Activation of temperature-dependent flagellar movement of demembranated fowl spermatozoa: involvement of an endogenous serine protease

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Summary. In the presence of ATP, the motility of demembranated fowl spermatozoa was vigorous at 30°C, but negligible at 40°C. Motility could be restored at 40°C by the addition of 10–100 ng trypsin ml^{-1}. Chymotrypsin also stimulated the motility, but neither papain nor carboxypeptidase B appreciably affected motility. Conversely, at 30°C, sperm motility was inhibited by aprotinin or phenylmethylsulphonyl fluoride (PMSF). These results suggest that endogenous protease, presumably serine protease, activity is instrumental in the regulation of fowl sperm motility. It seems likely that the site of action of this protease is axonemal, but a direct effect of added protease on dynein ATPase activity could not be demonstrated.

Introduction

Flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATP hydrolysis by dynein ATPase (for review, see Tash & Means, 1983; Lindemann & Kanous, 1989). Although this basic axonemal mechanism is fairly well understood, the factors and mechanisms of its regulation have still to be clarified. There seems to be several regulatory mechanisms that individually or synergistically control sperm motility: Ca^{2+} and Ca^{2+}–associated compounds, cAMP and intracellular pH (Majumder et al., 1990). In addition, current evidence suggests that a protease activity with a Lys– and Arg–ester bond specificity is required for sperm motility. It is assumed that this regulatory system is probably localized near the dynein arms, but not directly involve the force–generating dynein ATPase (de Lamirande et al., 1990).

Unlike mammalian spermatozoa, fowl spermatozoa show definitive temperature-dependent changes in their motility: in most synthetic diluents, they become immotile at the avian body temperature of 40-41°C, motility being restored by decreasing the temperature (Munro, 1938; Nevo & Schindler, 1968; Ashizawa & Nishiyama, 1978; Takeda, 1982; Ashizawa & Okauchi, 1984; Ashizawa & Wishart, 1987; Wishart & Ashizawa, 1987; Ashizawa et al., 1989a,b; Thomson & Wishart, 1989, 1991). The axoneme itself appears to be directly involved in this regulatory system, since the motility of demembranated spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C (Ashizawa et al., 1989a, b). Furthermore, cAMP-independent phosphorylation of a 43 kDa axonemal protein is likely to be a regulatory step in the maintenance of fowl sperm motility (Ashizawa et al., 1992).

If the activity of endogenous proteases is involved in the above temperature-dependent regulatory system, then fowl spermatozoa might be expected to be made immotile at 30°C by the addition of protease inhibitors and, conversely, motile at 40°C by the addition of appropriate exogenous proteases. In this study, the effects of some proteases and protease inhibitors on the motility of demembranated fowl spermatozoa were therefore examined at 30°C and 40°C, and information was obtained concerning the locus of action of such proteases.

Materials and Methods

Animals and preparation of spermatozoa

Commercial White Leghorn roosters (Babcock strain, Akagi Poultry Breeding Farm, Miyazaki, Japan) were used throughout the study. All birds were housed in individual cages and fed ad libitum on a commercial breeder diet. They were exposed to 14 h light per 24 h.

Semen was collected by the method of Bogdonoff & Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mmol NaCl l^{-1} with 20 mmol TES (N-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid) l^{-1} at pH 7.4 and centrifuged at 700 g for 13 min at room temperature (20-25°C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1 x 10^9 cells ml^{-1}. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Chemicals

Trypsin (approx. 10000 BALL A_{253} units mg^{-1} protein) was obtained from Mochida Pharmaceutical Co., Tokyo, Japan. Chymotrypsin (40-60 BTEE μmolar units mg^{-1} protein), papain (10-20 BAEE μmolar units mg^{-1} protein), carboxypeptidase B (approx. 100 HA μmolar units mg^{-1} protein), aprotinin, PMSF, adenosine 5'-triphosphate (ATP), dithiothreitol, potassium glutamate, TES, Triton X-100, phosphodiesterase and desiccated firefly tails were purchased from Sigma Chemical Co., St Louis, MO, USA. [γ-^{32}P]ATP was purchased from Du Pont-New England Nuclear. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were obtained from Bio-Rad Laboratories, Richmond, CA, USA. Other chemicals were of reagent grade from Nacalai Tesque, Inc., Kyoto, Japan.

Measurement of motility of demembranated spermatozoa

Intact spermatozoa were pre-incubated at 30°C or 40°C for 10 min. After the pre-incubation, demembranation and reactivation were performed according to the method described by Ashizawa et al. (1989b). The extraction medium used consisted of 0.1% Triton X-100, 0.2 mol sucrose l^{-1}, 25 mmol potassium glutamate l^{-1}, 1 mmol $MgSO_4$ l^{-1}, 1 mmol dithiothreitol l^{-1} and 20 mmol Tris-HCl buffer l^{-1} (pH 7.9). The reactivation medium consisted of 0.5 mmol ATP l^{-1}, 0.2 mol sucrose l^{-1}, 25 mmol potassium glutamate l^{-1}, 1.5 mmol $MgSO_4$ l^{-1}, 1 mmol dithiothreitol l^{-1} and 20 mmol Tris-HCl buffer l^{-1} (pH 7.9). To examine the effects of exogenous proteases or protease inhibitors, various concentrations of trypsin, chymotrypsin, papain or carboxypeptidase B at 40°C, and aprotinin or PMSF at 30°C were added to the reactivation medium. Addition of phosphodiesterase, EGTA or Ca^{2+} to trypsin-treated spermatozoa was also performed. The suspension of demembranated spermatozoa was viewed in a microscope slide chamber (Sekisui Chemical Co., Ltd., Tokyo, Japan, UR-157 type) on a thermostatically controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy at 30°C or 40°C (Katz & Overstreet, 1981).

Measurement of crude dynein ATPase activity

Crude dynein extract from fowl spermatozoa was obtained by the method described by Ashizawa & Hori (1990). ATPase activities were assayed by the reduction of ATP concentrations determined by firefly bioluminescence (Ashizawa & Hori, 1990). Protein concentrations of

crude dynein extract were determined according to published methods (Bradford, 1976), with bovine serum albumin as a standard. The rate of ATPase activity was expressed in terms of nmol ATP hydrolysis mg^{-1} protein min^{-1}.

Phosphorylation of endogenous proteins and electrophoresis

Phosphorylation reaction and electrophoresis on polyacrylamide gels of demembranated sperm proteins were carried out according to the methods described by Ashizawa et al. (1992). Briefly, demembranated spermatozoa with extraction-reactivation medium containing 0.1% Triton X-100, 0.2 mol sucrose l^{-1}, 25 mmol potassium glutamate l^{-1}, 1 mmol $MgSO_4$ l^{-1}, 1 mmol dithiothreitol l^{-1}, 20 mmol Tris-HCl buffer l^{-1} (pH 7.9), 0.1 mmol ATP l^{-1} and approximately 7000 cpm $pmol^{-1}$ [γ-^{32}P]ATP were incubated for 2 min at 30°C or 40°C. Aprotinin or trypsin, when incorporated, was present at final concentration of 0.24 TIU (trypsin inhibitor unit) ml^{-1} or 50 ng ml^{-1}, respectively. The phosphorylation reaction was terminated by the addition of equal volumes of concentrated (twofold) Laemmli (1970) sample buffer and boiling for 5 min. Samples containing protein from approximately 1.3 x 10^6 spermatozoa were loaded on 10% SDS-polyacrylamide slab gels, and electrophoresed. Autoradiography was performed at -80°C for 2-4 days exposure to X-ray film with an intensifying screen (Lightning plus, Du Pont, Wilmington, DE, USA).

Statistical analysis

Statistical comparisons were performed using Student's t test.

Results

Effects of proteases and protease inhibitors on the motility of demembranated fowl spermatozoa

Reactivation of fowl spermatozoa without the addition of trypsin was negligible at 40°C. In contrast, the presence of 10–100 ng trypsin ml^{-1} permitted reactivation of sperm motility at 40°C, with optimum reactivation occurring at 50 ng ml^{-1}. At higher concentrations, the percentages of reactivated spermatozoa decreased (Fig. 1a). Chymotrypsin also stimulated the motility of demembranated spermatozoa at 40°C, with maximum motility obtaining at 600 ng ml^{-1} (Fig. 1b). However, no stimulation of motility was observed following the addition of papain (Fig. 1a) or carboxypeptidase B (Fig. 1b), within the ranges 0–2μg ml^{-1} and 0–500μg ml^{-1}, respectively, and disintegration of sperm tails was observed at higher concentrations. The addition of ng ml^{-1} order of papain and carboxypeptidase B were also ineffective in initiating motility (data not shown).

On the other hand, the vigorous motility of demembranated spermatozoa at 30°C was inhibited in a dose-dependent manner by the addition of the protease inhibitors, aprotinin or PMSF (Fig. 2). However, the addition of 500 ng trypsin ml^{-1} released the inhibitory effect of aprotinin within 1 min (Fig. 3).

Neither the addition of phosphodiesterase to remove endogenous cAMP nor the addition of Ca^{2+} or EGTA to trypsin-treated spermatozoa had any significant effect on sperm motility at 40°C (Table 1).

Effects of proteases on dynein ATPase activity and the

phosphorylation state of proteins of demembranated fowl spermatozoa

The flagellar ATPase activity of crude dynein extract without the addition of proteases was approximately 22 nmol ATP hydrolysis mg^{-1} protein min^{-1}. This activity was not stimulated by the addition of proteases, including trypsin and chymotrypsin (Table 2).

A marked difference in the phosphorylation status of a 43 kDa protein was obtained in demembranated spermatozoa at 30°C and at 40°C. This protein was slightly phosphorylated at 40°C, but strongly phosphorylated at 30°C, confirming previous observation (Ashizawa et al., 1992). However, neither aprotinin at 30°C nor trypsin at 40°C affected the phosphorylated state of this or other phosphorylated proteins (Fig. 4).

Discussion

Whereas in the past, protease activities were mainly thought to be involved in the catabolism of proteins, recent evidence suggests that they also have a short term regulatory function, for example in the control of sperm motility (Gagnon & de Lamirande, 1987). The present study showed that temperature-dependent immobilization of demembranated fowl spermatozoa at 40°C was reversed by the addition of trypsin or chymotrypsin (Fig. 1a,b). Furthermore, sperm motility at 30°C was inhibited by aprotinin or PMSF (Fig. 2), but could be restored by the subsequent addition of trypsin (Fig. 3). It is therefore proposed that an endogenous, presumably serine, protease may be present in the fowl sperm axoneme, and play an important role in regulating sperm movement. The immobilization of fowl spermatozoa

at 40°C might be due in part to the reduction of activity of such protease, although the effects of temperature on the activity of such protease of fowl spermatozoa were not measured in this experiment.

Although this is the first report of the presence of such protease activity in avian spermatozoa, similar sperm proteases appear to be preserved throughout evolution from sea urchins to humans (de Lamirande et al., 1990), since protease inhibitors, including aprotinin, inhibited the motility of demembranated spermatozoa in sea urchins, fish and mammals (de Lamirande et al., 1983; de Lamirande & Gagnon, 1986; Cosson & Gagnon, 1988; Inaba & Morisawa, 1991).

The target and precise mechanisms of action of such proteases in the regulation of sperm motility remain to be elucidated. The inhibition of motility initiation by high concentrations of ATP was abolished by the inclusion of trypsin in demembranated hamster spermatozoa, and it is assumed that trypsin may involve the action of cAMP (Yeung, 1986), since a trypsin-like protease derived from bovine spermatozoa can stimulate adenylate cyclase (Johnson et al., 1985). In the present study, however, no decrease in motility was observed by the addition of phosphodiesterase to remove of endogenous cAMP from trypsin-treated spermatozoa. Moreover, trypsin-stimulated sperm motility was not affected by the removal or addition of Ca^{2+} (Table 1). From these results, it may be proposed that the proteases act directly on the axoneme in a cAMP- and Ca^{2+}-independent manner. However, present results also showed that ATPase activity of dynein extract of fowl spermatozoa was not stimulated by the addition of proteases tested (Table 2), suggesting that these proteases might not act directly on dynein ATPase. Similarly, trypsin did not stimulate

the steady-state activity of the axonemal dynein ATPase of sea urchin spermatozoa (Kamimura et al., 1985) and aprotinin, at 50-fold the concentration needed to block sperm motility, caused only a 30% inhibition of dynein ATPase isolated from bull spermatozoa (Gagnon & de Lamirande, 1987).

Recently, we suggested that cAMP-independent phosphorylation of a 43 kDa axonemal protein is likely to be a regulatory step in the maintenance of fowl sperm motility (Ashizawa et al., 1992). However, the phosphorylation state of this protein did not change in the presence of proteases or their inhibitors (Fig. 4), supporting the hypothesis that the site of action of endogenous protease may lie between the 43 kDa protein and the dynein-tubulin sliding system. This agrees with the suggestion that other sperm proteases may act near the site where dynein arms interact with microtubules, without affecting the dynein ATPase (de Lamirande et al., 1990).

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Legends for Figures:

Fig. 1. The motility of demembranated fowl spermatozoa in the reactivation medium at 40°C after addition of various concentrations of trypsin (●) or papain (○) (a) and chymotrypsin (●) or carboxypeptidase B (○) (b). Results are the mean (±SEM) from five samples of spermatozoa.

Fig. 2. The motility of demembranated fowl spermatozoa in the reactivation medium at 30°C after addition of various concentrations of aprotinin or PMSF. Results are the mean (±SEM) from five samples of spermatozoa.

Fig. 3. The motility of demembranated fowl spermatozoa in the reactivation medium at 30°C following addition of: 0.24 TIU aprotinin ml^{-1} (●); 500 ng trypsin ml^{-1} (○). Results are the mean (±SEM) from five samples of spermatozoa.

Fig. 4. 10% SDS-PAGE profile of demembranated fowl sperm proteins stained with the Coomassie blue (lane 5) and corresponding autoradiography of phosphorylated proteins (lanes 1-4). Triton X-100 extracted and reactivated samples were incubated at 30°C or 40°C for 2 min. Lane 1, 30°C in the absence of aprotinin; lane 2, 30°C in the presence of 0.24 TIU aprotinin ml^{-1}; lane 3, 40°C in the absence of trypsin; lane 4, 40°C in the presence of 50 ng trypsin ml^{-1}.

Table 1. The effects of phosphodiesterase, EGTA and Ca^{2+} on the motility of trypsin-treated demembranated fowl spermatozoa at 40°C

Treatments-substances	Motility (%)
None (control)	4.8 ± 1.9^{a}
Trypsin (50 ng ml^{-1})	62.1 ± 4.2^{b}
Trypsin (50 ng ml^{-1})+phosphodiesterase (2 units ml^{-1})	70.5 ± 5.3^{b}
EGTA (2 mmol l^{-1})	4.1 ± 1.8^{a}
Ca^{2+} (0.1 µmol l^{-1})	2.7 ± 1.3^{a}
Trypsin (50 ng ml^{-1}) + EGTA (2 mmol l^{-1})	72.1 ± 3.5^{b}
Trypsin (50 ng ml^{-1}) + Ca^{2+} (0.1 µmol l^{-1})	70.4 ± 2.6^{b}

Each value represents the mean (±SEM) of five samples of spermatozoa.

Values with different superscripts differ significantly ($P<0.01$) from each other.

Table 2. The effects of proteases on the ATPase activity of crude dynein extract of fowl spermatozoa at 40°C

Protease	ATPase activity (nmol ATP hydrolysis mg^{-1} protein min^{-1})
None (control)	21.8 ± 0.3[a]
Trypsin (50 ng ml^{-1})	20.5 ± 0.7[a]
Chymotrypsin (600 ng ml^{-1})	20.9 ± 0.9[a]
Papain (600 ng ml^{-1})	20.9 ± 0.7[a]
Carboxypeptidase B (40 µg ml^{-1})	21.0 ± 0.3[a]

Each value represents the mean (±SEM) of five samples of spermatozoa.

Values with different superscripts differ significantly ($P<0.01$) from each other.

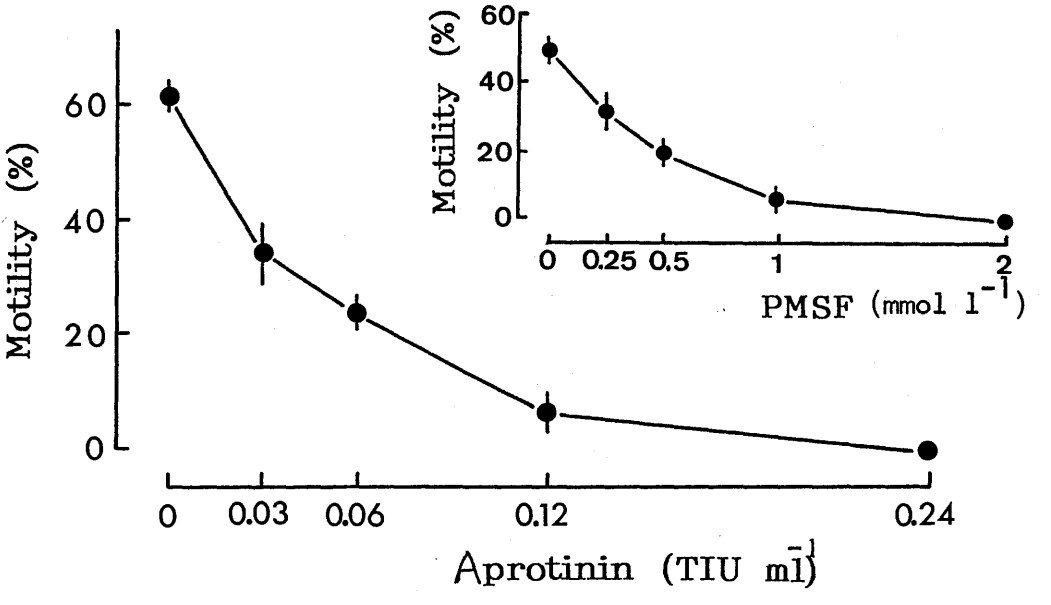
Motility (%)
60
40
20
0
0
0.03
0.06
0.12
0.24
Aprotinin (TIU ml⁻¹)
Motility (%)
60
40
20
0
0
0.25
0.5
1
2
PMSF (mmol l⁻¹)

Fig. 2.

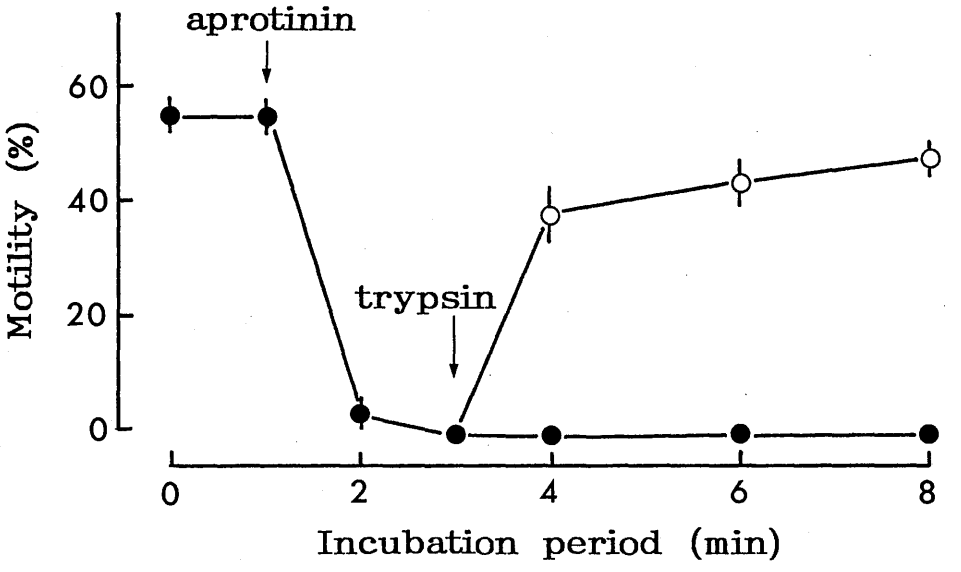
aprotinin
trypsin
Motility (%)
60
40
20
0
0
2
4
6
8
Incubation period (min)

Fig. 3.

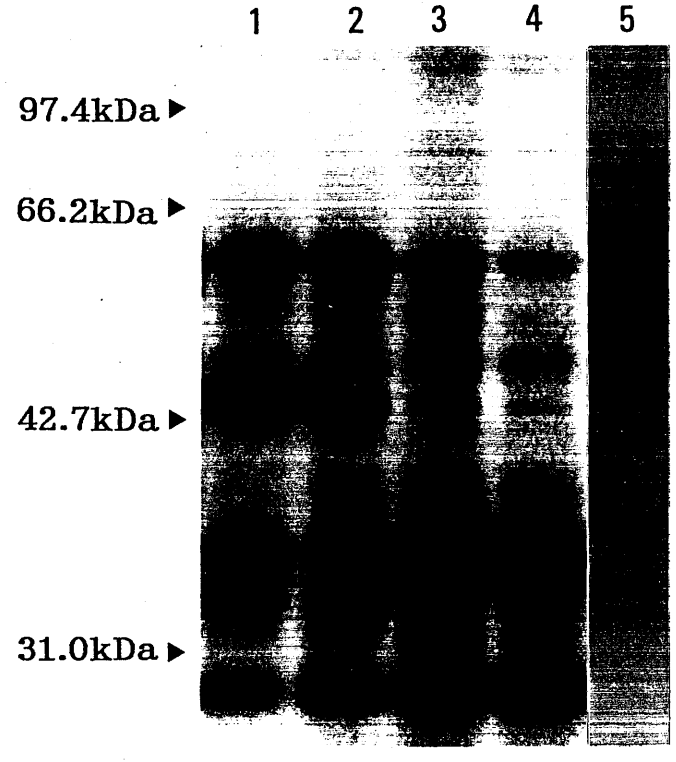

Fig. 4.

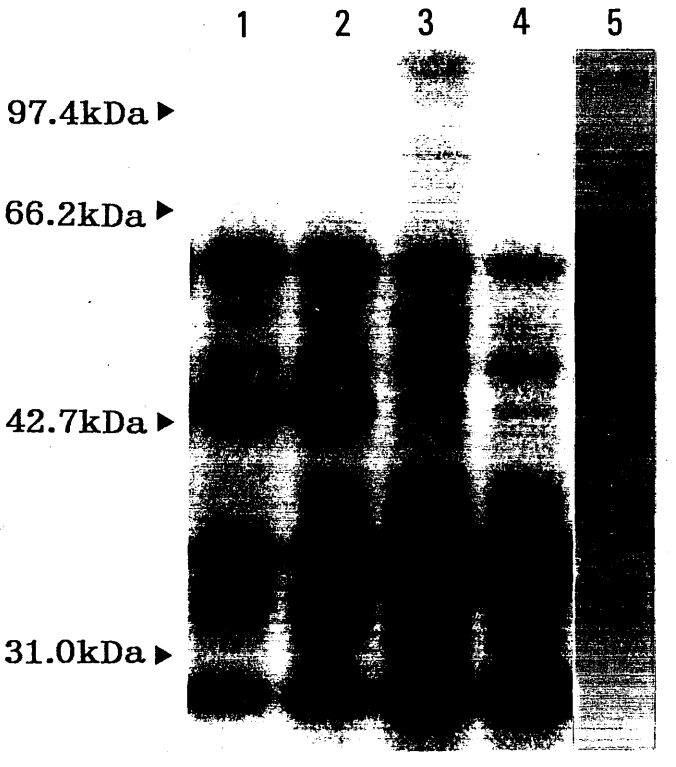

Fig. 4.