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Purification of proteasomes from salmonid fish sperm and their localization along sperm flagella

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SUMMARY

We have purified two chymotrypsin-like proteases from chum salmon sperm which have no apparent acrosome structure. Both of them were high molecular mass proteases (650 kDa and 950 kDa by gel filtration) and showed not only chymotrypsin-like activity but also trypsin-like activity. The 650 kDa protease was composed of at least eight or nine kinds of polypeptide with molecular masses ranging from 20 kDa to 30 kDa and was highly activated by low concentrations of SDS. Electron microscopy revealed that the 650 kDa protease was a ring-shaped particle. The 950 kDa protease was shown to contain at least one component that cross-reacts with an antibody against the 650 kDa protease. Finally, we revealed that the 650 kDa protease is located along the sperm flagella, by using immunofluorescence microscopy. The subunit composition, SDS-activation and molecular shape of 650 kDa salmonid protease were quite similar to those of the eukaryotic multicatalytic proteinase (proteasome), which is well known to participate in ATP-dependent degradation of ubiquitinated proteins; and, furthermore, the motility of demembranated sperm of salmonid fish is inhibited by chymotrypsin inhibitors in an ATP-dependent manner. Thus, the protease located in salmonid fish sperm flagella is a proteasome and is a strong candidate for the factor which regulates flagellar motility in an ATP-dependent manner.

Key words: proteasome, ATP-dependent proteolysis, multicatalytic proteinase, sperm motility, sperm flagella

INTRODUCTION

Cyclic AMP-dependent phosphorylation of axonemal proteins has been proposed as a step in the regulatory mechanism of sperm motility (Garbers and Kopf, 1980; Tash and Means, 1983). Direct evidence that cAMP may actually regulate sperm motility in salmonid fish was presented by Morisawa and Okuno (1982). In rainbow trout, spermatozoa demembranated with Triton X-100 remain immotile even in the presence of ATP and the addition of cAMP causes the initiation of motility. A 15 kDa protein was identified as a cAMP-dependent phosphorylated protein which was strongly phosphorylated within 1 s, a time period compatible with the normal initiation of sperm motility (Morisawa and Hayashi, 1985). Okuno and Morisawa (1982) further found that the movement of demembranated sperm did not require cAMP when they were activated by relatively low concentrations of ATP. This implies that not only cAMP-dependent phosphorylation of proteins, but also an ATP-dependent regulatory system, is involved in the control of trout sperm motility.

Sperm motility has been shown to be inhibited by some kinds of protease inhibitors and substrates in an ATP-dependent manner in mammals (de Lamirande and Gagnon, 1986), carp, sea urchin (Cosson and Gagnon, 1988) and salmonid fish (Inaba and Morisawa, 1991). The motilities of demembranated sperm of mammals, carp and sea urchin are inhibited by trypsin inhibitors when they are activated by the appropriate concentration of ATP. Chum salmon sperm activated by relatively high concentrations of ATP were also inhibited by protease inhibitors but the protease possibly involved appeared to be chymotrypsin-like, rather than trypsin-like (Inaba and Morisawa, 1991).

Some kinds of ATP-dependent proteases have been described and purified in eukaryotic cells. Among them, proteasomes (multicatalytic proteinases) have high molecular mass (600 kDa to 700 kDa) and are shown to be involved in ATP-dependent mechanisms of intracellular degradation of proteins, especially for ubiquitinated proteins (Rechsteiner, 1987; Rivetti, 1989). From the pattern of SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteasomes appear to be composed of at least ten different types of polypeptide with molecular masses ranging from 22 kDa to 34 kDa. The selective degradation of ubiquitinated proteins is considered to require proteasomes, which are associated with several other polypeptides (24 kDa to 125 kDa), to form a much larger complex (1000 kDa) (Waxman et al., 1987; Hough, et al., 1987; Eytan et al., 1989; Orino et al., 1991). Proteasomes have also been isolated and purified from sea urchin sperm (Matsumura and Aketa, 1991; Saitoh et al., 1991; Inaba et al., 1992). However, it is still not clear whether the proteasomes are

present in the acrosome or the flagella, and whether they are involved in the ATP-dependent regulatory mechanism of sperm motility.

Teleost sperm including salmonid fish sperm lack an apparent acrosome structure (Baccetti and Afzelius, 1976); therefore, study of them may supply important information concerning the involvement of proteasomes in the motility of sperm. In the present study, we isolated and purified two kinds of proteases, with molecular masses of 650 kDa and 950 kDa, from salmonid fish sperm. Studies using electron microscopy (EM) and immunofluorescence techniques showed that the 650 kDa protease was a proteasome and was located in sperm flagella, suggesting that proteasomes with chymotrypsin-like activity participate in ATP-dependent regulation of flagellar motility in salmonid fish.

MATERIALS AND METHODS

Materials

Synthetic fluorescent peptides were purchased from the Peptide Institute, Inc. (Osaka, Japan). Protease inhibitors were from Boehringer Mannheim (Mannheim, Germany); all the proteins for estimating molecular mass were from Sigma Chemical Co. (St. Louis, MO); mouse anti-tubulin monoclonal antibody was from Chemicon International Inc. (Temecula, CA); FITC- or biotin-labeled secondary antibody was from Vector Laboratories, Inc. (Burlingam, CA).

Purification of high molecular mass protease

The semen of chum salmon, *Oncorhynchus keta*, was collected by squeezing the abdomen of mature male chum salmon. The semen obtained was filtered through four layers of gauze to remove connective tissues and centrifuged at 3,000 ***g*** for 10 min, at 4˚C, to remove seminal plasma. The sperm pellet was suspended in three volumes of extraction medium containing 0.1% Triton X-100, 0.15 M KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM DTT and 10 mM Tris-HCl (pH 8.0) and stirred at 4˚C for 1 h. Then the suspension was centrifuged at 10,000 ***g*** for 30 min. The supernatant (crude extract) was stored at −80˚C until used. Sperm flagella were isolated by the method used for sea urchin sperm, as previously described (Inaba et al., 1988).

The frozen crude extract was thawed and centrifuged at 100,000 ***g*** for 1 h. The supernatant was charged onto a Bio-Beads SM2 (Bio-Rad, Richmond, CA) column (2 cm × 20 cm) to remove detergent and then washed with Triton-free extraction medium. The nonadsorbed fraction (approximately 290 mg protein) was collected and loaded onto a DEAE-Sephacel (Pharmacia LKB, Uppsala, Sweden) column (2 cm × 20 cm) and then washed with Triton-free extraction medium. Proteins were eluted with 140 ml of a 0.15 M to 0.6 M KCl linear gradient. The fractions (eluted with around 0.30 to 0.35 M KCl) which showed hydrolytic activity toward succinyl (Suc)-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) were pooled and dialyzed against 25 mM sodium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM DTT. The retentate was charged onto a hydroxylapatite column (1.5 cm × 8 cm) and proteins were eluted with 50 ml of a 25 mM to 250 mM linear gradient of sodium phosphate (pH 8.0). The fractions which showed hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA were pooled, concentrated with Aquacide III (Calbiochem Co., La Jolla, CA), and finally loaded onto a Superdex 200 gel filtration column (1 cm × 60 cm) (Pharmacia LKB, Uppsala, Sweden) and eluted with Triton-free extraction medium at 1 ml min^{-1}. The active fractions were examined by SDS-PAGE (12% acrylamide; Laemmli, 1970) and used for the experiments. Protein concentration was determined by the method of Bradford (1976).

Assay of peptidase activity

The synthetic fluorescent peptides were dissolved in dimethylsulfoxide to 1 mM and 10 μl of the solution was added to 1 ml of Triton-free extraction medium. For the examination of activation by SDS, KCl was replaced with NaCl. The hydrolytic activities towards synthetic peptides were measured as the fluorescence from released 7-amino-4-methylcoumarin using a fluorescence spectrophotometer (Hitachi, 650-10S) at excitation and emission wavelengths of 380 nm and 460 nm, respectively.

Preparation of antibody

The purified 650 kDa protease (200 μg) was mixed with an equal volume of Freund's complete adjuvant and injected into a rabbit. The subsequent injections were performed at 2-week intervals in the same way as the first injection. Antiserum was collected 10 days after the third injection. Affinity purification of anti-650 kDa antibody was performed by the method described previously (Inaba and Mohri, 1989) for the 20 kDa to 30 kDa subunits of the 650 kDa protease used as ligands. IgG from preimmune antiserum was prepared by precipitation with a 40% solution of saturated ammonium sulfate, followed by DEAE-Sephacel column chromatography. Immunoblotting was performed as described previously (Inaba and Mohri, 1989).

Immunofluorescence microscopy

Semen from the trout, *Oncorhynchus mykiss*, was diluted in 0.15 M KCl buffered with 10 mM HEPES (pH 7.4). Sperm that were completely immotile in the KCl solution were attached to a polylysine-coated coverslip by standing of diluted semen for 30 min at room temperature. Sperm were fixed and permeabilized by incubation in methanol at −20˚C for 20 min. After rinsing with PBS, sperm were further permeabilized with PBS containing 0.05% Tween 20. Then after blocking with 10 mg ml^{-1} of ovalbumin in PBS for 30 min, the coverslips were incubated with primary preimmune antiboby (50 μg ml^{-1}), primary anti-tubulin antibody (25 μg ml^{-1}) or primary anti-650 kDa antibody (25 μg ml^{-1}) in PBS for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20 (5 × 5 min), the coverslip was incubated with FITC-labeled secondary antibody for 30 min at room temperature. Then coverslips were rinsed with PBS containing Tween 20 (2 × 5 min), followed by washing with PBS (3 × 5 min), and then mounted in 50% glycerol in PBS. Sperm were observed with a fluorescence microscope (Nikon Optiphot, Japan) with a 100× glycerol immersion objective.

Electron microscopy

The solution of purified 650 kDa protease was deposited on a carbon-coated and discharged collodion film, and excess solution was removed by blotting. Then 1% uranyl acetate was applied to the sample and blotted. In the case of the 950 kDa protease, the sample solution and 2% uranyl acetate solution were deposited on the film at a ratio of 1:1 (v/v) and blotted within a few minutes. The samples were dried in air and examined with an electron microscope (JEM 1200EX, JEOL, Japan).

RESULTS

Purification of high molecular mass proteases

As preliminary experiments in the purification of chymotrypsin-like proteases from chum salmon sperm, we examined the chymotrypsin-like activity in the suspension

Table 1. Purification of chymotrypsin-like protease from chum salmon sperm

Stage	Volume (ml)	Protein (mg)	Specific activity* (nmole mg^{-1} min^{-1})	Total activity (nmole min^{-1})	Recovery (%)
Crude extract	300	290	0.014 (1.0)†	4.06	100
DEAE-Sephacel	16	53	0.067 (4.8)†	3.55	87
Hydroxylapatite	14	22	0.087 (6.2)†	1.91	47
Superdex 200	11	7.4	0.15 (11)†	1.11	27

*Hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA.
†The extent of purification. Pooled fraction from Superdex 200 chromatography contains both 950 kDa and 650 kDa proteases.

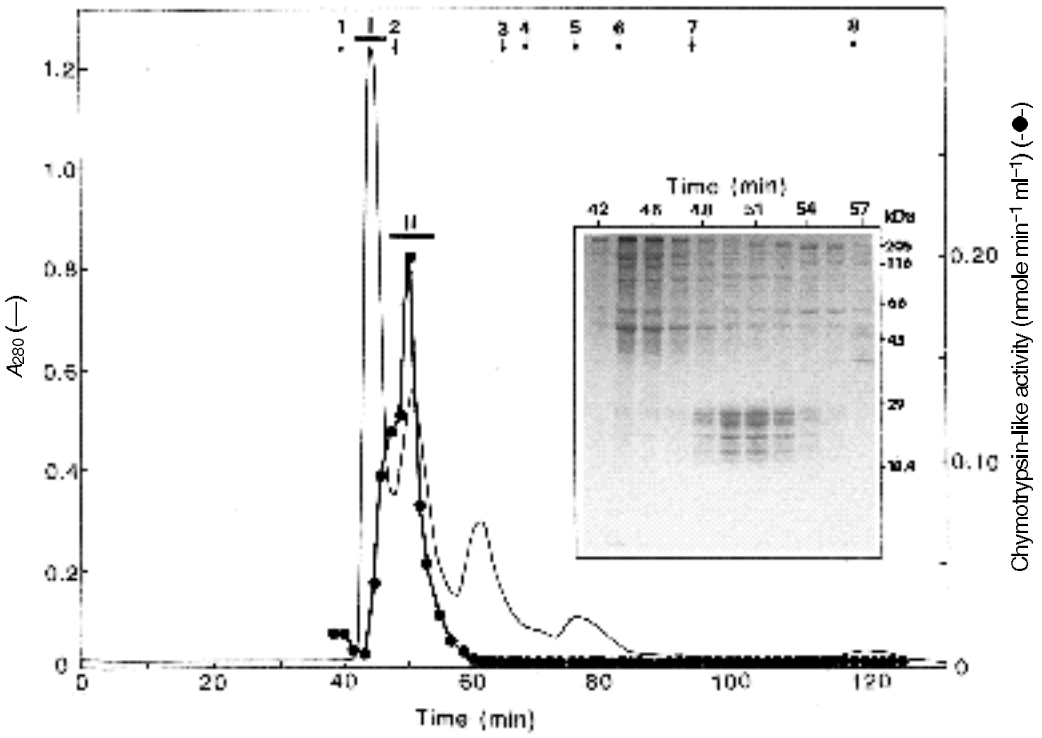

Fig. 1. Superdex 200 gel filtration of the pooled fraction from the hydroxylapatite column chromatography. The fraction pooled from the hydroxylapatite column was concentrated and loaded on a Superdex 200 gel filtration column. The flow rate was 1 ml min^{-1}. Fractions of 1.3 ml were collected. Chymotrypsin-like activity represents the hydrolytic activity towards a synthetic fluorescent peptide, Suc-Leu-Leu-Val-Tyr-MCA. Arrows indicate: the column void volume (1); the elution volume of thyroglobulin (669 kDa) (2); catalase (245 kDa) (3); alcohol dehydrogenase (150 kDa) (4); bovine serum albumin (66 kDa) (5); ovalbumin (43 kDa) (6); soybean trypsin inhibitor (20 kDa) (7); and the total bed volume of the column (8). Two bars at upper portions of the elution pattern show the fractions pooled for the 950 kDa (I) and 650 kDa (II) protease. A 20 μl sample of each fraction with elution times from 42 to 57 min was analyzed by 12% SDS-PAGE. The molecular mass markers for SDS-PAGE were beta-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactose dehydrogenase (36.5 kDa), triosephosphate isomerase (26.6 kDa), and soybean trypsin inhibitor (20 kDa).

of demembranated sperm using a synthetic peptide, Suc-Leu-Leu-Val-Tyr-MCA. The sperm suspension showed a certain hydrolytic activity towards this synthetic peptide and the activity could be extracted with a relatively long (1 h) exposure in 0.1% Triton X-100 solution. Therefore, we used a Triton X-100 extract as a starting crude extract for the purification of chymotrypsin-like proteases.

The steps for purification of chymotrypsin-like proteases are shown in Table 1. DEAE-Sephacel column chromatography of the extracts showed that the hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA was eluted as a broad single peak with around 0.30-0.35 M KCl. The activity was also recovered as a broad single peak around 100-130 mM sodium phosphate, on subsequent hydroxylapatite column chromatography. When the pooled fractions obtained from hydroxylapatite column were loaded onto a Superdex 200 gel filtration column, the hydrolytic activity was separated into two peaks, I and II, in the high molecular mass region (Fig.1). The molecular masses of the proteases corresponding to these two peaks were estimated as 950 kDa and 650 kDa using molecular mass markers. No other peak of hydrolytic activity was observed in the region of lower molecular mass. Rechromatography of these two peaks showed that these two peaks could be separated. The SDS-PAGE pattern of each fraction obtained from rechromatography of the 650 kDa (peak II) protease revealed that this protease was composed of at least eight or nine kinds of polypeptide with molecular masses ranging from 20 kDa

Table 2. Substrate specificities of the 950 kDa and 650 kDa proteases

Substrate	Activity (%)	
	950 kDa	650 kDa
Suc-Leu-Leu-Val-Tyr-MCA	100* (0.09)†	100* (0.48)†
Suc-Ala-Pro-Ala-MCA	0	0
Suc-Ala-Ala-Pro-Phe-MCA	0	0
Boc-Leu-Arg-Arg-MCA	56	128
Boc-Gln-Ala-Arg-MCA	8	14
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0	0
Arg-MCA	0	0
Leu-MCA	4	1
Ala-MCA	91	9

*The hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA was defined as 100%.

†The specific activity (nmole mg^{-1} min^{-1}) towards Suc-Leu-Leu-Val-Tyr-MCA.

to 30 kDa (see Fig.1). On the other hand, SDS-PAGE of each fraction obtained from rechromatography of the 950 kDa peak showed that this protease was composed of several polypeptides with molecular masses ranging from 22 kDa to 160 kDa, which was a quite different pattern from that of the 650 kDa protease (see Fig.1). Thus, it was shown that chum salmon sperm contained at least two distinct high molecular mass proteases.

Next we compared the substrate specificities and sensitivities to several inhibitors of both proteases (Tables 2 and 3). For the ten kinds of synthetic peptides examined in the present study, the substrate specificities of the 650 kDa and 950 kDa proteases were surprisingly similar to each other (Table 2). Both enzymes efficiently hydrolyzed a substrate for trypsin-like protease, *t*-butoxycarbony(Boc)-Leu-Arg-Arg-MCA, as well as a substrate for chymotrypsin-like protease, Suc-Leu-Leu-Val-Tyr-MCA. Both proteases also hydrolyzed Boc-Gln-Ala-Arg-MCA, Leu-MCA and Ala-MCA to a certain extent. Although the hydrolytic activity towards Ala-MCA differed considerably in both proteases, the profiles of substrate specificities of both enzymes were shown to be quite similar.

Then we compared these two proteases for sensitivity to several protease inhibitors of hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA (Table 3). Among twelve inhibitors examined, chymostatin showed the most inhibitory effects on the hydrolytic activity of both enzymes towards Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA. The hydrolytic activity towards Boc-Leu-Arg-Arg-MCA of both enzymes was inhibited by two kinds of trypsin inhibitor, leupeptin and antipain. An irreversible serine protease inhibitor, PMSF, also inhibited the activity of both enzymes to a certain extent. Thus there was much similarity in susceptibility to inhibitors between the 950 kDa and 650 kDa proteases. On the other hand, the susceptibility of the activity of both enzymes to *N*-tosyl-phenylalanine chloromethyl ketone and 3,4-dichloroisocoumarin was somewhat different. *N*-tosyl-phenylalanine chloromethyl ketone showed almost no effect on the hydrolytic activity towards both substrates in the case of the 950 kDa protease, whereas this protease inhibitor activated the hydrolysis of both substrates by the 650 kDa protease. Furthermore, 3,4-dichloroisocoumarin at 100 μM completely blocked the hydrolysis of both substrates by the 950 kDa protease, whereas this compound activated the hydrolytic activity towards Boc-Leu-Arg-Arg-MCA.

Activation by low concentrations of SDS

It is well known that proteasomes are activated by the addition of low concentrations of SDS. In order to obtain more information on the 950 kDa and 650 kDa proteases in relation to proteasomes, we compared the effects of SDS on the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA by both proteases. As shown in Fig.2, the 650 kDa protease was gradually activated with increase in the concentration of SDS. In the presence of 0.0125% SDS, the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA by the 650 kDa protease was activated up to more than fivefold. In contrast, the hydrolytic activity of the 950 kDa protease gradually decreased with increase in the concentration of SDS and no activation was observed.

Table 3. Effects of various inhibitors on the hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA of 950 kDa and 650 kDa proteases

Inhibitor	Concentration (μM)	Activity (%)			
		950 kDa		650 kDa	
		LLVY-MCA	LRR-MCA	LLVY-MCA	LRR-MCA
None	–	100	100	100	100
TPCK	100	108	94	355	288
TLCK	100	100	90	96	112
Leupeptin	100	120	0	69	3
Chymostatin	100	7	2	3	3
Antipain	100	85	20	128	6
PMSF	2000	78	38	46	35
E-64	10	123	105	115	89
Pepstatin	10	115	119	69	81
3,4-DIC	100	0	0	10	477
Bestatin	10	117	103	127	105
Amastatin	10	103	123	107	134

LLVY-MCA, Suc-Leu-Leu-Val-Tyr-MCA; LRR-MCA, Boc-Leu-Arg-Arg-MCA; TPCK, *N*-tosylphenylalanine chloromethyl ketone; TLCK, *N*-tosyllysine chloromethyl ketone; 3,4-DIC, 3,4-dichloroisocoumarin.

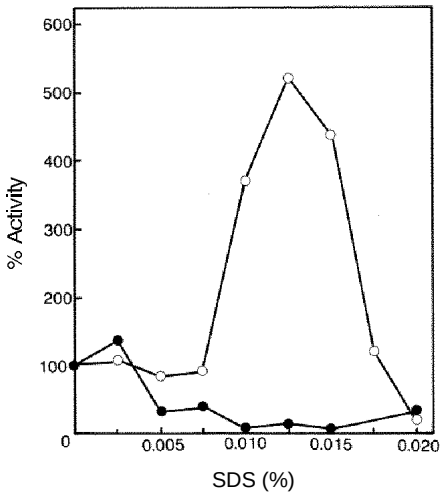

Fig. 2. Effects of SDS on the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA by 950 kDa and 650 kDa proteases. The purified 950 kDa (●) and 650 kDa (○) proteases were incubated with 10 μM Suc-Leu-Leu-Val-Tyr-MCA in the presence of several concentrations of SDS. % Activity shows the hydrolytic activity relative to that in the absence of SDS.

Molecular shape of the high molecular mass proteases

Proteasomes are usually observed by EM as symmetrical ring-shaped particles and such shapes have been considered to be one of the characteristics of proteasomes. In the present study, we examined the molecular shapes of the 950 kDa and 650 kDa proteases by EM. The subunit composition and the profiles of the activation by SDS imply that the 650 kDa protease is a proteasome. EM of the purified 650 kDa protease showed that this protease was a ring-shaped particle with a diameter of approximately 10 nm (Fig.3B). This feature was quite similar to that reported for proteasomes. On the other hand, the molecular shape of the 950 kDa protease was revealed to be quite different from that of the 650 kDa protease. The fraction of purified 950 kDa protease contained heterogeneous molecular species. The most predominant species of molecular shape seemed to be a slender shape with one or both ends swollen (Fig.3A). But we could not conclude much about the exact molecular shape of the 950 kDa protease from the present results.

Immunological studies of high molecular mass proteases

The similarity of both high molecular mass proteases in substrate specificity and susceptibility to inhibitors implies that these two enzymes have common polypeptides as active subunits. To elucidate this, we prepared a polyclonal antibody from rabbit against the 650 kDa protease. The affinity-purified antibody reacted with at least three or four polypeptides in the 650 kDa protease (Fig.4). This polyclonal antibody strongly recognized 29 kDa and 28 kDa polypeptides in a crude extract of sperm from *Oncorhynchus keta.* Furthermore, the antibody revealed that the 29 kDa and 28 kDa polypeptides were also present in a crude extract from *Oncorhynchus mykiss.* Therefore this antibody would be a useful probe for detecting the subunits of 650 kDa protease. We then looked for the presence of the subunits of 650 kDa protease in the preparation of 950 kDa protease. Gel filtration of crude extracts of sperm showed two peaks of hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA, as shown in Fig.1. Immunostaining of each fraction obtained from rechromatography of the 950 kDa peak revealed that the major peak of the 29 kDa and 28 kDa polypeptides was eluted at 650 kDa, whereas another peak of the 29 kDa polypeptide was clearly observed in the 950 kDa region (Fig.5A). This peak of the

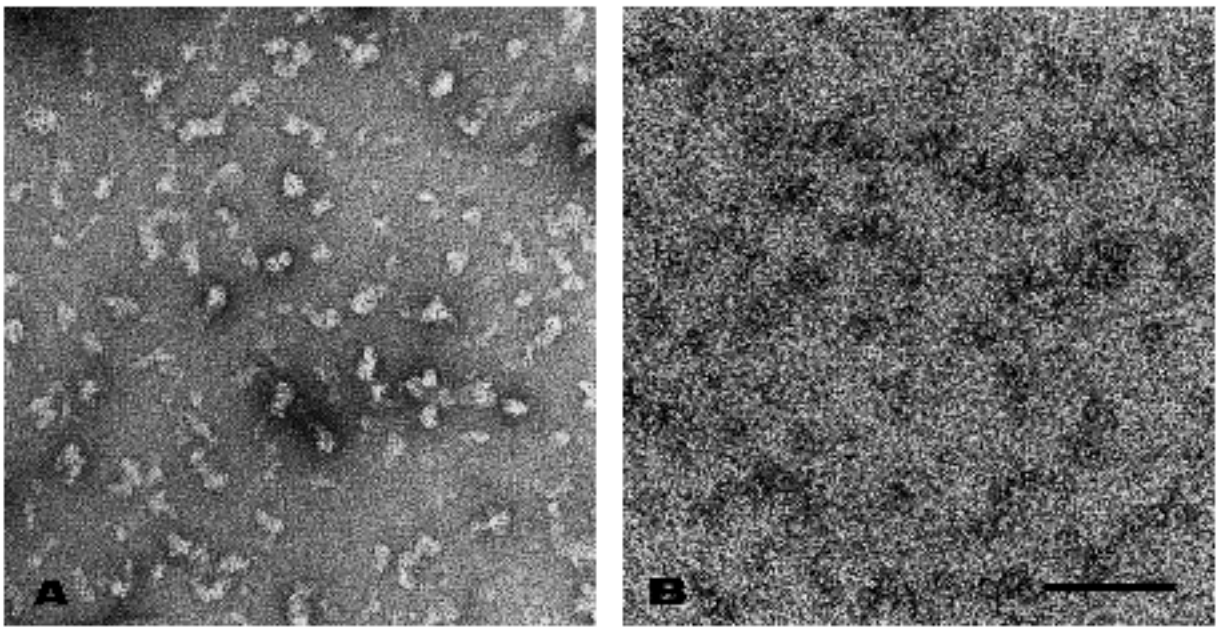

Fig. 3. Electron micrographs of the purified 950 kDa (A) and 650 kDa (B) proteases negatively stained with 1% uranyl acetate. Bar, 100 nm.

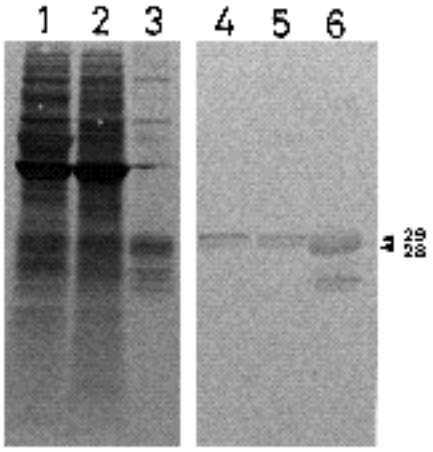

Fig. 4. Western blots of crude extracts of sperm from *Oncorhynchus keta* and *Oncorhynchus mykiss*, and purified 650 kDa protease from *Oncorhynchus keta* sperm, using affinity-purified anti-650 kDa antibody. Proteins separated by SDS-PAGE were electrophoretically transferred to a hydrophobic polyvinylidine difluoride membrane and stained with Coomassie Brilliant Blue R-250 (lanes 1, 2 and 3) or with affinity-purified anti-650 kDa antibody (lanes 4, 5 and 6) using a Vectastain ABC kit. Lanes 1 and 4, crude extract from *Oncorhynchus keta* sperm (32 μg); lanes 2 and 5, crude extract from *Oncorhynchus mykiss* sperm (32 μg); lanes 3 and 6, purified 650 kDa protease (7 μg). Numbers at the right represent the molecular masses (kDa) of the major polypeptides recognized by the antibody.

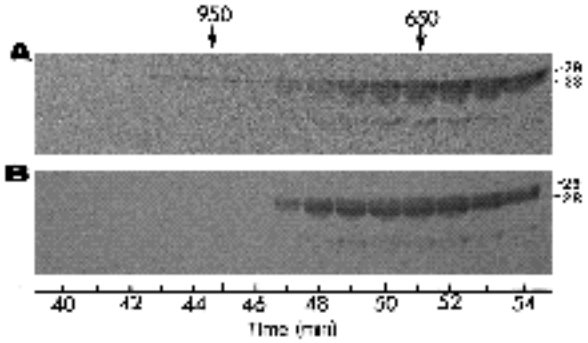

Fig. 5. Immunoblotting analysis 950 kDa and 650 kDa proteases with anti-650 kDa antibody. Both proteases were separated on a Superdex 200 gel filtration column. After peak I (950 kDa) and peak II (650 kDa), which were obtained as shown in Fig.1, were subjected to rechromatography on the same column, fractions of 1 ml each were collected and 25 μl of each fraction was separated by SDS-PAGE, transfered onto a polyvinylidine fluoride membrane and stained with anti-650 kDa antibody. (A) Elution pattern of peak I, (B) elution patter of peak II. Elution time of each fraction and eluted positions of 950 kDa and 650 kDa protease are indicated at the bottom and the top, respectively. The major polypeptides which were recognized by the antibody are shown by arrows and by molecular masses (kDa).

29 kDa polypeptide was revealed not to be due to contamination by the 650 kDa protease, since the immunostaining of each fraction from rechromatography of the 650 kDa protease (peak II) detected no peak of 29 kDa polypeptide in the 950 kDa region (Fig.5B). Therefore it was shown that the 950 kDa protease contained at least one subunit with molecular mass of 29 kDa that cross-reacted with anti-650 kDa antibody.

Subcellular localization of high molecular mass proteases

In order to clarify the involvement of proteasomes in sperm motility, we looked for the presence of proteasomes in isolated sperm flagella by immunoblotting (Fig.6). After heads and tails of sperm were dissociated by homogenization, the suspension was centrifuged to sediment the sperm heads. Most of the flagella were found in the supernatant and recovered as a pellet by subsequent centrifugation (Fig.6A,B). SDS-PAGE of proteins in the sperm flagella showed many kinds of protein bands containing tubulin as the main component (Fig.6C). Western blotting with anti-650 kDa antibody revealed that the 29 kDa and 28 kDa subunits of the 650 kDa protease were clearly present in the isolated sperm flagella (Fig.6C).

Since some parts of the sperm flagella contaminated the pellet of sperm heads, separation of head and tail by centrifugation seemed to be suitable for preparing pure tail fraction but not for isolating pure head fraction. Actually, the head fraction obtained by centrifugation showed considerable hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA. However, when we compared the activity per volume of pellet between head and tail fractions, the activities were estimated as 360 pmole min^{-1} ml^{-1} for head fraction and 1150 pmole min^{-1} ml^{-1} for tail fraction, showing that the chymotrypsin activity is concentrated in the sperm tail. Furthermore, using the value of specific activity of purified protease shown in Table 1, the relative amount of the chymotrypsin-like protease in sperm tail could be estimated as 2% of the total flagellar proteins.

To obtain further information about the localization of the high molecular mass proteases in sperm, sperm were fixed, permeabilized and treated with anti-650 kDa antibody. Affinity-purified anti-650 kDa antibody recognized at least two subunits of the 650 kDa protease in the crude extract from sperm in *Oncorhynchus mykiss*, as shown in Fig.5. Immunofluorescence microscopy revealed that the 650 kDa protease was located predominantly in the sperm tail (Fig.7B). The head region was also slightly stained but the basal part of the flagellum was rather strongly stained. Surprisingly, the anti-650 kDa antibody did not stain all of the sperm tail evenly but showed patches in staining pattern along the flagellum. In order to examine whether such a staining pattern of flagellum resulted from some artifacts in the procedure of fixation or permeabilization of sperm, they were subjected to staining with anti-tubulin antibody. The result showed a uniform staining pattern along the flagellum without any patches (Fig.7C), indicating that the procedure for fixation and permeabilization of sperm was suitable for the access of primary and secondary antibodies. Western blot analysis showed that the preimmune antibody did not stain any protein bands (data not shown), and it only weakly stained the head region and the basal part of the flagellum (Fig.7D).

DISCUSSION

High molecular mass, multicatalytic proteases which sedi-

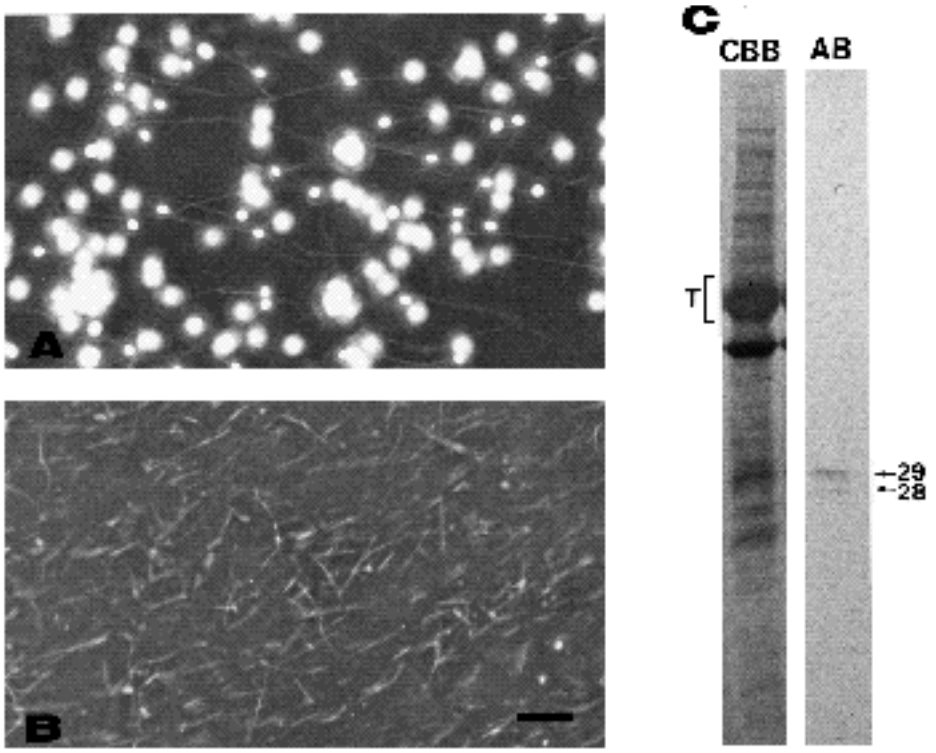

Fig. 6. Immunoblot of isolated sperm flagella proteins by anti-650 kDa antibody. Phase-contrast microscopic images of *Oncorhynchus keta* sperm (A) and flagella (B). CBB and AB show the SDS-PAGE patterns of flagellar proteins (25 μg loaded) visualized by Coomassie Brilliant Blue and the corresponding immunoblot, respectively (C). Numbers on the right show the molecular masses (kDa) of the proteins recognized by the anti-650 kDa antibody. T indicates tubulin bands. Bar, 20 μm.

ment at around 20 S and 26 S have been isolated from several mammalian cells (Waxman et al., 1987; Hough et al., 1987; Eytan et al., 1989; Orino et al., 1991). The 20 S protease (600 kDa to 700 kDa), the proteasome, is a ring-shaped particle which is composed of at least ten polypeptides with molecular masses ranging from 22 kDa to 34 kDa. The larger 26 S protease complex (1,000 kDa) can be isolated from crude extracts of mammalian cells in the presence of ATP. Immunological studies showed that 26 S protease contains the 20 S proteasome along with other polypeptides with molecular masses ranging from 24 kDa to 125 kDa. Although both proteases show multicatalytic activities towards synthetic peptides, they differ in some enzymatic properties: the 20 S protease is activated by low concentrations of SDS but is inhibited by 3,4-dichloroisocoumarin, whereas the 26 S protease is inhibited by SDS but activated by 3,4-dichloroisocoumarin (Waxman et al., 1987; Hough et al., 1987; Orino et al., 1991).

We isolated two kinds of multicatalytic proteases from sperm of chum salmon. The 650 kDa protease was revealed to be a proteasome, judged by its molecular composition, molecular shape and some enzymatic properties. Furthermore, immunological studies suggest that the other one, the 950 kDa protease, is a modified form of the 650 kDa proteasome. The relationship between salmonid 950 kDa and 650 kDa proteases is similar to that between mammalian 26 S and 20 S proteasomes in sensitivity to SDS and 3,4-dichloroisocoumarin (Waxman et al., 1987). However, all the components of the 650 kDa protease could not be immunologically detected in the preparation of 950 kDa protease in the present study. Therefore, although it might be still possible that the 950 kDa protease could correspond to the mammalian 26 S proteasome, it appears to be a modified form of proteasome other than the mammalian 26 S proteasome.

The 650 kDa protease isolated in the present study showed a subunit composition peculiar to proteasomes and EM revealed that the sample of 650 kDa protease had been almost completely purified. However, the molecular shape of the 950 kDa protease examined by EM was heterogeneous. It is possible that all of the molecules seen in the EM images might be the 950 kDa protease itself or its dissociated products as reported for mammalian 26 S proteasome (Ikai et al., 1991). Since the immunoblot of the 950 kDa protease with anti-650 kDa antibody revealed that 950 kDa protease contains the 29 kDa polypeptide in common with the 650 kDa protease, the 950 kDa protease might be a product dissociated from a large complex containing the 650 kDa protease, during extraction or purification.

It is well known that proteasomes are abundantly distributed in the nucleus and cytoplasm of eukaryotic cells and their biochemical properties have been widely investigated. Although proteasomes are likely to participate in ATP-dependent intracellular proteolysis, the intrinsic functions of the proteasome remain to be solved, except for the recent description of its participation in antigen processing in the immune system (Driscoll and Finley, 1992). Sperm are highly differentiated cells with simple structure and function, therefore they may be helpful in understanding the function of proteasomes.

Studies on sperm proteases have been mainly focused on the process of the acrosome reaction. Actually, some protease inhibitors block the acrosome reaction or fertilization in tunicates and sea urchins (Hoshi et al., 1981; Green and Summers, 1982). Some kinds of proteases which have been isolated from mammalian and ascidian sperm appear to be

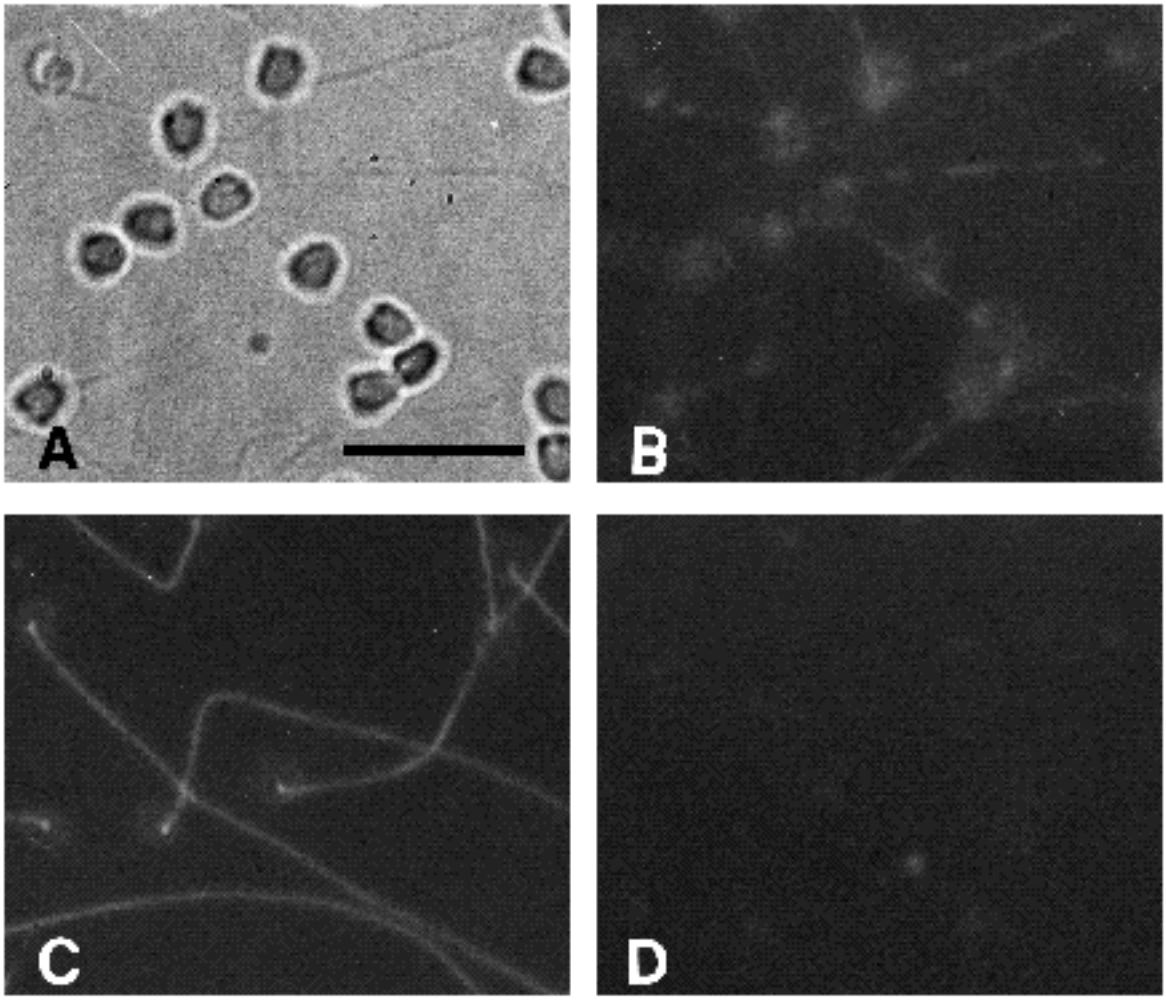

Fig. 7. Immunofluorescence analysis of sperm from *Oncorhynchus mykiss* using anti-650 kDa antibody. Sperm were fixed, permeabilized and treated with anti-650 kDa antibody (B), anti-tubulin antibody (C) or preimmune IgG (D). The image of bright-field microscopy corresponding to (B) is shown in (A). The photographs show typical images from three individual experiments. Bar, 10 μm.

acrosome-related proteases, such as acrosin (Parrish and Polakoski, 1979) and spermosin (Sawada et al., 1984). Recently, several investigators have isolated proteasomes, from sea urchin sperm: with a molecular mass of 700 kDa from *Strongylocentrotus intermedius* (Matsumura and Aketa, 1991) and of 950 kDa and 650 kDa from *Anthocidaris crassispina* (Inaba et al., 1992). However, the precise function and localization of proteasomes in sperm are unclear.

Gagnon and his coworkers first described the significance of proteases in flagellar motility of sperm (de Lamirande and Gagnon, 1986; Cosson and Gagnon, 1988). They concluded that a serine protease with Lys- and Arg-ester bond specificity is involved in the control of sperm motilty in mammals, carp and sea urchin. We have previously shown that some chymotrypsin inhibitors block chum salmon sperm motility, and the extent of inhibition by the protease inhibitors depends on the concentration of ATP used to reactivate demembranated sperm (Inaba and Morisawa, 1991). In the present study, we isolated proteasomes from chum salmon sperm which lack an apparent acrosome structure (Baccetti and Afzelius, 1976). Proteasomes showed a certain hydrolytic activity towards a substrate for chymotrypsin-like proteases and were immunologically detected along sperm flagella. Taking into account the finding that proteasomes in mammalian cells are activated by high concentrations of ATP (Driscoll and Goldberg, 1989), proteasomes present in flagella of chum salmon sperm would be strong candidates for a role in ATP-dependent regulation of sperm motility.

Sperm motility basically depends on the active sliding of the outer doublet microtubules in the flagellar axoneme (Gibbons, 1981). To produce sliding of each outer doublet microtubule in the sperm axoneme, it is necessary to preincubate the axoneme with trypsin to digest the structures causing resistance, such as nexin links and radial spokes (Summers and Gibbons, 1971). The localization of proteasomes along sperm flagella revealed in the present study leads us to speculate that the destruction of such structures by proteasomes is a prerequisite for producing bend propagation of sperm flagella. In our observations, a certain amount of the proteasomes in sperm from chum salmon and sea urchin could be extracted by incubation with 0.1% Triton X-100 at 4˚C for 1 h. However, after a short extrac-

tion, for example in 0.04% Triton X-100 at 4˚C for 5 min, approximately 60% of the hydrolytic activity towards Suc-Leu-Leu-Val-Tyr-MCA was still retained by the sperm, although most of the detergent-soluble proteins were extracted (K. Inaba and M. Morisawa, unpublished data). Therefore, proteasomes might bind weakly to the axoneme and regulate the sliding of microtubules. In order to clarify the role of the proteasome in the regulation of sperm motility, further studies are necessary to understand the process of interaction of proteasomes with the axoneme, to identify the natural substrates of proteasomes in flagellar axonemes and to examine the observation of the periodical distribution of proteasomes along sperm flagella.

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