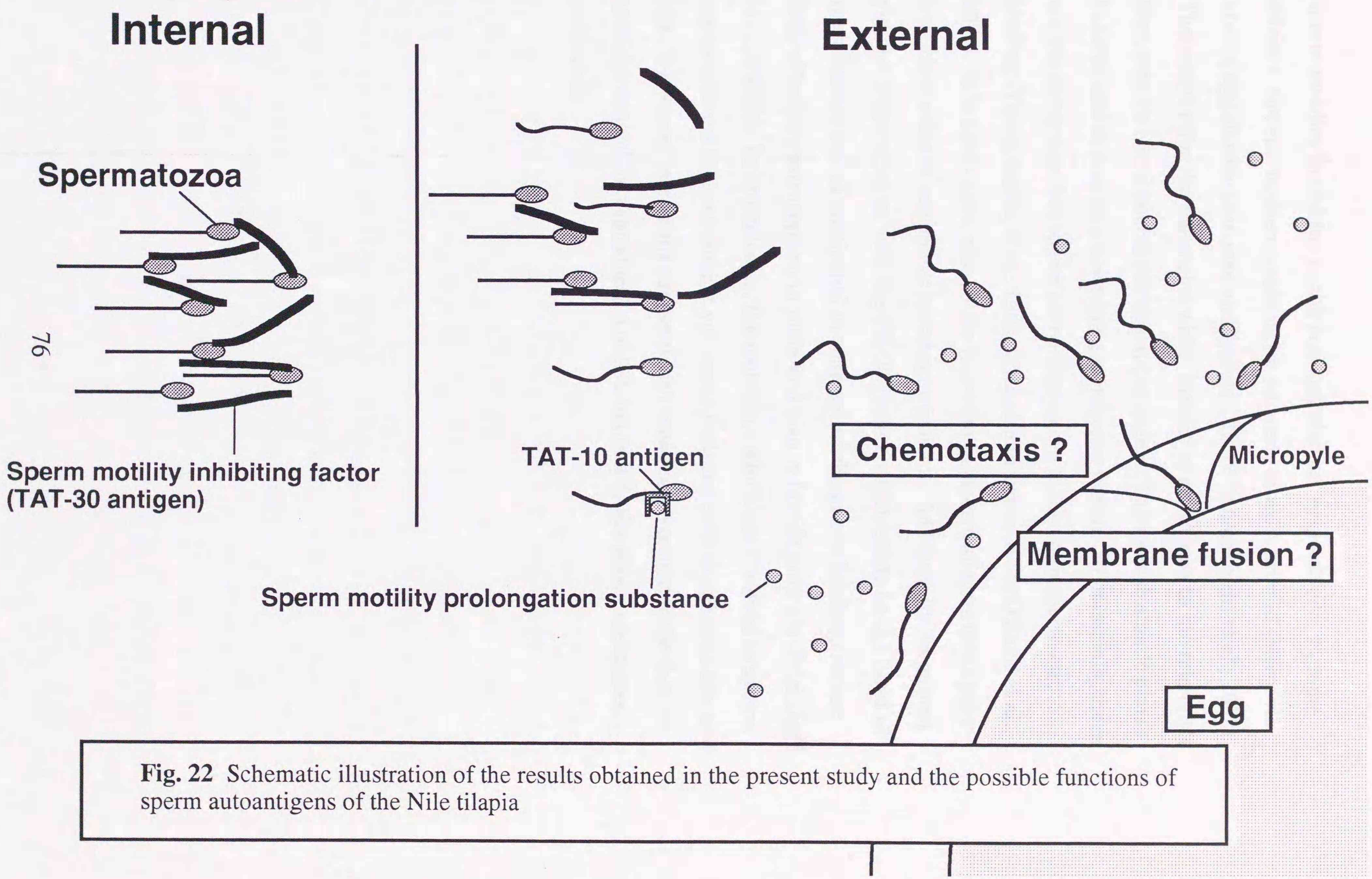


Fig. 22 Schematic illustration of the results obtained in the present study and the possible functions of sperm autoantigens of the Nile tilapia

sperm motility inhibition in the seminal plasma, respectively. In some teleosts, the sperm chemotaxis factor suggests to exist around micropyle area of egg chorion (Amanze and Iyengar, 1990; Yanagimachi et al., 1992). The receptor for the molecules which attract spermatozoa to the micropyle area may be also a sperm surface autoantigen. The most dramatic phase in fertilization may be a sperm-egg membrane fusion. In mammals, there are many reprots that sperm autoantigens are involved in sperm-egg binding (Yanagimachi et al., 1981; O'Rand and Irons, 1984; O'Rand et al., 1984). It is likely that, also in the Nile tilapia, sperm autoantigens play important roles in sperm-egg membrane fusion. Additionally, in guinea pig, the immunization with the PH-20 protein which exists on the head of spermatozoa and is implicated in zona pellucida- sperm binding induce fully effective contraception in male and also in female animals (Primakoff et al., 1988b). In any teleosts, the molecules which are involved in sperm-egg membrane fusion have not yet been identified both in spermatozoa and egg. It is one of important subject which must be investigated for the artificial control for reproduction of fish to identify the above mentioned molecules.

VII. Summary

The present study was conducted to obtain knowledge about the functions of sperm autoantigens of teleosts, using the Nile tilapia, Oreochromis niloticus, as an experimental animal. In order to investigate the functions of the sperm autoantigens, four of monoclonal antibodies against these antigens were produced. Furthermore, the functions and the localizations of especially 27kDa and 120kDa sperm autoantigens recognized by the monoclonal antibodies were analyzed to be described and discussed.

1. Balb/c mice were immunized with the autoantigens purified from the detergent-solubilized sperm protein by antisperm autoantibody affinity chromatography.

2. Four hybridomas producing specific antibodies to the autoantigens were obtained. The monoclonal antibody, Testicular Antigen of Tilapia (TAT) -10 recognized a 27kDa antigen. This antigen was localized in A and early B type of spermatogonia, spermatids in late spermiogenesis, and spermatozoa. This antigen was located primarily in the mid piece of the spermatozoa. Another antibody, TAT-20, recognized a 80kDa polypeptide and another of about 40kDa. TAT-21 recognized only a 80kDa antigen. The antigens recognized by both TAT-20 and 21 were localized in spermatids in late spermiogenesis and in spermatozoa. TAT-30 reacted with a 120kDa antigen which was localized in the epithelium of seminal ducts, Sertoli cells and spermatozoa. The antigens recognized by TAT-20, 21 and 30 were primarily on the head of spermatozoa.

3. In order to investigate the interaction between the phenomena that sperm motility activation during fertilization and sperm antigens, several experiments using previously produced monoclonal antibody to the 27kDa antigen (TAT-10) were carried out. First, an attempt was made at

detecting sperm motility regulating substance in ovarian fluid, and demonstrated sperm motility prolongation substance (SMPS) in the ovarian fluid. The SMPS showed to be a M_r 2000 by gel filtration and further to be heat-stable. Further, The TAT-10 inhibited the activity of the SMPS, suggesting the 27kDa antigen on spermatozoa was implicated in a signal transduction of the motility prolongation by the SMPS.

4. The analysis of tissue specificity of the TAT-10 antigen by Western blot analysis showed that the antigen also localized on olfactory organ and ovary other than testis as a same M_r 27kDa polypeptide. Immunohistochemical study demonstrated that the antigen localized in the olfactory neurons, nerve bundles and olfactory bulb in the olfactory organ, and localized in previtellogenic oocytes in the ovary. Further immunoelectron microscopic observation showed that the TAT-10 antigen was actively synthesized in spermatids. These observations also suggested that the TAT-10 antigens played important roles in primary growth and final maturation of germ cells.

5. In order to investigate the relationship between sperm motility inhibiting factors in the seminal plasma and testicular antigens, several experiments using previously produced monoclonal antibodies (TAT-30) was carried out. First, sperm motility in the seminal plasma protein fraction (SPP) was investigated. The result revealed that the sperm motility was inhibited by the SPP concentration-dependent manner. Additionally, the TAT-30 treatment to the diluent prominently recovered the sperm motility, suggesting that the TAT-30 antigen was involved in the immobilization of spermatozoa in seminal plasma.

6. Gel filtration and electrophoretic analysis demonstrated that the sperm motility inhibiting factor (SMIF) was M_r 2,000,000 and also suggested the SMIF was composed of 120kD TAT-30 antigen, 27kDa, and

18kDa polypeptides at molar ratio 1: 1: 1. Furthermore the TAT-30 antigen had sugar residues which had an affinity for the LCA.

7. The analysis of tissue specificity of the TAT-30 antigen by Western blot analysis showed that the antigen expressed testis-specific. Immunohistochemical studies showed that, although almost all of the staining patterns by the LCA on the testis were similar to those by the TAT-30, some of spermatozoa in the seminal lobule lumen were recognized by the TAT-30 but not by the LCA. These results suggested the relationship between the attachment of the sugar residues on the SMIF and the acquirement of potential for motility of spermatozoa. Electron microscopic study demonstrated that the SMIF actively synthesized to secreted by the Sertoli cells. However, it is likely that excess and degenerated SMIF were endocytosed by the Sertoli cells

8. The above results demonstrated that the sperm autoantigens analyzed in the present study had some sperm-specific functions, such as motility regulation in the Nile tilapia. A possibility of the analysis of sperm autoantigens for the control of fish reproduction was suggested and discussed.

VIII. References

Acott, T. S. and Hoskins D. D. (1981). Bovine sperm forward motility protein: binding to epididymal spermatozoa. Biol. Reprod. 24, 234-240.

Acott, T. S., Katz, D. F. and Hoskins, D. D. (1983). Movement characteristics of bovine epididymal spermatozoa: effects of forward motility protein and epididymal maturation. Biol. Reprod. 29, 389-399.

Acott, T. S. and Carr, D. W. (1984). Inhibition of bovine spermatozoa by caudal epdidymal fluid: II. interaction of pH and quiescence factor. Biol. Reprod. 30, 926-935.

Aitken, R. J., Clarkson, J. S., Hulme, M. J. and Henderson, C. J. (1988). Analysis of calmodulin acceptor proteins and the influence of calmodulin antagonists on human spermatozoa. Gamete Res. 21, 93-111.

Amanze, D. and Iyengar, A. (1990). The micropyle: a sperm guidance system in teleost fertilization. Development 109, 495-500.

Araki, Y., Vierula, M. E., Rankin, T. L., Tulsiani, D. R. P. and Orgebin-Crist, M.-C. (1992). Isolation and characterization of a 25-kilodalton protein from mouse testis: sequence homology with a phospholipid-binding protein. Biol. Reprod. 47, 832-843.

Audhya, T., Reddy, J. and Zaneveld, L. J. D. (1987). Purification and partial chemical characterization of a glycoprotein with antifertility activity from human seminal plasma. Biol. Reprod. 36, 511-521.

Baas, J. W., Molan, P. C. and Shannon, P. (1983). Factors in seminal plasma of bulls that affect the viability and motility of spermatozoa. J. Reprod. Fert. 68, 275-280.

Balarin, J. D. (1979). "Tilapia: a guide to their biology and culture in Africa" pp. 40-42, 57-67. University of Stirling, Stirling.

Bergamo, P., Venditti, P., Sanosone, G., Ferrara, L. and Abrescoa, P. (1992). Detection of a sperm-coating antigen in the semen of Bubalus Bubalis. Comp. Biochem. Physiol. 102B, 733-742.

Bibring, T., Baxandall, J. and Harter, C. C. (1984). Sodium-dependent pH regulation in active sea urchin sperm. Dev. Biol. 101, 425-435.

Brokaw, C. J. and Nagayama, S. M. (1985). Modulation of the asymmetry of sea urchin sperm flagellar bending by calmodulin. J. Cell Biol. 100, 1875-1883.

Bronson, R. A., Cooper, G. W. and Rosenfeld, D. L. (1982). Correlation between regional specificity of antisperm antibodies to the spermatozoan surface and complement-mediated sperm immobilization. Am. J. Reprod. Immunol. 2, 222-224.

Brooks, D. E. and Tiver, K. (1983). Localization of epididymal secretory proteins on rat spermatozoa. J. Reprod. Fert. 69, 651-657.

Carr, D. W. and Acott, T. S. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid: I. studies of a sperm motility quiescence factor. Biol. Reprod. 30, 913-925.

Carré, D. and Sardet, C. (1981). Sperm chemotaxis in Siphonophores. Biol. Cell 40, 119-128.

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, Strongylocentrotus purpuratus. J. Biol. Chem. 257, 14881-14890.

Clapper, D. L. and Brown, G. G. (1980). Sperm motility in the horseshoe crab, Limulus polyphemus L. I. sperm behavior near eggs and motility initiation by egg extracts. Dev. Biol. 76, 341-349.

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

Cuasnicu, P. S., Echeverria, F. G., Piazza, A. D., Cameo, M. S. and Blaquier, J. A. (1984). Antibodies against epididymal glycoproteins block fertilizing ability in rat. J. Reprod. Fert. 72, 467-471.

Danahey, D. G., Letscher, R. L. and Dephilip, R. M. (1986). Evidence for the establishment of functional polarity by Sertoli cells in vitro. J. Cell Biol. 103, 484a.

Dangott, L. J. and Garbers, D. L. (1984). Idenification and partial characterization of the receptor for speract. J. Biol. Chem. 259, 13712-13716.

Depeche, J. and Sire, O. (1982). In vitro metabolism of progesterone and 17α-hydroxyprogesterone in the testis of the rainbow trout, Salmo gairdneri Rich., at different stages of spermatogenesis. Reprod. Nutr. Develop. 22, 427-438.

Feinberg, J., Weinman, J., Weinman, S., Walsh, M. P., Harricane, M. C., Gabrion, J. and Demaille, J. G. (1981). Immunocytochemical and biochemical evidence for the presence of calmodulin in bull sperm flagellum. Isolation and characterization of sperm calmodulin. Biochim. Biophys. Acta 673, 303-311.

Flickinger, C. J., Herr, J. C. and Klotz, K. L. (1988). Immunocytochemical localization of the major glycoprotein of epididymal fluid from the cauda in the epithelium of the mouse epididymis. Cell Tissue Res. 251, 603-610.

Garbers, D. L. (1989). Molecular basis of fertilization. Annu. Rev. Biochem. 58, 719-742.

Garbers, D. L. (1992). Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. Cell 71, 1-4.

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

Hardy, D. M., Huang, T. T. F., Driscoll, W. J. J., Tung, K. S. K. and Wild, G. C. (1988). Purification and characterization of the primary acrosomal autoantigen of guinea pig epididymal spermatozoa. Biol. Reprod. 38, 423-437.

Harvey, B. and Kelley, R. N. (1984). Control of spermatozoan motility in a euryhaline teleost acclimated to different salinities. Can. J. Zool. 62, 2674-2677.

Hermo, L., Barin, K. and Oko, R. (1994). Developmental expression of immobilin in the rat epididymis. Anat. Rec. 240, 86-103.

Hermo, L., Oko, R. and Robaire, B. (1992). Epithelial cells of the epididymis show regional variations with respect ot the secretion or endocytosis of immonilin as revealed by light and electron microscope immunocytochemistry. Anat. Rec. 232, 202-220.

Hermo, L., Wright, J., Oko, R. and Morales, C. R. (1991). Role of epithelial cells of the male excurrent duct system of the rat in he endocytosis or secretion of sulfated glycoprotein-2 (Clusterin). Biol. Reprod. 44, 1113-1131.

Holland, M. K. and Orgebin-Crist, M.-C. (1988). Characterization and hormonal regulation of protein synthesis by the murine epididymis. Biol. Reprod. 38, 487-496.

Hyne, R. V. and Garbers, D. L. (1979). Regulation of guinea pig sperm adenylate cyclase by calcium. Biol. Reprod. 21, 1135-1142.

Hynes, R. O. (1987). Integrins: a family of cell surface receptors. Cell 48, 549-554.

Jones, H. P., Lenz, R. W., Palevitz, B. A. and Cormier, M. J. (1980). Calmodulin localization in mammalian spermatozoa. Proc. Natl. Acad. Sci. USA 77, 2772-2776.

Kallajoki, M., Parvinen, M. and Suominen, J. J. O. (1986). Expression of acrosin during mouse spermatogenesis: a biochemical and immunocytochemical analysis by a monoclonal antibody C11H. Biol. Reprod. 35, 157-165.

Killian, G. J., Chapman, D. A. and Rogowski, L. A. (1993). Fertility-associated proteins in holstein bull seminal plasma. Biol. Reprod. 49, 1202-1207.

Kopf, G. S. and Garbers, D. L. (1980). Calcium and a fucose-sulfate-rich polymer regulate sperm cyclic nucleotide metabolism and the acrosome reaction. Biol. Reprod. 22, 1118-1126.

Koshimoto, H., Katoh, K., Yoshihara, Y. and Mori, K. (1992). Distribution of putative odour receptor proteins in olfactory epithelium. Neuroreport 3, 521-523.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laird, L. M., Ellis, A. E., Wilson, A. R. and Holliday, G. T. (1978). The development of the gonadal and immune systems in the Atlantic salmon (Salmo salar L.) and consideration of the possibility of inducing autoimmune destruction of the testis. Ann. Biol. Anim. Biochim. Biophys. 18, 1101-1106.

Laird, L. M., Wilson, A. R. and Holliday, F. G. T. (1980). Field trials of a method of induction of autoimmune gonad rejection in Atlantic salmon (Salmo salar L.). Rrprod. Nutr. Develop. 20, 1781-1788.

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

Lou, Y.-H. and Takahshi, H. (1987). Induction of autoimmune responses to testes in a tilapia, Oreochromis niloticus. Bull. Fac. Fish. Hokkaido Univ. 38, 14-26.

Lou, Y.-H. and Takahashi, H. (1988). Chronic changes of autoimmune responses to testis material in male Nile tilapia, Oreochromis niloticus. Bull. Fac. Fish. Hokkaido Univ. 39, 201-209.

Lou, Y.-H. and Takahashi, H. (1989a). The blood-testis barrier and its breakdown following immunization to tesits material in the Nile tilapia, Oreochromis niloticus. Cell Tissue Res. 258, 491-498.

Lou, Y.-H. and Takahashi, H. (1989b). Spermiogenesis in the Nile tilapia Oreochromis niloticus with notes on a unique pattern of nuclear chromatin condensation. J. Morph. 200, 321-330.

Lou, Y.-H. and Takahashi, H. (1991). Highly specialized sperm surface antigens in the Nile tilapia, Oreochromis niloticus, as revealed by conventional antisperm antibody and autoantibody. J. Exp. Zool. 258, 255-262.

Lou, Y.-H., Hara, A. and Takahashi, H. (1989). Induction of autoantibodies against spermatozoa by injection of allogeneic sperm in the Nile tilapia, Oreochromis niloticus. Comp. Biochem. Physiol. 94B, 829-836.

Lou, Y.-H., Yamauchi, K. and Takahashi, H. (1990). Isolation and partial characterization of sperm plasma membrane of masu salmon, Oncorhynchus masou. Comp. Biochem. Physiol. 95B, 187-192.

Mack, S. R., Everingham, J. and Zaneveld, L. J. D. (1986). Isolation and partial characterization of the plasma membrane from human spermatozoa. J. Exp. Zool. 240, 127-136.

Menge, A. C., Medley, N. E., Mangione, C. M. and Dietrich, J. W. (1982). The incidence and influence of antisperm antibodies in infertile couples on sperm-cervical mucus interactions and subsequent fertility. Fertil. Steril. 38, 439-446.

Miller, D. J., Macek, M. B. and Shur, B. D. (1992). Complementarity between sperm surface β-1,4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. Nature 357, 589-593.

Miller, R. L. (1985). Demonstration of sperm chemotaxis in Echinodermata: Asteroidea, Holothuroidea, Ophiuroidea. J. Exp. Zool. 234, 383-414.

Mita, M. and Yasumasu, I. (1984). The role of external potassium ion in activation of sea urchin spermatozoa. Develop. Growth. Differ. 26, 489-495.

Miura, T., Yamauchi, K., Takahashi, H. and Nagahama, Y. (1992). The role of hormones in the acquisition of sperm motility in salmonid fish. J. Exp. Zool. 261, 359-363.

Moore, A., Hall, L. and Hamilton, D. W. (1990). An 18KDa androgen-regulated protein that modifies galactosyltransferase activity in synthesized by the rat caput epididymides, but has no structural similarity to rat milk alphalactalbumin. Biol. Reprod. 43, 497-506.

Moore, P. B. and Dedman, J. R. (1984). Calmodulin, a calmodulin acceptor protein, and calcimedins: unique antibody localizations in hamster sperm. J. Cell. Biochem. 25, 99-107.

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

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.

Morisawa, M. and Suzuki, K. (1980). Osmolality and potassium ion: Their roles in initiation of sperm motility in teleosts. Science 210, 1145-1146.

Morisawa, M. Suzuki, K., and Morisawa, S. (1983). Effects of potassium and osmolality on spermatozoan motility of salmonid fishes. J. Exp. Biol. 107, 105-113.

Murofushi, H., Ishiguro, K., Takahashi, D., Ikeda, J. and Sakai, H. (1986). Regulation of sperm flagellar movement by protein phosphorylation and dephosphorylation. Cell Motil. Cytockeleton 6, 83-88.

Naz, R. K., Saxe, J. M. and Menge, A. C. (1983). Inhibition of fertility in rabbits by monoclonal antibodies against sperm. Biol. Reprod. 28, 249-254.

Ngai, J., Chess, A., Dowling, M. M., Necles, N., Macagno, E. R. and Axel, R. (1993a). Coding of olfactory information: topography of odorant receptor expression in the catfish olfactory epithelium. Cell 72, 667-680.

Ngai, J., Dowling, M. M., Buck, L., Axel, R. and Chess, A. (1993b). The family of genes encoding odorant receptors in the channel catfish. Cell 72, 657-666.

O'Brien, D. A., Gabel, C. A. and Eddy, E. M. (1993). Mouse Sertoli cells secrete mannose 6-phosphate containing glycoproteins that are endocytosed by spermatogenic cells. Biol. Reprod. 49, 1055-1065.

O'Rand, M. G. and Irons, G. P. (1984). Monoclonal antibodies to rabbit sperm autoantigens. II. inhibition of human sperm penetration of zona-free hamster eggs. Biol. Reprod. 30, 731-736.

O'Rand, M. G. and Romrell, L. J. (1981). Localization of a single sperm membrane autoantigen (RSA-1) on spermatogenic cells and spermatozoa. Dev. Biol. 84, 322-331.

O'Rand, M. G., Irons, G. P. and Porter, J. P. (1984). Monoclonal antibodies to rabbit sperm autoantigens. I. inhibition of in vitro fertilization and localization on the egg. Biol. Reprod. 30, 721-729.

Ohta, H., Matsubara, T. and Imada, K. (1989). Sperm motility of masu salmon, Oncorhynchus masou, in coelomic and artificial coelomic fluids (in Japanese with English abstract). SUISANZOSHOKU 37, 235-239.

Okamura, N., Dacheux, F., Venien, A., Onoe, S., Huet, J.-C. and Dacheux, J.-L. (1992). Localization of a maturation-dependent epididymal sperm surface antigen recognized by a monoclonal antibody raised against a 135-kilodalton protein in procine epididymal fluid. Biol. Reprod. 47, 1040-1052.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H. and Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spematozoa through direct activation of adenylate cyclase. J. Biol. Chem. 260, 9699-9705.

Orgebin-Crist, M. C. and Delpech-Fournier, S. (1982). Sperm-egg interaction: evidence for maturational changes during epididymal transit. J. Androl. 3, 429-433.

Orgebin-Crist, M. C., Danzo, B. J. and Davies, J. (1975). Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In: "Handbook of physiology" (R. Greep, and D. W. Hamilton, Eds.) Vol. 5, pp. 319-338. Williams and Wilkins, Baltimore.

Papp, S., Robaire, B. and Hermo, L. (1994). Developmental expression of the glutathione S-transgerase Yo subunit in the rat testis and epididymis using light microscope immunocytochemistry. Anat. Rec. 240, 345-357.

Parmentier, H. K., Timmermans, L. P. M. and Egberts, E. (1984). Monoclonal antibodies against spermatozoa of the common carp (Cyprinus carpio L.) I. A study of germ cell antigens in adult males and females. Cell Tissue Res. 236, 99-105.

Parmentier, M., Libert, F., Schurmans, S., Schiffman, S., Lefort, A., Eggerickx, D., Ledent, C., Mollereau, C., Gerard, C., Perret, J., Grootegoed, A. and Vassart, G. (1992). Expression of members of the putative olfactory receptor gene family in mammalian germ cells. Nature 355, 453-455.

Peterson, R., Russell, L., Bundman, D. and Freund, M. (1980). Evaluation of the purity of boar sperm plasma membranes prepared by nitrogen cavitation. Biol. Reprod. 23, 637-645.

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 pallasi. J. Exp. Zool. 265, 336-342.

Primakoff, P., Cowan, A., Hyatt, H., Tredick-Kline, J. and Myles, D. G. (1988a). Purification of the guinea pig sperm PH-20 antigen and detection of a site- specific endoproteolytic activity in sperm preparations that cleves PH-20 into two disulfide-linked fragments. Biol. Reprod. 38, 921-934.

Primakoff, P., Hyatt, H. and Myles, D. G. (1985). A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg-zona pellucida. J. Cell Biol. 101, 2239-2244.

Primakoff, P., Hyatt, H. and Tredick-Kline, J. (1987). Identification and purification of a sperm surface protein with a potential role in sperm-egg membrane fusion. J. Cell Biol. 104, 141-149.

Primakoff, P., Lathrop, W., Woolman, L., Cowan, A. and Myles, D. (1988b). Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. Nature 335, 543-546.

Ralt, D., Goldenberg, 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. USA 88, 2840-2844.

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

Rankin, T. L., Holland, M. K. and Orgebin-Crist, M. C. (1989). Lectin binding characteristics of mouse epididymal fluid and sperm extracts. Gamete Res. 24, 439-451.

Rankin, T. L., Tsuruta, K. J., Holland, M. K., Griswold, M. D. and Orgebin-crist, M.-C. (1992). Isolation immunolocalization, and sperm-association of three proteins of 18,25, and 29 kilodaltons secreated by the mouse epididymis. Biol. Reprod. 46, 747-766.

Reddy, E. S. P. and Bhargave, P. M. (1979). Seminal plasmin-an antimicrobial protein from bovine seminal plasma whichiacts in E. coli by specific inhibition of rRNA synthesis. Nature 279, 725-728.

Ruiz-Bravo, N. (1988). Tissue and cell specificity of immobilin biosynthesis. Biol. Reprod. 39, 901-911.

Saling, P. M. and Lakoski, K. A. (1985). Mouse sperm antigens that participate in fertilization. II. inhibition of sperm penetration through the zona pellucida using monoclonal antibodies. Biol. Reprod. 33, 527-536.

Saling, P. M., Irons, G. and Waibel, R. (1985). Mouse sperm antigens that participate in fertilization. I. inhibition of sperm fusion with the egg plasma membrane using monoclonal antibodies. Biol. Reprod. 33, 515-526.

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

Scheit, K. H., Reddy, E. S. P. and Bhargava, P. M. (1979). Seminalplasmin is a potent inhibitor of E. coli RNA polymerase in vitro. Nature 279, 728-731.

Secombes, C. J., Lewis, A. E., Needham, E. A., Laird, L. M. and Priede, I. G. (1985a). Apperance of autoantigens during gonad maturation in the rainbow trout (Salmo gairdneri). J. Exp. Zool. 233, 425-431.

Secombes, C. J., Needham, E. A., Laird, L. M., Lewis, A. E. and Priede, I. G. (1985b). The long-term effects of auto-immunologically induced granulomas on the testes of rainbow trout, Salmo gairdneri Richardson. J. Fish Biol. 26, 483-489.

Secombes, C. J., Van Winkoop, A., Van Den Boogaart, J. G. M., Timmermans, L. P. M. and Priede, I. G. (1986). Immunological approaches to control maturation in fish. 1. cytotoxic reactions against germ cells using monoclona antibodies. Aquaculture 52, 125-135.

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.

Stacy, N. and Sorensen, P. (1991). Function and evolution of fish hormonal pheromones. *In* "Biochemistry and molecular biology of fishes"(Hochachka and Mommsen, Ed.), Vol. 1, pp.109-135. Elsevier Science Publishers B. V.

Suzuki, N., Kajiura, H., Nomura, K., Garbers, D. L., Yoshino, K., Kurita, M., Tanaka, H. and Yamaguchi, M. (1988a). Some more speract derivatives associated with eggs of sea urchins Pseudocentrotus Depressus, Strongylocentrotus purpuratus, Hecentrotus pulcherrimus and Anthocidaris crassispina. Comp. Biochem. Physiol. 89B, 687-693.

Suzuki, N., Kurita, M., Yoshino, K., Kajiura, H., Nomura, K. and Yamaguchi, M. (1987). Purification and structure of mosact and its derivatives from the egg jelly of the sea urchin Clypeaster japonicus. Zool. Sci. 4, 649-656.

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. Commun. 99, 1238-1244.

Suzuki, N., Shimomura, H., Radany, E. W., Ramarao, C. S., Ward, G. E., Bentley, J. K. and Garbers, D. L. (1984). A peptide associated with eggs causes a mobility shift in a major plasma membrane protein of spermatozoa. J. Biol. Chem. 259, 14874-14879.

Suzuki, N., Yoshino, K., Kurita, M., Nomura, K. and Yamaguchi, M. (1988b). A novel group of sperm activating peptides from the sea urchin Glyptocidaris crenularis. Comp. Biochem. Physiol. 90C, 305-311.

Suzuki, R. (1958). Sperm activation and aggregation during fertilization in some fishes I. Behavior of spermatozoa around the micropyle. Embriologia 4, 93-102.

Suzuki, R. (1959). Sperm activation and aggregation during fertilization in some fishes III. non species-specificity of stimulating factor. Annot. Zool. Japon. 32, 105-111.

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.

Thaler, C. J., Vanderpuye, O. A., Mcintyre, J. A. and Faulk, W. P. (1990). Lactoferrin binding molecules in human seminal plasma. Biol. Reprod. 43, 712-717.

Tombes, R. M. and Shapiro, B. M. (1985). Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. Cell 41, 325-334.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Tsuchiya, T. and Saito, S. (1984). Use of n-octyl-β-D-thioglucoside, a new noionic detegent, for solubilization and reconstitution of membrane proteins. J. Biochem. 96, 1593-1597.

Turner, T. T. and Giles, R. D. (1982). Sperm motility-inhibiting factor in rat epididymis. Am. J. Physiol. 242, R199-R203.

Ueda, H., Tres, L. L. and Kierszenbaum, A. L. (1988). Culture patterns and sorting of rat Sertoli cell secretory proteins. J. Cell Sci. 89, 175-188.

Usselman, M. C. and Cone, R. A. (1983). Rat sperm are mechanically immobilized in the caudal epididymis by "immobilin" a high molecular weight glycoprotein. Biol. Reprod. 29, 1241-1253.

Usselman, M. C., Cone, R. A. and Rossignol, D. P. (1985). Rat cauda epididymal fluid is mucus. J. Androl. 6, 315-320.

Vanderhaeghen, P., Schurmans, S., Vassart, G. and Parmentier, M. (1993). Olfactory receptors are displayed on dog mature sperm cells. J. Cell Biol. 123, 1441-1452.

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

Vreeburg, J. T. M., Holland, M. K., Cornwall, G. A. and Orgebin-Crist, M. (1990). Secretion and transport of mouse epididymal proteins after injection of 35S-methionine. Biol. Reprod. 43, 113-120.

Wagner, C. L. and Kistler, W. S. (1987). Analysis of the major large polypeptides of rat seminal vesicle secretion: SVS I, II, and III. Biol. Reprod. 36, 501-510.

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

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

Wong, P. Y. D. and Tsang, A. Y. F. (1982). Studies on the binding of a 32K rat epididymal protein to rat epididymal spermatozoa. Biol. Reprod. 27, 1239-1246.

Yanagimachi, R. (1957). Studies of fertilization in Clupea pallasii-IV. some properties of the sperm-stimulating factor in the micropyle area of the mature egg. NIPPON SUISAN GAKKAISHI 23, 81-85.

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 Differ. 34, 447-461.

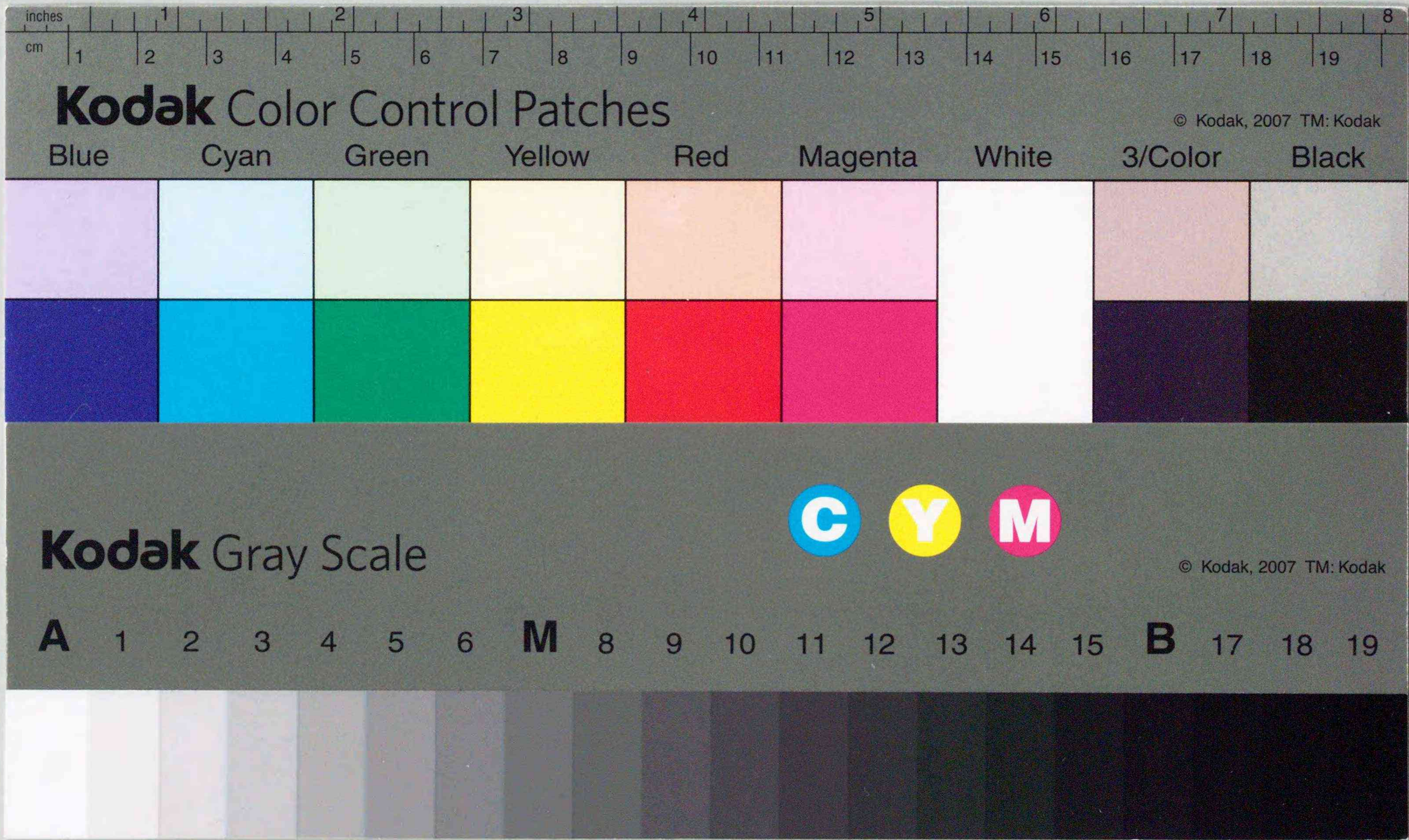
Yanagimachi, R., Okada, A. and Tung, K. S. K. (1981). Sperm autoantigens and fertilization: II. effects of anti-guinea pig sperm autoantibodies on sperm-ovum interactions. Biol. Reprod. 24, 512-518.

Yoshida, M., Inaba, K. and Morisawa, M. (1993). Sperm chemotaxis during the process of fertilization in the ascidians Ciona savignyi and Ciona intestinalis. Dev. Biol. 157, 497-506.

Yoshida, T. and Nomura, M. (1972). A substance enhancing sperm motility in the ovarian fluid of rainbow trout. NIPPON SUISAN GAKKAISHI. 38, 1073.

Yoshino, K., Takao, T., Shimonishi, Y. and Suzuki, N. (1992). Sperm-activating peptide type-V (SAP-V), a fifth member of the sperm-activating peptide family, purified from he egg-conditioned media of the heart urchin Brissus agassizii. Comp. Biochem. Physiol. 102B, 691-700.

Yoshino, K., Takao, T., Yamaguchi, M., Shimomura, Y. and Suzuki, N. (1990). Derivatives of sperm-activating peptide III (SAP-III) associated with eggs of sand dollars, Clypeaster japonicus and Astriclypeus manni. Comp. Biochem. Physiol. 97B, 679-685.


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue Cyan Green Yellow Red Magenta White 3/Color Black
Kodak Gray Scale
C Y M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19