Oocyte Proteasomes: The Structure and Role in the Regulation of Meiotic Maturation and Fertilization in the Goldfish, *Carassius auratus*

Toshinobu Tokumoto

Doctor of Science

Department of Molecular Biomechanics

School of Life Science

The Graduate University for Advanced Studies

(1993)

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INTRODUCTION

It is generally accepted that proteolysis plays an important role in the regulation of the eukaryotic cell cycle. Eukaryotic cells contain a nonlysosomal large protease called the proteasome (or the multicatalytic protease) which is found in all eukaryotes, from yeast to man [Orlowski, 1990]. Proteasomes have been isolated as latent forms which can be activated by several agents such as SDS , fatty acids [Dahlman et al.,1985], poly-lysine [Tanaka et al., 1986] and by heat treatment [Mykles, 1989]. Although it has been suggested that proteasomes are involved in the ubiquitin-dependent proteolytic system, their exact role in intracellular protein breakdown is still uncertain.

In teleost fishes, like in most other vertebrates, full-grown postvitellogenic oocytes in the ovary are physiologically arrested at the G2/M border in first meiotic prophase and cannot be fertilized. For the oocytes to be fertilizable, they must complete the first meiotic division. Under an appropriate hormonal stimulation, full-grown oocytes resume their first meiotic division which involves breakdown of the germinal vesicle (GVBD), chromosome condensation, assembly of the first meiotic spindle, and extrusion of the first polar body. Meiosis is again arrested at the second metaphase. Shortly thereafter, mature fertilizable oocytes are ovulated. The meiotic process leading to extrusion of the second polar body is resumed again at the time of fertilization, immediately after sperm penetration (egg activation). The time period between the resumption of meiosis and the second meiotic metaphase has been referred to as the period of oocyte maturation. Thus, the process of oocyte maturation and egg activation is a prerequisite for successful fertilization and normal development.

Recent studies using various protease inhibitors suggest that proteasomes are involved in meiotic maturation of animal oocytes. For example, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a synthetic inhibitor of chymotrypsin, has been reported to prevent progesterone-induced meiotic maturation of frog oocytes [Ishikawa et al., 1989; Azuma et al., 1991]. More recent studies have demonstrated that the termination of mitosis and meiosis, tran-

sition from metaphase to anaphase is induced by the degradation of cyclin, a regulatory subunit of maturation-promoting factor or M-phase promoting factor (MPF) [Maller, 1991]. Furthermore, it has been suggested that cyclin is degraded by an ubiquitin-dependent proteolytic pathway [Glotzer, 1991]. Since proteasomes are known to be a component of the ubiquitin-dependent proteolytic system, it can be hypothesized that proteasomes play a role in cyclin degradation. However, there is no direct evidence for this hypothesis at present.

Fish oocytes provide an appropriate experimental system to investigate the above problems. In recent years, considerable progress has been made in identifying the factors responsible for the regulation of meiotic maturation of fish oocytes; these include the isolation and characterization of a fish maturation-inducing hormone (17α,20β-dihydroxy-4-pregnen-3-one, 17α,20β-DP) [Nagahama and Adachi, 1985] and the components of MPF ($p34^{cdc2}$, the catalytic subunit and cyclin, the regulatory subunit [Yamashita et al., 1992a; Yamashita et al., 1992b; Hirai et al., 1992].

This study was designed to investigate the possible role of proteasomes in the regulation of meiotic maturation and egg activation, in particular cyclin degradation. A teleost fish, the goldfish, *Carassius auratus*, was used, since in this species maturation-inducing hormone (17α,20β-DP) has been identified and the two components of MPF, cdc2 kinase and cyclin B, have also been characterized. I first purified and characterized a SDS-dependent (latent) proteasome (20 S proteasome) from goldfish oocyte cytosols and raised monoclonal antibodies against this enzyme. During the course of this study, I also found that goldfish oocyte cytosols contain a SDS-independent (active) proteasome (26 S proteasome) which is quite labile and easily coverts to the latent form. A newly developed procedure was used to purify active proteasome. I then produced several monoclonal antibodies against latent proteasome, and used these antibodies to examine cyclic changes in the activity and protein levels of proteasomes in oocytes during 17α,20β-DP-induced meiotic maturation [Tokumoto et al., 1993a] and egg activation. Finally, the role of proteasomes in the regulation of cyclin degradation was investigated using bacterially produced goldfish cyclin B and purified active proteasome.

ABBREVIATIONS

Boc, butyloxycarbonyl; DMSO, dimethyl sulfoxide; HCG, human chorionic gonadotropin; -MCA, -4-methlcoumaryl-7-amide; -NA, -2-naphthylamide; Suc, succynyl; TBS, Tris buffered saline; Z, carbobenzoxy.

MATERIALS AND METHODS

Materials

Goldfish were purchased from a local supplier (Yatomi, Japan) and maintained at 15°C until use. Femail gravid African clawed frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu Kyozai Corp. and maintained at 20°C. Fluorogenic peptide substrates (*ex.*, Suc-LLVY-MCA, Suc-AAPF-MCA, Boc-FSR-MCA, Boc-QRR-MCA, Boc-LRR-MCA, *etc.*) were purchased from Peptide Inc. Corp. except for Z-LLE-βNA and Z-GGL-βNA which were from Sigma. Arginine-Sepharose 4B, Blue-Sepharose CL-6B, Mono-Q, Sepharose CL-4B, Phenyl Sepharose Fast Flow, Superose 6, Activated-CH Sepharose and ECH-Sepharose were from Pharmacia. DEAE-cellulose (DE52) and CM-cellulose (CM52) were purchased from Whatman. TSK gel Phenyl 5PW-RP and TSK gel G4000 SWXL were from Tosoh Corp. Dried polyacrylamide beads were from Atto Corp. All other chemicals were of reagent grade.

Purification of latent proteasome (20 S proteasome)

Ovaries were dissected into fragments of 50-100 oocytes in goldfish Ringer's solution (125 mM NaCl, 2.4 mM KCl, 0.28 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2 mM HEPES, 5.6 mM glucose, 100,000 IU/l penicillin, 0.2 g/l streptomysin, pH 7.5). Oocytes allowed to settle in centrifuge tubes and after removal of excess medium, they were centrifuged at 150,000 g for 60 min. The cytosol fraction between the lipid layer and the packed components was applied to a DEAE-cellulose column (2.6 x 12.0 cm) equilibrated with 50 mM Tris-HCl, pH 7.5 containing 20% glycerol and 10 mM 2-mercaptoethanol (TGM buffer). The column was washed with TGM buffer and bound materials were eluted with a step elution of 0.2 M NaCl in TGM buffer at a flow rate of 60 ml/h (fraction volume: 10 ml). Active fractions were pooled and concentrated to less than 10 ml by adding dried polyacrylamide beads and were chromatographed on a Sepharose CL-4B column (2.6 x 84.0 cm) equilibrated with TGM buffer at a flow rate of 40 ml/h (fraction volume: 5 ml). Active fractions were pooled and applied to a Mono-Q column (HR 5/5) equilibrated with TGM buffer. Proteins were eluted using a linear gradient (total volume: 40 ml)

of 0 ~ 0.5 M NaCl in TGM buffer at a flow rate of 30 ml/h (fraction volume: 2 ml). Active fractions were pooled and bound to DEAE-cellulose (0.9 x 4.0 cm) after dilution with 20 mM Tris-HCl, pH 8.0 (TN buffer). The column was then washed extensively with TN buffer to remove glycerol. Bound proteins were eluted with a high salt buffer (TN buffer containing 2 M NaCl) and the concentration of NaCl in active fractions was adjusted to 2 M by adding solid NaCl. The fractions were then passed through a Phenyl Sepharose Fast Flow column (1.0 x 6.4 cm) equilibrated with TN buffer containing 2 M NaCl (fraction volume: 2 ml). Active fractions were pooled, dialyzed overnight against TGM buffer, and stored at 4°C. All procedures were performed at 4°C.

Purification of active proteasome (26 S proteasome)

Ovaries were homogenized with four volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.25 M sucrose and the homogenate was centrifuged at 150,000 g for 1 hour. The supernatant was applied to a DEAE-cellulose column (2.6 x 12.0 cm) equilibrated with TGM buffer. The column was washed with ten volumes of the same buffer and bound materials were eluted with TGM buffer containing 0.2 M NaCl, and 10 ml fractions were collected. Active fractions were pooled, diluted four-fold with TGM buffer, and supplemented with 0.1 mM ATP (TGMA) and applied to Arginine-Sepharose 4B column (1.6 x 10.0 cm) equilibrated with TGMA buffer containing 50 mM NaCl. Absorbed materials were eluted with TGMA buffer containing 0.2 M NaCl, and fractions of 10 ml were collected. Active fractions were pooled and concentrated to less than 10 ml with dried polyacrylamide beads. Concentrated fractions were then chromatographed on a Sepharose CL-4B column (2.6 x 84.0 cm) equilibrated with TGMA buffer, and fractions of 10 ml were collected. Fractions with a high SDS-independent activity were pooled and passed through a Blue-Sepharose column (1.0 x 6.4 cm) equilibrated with TGMA buffer, and the enzymes were concentrated by DEAE-cellulose column (1.0 x 2.5 cm). The enzymes were then applied to a Superose 6 column (1.0 x 30.0 cm) equilibrated with TGMA buffer, and fractions of 0.5 ml were collected. Active fractions were obtained at the position near the void volume and peak fractions were collected as purified

enzyme. The purified enzyme was analyzed by gel chromatography on a TSK gel G4000SWXL column (0.78 x 30.0 cm). Fractions of 0.5 ml were collected.

Purification of ubiquitin

Goldfish ovaries were dissected into fragments of 50 - 100 oocytes in goldfish Ringer's solution (125 mM NaCl, 2.4 mM KCl, 0.28 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2 mM HEPES, 5.6 mM glucose, 100,000 IU/l penicillin, 0.2 g/l streptomysin, pH 7.5) and washed with the Ringer's solution three times. Oocytes were homogenized with four volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.25 M sucrose and the homogenate was centrifuged at 150,000 g for 1 hr. The supernatant was applied to a DEAE-cellulose column (DE52, 2.6 x 12.0 cm) equilibrated with TGM buffer. Flow-through and first one column volume eluate were collected and used for ubiquitin purification. After pH was adjusted to 4.5 with HCl, the DEAE flow-through fraction was applied to a CM-cellulose column (CM52, 2.6 x 18.8 cm) equilibrated with 25 mM ammonium acetate at pH 4.5 (buffer A, pH adjusted at room temperature). The column was washed with five column volumes of buffer A and bound materials were eluted with 50 mM ammonium acetate at pH 5.5 at room temperature, and 20 ml fractions were collected. Fractions were assessed by SDS-PAGE or immunoblotting with anti-bovine ubiquitin anti-serum (Sigma) and the fractions containing ubiquitin were collected and concentrated with a CM-cellulose column as following; pH of fraction was adjusted to 4.5 and applied to column (1.0 x 6.4 cm), then proteins were eluted with buffer A containing 500 mM NaCl. The concentrated fraction was applied to Bio Gel P-30 column (Bio Rad, 1.6 x 50.0 cm) equilibrated with buffer A, and 5 ml fractions were collected. Fractions containing ubiquitin were pooled and applied to a Mono-S column (Pharmacia, HR 5/5) equilibrated with buffer A. Proteins were eluted using a linear gradient (total volume: 30 ml) of 0 - 0.3 M NaCl in buffer A (fraction volume: 2 ml). The peak fraction at a concentration of 0.15 M NaCl was collected as purified ubiquitin. All procedures were performed at 4 °C.

Molecular weight determination

Purified enzyme was applied to TSKgel G4000SWXL equilibrated with TGMA buffer, and fractions of 0.5 ml were collected. Molecular weight was calculated using molecular weight markers.

Protease assay

In all assays, 100 µl of the reaction mixture, containing 100 mM Tris and 10 or 100 µM fluorogenic substrate with or without SDS (0 ~ 0.15%), was preincubated for 10 min at 37°C. Ten µl of one of the chromatography fractions or of the purified proteasome (0.1 µg) was added and the incubation continues for 10 min at 37°C. The reaction was terminated by the addition (100µl) of 10% SDS (wt/v) and 2.0 ml of 0.1 M Tris-HCl, pH 9.0. Fluorescence was determined on a fluorescence spectrophotometer (F-5000, Shimazu) or fluorescence microplate reader (MTP 100-F, Corona) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm for MCA-substrate or an excitation wavelength of 335 nm and an emission wavelength of 410 nm for NA-substrate.

Effect of pH on protease activity

The pH of the reaction mixture was adjusted with hydrochloric acid or sodium hydrochloride and the volume of reaction mixture was adjusted to 200 µl. After the reaction, one half of the reaction mixture was diluted five-fold with distilled water and the pH was measured. The other half of the mixture was used to determine protease activity.

Protein assay

The protein concentration of pooled fractions was measured by the method of Bradford [Bradford, 1976] using bovine serum albumin as a standard.

Electron microscope observations

The purified enzyme was observed with electron microscope by the method described

previously [Ikai, 1991]. The enzyme was diluted to 50 μg/ml with TGMA buffer. The sample solution was then deposited on the film and excess solution was removed by blotting. Three percent uranyl acetate solution was applied to the sample and blotted within 30 sec. The sample was dried and observed by a electron microscope (JEOL-100CX).

Preparation of monoclonal antibodies against goldfish latent proteasome

a) Immunization: Protein was diluted with HEPES buffer (20 mM, pH 7.5) and emulsified in an equal volume of Freund's adjuvant (complete for the initial injection, incomplete for subsequent injections). For each injection, 100 μg of protein in a total volume of 0.5 ml was used per mouse. BALB/c mice were injected at 2 week intervals. Two weeks after the second injection, titer of sera was checked by ELISA. Three days prior to fusion, mice were injected with 200 μg of protein in a total volume of 0.5 ml. Mice with higher titers were used for fusion.

b) Hybridoma production: Mice were killed by cervical dislocation. Spleens were removed, minced in Dulbecco's medium and washed with serum-free medium. Mouse myeloma cells in log-phase growth were also washed using serum-free medium and low speed centrifugation. The cells were combined and added to 1 ml of polyetylenglycol 4000 (50%) with gentle shaking. The cells were diluted to 60 ml with Iscove's-supplemented HAT selection medium (containing 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine) and plated in 96 well plates. Culture supernatants were tested for reactivity to antigen by ELISA and positive wells were cloned by the limiting-dilution method. Cells were frozen in medium containing 10% fetal bovine serum and 10% DMSO at -80°C. Pristane-treated BALB/c mice were used to generate ascites fluid.

ELISA

Antigen in HEPES buffer was absorbed on a Xenobind plate (Bokusui Brown) during an overnight incubation at 37°C. Nonspecific binding was blocked by incubation of wells with 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TTBS). Serum culture supernatant was incubated on the antigen-coupled Xenobind plate for 1 hour at 37°C, and antigen-antibody

complexes were detected using alkaline phosphatase conjugated anti-mouse immunoglobulins (Zymed).

Preparation of IgG-Sepharose and immunoprecipitation

Anti-proteasome IgG was purified from ascites fluid using a protein A-Sepharose column and was dialyzed against distilled water. Pre-immune (control) and anti-proteasome IgG (0.2 mg) were coupled to ECH-Sepharose (1 ml) according to the manufacturer's instructions. Cytosol fractions (150 µl) were incubated with non-immune (control) IgG-Sepharose or anti-goldfish proteasome IgG-Sepharose (800 µl) for 2 hours at 4°C. After centrifugation at 1,200 g for 5 min, the supernatants were used for protease assay. Precipitated beads were washed three times with TTBS and mixed with SDS sample buffer and boiled for 5 min. Proteins in supernatants and in extracts from precipitated beads were separated on SDS-PAGE followed by immunoblotting with anti-proteasome antibodies.

Production of polyclonal antibodies against active proteasome

a) Preparation of antigens: A large quantity of active proteasome was purified as follows: The Sepharose CL-4B fraction (the third step in purification of active proteasome) was applied to a Fast Flow Q-Sepharose column (1.0 x 12.7 cm). Bound proteins were eluted with a linear gradient (total volume: 150 ml) of 0.2-0.5 M NaCl in TGMA buffer. Although a SDS-independent protease activity was almost lost during this step, highly purified components of active proteasome were obtained in large quantities at the 0.3 M NaCl fraction.

b) Preparation of antiserum: An antiserum against purified goldfish active proteasome was prepared by the procedure of Winberry and Holten [Winberry and Holton, 1977]. Purified enzyme (5 mg) was emulsified in an equal volume of complete Freund's adjuvant (Difco), and injected into the back of female white rabbit weighing about 2.5 kg. A booster injection of purified enzyme emulsified in an equal volume of incomplete adjuvant was given 2 weeks later and 2 week after the second booster injection, blood was collected from the carotid artery and the heart, and the serum was separated by centrifugation. The IgG fraction was purified by a protein

A-Sepharose affinity chromatography.

Electrophoresis and immunoblotting

Electrophoresis followed the method of Laemmli [Laemmli, 1970]. Proteins were then transferred to an Immobilon membrane (Millipore) using a semi-dry electroblotter. The membrane was blocked with 5% non-fat dry milk and incubated with culture supernatant containing anti-proteasome antibodies for 1 hour at room temperature. Immunocomplexes were visualized using alkaline phosphatase conjugated anti-mouse immunoglobulin, as described previously [Yamashita, 1992a] or peroxidase conjugated immunoglobulin using an ECL detection kit (Ammersham).

Separation of components of latent proteasome

Purified proteasome was separated into components by reversed-phase high performance liquid chromatography on a TSK-gel Phenyl-5PW-RP column as previously described [Tanaka, 1989].

Glycerol density gradient centrifugation

A linear gradient of glycerol (10 ~ 35%) was made in a total volume of 12.5 ml. The glycerol concentration of samples was adjusted to 10% and 100 µl was centrifuged at 152,000 g for 12 hour at 4°C. Samples were fractionated (0.5 ml/fractions) from top to bottom.

Amino acid analysis

Samples (32 and 14 µg of latent and active proteasomes) were hydrolyzed with 0.1 ml of 6N HCl in sealed, evacuated glass tubes at 110°C for 24, 48, and 72 hr. The hydrolysates were analyzed a in Hitachi 835 amino acid analyzer. Tryptophan was determined spectrophotometrically [Edelhoch, 1967].

Determination of sedimentation coefficient

Purified enzyme in TGM buffer was concentrated by centrifugation (372,000 g for 2 hr) and dialyzed against TGMA buffer and diluted to absorvance at 280 nm between 0.2 to 0.5. The sample was centrifuged in double sector cells with buffer used in dialysis at 60,000 rpm (Hitachi model 282 analytical ultracentrifuge). The sedimentation coefficient of the enzyme was calculated using a partial specific volume of 0.725 and 0.733 ml/g for latent and active proteasomes estimated from its amino acid composition.

Culture of oocytes

Oocyte maturation was induced *in vitro* by incubating fragments of ovaries (each contains 20-40 oocytes) in goldfish Ringer's solution (125 mM NaCl, 2.4 mM KCl, 0.28 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2 mM HEPES, 5.6 mM glucose, 100,000 IU/l penicillin, 0.2 g/l streptomycin, pH 7.5) containing 1 μM of 17α,20β-DP, as described previously [Yamashita et al., 1992a]. Maturation processes were assessed by immersing the oocytes in a clearing solution [Lessman and Kavumpurath, 1984], enabling easy microscopic examination for the presence or absence of a germinal vesicle.

Production of goldfish cyclin B in bacteria

Wild type (Δ0) and N-terminal deleted (Δ41, Δ68, and Δ96) goldfish cyclin Bs were produced in *Escherichia coli* using the T7 promoter system [Studier et al., 1986; Rosenberg et al., 1987]. *E. coli* BL21(DE3) were grown to an OD_{600} of 0.7 in 1 liter of NZCYM or LB medium containing 100 μg/ml ampicillin. Then cyclin synthesis was induced by 1 mM isopropylthio-β-galactoside (IPTG) for 3 hr. The bacteria were collected, suspended in 6 ml lysis buffer (50 mM Tris-HCl, 1mM EDTA, 100 mM NaCl, 1 mM DTT, 100 mM p-amidinophenylmethanesulfonyl fluoride, pH 8.0) containing 1.6 mg lysozyme, stirred at 4°C for 20 min, and lysed with a sonicator (model W-225R; Heat-Ultrasonics). The lysate was centrifuged at 12,000 g for 15 min at 4°C. Pellets were washed with lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, and insoluble inclusion bodies were separated on SDS-

PAGE, and the cyclin band was excised after being visualized with 250 mM KCl. For the large scale preparation, proteins were separated by Prep Cell Model 491 (BIO RAD). Cyclin proteins were electroeluted from the gel (Max Yield-NP; ATTO) in a buffer containing 2.5 mM Tris and 19.2 mM glycine, dialyzed against 1 mM HEPES (pH 7.0), and then lyophilized. Cyclins were stocked at -80°C until used.

Preparation of cell-free extracts from Xenopus eggs

Xenopus laevis females were primed with 50 I.U.pregnant mare serum gonadotropin at day 1 and 25 I.U. at day 3. Eggs were collected in modified modified ringer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM Na-EGTA, 5 mM NaHEPES, pH 7.8; MMR) after injection with 1000 I.U. human chorionic gonadotropin and de-jellied in 2% cysteine (pH 7.8). Dejellied eggs were washed four times in an extraction buffer (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM Sucrose, 10 mM HEPES, pH7.7), transferred to 2 ml tubes containing the extraction buffer supplemented with 1 mM $MgCl_2$, 5 mM EGTA, 10 μg/ml protease inhibitors (chymostatin, leupeptin, and pepstatin) and 100 μg/ml cytochalasin B, overlaid with 0.25 ml Versilube oil and packed during a 1 min 600 g spin. Displaced buffer above the oil were removed and the eggs were crushed by a 10 min centrifugation at 15,000 g at 16°C. The material between the lipid cap and the yolk in the pellet was collected, cytochalasin B and protease inhibitors were added to 10 μg/ml, and the extracts were centrifuged again at 4°C. The supernatant was used or freezed by liquid N_2 immediately. Activation of extract was induced by addition of Ca^{2+} to a final concentration of 0.2 or 0.4 mM at room temperature.

Kinase assay

Kinase activity of $p34^{cdc2}$ was measured according to Yamashita et al.[1992b], using a synthetic peptide (SP-peptide: KKAAKSPKKAKK), which includes the consensus sequence of the site phosphorylated by $p34^{cdc2}$. The samples were incubated for 2 min at 30°C in the presence of the followings : 100 mM SP-peptide, 500 mM ATP, 1.5 mCi [γ-^{32}P]ATP, 1 mM EGTA, 10 mM $MgCl_2$, 4.5 mM β-mercaptoethanol, 20 mM Tris- HCl (pH 7.4). The reaction

was stopped by phosphoric acid, and the SP-peptide was absorbed on P81 phosphocellulose paper (Whatman). The paper was washed in phosphoric acid, and the radioactivity remaining on the paper was measured by a scintillation counter.

RESULTS

1. Purification and characterization of latent proteasome (20 S proteasome)

Purification

The 150,000 g supernatant prepared from goldfish ovaries possessed Suc-LLVY-MCA hydrolyzing activity at neutral pHs (Fig. 1A). Since proteasomes are known to be activated by a low concentration of SDS, the effect of SDS on Suc-LLVY-MCA hydrolyzing activity was examined. At lower and higher concentrations of SDS (0.02 ~ 0.06%: higher than 0.12% respectively), activity decreased. However, at 0.08 ~ 0.1% SDS, activity was similar to that in the absence of SDS (Fig. 1B). During the following purification steps, the proteasome activity was determined using two assay systems (in the presence and absence of SDS). Four steps of chromatography were used. Using a step elution in a DEAE-cellulose, these two activities eluted at 0.2 M NaCl (Fig. 2A). Both activities eluted as a broad peak after a Sepharose CL-4B gel chromatography (Fig. 2B), these fractions were pooled and applied to a Mono-Q column. Using a linear gradient of NaCl, activities eluted at 0.25 M NaCl with a symmetrical peak, and with a marked decrease in the SDS-independent activity (Fig. 2C). Since fractions from the Mono-Q column chromatography contained several high molecular weight proteins (M.W. 60-100 kDa), these proteins were removed by a Phenyl-Sepharose column (Fig. 2D). By these chromatographic steps, one mg proteasome was purified from 79 g of goldfish ovaries (Table 1). The purified proteasome migrated as a single band on native PAGE and as ten bands on one dimensional SDS-PAGE, with molecular weights ranging from 23.5 to 31.5 kDa (Fig. 3). The molecular weight and sedimentation coefficient of the purified proteasome were 620 kDa (Fig. 4) and 19.2 S, respectively. The amino acid composition of the goldfish proteasome was similar to that of proteasomes from other species [Tanaka et al., 1988] except that serine and tryptophane content was high (Table 2). The purified enzyme hydrolyzed peptides containing Tyr, Arg and Glu, thus displaying chymotrypsin-like, trypsin-like, and V8 protease-like activities, respectively. Among these activities, chymotrypsin-like and V8 protease-like activities were increased in the presence

of 0.03 ~ 0.04% SDS (Fig. 5), a finding similar to those by Tanaka *et al* and Ozaki *et al* [Tanaka et al., 1988; Ozaki et al., 1992]. At the ultrastructural level, purified goldfish proteasome appeared as a ring-shaped particle with cylindrical structure (Fig. 17).

Production of monoclonal antibodies

Monoclonal antibodies were raised against purified goldfish latent proteasome. Three positive clones were obtained; these clones recognized a single species of protein on immunoblots of native PAGE. However, each of these clones recognized three separate proteins (p-25, p-31.5 and p-30) on immunoblots of SDS-PAGE (Fig. 6). To further characterize these antibodies, the purified proteasome was separated into components by reversed phase HPLC on TSK gel Phenyl 5PW-RP (Fig. 7). Ten major peaks were obtained, namely goldfish components 1 to 10 (GC1-10). On SDS-PAGE, proteins of some of these peaks (GC1, 2, 4, 6, 8, and 10) exhibited only one component per individual peak. However, proteins of each of the remaining peaks (GC3, 5, 7, and 9) consisted of several components. Some of these were identified as α, β, γ and so on according to decreasing molecular size on SDS-PAGE: *e.g.* GC3α, β, γ and so on; GC4α, β, γ and so on. A total of 15 major components were obtained by HPLC including several minor bands. In Western blots of the Phenyl 5PW-RP fraction, the anti-25K, 31.5K, and 30K antibodies recognized GC4 and GC5, GC3α, and GC3β, respectively.

Immunoprecipitation analysis

Cytosol fractions from goldfish ovaries possessed some peptidase activities. These activities were not inhibited by the addition of the antibodies to the cytosol, but could be removed by immunoadsorption with the antibody-Sepharose (Table 3). Proteasome removal was confirmed by the presence of the subunits of the proteasome in the immunoprecipitates and their absence from the supernatant (Fig. 8). Table 3 compares hydrolyzing activities against various substrates between the cytosol fraction and the purified latent proteasome. Hydrolyzing activities in the cytosol against Suc-LLVY-MCA and substrates containing Arg or Glu at the P1 position, in the absence of SDS, were found to significantly higher than those of the purified latent proteasome.

Accordingly it is thought that these activities were lost during purification.

Fractionation of active and latent proteasomes

Reduction of the SDS-independent activity was also observed after incubation of the cytosol at 37°C for one hour (Fig. 9) or after storage at 4°C for several hours (data not shown). In contrast, the SDS-dependent activity was maintained after these treatments.

Proteasomes from temperature (37°C) -treated and non-treated cytosols were fractionated by DEAE-cellulose and glycerol density gradient centrifugation. When the non-treated cytosol was fractionated, Suc-LLVY-MCA hydrolyzing activity was recovered in a fraction with a sedimentation coefficient higher than that of purified latent proteasome; the hydrolyzing activity in the presence or absence of SDS was almost identical. In contrast, the temperature-treated cytosol exhibited only a SDS-dependent activity in the same fraction as that of purified latent proteasome (Fig. 10A, B). Immunoblot analyses revealed that proteasome from the non-treated cytosol was different from either purified latent proteasome or proteasome from the temperature-treated cytosol (Fig. 10C, D).

2. Purification and characterization of active proteasome (26 S proteasome)

Purification

As described in the preceding section, cytosol fractions of goldfish ovaries exhibited high peptidase activities against several kinds of peptide substrates. It was demonstrated by immuno-precipitation assay that these activities were mainly attributable to proteasome (Table 3). However, the activities were lost during a series of chromatographic steps, except for the Bz-GGL-βNA hydrolyzing activity. The loss of activities occurred mainly during a linear gradient elution on ion-exchange or affinity chromatography resins. I also tested several other resins such as DEAE-cellulose, hydroxylapatite, arginine-Sepharose, phosphocellulose, *etc.* In all cases however, the SDS-independent activity was greatly reduced by a linear gradient elution. In

contrast, the SDS-dependent activity against Suc-LLVY-MCA was quite stable and there was no reduction in activity with this substrate.

It was then found that the loss of SDS-independent activity was greatly reduced when the enzyme was eluted using a step elution at a concentration of ions higher than that used to elute SDS-dependent 20 S proteasome activity by a linear gradient in each resin. I also found that the presence of ATP prevents the reduction of activity during gel filtration. Based on these properties, a purification procedure was developed as follows. On an DEAE-cellulose and Arginine-Sepharose chromatography, the SDS-dependent activity was eluted by a linear gradient at 0.18 and 0.13 M, respectively. On the DEAE-cellulose column chromatography, the SDS-independent activity was recovered with step elution at 0.2 M NaCl, but lost during elution with linear gradient, irrespective of the absence or presence of ATP (Fig. 11). When enzymes were eluted with a step elution of 0.2 M, a high yield of SDS-independent activity was also obtained on the Arginine-Sepharose 4B (Fig. 12). Further purification was carried out by gel filtration using a buffer supplemented with ATP (Figs. 13, 14). On the Superose 6 chromatography, active fractions were obtained at a position following the void volume and peak fractions were collected as purified enzyme (Fig. 14, Table 4). Purified enzyme was analyzed by gel chromatography on a TSKgel G4000SWXL column, and showed a symmetrical peak of protein and enzyme activity at a position corresponding to a molecular mass of 1,200 kDa (Fig. 15).

Molecular characteristics

On native PAGE, active proteasome had two high molecular weight bands (Fig. 16A). These two bands were excised from the gel and analyzed by SDS-PAGE. Subunits with molecular weights ranging from 35.5 to 110 kDa overlapped between these two bands (Fig. 16C). However, subunits corresponding to 20 S proteasome were found only in the lower band. These results are consistent with the observation that the antibodies recognized only the lower band in Western blots after native PAGE (Fig. 16A). When extracted from the gel, these two bands showed no SDS-independent Suc-LLVY-MCA hydrolyzing activity (data not shown).

The SDS-PAGE analysis demonstrated that active proteasome consists of multiple subunits

with molecular weights ranging from 23.5 to 140 kDa (Fig. 16B). Subunits with molecular weights ranging from 23.5 to 31.5 kDa resemble those of 20 S proteasome. Western blot analyses revealed that three kinds of monoclonal antibodies against goldfish 20 S proteasome recognizes a band which is the counterpart of 20 S proteasome (Fig. 16B).

In electron micrographs, active proteasome appeared as several forms; these include a cylindrical dumbbell image, a smaller complex with only one terminal domain, and a particle with similar size of terminal domain (Fig. 17). The sedimentation coefficient of active proteasome was 29.4 S. Amino acid composition of active proteasome was similar to those of latent proteasome except the contents of serin and tryptophan residue were lower than those of latent one [Table 2].

Enzymatic properties

The hydrolytic activities of the purified active and latent proteasomes were compared using various peptide substrates (Table 5). The active proteasome hydrolyzed four kinds of peptides, all of which have Tyr, Leu, Arg, and Glu at P1 position, but did not hydrolyze their sinble peptide substrates. Hydrolysis of peptides containing Tyr or Leu, Arg, and Glu indicated that the active proteasome possesses chymotrypsin-like, trypsin-like, and V8-protease-like activities, respectively [Orlowski, 1990; Haas et al., 1990]. Both the Suc-LLVY-MCA and Z-GGL-βNA hydrolyzing activities are chymotrypsin-like; however, these two activities could be separated by their SDS dependency (Fig. 18) and inhibitor sensitivity (will be described later). The Suc-LLVY-MCA hydrolyzing activity and V8 protease-like activity of 20 S proteasome are known to be stimulated by a low concentration of SDS. These activities were also stimulated by addition of 0.03 ~ 0.04% SDS to goldfish 20 S proteasome. The SDS-independent Suc-LLVY-MCA hydrolyzing activity of active proteasome was similar to that of the cytosol fraction (Fig. 1 and Fig. 18). The V8 protease-like activity of the active proteasome was stimulated by SDS, but the activity in the absence of SDS was higher than that of the latent proteasome. The trypsin-like activity of the active proteasome was also significantly higher than that of the 20 S proteasome. Another kind of chymotrypsin-like activity, recognizing the peptide sequence GGL, was not

stimulated by SDS. Latent and active goldfish proteasomes hydrolyzed this peptide to the same degree.

Effect of protease inhibitors on hydrolyzing activity

Among the competitive inhibitors tested in this study, both chymostatin and elastatinal were very effective in inhibiting Suc-LLVY-MCA hydrolyzing activity (Table 6). Similarly, leupeptin and antipain strongly inhibited the trypsin-like activity. In almost every case, the divalent cation chelating reagents, EDTA and EGTA, inhibited all activities by 20 ~ 60%. Among the irreversible serine protease inhibitors, APMSF strongly inhibited the Suc-LLVY-MCA hydrolyzing activity and trypsin-like activity. DFP also inhibited the Suc-LLVY-MCA and Z-GGL-βNA hydrolyzing activity. TLCK exhibited a strong inhibitory effect on these activities, especially the V8 protease-like activity and Z-GGL-βNA hydrolyzing activity.

Effect of ATP on the chymotrypsin-like activity

The reaction was performed in the presence of various concentrations of ATP. The Suc-LLVY-MCA hydrolyzing activity of active proteasome and cytosol fractions was reduced to about 30% of the initial level in the presence of 1 mM ATP (Fig. 19). This reduction of activity was found to be reversible. The Suc-LLVY-MCA activity was restored by the incubation of the cytosol for several times at 37°C (data not shown), as reported in *Xenopus* oocyte extracts [Tokumoto and Ishikawa, 1993a]. Restration was thought to be caused by the consumption of ATP due to the action of an endogenous ATPase.

Effect of pH and temperature on the chymotrypsin-like activity

The optimal pH for active proteasome was ranging 7.2 ~ 7.6 (Fig. 20), a finding consistent with the pH dependence for proteasome activity in the cytosol (Fig. 1). Active proteasome was stable for at least 10 min at temperatures lower than 37°C in TGM buffer containing 0.1 mM ATP, but was inactivated at temperatures higher than 40°C (Fig. 21A). It was found that the temperature optimum for the Suc-LLVY-MCA degrading activity was between 34 and 37°C

(Fig. 21B).

3. Changes in the activity and protein levels of active proteasome during oocyte maturation and egg activation

In goldfish, like in other vertebrates, the fully grown oocyte possesses a large nucleus (germinal vesicle) in meiotic prophase. The germinal vesicle of this stage is located centrally. The first visible event associated with 17α,20β-DP-induced final maturation is the migration of the germinal vesicle to the animal pole where the micropile is situated; at this stage their germinal vesicle becomes visible under the dissecting microscope. The membrane of the germinal vesicle then breaks down (germinal vesicle breakdown) (Fig. 22A).

It was shown previously that the proteasome activity could be detected only in the presence of a low concentration of SDS in the reaction mixture. However, in this study the 150,000 g supernatant of crushed goldfish oocytes exhibited the proteasome activity regardless of the addition of SDS to the reaction mixture. Therefore, in this study the proteasome activity was determined in the absence of SDS. The proteasome activity in oocyte extracts prepared during oocyte maturation was measured with a synthetic peptide substrate specific to proteasomes, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA). Since the proteolytic activity measured with this substrate was completely precipitated with the antibodies against proteasome (Table.3), the obtained activity can be considered to be solely derived from active proteasome.

Upon stimulation of oocytes with 17α,20β-DP, there was a 2-fold increase in the proteasome activity within 1 hr that gradually decreased to the lowest level by 6 hr post 17α,20β-DP. The proteasome activity elevated again at 7 hr after 17α,20β-DP treatment, followed by a sharp drop at 8 hr (Fig. 22A). The data presented in Fig. 22A are derived from a representative experiment utilizing oocytes from a single gravid female goldfish. Similar experiments were repeated several times with oocytes from different females to confirm the reproducibility of the

data. Although the absolute values varied between experiments, the relative pattern of changes was consistent between experiments.

Changes in protein levels of proteasomes were examined using three different monoclonal antibodies (GC4/5, GC3α and GC3β) against purified goldfish latent proteasome. An analysis by immunoblots of native PAGE revealed that all of the three antibodies recognize a single species of protein. The density of the band was proportional to the proteasome activity throughout the maturational processes (Fig. 22B). On immunoblots of SDS-PAGE, each of these three antibodies recognized three separate components of subunits of goldfish 20S proteasome. Again, their density fluctuated in harmony with the changes in the activity (Fig. 22C). Changes in the density of the three bands were synchronous, showing no specific changes in a specific band.

Activation of goldfish eggs can easily be induced by immersing ovulated oocytes in water. Upon egg activation, proteasome activity increased two-fold within a few minuites, and then significantly decreased (Fig. 23A). The immunoblot analysis of extracts from activated eggs did not appear to show a direct correlation between the increase in the activity and the intensity of protein bands. Nevertheless, the sharp drop of the enzyme activity were found to be associated with the disappearance of the protein bands (Fig. 23B). It is of interest that a new anti-GC4/5-positive band appeared after egg activation (Fig. 23B,b). This protein continuously existed in soluble fraction. It was shown by immunoblotting with an anti-cyclin B that cyclin B is degraded within 3 minuites after egg activation in goldfish (Fig. 28). Proteasome activity remained high throughout the period of cyclin B degradation.

4. Role of active proteasome in the regulation of cyclin B degradation

In vitro digestion of E. coli-produced goldfish cyclin B by the active proteasome

It has been proposed that active proteasome (26 S proteasome) containing a 20 S latent proteasome is an ubiquitin-dependent protease in eukaryotic cells. However, active proteasome has also been reported to mediate an ATP-dependent and ubiquitin-independent proteolysis [Tanaka et al, 1983; Matthews et al., 1989]. In fact, ornithine decarboxylase, a key enzyme in polyamine biosynthesis, was shown to be degraded by 26 S proteasome without ubiquitination [Murakami et al., 1992]. In the next series of experiments, I examined whether the active proteasome is involved in the process of cyclin B degradation.

To this end, the wild type cyclin B (Δ0) and three kinds of N-terminal truncated cyclins were used. These truncated cyclins incude cyclin B Δ41 missing the 41 N-terminal amino acids including consensus three amino acids in the destruction box, cyclin B Δ68 missing the 68 N-terminal amino acids with a half of the lysine-rich stretch, and cyclin B Δ96 missing the 96 N-terminal amino acids without both the destruction box and lysine-rich stretch (Fig. 24). Figure 25A shows the results of digestion of goldfish cyclin B Δ0 by purified active proteasome, producing two intermediate proteins which could be detected by the anti-cyclin B antibody (42 and 45 kDa proteins). The major 42 kDa intermediate (band 3 in Fig. 25) was found to be specific to active proteasome. In contrast, the minor 45 kDa intermediate (band 2 in Fig. 25) was not specific to active proteasome, since this protein also appeared after incubation with latent proteasome. Therefore, it is most likely that the minor intermediate was produced by latent proteasome which was derived from the conversion of active proteasome during incubation.

In the next series of experiments, I focused my attention upon the 42 kDa intermediate cyclin B, since active proteasome was thought to be a form of proteasome in the physiological state. I first determined whether active proteasome-induced cyclin B degradation occurs in the N-terminal or C-terminal region of the cyclin B molecule. To this end, two kinds of anti-cyclin B antibodies were used; the anti-cyclin B63 recognizes the C-terminal of cyclin B, whereas the anti-cyclin B112 recognizes its N-terminal. Immunoblotting with these two antibodies clearly

revealed that the former antibody recognizes the 42 kDa intermediate and the N-terminal deletion mutant of cyclin B. In contrast, the latter did not cross-react with the intermediate, but recognized the cyclin B Δ0 (Fig. 26). The N-terminal deletion mutants of goldfish cyclin B lacking the first 41 (Δ41), 68 (Δ 68), or 96 (Δ 96) amino acids including the destruction box were not digested by purified active proteasome (Fig. 24 and 26). The molecular weight of the intermediate was estimated to be smaller than that of Δ41, but larger than that of Δ68.

The effects of various protease inhibitors on cyclin B degradation *in vitro* were also determined. Antipain and elastatinal, which are known to inhibit trypsin-like enzymes, were found to be very effective in blocking the action of active proteasome on cyclin B degradation. In contrast, neither chymostatin nor pepstatin, which inhibits the chymotrypsin-like and calboxyendopeptidase-like activity, respectively, was ineffective (Fig. 27A). These results suggest that the trypsin-like activity of proteasome may be responsible for cyclin B digestion.

It was also found that the addition of a N-terminus of *Xenopus* cyclin B2 (B2Nt, a gift from Dr. Lohka)[Velden and Lohka, 1993], prevented the active proteasome-induced digestion of goldfish cyclin B in a dose-dependent manner. However, lysozyme, a basic and low molecular weight protein like B2Nt, did not compete this reaction (Fig. 27B). These results suggest that the N-terminus of cyclin interacts specifically with active proteasome.

The 42 kDa intermediate cyclin B was also detected in extracts from goldfish activated eggs. As shown in Figure 28A, cyclin B was degraded within a few minutes after egg activation. In a partially purified and highly concentrated fraction from egg extracts, the intermediate cyclin B was detected 3 min after activation (Fig. 28B). Active proteasome also digested the natural cyclin B/cdc2 kinase complex. As shown in Fig. 29, cyclin B which was included within the active MPF complex (precipitated with Suc-1 beads) prepared from carp unfertilized oocyte extracts was digested by goldfish active proteasome, giving an intermediate protein similar to that seen in the case of the goldfish recombinant cyclin B. These results suggest that digestion by active proteasome is an initial reaction of *in vivo* cyclin B degradation.

The next experiment was designed to determine the site of cyclin B which is cleaved by active proteasome. For this purpose, a large scale purification of cyclin B was conducted using a

preparative SDS-PAGE system (Prep Cell Model 491), followed by a long term digestion with active proteasome. Although purified cyclin B was completely digested after this treatment (Fig. 30), I cound not purify the intermediate cyclin B from the reaction mixture even using either either liquid chromatography or reverse-phased chromatrography. Nethertheless, the use of SDS-PAGE (Multi gel 12.5, Daiichi Pure Chemicals) mede it possible to separate the band of the intermediate cyclin B from those of active proteasome subunits (Fig. 30A). The N-terminal amino acid sequence of the intermediate was determined directly by excision of the electro-blotted band. Amino acid sequence anylysis revealed that the site of cyclin B cleaved by active proteasome is the C-terminal peptide bond of lysine 57 (Fig. 30B). These results are consistent with those of the inhibitor experiments, suggesting that the trypsin-like activity of the proteasome is responsible for the digestion of cyclin B.

Degradation of E. coli-produced goldfish cyclin B in Xenopus cycling extracts

Next, I examined whether *E. coli*-produced goldfish cyclin B can be degraded in a *Xenopus* cycling extract, a cell-free system widely used for the studies of cell cycle, which contains a complete system necessary for cyclin B degradation. It is well known that cell-free extracts of unfertilized *Xenopus* eggs, which are naturally arrested in metaphase of meiosis II, retain many of the properties of intact metaphase cells. The extracts possess high MPF activity (protein kinase activity of the $p34^{cdc2}$/cyclin B), and can induce nuclear envelope disassembly and chromosome condensation both when added to the cell-free system and injected into oocytes. The addition of low amounts of Ca^{2+} to the extracts causes the cyclin B degradation and loss of histone H1 kinase activity, leading to the release of metaphase arrest. To confirm the results obtained hypothesis from *in vitro* studies, degradation of goldfish cyclin B in extracts of *Xenopus* eggs was examined. As shown previously, egg extracts prepared according to the procedure of Marray et al. [1990] exhibited a cyclic change in H1 kinase activity after addition of Ca^{2+} (Fig. 31A). Full-length goldfish cyclin B (cyclin B Δ0) was completely degraded within 30 min after addition of Ca^{2+}, but was not degraded in none-activated extracts (Fig. 31B). However, N-terminus deleted cyclins (cyclin B Δ41 and Δ68) were not degraded or degraded at a slow rate

(Fig. 32). The degradation of cyclin B Δ0 was also prevented by antipain (Fig. 33). These results indicate that the behavior of goldfish cyclin B in the *Xenopus* egg extracts was the same as that seen under *in vitro* conditions. Finally, the degradation of cyclin B was prevented by immuno-precipitation by an affinity purified anti-active proteasome polyclonal antibody. As in Figure 34A, this polyclonal antibody could precipitate more than half of *Xenopus* proteasome from highly concentrated egg extracts. In control-IgG treated extracts, goldfish cyclin B was still degraded after Ca^{2+} addition, whereas only a small amount of degradation was observed in anti-IgG treated extracts (Fig. 34B).

Purification and characterization of ubiquitin

Ubiquitin is an 8,600 dalton heat stable protein widely distributed in eukaryotic cells and its amino acid sequence exhibits a high degree of evolutionary conservation [Gavilanes et al., 1982; Goldstein, 1975; Ozkaynak et al., 1984; Schlesinger et al., 1975; Watoson et al., 1978]. Within the cells ubiquitin occurs either free or as a covalent adduct to various target proteins [Ciechanover et al., 1984], with the linkage occurring between the carboxyl terminal of ubiquitin and the lysyl ε-amino groups of the target protein [Hershko et al., 1980]. Although there are numerous studies describing the mechanisms of ubiquitin conjugate formation, the physiological role of this post-ribosomal modification is still unclear. A widely accepted candidate for the role of ubiquitin conjugation is ubiquitin-dependent proteolysis. In the cytoplasm, ubiquitin conjugation marks the target proteins for degradation by the ubiquitin-dependent proteolytic system [Haas and Rose, 1985].

In this study, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was used to assay ubiquitin, since ubiquitin is known to migrate as a 5.5 kDa band [Haas and Wilkinson, 1985]; bovine ubiquitin was used as a standard. Immunoblotting using an anti-ubiquitin antibody was also used for assaying ubiquitin. Ubiquitin was purified from the 150,000 xg supernatant prepared from goldfish ovaries containing full-grown postvitellogenic oocytes. Four steps of

column chromatography (DEAE-cellulose, CM-cellulose, Bio Gel P-30 and Mono-S) were employed. On Bio Gel P-30 gel chromatography, ubiquitin was eluted at the position as the free form (Fig. 35A). In the final step of purification on Mono-S column chromatography, goldfish ubiquitin was eluted at 0.15 M NaCl with a symmetrical peak (Fig. 35B). By this procedure, 1.2 mg of ubiquitin was purified from 80 g goldfish ovary. Purified ubiquitin gave a single band with a molecular weight of 5.5 kDa (Fig. 36A). This 5.5 kDa band reacted with the anti-bovine ubiquitin antibody (Fig. 36B).

The primary structure of ubiquitin has been reported in bovine [Schlesinger et al., 1975], human [Goldstein, 1975], trout [Watson et al., 1978], insect [Gavilanes et al., 1982] and yeast [Ozkaynak et al., 1984]. The amino acid sequence of these ubiquitins is completely identical, except for that of yeast ubiquitin which differs in only three of 76 residues from that of the animal ubiquitins. I determined the first 40 amino acid residues of the N-terminal sequence of the purified goldfish ubiquitin. To this end the purified ubiquitin was further purified by a reverse-phased HPLC. As shown in Fig. 37, the N-terminal 40 amino acid sequence of goldfish ubiquitin is identical with that of the animal ubiquitins and differs in only three amino acid residues from that of yeast ubiquitin.

DISCUSSION

1. Purification and characterization of oocyte proteasomes

In most cases, proteasomes have been isolated as latent forms; these inactive forms can be activated by several agents such as SDS, poly-lysine and fatty acids [Orlowski, 1990]. In the present study, chymotrypsin-like activity of goldfish latent proteasome was markedly activated by a low concentration of SDS. Furthermore, it was found that purified goldfish latent proteasome showed hydrolyzing activity only in the presence of SDS. These results are consistent with the view that proteasomes are present in a latent state in cells.

In the present study, I used a fluorogenic peptide substrate, Suc-LLVY-MCA, to assay hydrolyzing activity during the purification of latent proteasome from the 150,000 g supernatant of goldfish oocytes. The four steps of chromatography employed resulted in a 135-fold purification of latent proteasome. The structural and enzymatical properties of purified latent proteasome are as follows: 1) the enzyme is composed of several protein subunits with molecular masses ranging 23.5-31.5 kDa; 2) the molecular weight and sedimentation coefficient of the enzyme are estimated to be 620 kDa and 19.2 S, respectively; 3) the enzyme shows chymotrypsin-like, trypsin-like and *S. aureus* V8 protease-like activities; and 4) the enzyme appears to be a ring-shaped particle with a small central hole and cylindrical structure. It is concluded from these properties that the purified goldfish latent proteasome corresponds to 20 S proteasomes previously purified from a variety of eukaryotic cells [Orlowski, 1990].

The demonstration that the 150,000 g supernatant possesses a high Suc-LLVY-MCA hydrolyzing activity in the absence of SDS is of great importance. This form of proteasome possessed three distinct protease activities (chymotrypsin-like, trypsin-like and V8 protease-like activities) even in the absence of SDS. In fact, we could not detect any latent (SDS-dependent) proteasome in freshly prepared goldfish oocyte cytosol preparations. Immunoprecipitation experiments also confirmed the existence of a high SDS-independent proteasome activity in the oocyte cytosol. Similarly, I have also shown that the cytosol fractions (150,000 g supernatant) of *Xenopus laevis* and *Bufo japonicus* oocytes possess a high Suc-LLVY-MCA activity in the

absence of SDS [Tokumoto and Ishikawa, 1993a; M. Takahashi et al., 1993]. I thus consider these proteasomes present in the oocyte cytosol to be "active" proteasomes. As far as I aware, this is the first demonstration of active (SDS-independent) proteasome in cells under physiological conditions.

In this study, the SDS-independent proteasome activity was lost during purification by a series of chromatographic steps, especially during a linear gradient elution. Thus, it seems most likely that the loss of the activity is caused by the instability of this enzyme. In this respect, it is of great interest to note that cytosol fractions containing the SDS-independent proteasome activity had a band which migrated slower than that of the purified latent proteasome on native PAGE. During glycerol density gradient centrifugation, active proteasome migrated faster than latent proteasome, indicating that the molecular weight of active proteasome is larger than that of latent proteasome. Furthermore, the SDS-independent proteasome activity was removed by immunoadsorption with the antibody-Sepharose. Thus, it is conceivable that active proteasome was converted to the latent form during purification, in accord with an earlier finding that 20 S proteasome assembles to form 26 S proteolytic complex [Driscoll et al., 1990; Armon et al., 1990]. These results, together with the accepted view that the 26 S proteolytic complex is active in the absence of SDS, suggest that the active proteasome present in the goldfish oocyte cytosol is closely related to the 26 S proteolytic complex reported from other sources [Drriscoll and Goldberg, 1990; Armon et al., 1990; Kanayama et al., 1992].

I have shown that the 150,000 g supernatant of goldfish ovarian homogenates exhibits high hydrolyzing activities in the absence of SDS. In this stidy, a method to stabilize active proteasome for further purification was depeloped. This method was based on the use of a step elution and ATP. Using this method, I was able to purify the active proteasome to homogeneity. The properties (subunit composition, structure, molecular weight and enzymatic activity) of active proteasome purified in this study is identical to, or closely related to those of the 26 S proteolytic complex reported from rabbit reticulocyte [Haas et al., 1990; Driscol and Goldberg, 1990; Armon, 1990] and human kidney [Kanayama et al., 1992].

Goldfish active proteasome purified in this study exhibited two bands (lower and higher) on

native PAGE. It was shown on SDS-PAGE that the lower band contained all of the components of this complex except for the 140 kDa band. The higher band possessed subunits with molecular weights ranging from 35.5 to 110 kDa, which overlapped with those of the lower band, as well as the unique 140 kDa band. The subunit composition of the higher band is closely related to the subunit pattern of the ball structure reported for rabbit reticulocyte lysate [Hoffman et al., 1992]. The ball structure is associated with 20 S proteasome and other proteins to form the 26 S proteolytic complex and is thought to be the major part of the terminal domain of active proteasome. Thus, it seems likely that the higher band appearing on native PAGE is a terminal domain of 26 S proteasome or a particle structure observed in electron micrographs. Thus, it is possible that during electrophoresis, the cylindrical dumbbell structure (the 26 S proteolytic complex) was cleaved into the complexes with only one terminal domain (the lower band on native PAGE) and particles (the higher band). This is supported by the fact that neither of these two bands showed Suc-LLVY-MCA hydrolyzing activity in the absence of SDS. Although the reason why only one side was cleaved is unclear, it is possible that this was caused by the structural asymmetry due to the presence of the unique 140 kDa component in the higher band.

Immunoblot analyses using monoclonal antibodies against the latent goldfish proteasome revealed that the cytosol fraction of goldfish oocytes exhibited only one band which corresponded to the lower band of the active proteasome. There was no band which corresponded to that of the latent proteasome (Figs. 10 and 16). These findings are consistent with the above finding that goldfish oocytes mainly contain active proteasome, but not free, latent (20S) proteasome.

It has been shown that ATP is required for the formation and functioning of 26S proteolitic complex [Driacol and Goldberg, 1990; Armon et al., 1990; Kanayama et al., 1992] . ATP was also reported to promote the degradation of abnomal proteins and ^{125}I-lysozyme-Ub conjugates in reticulocyte lysate [Etlinger and Goldberg, 1977; Hershko et al., 1984]. Furthermore, ATP enhanced the Suc-LLVY-MCA hydrolyzing activity in K562 cell lysate [Tsukahara et al., 1988]. In contrast, in the present study there was a marked reduction of the chymotrypsin-like activity of active proteasome in the presence of higher concentations of ATP. Similar observations were

reported in *Xenopus* oocytes [Tokumoto and Ishikawa, 1993a]. These inhibitory effects of ATP may be specific to the oocytes. However, more recently it was shown that an inhibitor which supresses the hydrolyzing activity of 20S proteasome is a component of 26S proteasome complex [Driscoll et al., 1992]. Thus, it is possible that the inhibitory effect of ATP on oocyte proteasomes is due to the presence of this inhibitor. If so, the inhibitor is inactive under the condition of a low concentration of ATP (0.1 mM), but can be activated in the presence of higher concentrations of ATP (1 mM). I have shown that proteasomes in fish and frog oocytes consist mainly of 26S proteasome. Thus, it seems likely that proteasome activity in oocytes is not due to the conversion of 20S protesome to 26S proteasome, but is rather related to a ATP-dependent mechanism.

The purified active proteasome possessed various peptide hydrolyzing activities. However, the sensitivity of these activities to various inhibitors differed. It seems likely that the activities were catalyzed by different catalytic sites as previously described for the 20S proteasome [Tanaka et al., 1988]. Although the purified 20 S proteasome showed only a weak activity toward most of the peptides in the absence of the activator, the activity toward Z-GGL-βNA was the same as that of the active proteasome. It has been reported previously that mammalian and *Xenopus* 20 S proteasomes could not hydrolyze the C-terminal of Tyr and Arg residues but hydrolyzed the C-terminal of Leu when oxidized insulin-B chain was used as a substrate [Rivett, 1985; Dick et al., 1991; T. Takahashi et al., 1993]. These results suggest that 20 S proteasomes possess higher activity against a Leu-containing sequence.

2. The role of oocyte proteasomes in the regulation of meiotic maturation and fertilization.

This study demonstrates, for the first time, the changes in the activity and protein levels of proteasomes during oocyte maturation and egg activation. Both the activity and protein levels exhibited a similar pattern of fluctuation during the 17α,20β-DP-induced oocyte maturation and egg activation in goldfish. These results suggest that the activity of proteasome is dependent on the amount of the protein. There were two peaks of the activity and protein levels of proteasome during oocyte maturation: the first peak immediately prior to the migration of germinal vesicle and the second one just after the completion of germinal vesicle breakdown. At present there is no experimental data to explain the functional significance of the first peak. However, some discussion could be made on the second peak. The time of the second peak appears to correspond to the time of the first polar body extrusion (between the first and second meiotic metaphase). It is also of great interest to note that the time of the increase in the activity and protein levels after egg activation corresponds to the time of the transition from metaphase to anaphase. At present less is known about exit from mitosis than entry into mitosis. In *Xenopus* oocytes, it was found that MPF activity decreased rapidly after fertilization (Murray et al., 1987). More recently, it has been suggested that this rapid inactivation of MPF is induced by the degradation of cyclin B, the regulatory subunit of MPF [Glotzer et al., 1990; Kobayashi et al., 1991; Maller, 1991]. In fact, goldfish oocytes H1 histone kinase (MPF) activity decreased transiently when the first polar body was eliminated [Yamashita et al., 1992a]. Thus, it is possible that proteasomes are responsible for the decrease in the MPF activity, probably by inducing the degradation of the regulatory subunit of MPF, cyclin B.

Immediately prior to the transition from metaphase to anaphase the protein kinase activity of MPF is inactivated by a mechanism that involves the degradation of the cyclin B subunit (Nurse, 1990; Murray et al., 1990). This was confirmed by the present study using goldfish oocytes. The mechanism of cyclin degradation, which must be a highly selective process since few other proteins are degraded only at this time, is only poorly understood. In this study, the availability of *E. coli*-produced goldfish cyclin B and purified goldfish active proteasome has

made it possible for the first time in any animal system to test the role of proteasomes in the regulation of cyclin degradation during egg activation. It was shown clearly in this study that purified goldfish active proteasome can degrade not only bacterially produced goldfish cyclin B, but also native cyclin B. These results were complemented with *Xenopus* cycling extracts. Finally, the immunodepletion of proteasomes from the *Xenopus* extracts prevented the degradation of goldfish cyclin B. Recently, I also purified and characterized both latent [Tokumoto and Ishikawa, 1993b] and active proteasomes [Tokumoto et al., in preparation] from *Xenopus* oocyte extracts, and used these proteasomes to test whether *Xenopus* proteasomes can digest goldfish cyclin B. The results clearly indicate that *Xenopus* active proteasome is able to digest goldfish cyclin B (data not shown), suggesting a similar role of goldfish and *Xenopus* proteasomes in the regulation of cyclin degradation.

It has been suggested that the N-terminal sequences of cyclin B, including a consensus sequence that is called the destruction box, have been shown to play a critical role in targeting cyclins for degradation, since truncated sea urchin (Marray et al., 1989), human (Lorca et al., 1991) and clam [Luca et al., 1991] B-type cyclins missing the first 90, 72 or 97 amino acids, respectively, and clam [Luca et al., 1991] and *Xenopus* (Kobayashi et al., 1992) A-type cyclins missing the N-terminal 60 or 62 amino acids are resistant to degradation. Each of these truncated cyclins continuously activates $p34^{cdc2}$, which prevents cells or cellular extracts from leaving mitosis. A truncated protein containing only the first 89 amino acids of *Xenopus* cylcin B2 (B2Nt), including sequences known to be essential for cyclin degradation in other species, also inhibited cyclin degradation, even though the trancated protein was stable in extracts following Ca^{2+} addition [Velden and Lohka, 1993].

The results of the present study using various goldfish N-terminus cyclin B mutants confirm the importance of the N-terminal amino acids for cyclin B to be degrated by active proteasome. The present finding that active proteasome first digests the C-terminal peptide bond of Lys 57 further confirms the important role of the N-terminal sequence of cyclin B. However, it is important to note that the cyclin B Δ41 lacking the first 41 N-terminal amino acids was not digested by purified active proteasome. These results are consistent with the suggestion that the

destruction box known to be essential for cyclin degradation in other species is also necessary for cyclin B degration in goldfish eggs.

It was found in this study that goldfish cyclin B was completely degraded in *Xenopus* cycling extracts and no intermediate cyclins were detected. Mechanisms of further degradation of cyclin B after the first cutting by active proteasome remain to be determined. The most likely candidate will be ubiquitination of cyclin B, because goldfish cyclin B Δ41 and Δ68 mutants were not digested either *in vitro* or in *Xenopus* cycling extracts. In an earlier study, it was shown that proteins to be degraded by the ubiquitin pathway are ligated to ubiquitin through their lysine amino acid groups and then degraded by the 26 S proteolytic complex [Hershko and Ciechanover, 1982]. More recently, it has been suggested that the degradation of cyclin B is induced by the ubiquitin-dependent proteolysis [Glotzer et al., 1990]. Other support for the involvement of a ubiquitin-dependent pathway in the cyclin degradation comes from observation that methylated ubiquitin, which prevents the polyubiquitination of proteins destined for degradation, delays cyclin degradation in an extract from clam embryos [Hershko et al., 1991]. These results, taken together with the findings of the present study, suggest that the cell-cycle specific cyclin degradation is mediated by an ubiquitin-dependent proteolytic system and the N-terminus of cyclin is a recognition site for ubiquitination. The present study also indicates that a large amount of ubiquitin occurs as a free polypeptide in immature oocytes of goldfish [Tokumoto et al., 1993b]. I have also shown that approximately the same amount of proteasome as ubiquitin is present in immature oocytes of goldfish (Table 1 and Table 4, see also the result section, p. 30). Thus, these results are consistent with the notion that both proteasome and ubiquitin are involved in cyclin B degradation during meiotic cell cycle of goldfish oocytes, which in turn leads to the inactivation of MPF.

In conclusion, this study provides the first evidence that proteasomes play an important role in triggering cyclin B degradation, the event which is crucial in exiting metaphase and entering the next interphase during the cell cycle. It was also suggested that the first cut of cyclin B by proteasome is a crucial step for cyclin B to be recognized by the ubiquitin-conjugating system. An understanding of this recognition step may be required for a complete understanding

of the mechanisms regulating cyclin B degradation that leads to the completion of mitosis and meiosis.

SUMMARY

Evidence is now accumulating to suggest that proteolysis plays an essential role in the regulation of the eukaryotic cell cycle. In this study, the structure of proteasomes, a nonlysosomal large protease, and their role in the regulation of oocyte maturation and egg activation were investigated using the goldfish (*Carassius auratus*) as an experimental animal. The results obtained in this study are as follows.

(1) Latent proteasome was purified and characterized from cytosol fractions (150,000 g supernatants) of goldfish ovaries using four steps of column chromatography. The cytosol hydrolyzed a fluorogenic peptide, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide, a well-known substrate for proteasome, regardless of the addition of SDS to the reaction mixture. The purified latent proteasome possessed weak hydrolyzing activity (chymotrypsin-, trypsin-, and V8 protease-like activities) even in the absence of SDS; the chymotrypsin- and V8 protease-like activities were significantly increased in the presence of SDS. Its molecular weight and sedimentation coefficient were estimated to be 620 kDa and 19.2 S, respectively. Three kinds of monoclonal antibodies were raised against the purified latent proteasome. Western blot analyses revealed that these antibodies recognized a single species of protein on native PAGE, but recognized several subunits ranging in molecular mass from 23.5 to 31.5 kDa on SDS-PAGE. Cytosol fractions containing the SDS-independent activity had a band which migrated slower than that of purified latent proteasome. The SDS-independent protease activity was depleted when the cytosol fraction was immunoprecipitated with the anti-proteasome antibody. From these structural and enzymatical properties it is concluded that the purified latent proteasome corresponds to 20 S proteasomes reported in other eukaryotic cells.

(2) With the use of a newly developed purification procedure involving five steps of chromatography, active proteasome was purified to homogeneity from the ovarian supernatants. The purified active proteasome had chymotrypsin-like, trypsin-like, and V8 protease-like activities even in the absence of SDS. The enzyme exhibited two bands on native PAGE. Electrophoresis and Western blot analyses showed that the enzyme consisted of at least 15

protein components ranging in molecular mass from 35.5 to 140 kDa, as well as the multiple subunits of the latent proteasome (20 S proteasome) ranging in molecular mass from 23.5 to 31.5 kDa. The molecular weight and sedimentation coefficient of the active proteasome were estimated to be 1,200 kDa and 29.4 S, respectively, both of which are larger than those of the latent proteasome of the same species. In electron micrographs, the active proteasome appeared as a dumbbell-like image. It is concluded that the active proteasome purified from goldfish oocyte cytosol is identical to the 26 S proteolytic complex reported in human and rabbit.

(3) Using the monoclonal antibodies against the goldfish proteasome, protein levels of proteasome during oocyte maturation and egg activation were determined. Proteasome activity was also measured using the fluorogenic peptide. During oocyte maturation, the activity and protein levels of proteasome changed synchronously with two peaks, one prior to the migration of germinal vesicle and the other just after the completion of germinal vesicle breakdown. Upon egg activation, the activity increased two-fold then significantly decreased after cyclin degradation occurred. Protein levels of proteasome components detected by antibodies also decreased as the activity declined, except for one component that weakly cross-reacts with anti-GC4/5. These results suggest that proteasome is involved in oocyte maturation and egg activation.

(4) The possible role of active proteasome in the regulation of cyclin B degradation was investigated, for the first time, using *E. coli* produced goldfish cyclin B and purified goldfish active proteasome. It was found that active proteasome can digest the wild type cyclin B (cyclin B Δ0), producing an intermediate cyclin B (42 kDa). In contrast, cyclin B mutants lacking the first 42, 68, and 96 N-terminal amino acids (cyclin B Δ42, Δ68, and Δ96, respectively) were not digested by active proteasome, suggesting that the N-terminal amino acids are necessary for cyclin B degradation. Amino acid sequence analysis of the 42 kDa intermediate protein revealed that active proteasome cuts the C-terminal peptide bond of lysine 57. Experiments using various protease inhibitors suggest that trypsin-like activity may be responsible for cyclin B degradation. Full-length goldfish cyclin B was also degraded in *Xenopus* egg extracts after activation by the addition of Ca^{2+}. Taken together, these results provide the first evidence to indicate that

proteasomes are involved in the regulation of cyclin B degradation.

(5) Ubiquitin was purified from the cytosol fraction (150,000 g supernatant) of goldfish ovaries containing full-grown postvitellogenic oocytes using four steps of column chromatography. The purified goldfish ubiquitin gave a single band with a molecular weight of 5.5 kDa on denaturing polyacrylamide gel electrophoresis and reacted with an anti-bovine ubiquitin antibody on Western blot. The first 40 amino acid residues of the N-terminal sequence of goldfish ubiquitin are identical with those of ubiquitins in other higher eukaryotes. These results indicate that ubiquitin exists and occurs as a free polypeptide in immature oocytes of goldfish.

(6) Figure 38 illustrates a current hypothetical model for the possible participation of proteasomes in the regulation of cyclin B degradation. This scheme is derived from data produced using not only goldfish but also other species. Upon fertilization (egg activation), an increase in Ca^{2+} activates a Ca^{2+}/calmodulin-dependent protein kinase [Lorca et al., 1993]. This serine/threonine kinase either directly activates proteasome or makes cyclin B a better substrate for the proteasome through an unknown mechanism. Proteasome then cuts the N-terminus (Lys 57) of cyclin B and exposes an ubiquinating site of cyclin B, thus enabling cyclin B to interact with ubiquitinating enzymes. After ubiquitination, cyclin B-ubiquitin complexes are degraded by active proteasome to small peptides, leading to the inactivation of MPF.

ACKNOWLEDGMENTS

I am very grateful for the guidance, support and encouragement of my supervisor, Professor Yoshitaka Nagahama, throughout my graduate research. I also thank my graduate committee: Professor Goro Eguchi, Professor Yoshiaki Suzuki, and Professor Masaharu Noda, for their advice. I wish to thank Professor Katsutoshi Ishikawa, Sizuoka University, for his stimulating discussions and constant encouragement, Dr. M. Yamashita, Dr. M. Yoshikuni, Dr. M. Tanaka and Dr. T. Kobayashi for their invariable advices throughout this study, Miss H. Kajiura for her technical assistance in amino acid sequence, Mrs. Y. Kabeya for her technical assistance in amino acid analysis, and Mr. H. Maebashi for his technical advice in electron microscope studies. My thanks are extended to the staff and students of the Laboratory of Reproductive Biology.

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TABLES AND FIGURES

Table 1. Purification of proteasome from goldfish ovary. Activity of pooled fractions were determined by Suc-LLVY-MCA hydrolyzing activity in the presence of optimal concentration SDS for each fraction.

	Protein (mg)	Total activity (μ mol AMC / min)	Specific activity (μ mol AMC / min / mg)	-fold	Yield (%)
Cytosol	784.0	235.0	0.30	1.0	100.0
DEAE-cellulose	176.4	100.0	0.57	1.9	42.6
Sepharose-CL-4B	19.5	120.1	6.16	20.5	51.1
Mono-Q	1.9	65.1	34.26	114.2	27.7
Phenyl-Sepharose	1.0	40.6	40.60	135.3	17.3

Table 2. Amino acid composition of the proteasomes

	Latent(20S)	Active(26S)
	mol %	
Asn or Asp	9.8	11.3
Gln or Glu	10.6	11.5
Arg	4.7	4.1
Lys	4.4	5.6
His	1.4	1.6
Ala	8.1	8.1
Gly	6.7	6.8
Leu	7.4	9.4
Ile	4.8	5.2
Val	5.9	6.6
Phe	2.4	2.7
Tyr	5.6	4.8
Trp	3.7	1.0
Ser	10.2	6.4
Thr	7.9	6.8
Half-Cys	N.D.	N.D.
Met	1.9	2.4
Pro	4.5	5.7

Table 3. Substrate specificity of proteasomes in the cytosol fraction and purified proteasome. Activities toward fluorogenic peptides are given in arbitrary units with values for Suc-LLVY-MCA hydrolyzing activity in the presence of SDS set at 100. Inhibition by immunoprecipitation was calculated from the remaining activity in the supernatant of control-IgG and anti-IgG, and the deduced proteasome activity in the cytosol fraction was defined from initial activity and %inhibition by immunoprecipitation.

substrate	SDS	cytosol	%inhibition	deduced proteasome activity in cytosol	purified proteasome
LLVY	+	100.0	79.5	100	100
	-	93.5	100	117.6	1.4
AAPF	-	9.0	0	0	0
LRR	-	150.5	51.8	98.1	1.9
FSR	-	42.7	47.4	25.5	0.5
QRR	-	34.5	38.3	16.6	0
LLE	-	10.0	67.6	8.5	1.4

Table 4. Purification of active proteasome from goldfish ovary. Activity of pooled fractions were determined by Suc-LLVY-MCA hydrolyzing activity in the presence and absence of an optimal concentration of SDS.

	Protein (mg)	SDS	Total activity (μ mol AMC / min)	Specific activity (μ mol AMC / min / mg)	-fold	Yeild (%)
Cytosol	1800	-	340.9	0.19	1.0	100
		+	238.1	0.13	1.0	100
DEAE-cellulose	648	-	59.2	0.09	0.5	17.4
		+	84.0	0.13	1.0	35.3
Arginine-Sepharose	36.5	-	32.3	0.88	4.6	9.5
		+	90.7	2.48	19.1	38.1
Sepharose-CL-4B	4.2	-	23.8	5.67	29.8	7.0
		+	55.3	13.17	101.3	23.2
Blue-Sepharose	1.5	-	12.4	8.27	43.5	3.6
		+	21.5	14.33	110.2	9.0
Superose 6	0.23	-	1.9	8.26	43.5	0.6
		+	3.0	13.04	100.3	1.3

Table 5. Protease activity of active proteasome and 20 S proteasome. Activities toward fluorogenic peptides are given in the unit and arbitrary value designated the value for Suc-LLVY-MCA hydrolyzing activtiy in the presence of SDS as 100.

Substrate	SDS	Activity (μ mol/min/mg)		Ralative activity	
		Active	Latent	Active	Latent
Suc-LLVY-MCA	-	6.8	0.30	56.7	1.4
	+	12.0	21.0	100	100
Bz-Y-pNA	-	0	0	0	0
Suc-AAPF-MCA	-	0	0	0	0
F-MCA	-	0	0	0	0
Boc-AGPR-MCA	-	0	0	0	0
Boc-FSR-MCA	-	0	0.1	0	0.5
Boc-LRR-MCA	-	3.4	0.4	28.3	1.9
Boc-QRR-MCA	-	1.6	0	13.3	0
Bz-R-MCA	-	0	0	0	0
Z-LLE-βNA	-	0.6	0.3	5.0	1.4
	+	2.7	1.6	22.5	7.6
E-pNA	-	0	0	0	0
Z-GGL-βNA	-	0.3	0.5	2.5	2.4
L-MCA	-	0.2	0	1.7	0

Table 6. Effect of various inhibitors on protease activity. The activity is given in the arbitrary unit designated the value in the absence of the inhibitors as 100.

	(mM)	LLVY	LRR	LLE	GGL
Control		100	100	100	100
Leupeptin	0.125	89	9	151	106
Antipain	0.125	89	27	128	93
Chymostatin	0.125	36	69	111	131
Pepstatin	0.125	79	96	116	163
Elastatinal	0.125	12	56	111	94
TAME	0.125	89	96	102	86
E64	0.125	115	148	170	59
NEM	2	92	90	153	50
Iodoacetamide	2	107	113	173	53
EDTA	5	60	66	58	55
EGTA	5	76	89	58	59
APMSF	2	8	0	48	78
DFP	0.63	70	92	91	77
	2	58	85	78	37
	10	16	54	65	0
TLCK	0.25	39	37	0	0
TPCK	0.5	66	33	76	96

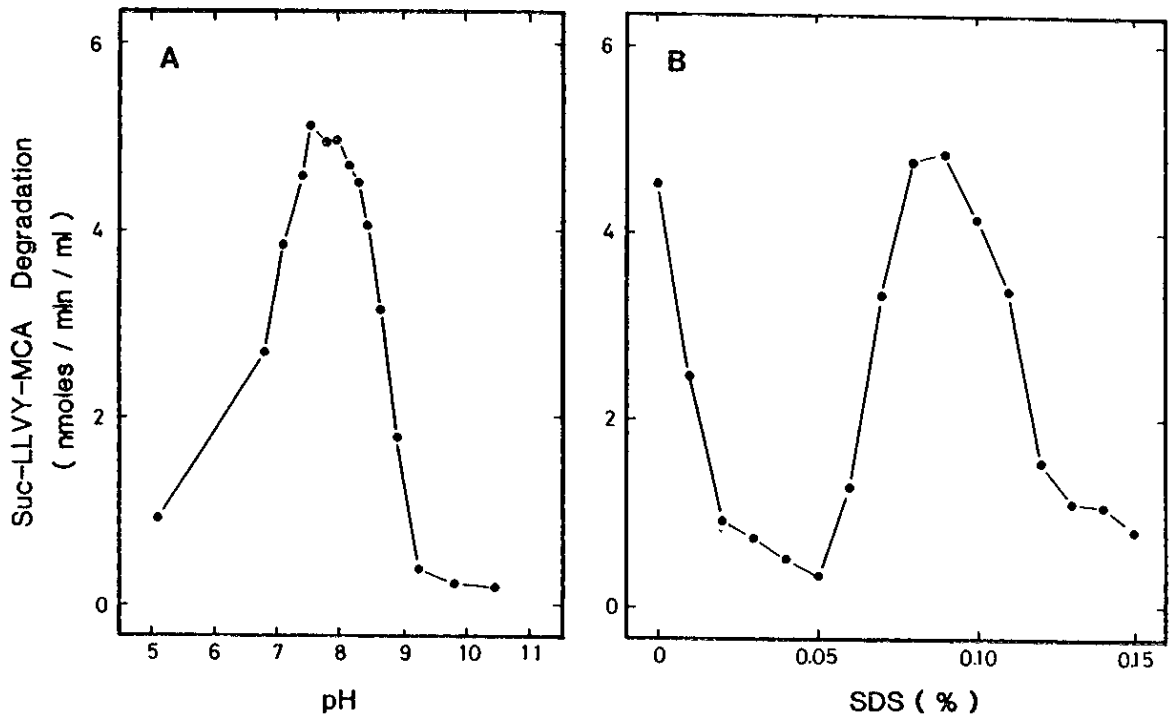


Fig. 1. Effect of pH and SDS on Suc-LLVY-MCA hydrolyzing activity in cytosol fraction.

The Suc-LLVY-MCA hydrolyzing activity of the cytosol fraction was determined at various pH (A) or in the presence of 0 ~ 0.15% of SDS (B).

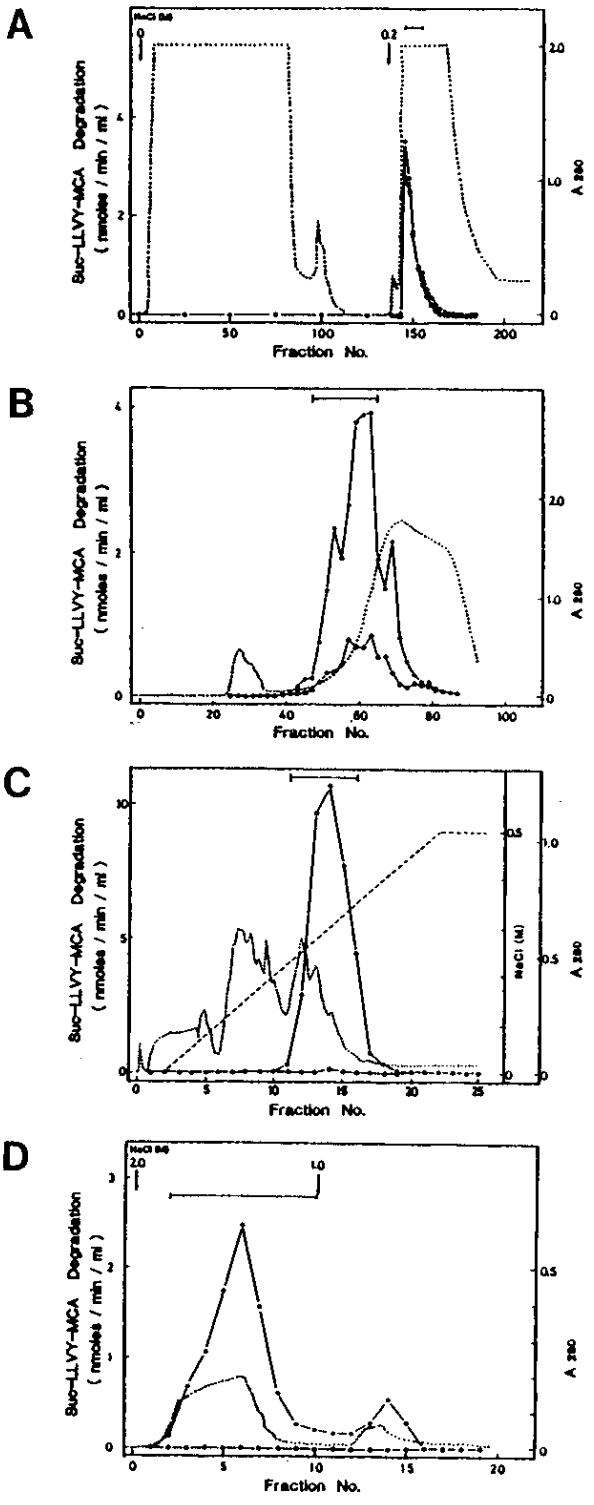
Fig. 2. Purification of latent (20 S) proteasome.

A) DEAE-cellulose column chromatography: cytosol fraction (18 ml) was applied to a DEAE-cellulose column (2.6 x 12.0 cm), and bound proteins were eluted with 0.2 M NaCl in a step-wise gradient.

B) Sepharose CL-4B gel chromatography: active fractions from a DEAE-cellulose column were concentrated to 10 ml and applied to a Sepharose CL-4B column (2.6 x 84.0 cm) equilibrated with TGM Buffer.

C) Mono-Q column chromatography: active fractions from a Sepharose CL-4B gel were pooled and applied to a Mono-Q column (0.5 x 5.0 cm). Proteins were eluted with a linear gradient of NaCl and the 0 ~ 0.5 M eluate was collected in 2 ml fractions.

D) Phenyl Sepharose Fast Flow: buffer of active fractions was exchanged as described in "Materials and Methods" and applied to a Phenyl-Sepharose Fast Flow column (1.0 x 6.4 cm) equilibrated with TN buffer containing 2 M NaCl. In each purification step, Suc-LLVY-MCA hydrolyzing activity was determined using 10 µl of fractions both in absence (○) and presence (●) of 0.04 % of SDS. Elution profile was monitored by absorbance at 280 nm (----). Pooled fractions are indicated by the horizontal line.


A
B
C
D
Suc-LLVY-MCA Degradation
(nmoles / min / ml)
A 280
Fraction No.
NaCl (M)

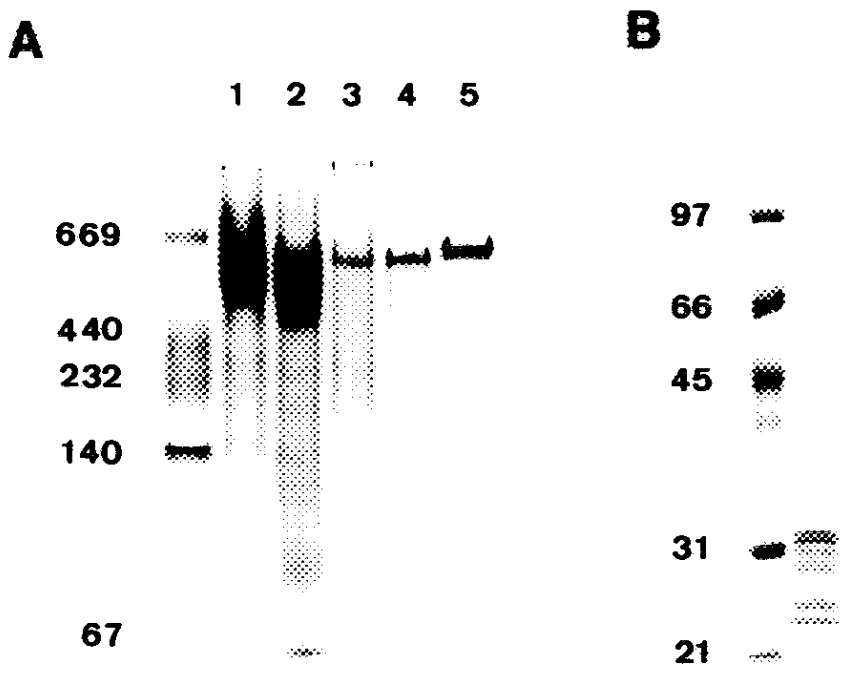


Fig. 3. Polyacrylamide gel electrophoresis of fractions during purification and of purified latent proteasome

A) Fractions during purification were separated on 5% native PAGE and stained with CBBR-250. Lane 1, cytosol; lane 2, DEAE-cellulose; lane 3, Sepharose CL-4B; lane 4, Mono-Q; lane 5; Phenyl-Sepharose Fast Flow (purified proteasome). Molecular weight of standard proteins were indicated at the left.

B) Purified latent proteasome (20 μg) was separated on 12.5 % SDS polyacrylamide gel and stained with CBBR-250. Molecular weight of standard proteins are indicated at the left.

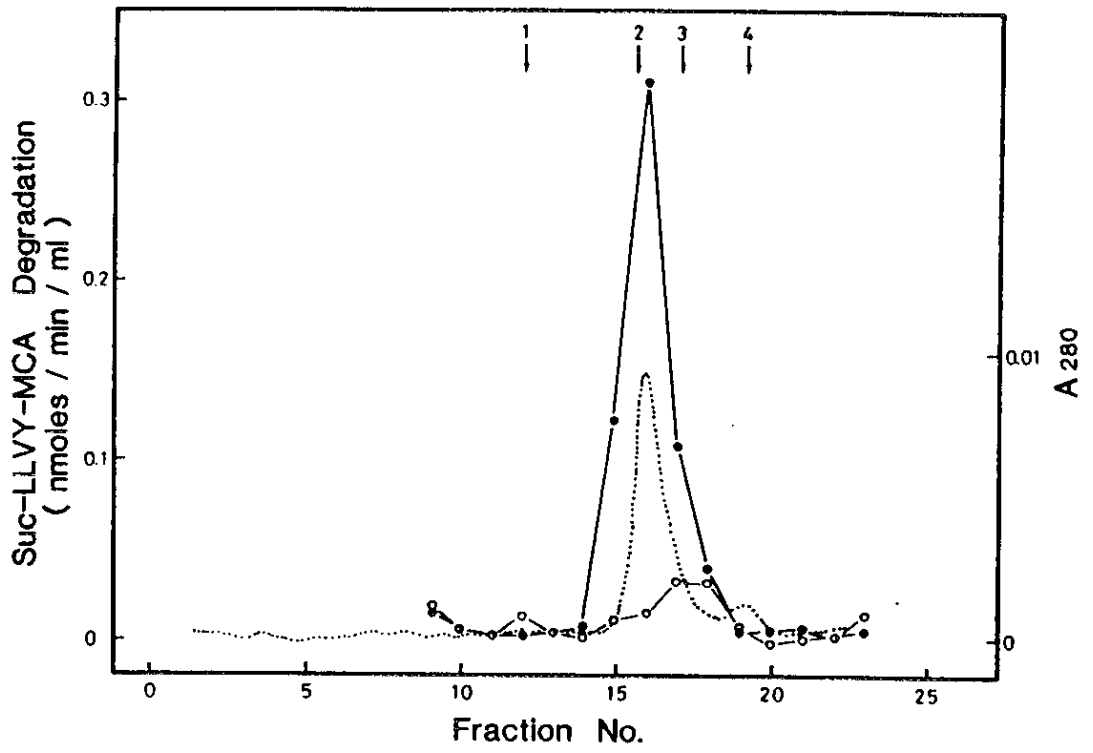


Fig. 4. TSK Gel G4000SWXL chromatography.

The purified enzyme fraction (50 µl) was chromatographed in a TSK Gel G4000SWXL column (0.78 x 30.0 cm) equilibrated with TGM buffer, and fractions of 0.5 ml were collected. Suc-LLVY-MCA hydrolyzing activity was determined using 10 µl of fractions in the absence (○) or presence (●) of 0.04% SDS. Elution profile was monitored by absorbance at 280 nm (----). Arrows indicate the eluted position of molecular weight standards as follow: 1, blue-Dextran 2000; 2, thyrogloblin; 3, ferritin; 4, aldolase.

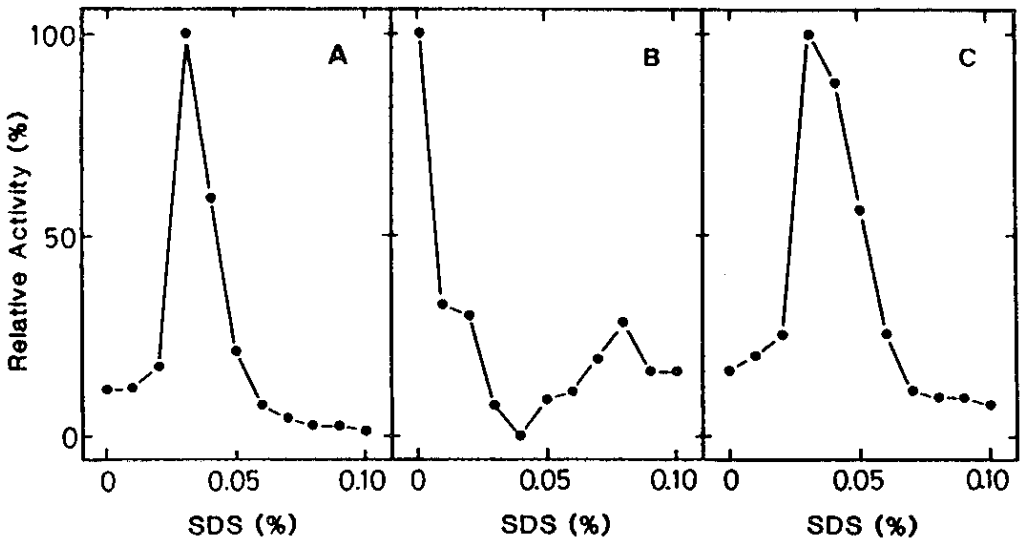


Fig. 5. Effect of SDS on three kinds of protease activity of latent proteasome.

Hydrolyzing activities of Suc-LLVY-MCA (A), Boc-LRR-MCA (B) and Z-LLE-βNA (C) were measured at various concentrations of SDS. Activities are indicated as the percentage of the maximum activity.

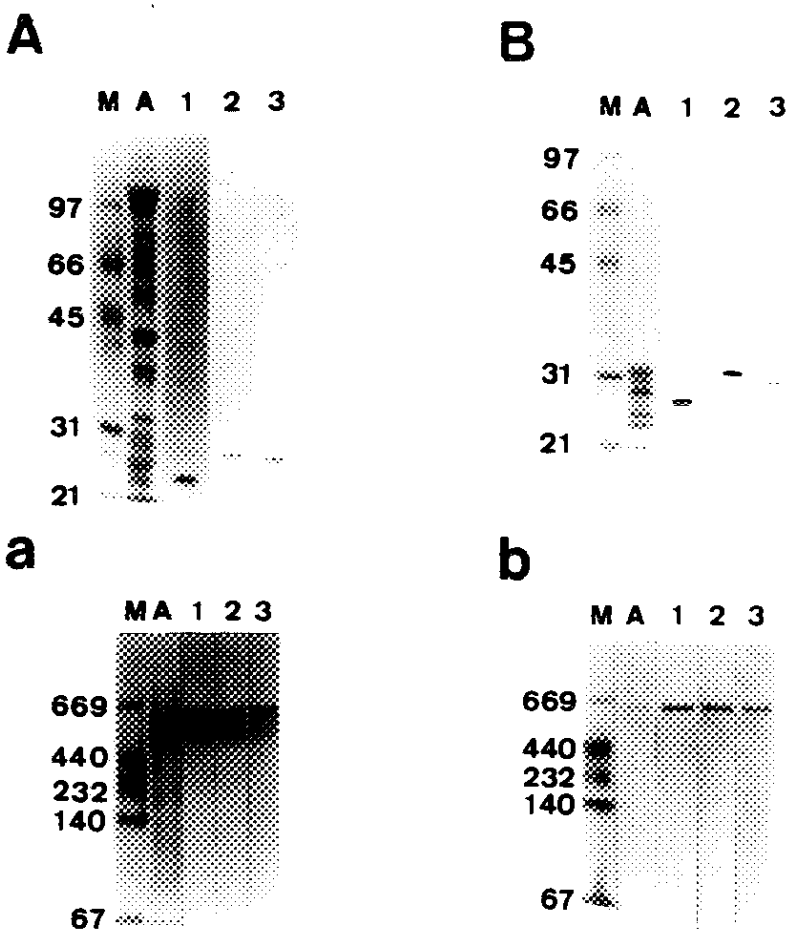


Fig. 6. Immunoblotting of cytosol fraction and purified latent proteasome using anti-goldfish latent proteasome monoclonal antibodies.

Immunoblotting of cytosol fraction (A, a) and purified latent proteasome (B, b). Panels A and B were immunostained after SDS-PAGE and a and b were immunostained after native PAGE. Lanes M and A, amide black staining of standard proteins (M) and sample (A), lanes 1 ~ 3, immunostaining by anti-25k (1), anti-31.5k (2), and anti-30k (3).

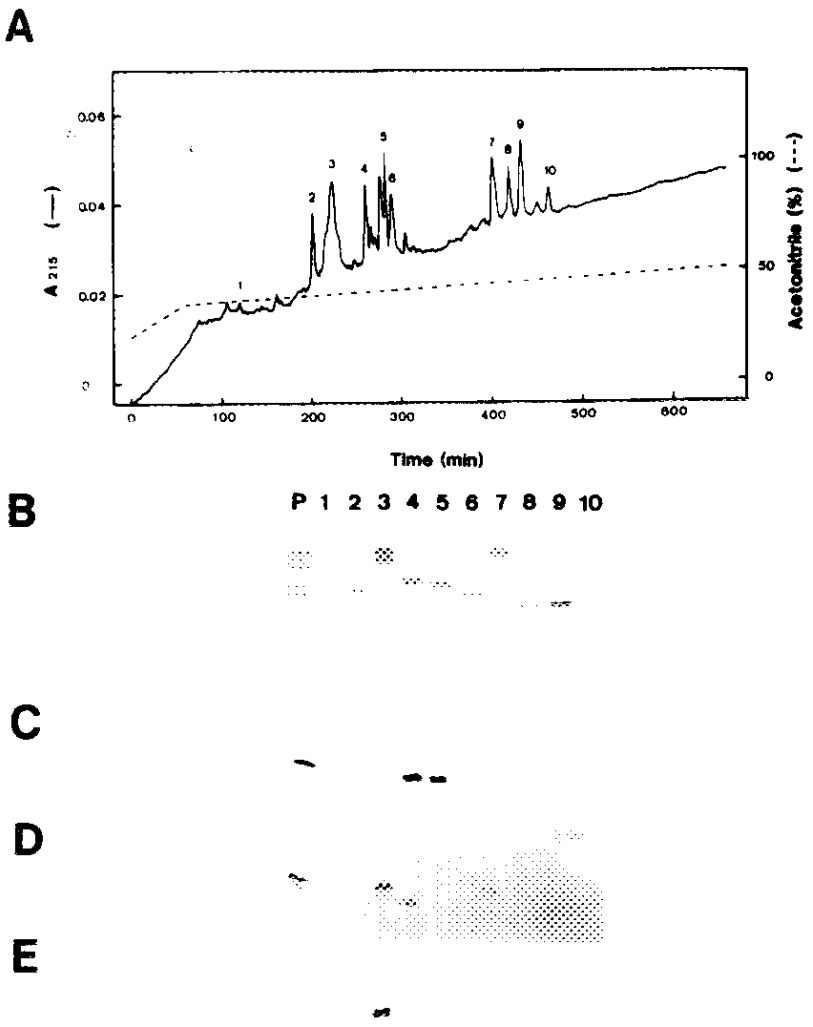


Fig. 7. Separation of components of proteasomes by reversed-phase HPLC and immunoblotting of components.

Purified latent proteasome (1 mg) was dialyzed against 0.05% TFA solution and adsorbed to a Phenyl 5PW-RP column. Proteins were eluted with linear gradient of acetonitrile (35 ~ 50%). The elution profile was monitored by absorbance at 215 nm (A). Peak fractions were lyophilized, subjected to 12.5% SDS-PAGE and stained with CBBR-250 (B) or immunoblotted with three antibodies: anti-25k (C), anti-31.5k (D), and anti-30k (E). Purified proteasome was electroloaded in lane P; and other lanes correspond to the peaks of the elution profile.

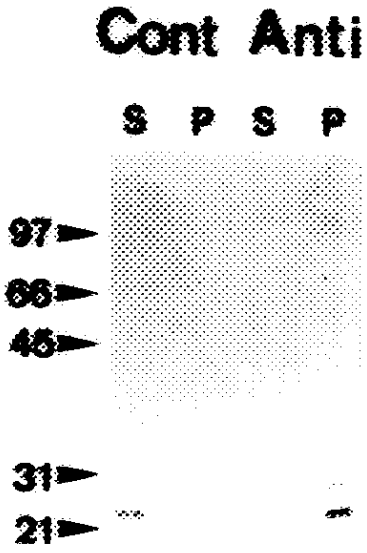


Fig. 8. Immunoprecipitation of proteasome by anti-goldfish latent proteasome. Cytosol from goldfish ovaries (150 µl) was incubated with anti-proteasome-Sepharose or control-IgG-Sepharose for 2 hours at 4°C. Immunocomplexes were precipitated by centrifugation and protease activity in the soluble fraction was determined. Proteins in each fraction were separated on SDS-PAGE and detected by immunoblotting using a mixture of three anti-proteasome antibodies.

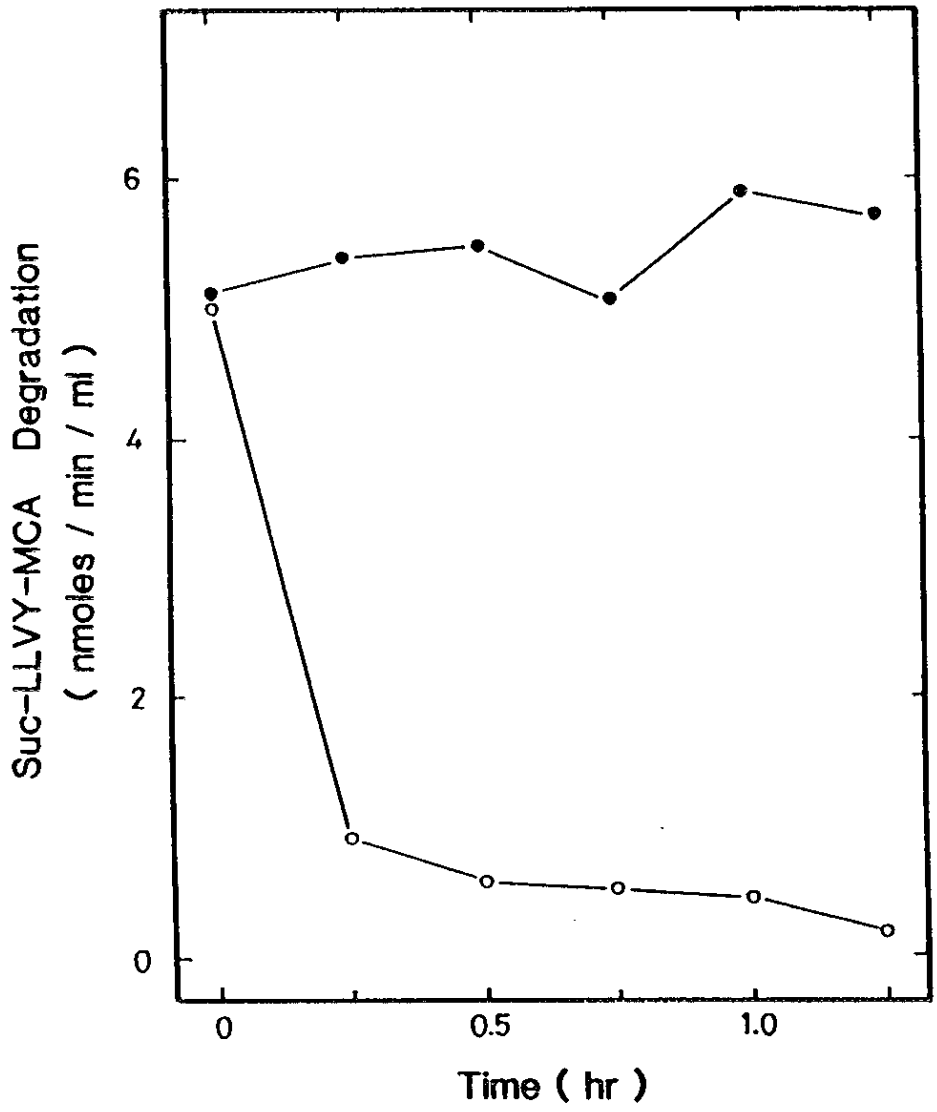
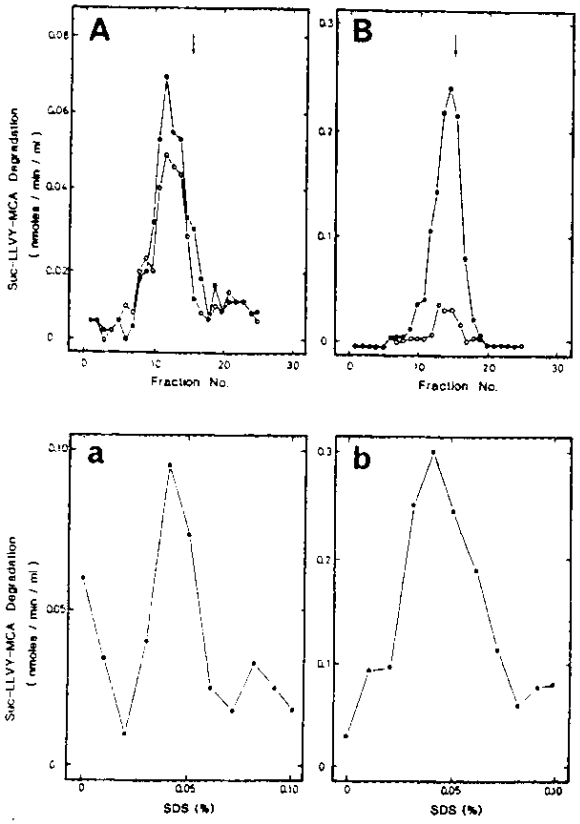


Fig. 9. Changes in Suc-LLVY-MCA hydrolyzing activity during incubation at 37°C. Freshly prepared cytosol fractions were incubated at 37°C. During incubation, Suc-LLVY-MCA hydrolyzing activity in the absence (○) or presence (●) of 0.09% SDS was determined every 15 min.

Fig. 10. Fractionation of proteasomes from cytosols before and after temperature treatment.

Cytosol fractions incubated either at 37°C for one hour or without temperature-treatment were separated on a DEAE-cellulose column by 0.2 M NaCl step wise elution. Active fractions from DEAE-cellulose chromatography were subjected to glycerol density gradient centrifugation (10-35%) as described in "Materials and Methods." Suc-LLVY-MCA hydrolyzing activity in the absence (○) or presence (●) of 0.04 % SDS was determined (A, non-treated; B, incubated). The peak fraction of purified proteasome is indicated by arrows. The effect of SDS on Suc-LLVY-MCA hydrolyzing activity of peak fractions was also determined (non-temperature-treated, a; treated, b). Cytosol fractions (C) and the peak fractions from glycerol density gradient centrifugation (D) were analyzed by immunoblotting after native-PAGE (lane 1, purified latent proteasome; lane 2, non-treated cytosol; lane, 3, temperature-treated cytosol; lane 4, purified latent proteasome with non-treated cytosol; lane 5, non-treated cytosol with incubated cytosol).


A
B
a
b
Suc-LLVY-MCA Degradation
(nmoles / min / ml)
Fraction No.
SDS (%)
C
D
1 2 3 4 5
1 2 3 4 5

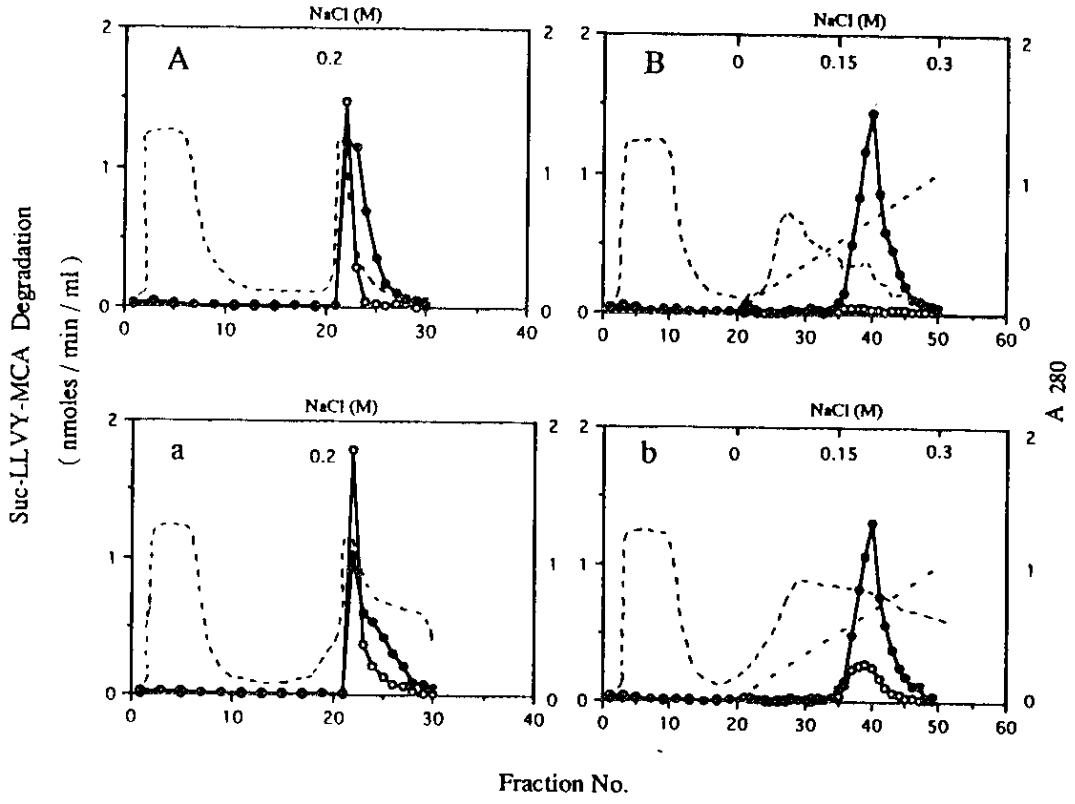


Fig. 11. DEAE-cellulose column chromatography of cytosol.

Cytosol fraction (10 ml) was applied to DEAE-cellulose column (2.0 x 10.0 cm) equilibrated with TGM buffer (A, B) or TGM buffer containing 2 mM ATP (a, b). After washing with the same buffer, proteins were eluted by step elution with TGM buffer containing 0.2 M NaCl in the absence (A) or presence (a) of 2 mM ATP, or eluted by linear gradient of NaCl (0 - 0.3 M) in the absence (B) or presence (b) of 2 mM ATP. The activity toward Suc-LLVY-MCA in the presence (●) or absence (○) of 0.04% SDS and the absorbance at 280 nm (----) were measured.

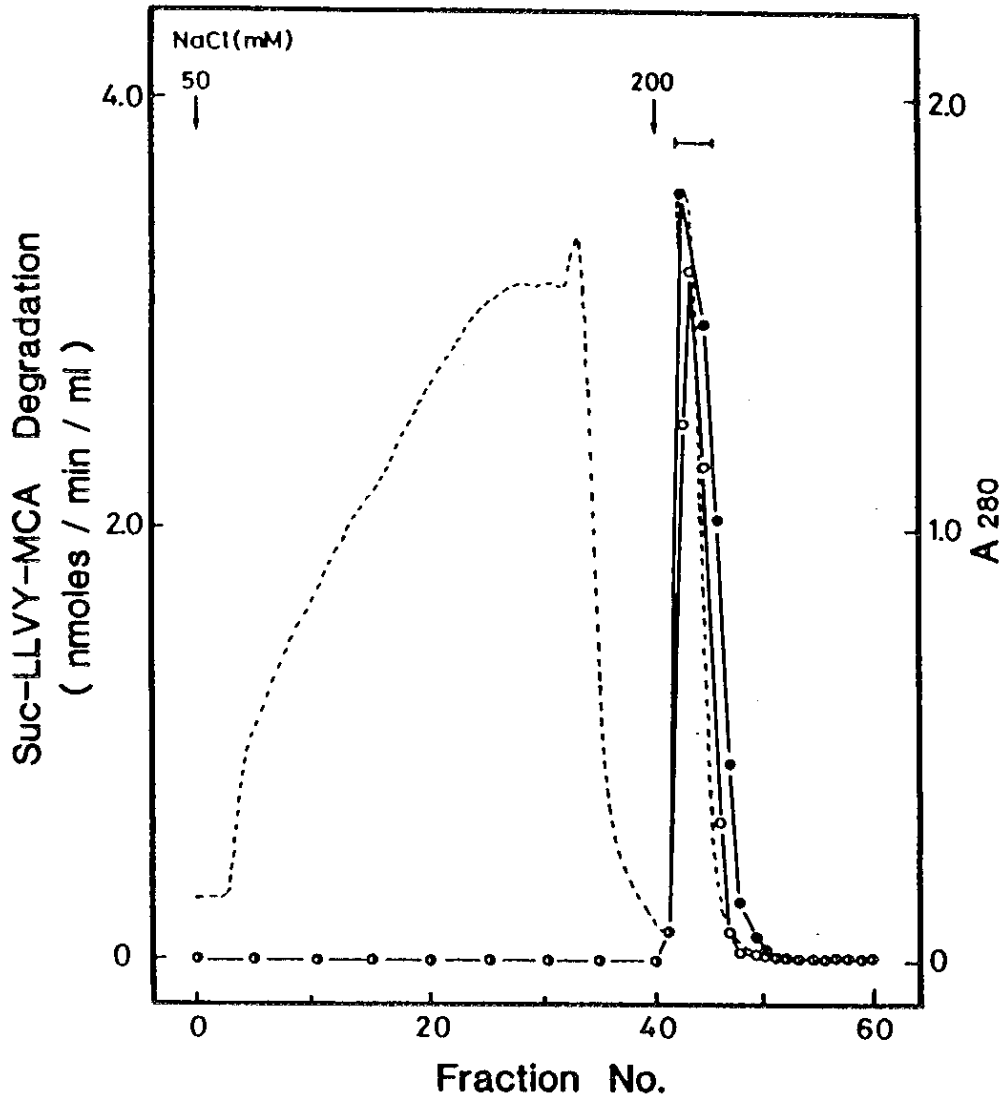


Fig. 12. Arginine-Sepharose 4B chromatography.

Active fractions from DEAE-cellulose chromatography were separated by Arg-Sepharose 4B column as described under "Materials and Methods." The activity toward Suc-LLVY-MCA in the presence (●) or absence (○) of 0.04% SDS and the absorbance at 280 nm (----) were measured. The bar at the top indicates the fractions pooled.

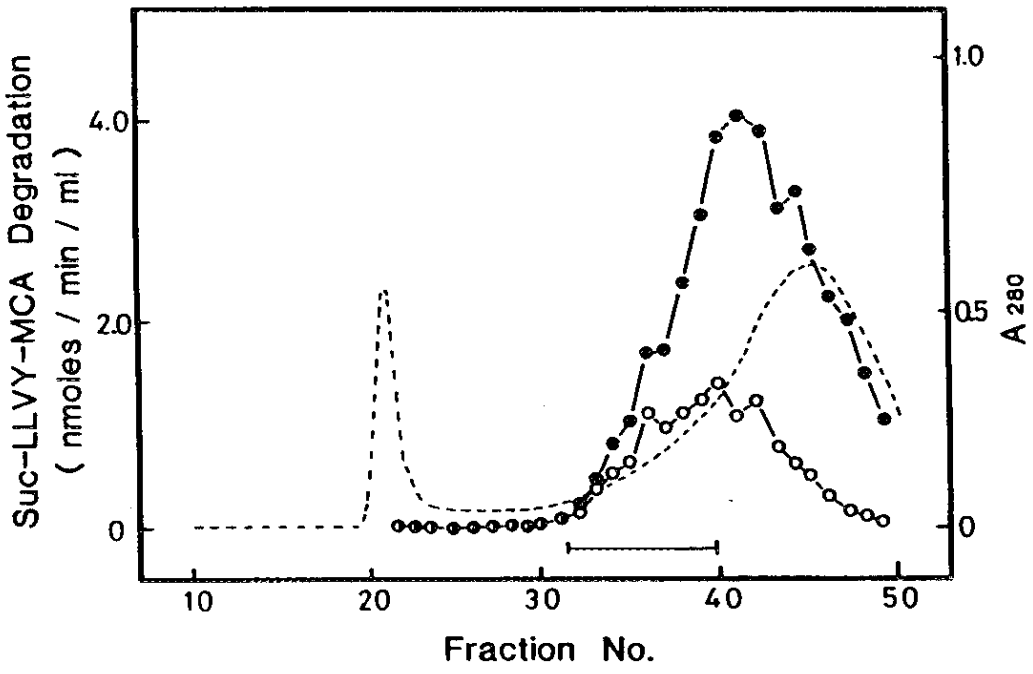


Fig. 13. Sepharose CL-4B chromatography.

Details of the procedures are described under "Materials and Methods." The activity toward Suc-LLVY-MCA in the presence (●) or absence (○) of 0.04% SDS and the absorbance at 280 nm (----) were measured. The bar at the bottom indicates the fractions pooled.

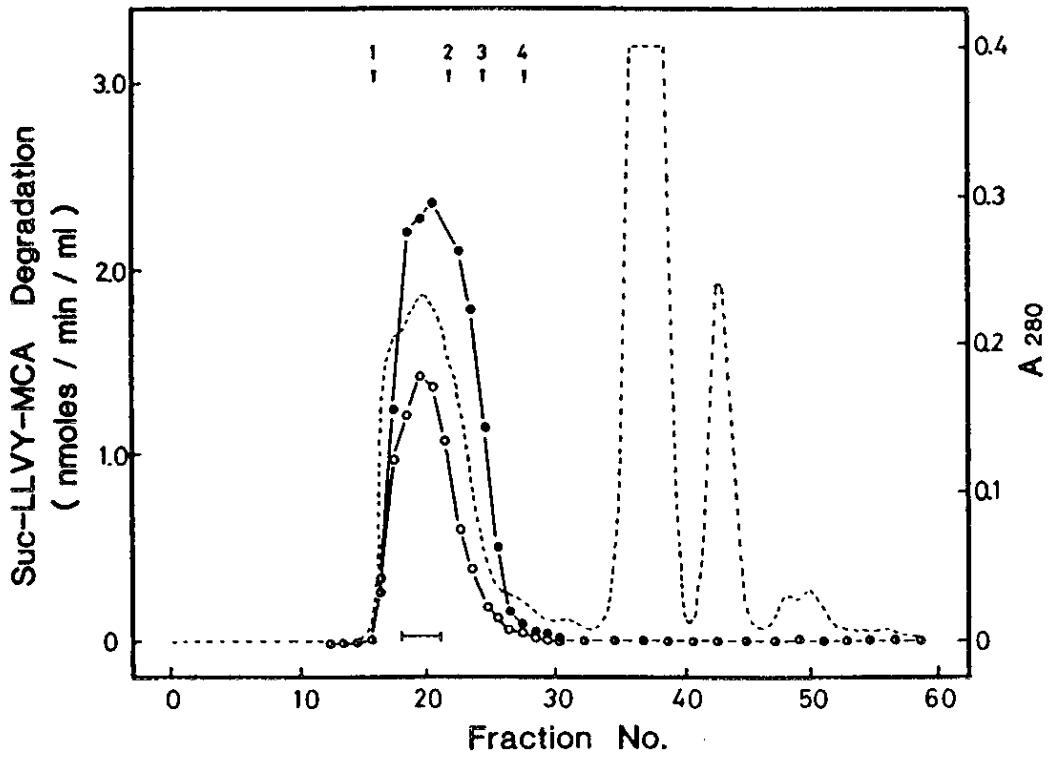


Fig. 14. Superose 6 chromatography.

Details of the procedures are described under "Materials and Methods." The activity for Suc-LLVY-MCA in the presence (●) or absence (○) of 0.04% SDS and the absorbance at 280 nm (----) were measured. The bar at the bottom indicates the fractions pooled.

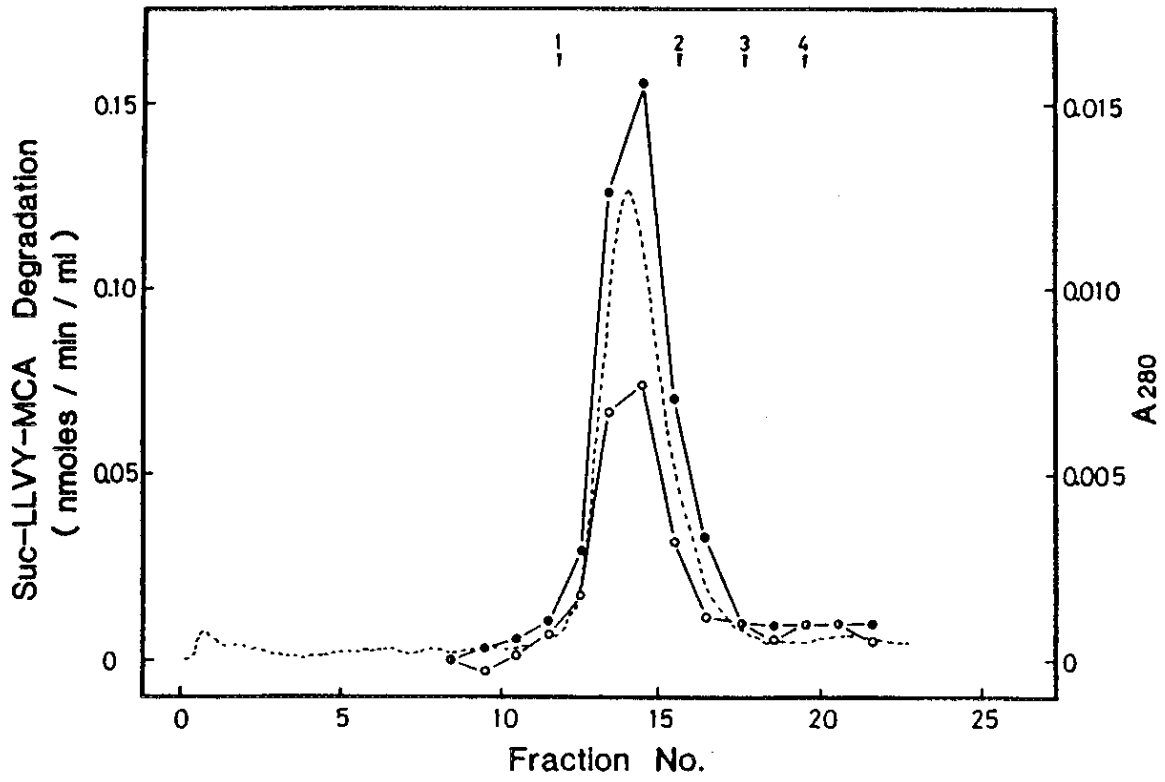


Fig. 15. TSK gel G4000SWXL chromatography.

Details of the procedures are described under "Materials and Methods." The activity toward Suc-LLVY-MCA in the presence (●) or absence (○) of 0.04% SDS and the absorbance at 280 nm (----) were measured.

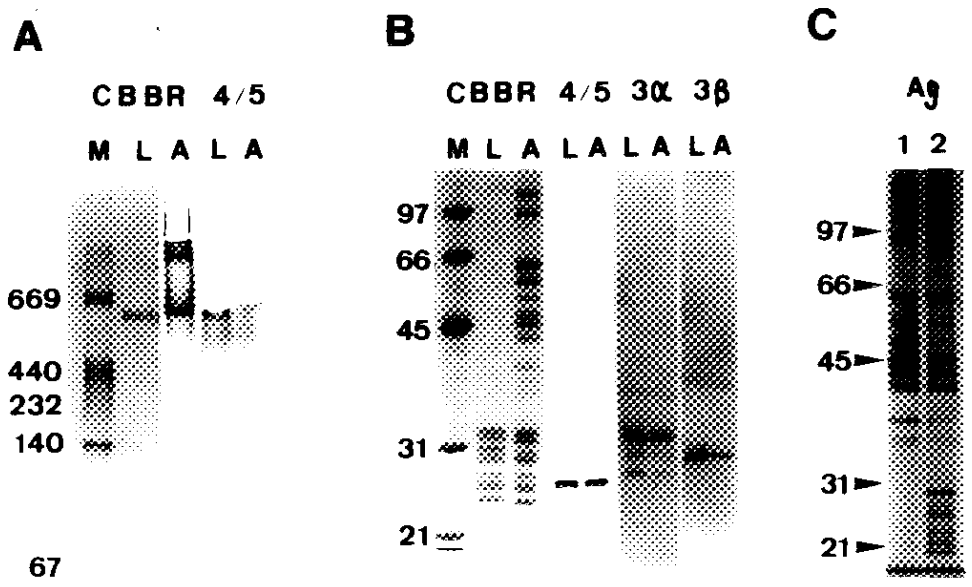


Fig. 16. Polyacrylamide gel electrophoresis and immunoblotting of purified latent and active proteasome.

A) Latent (10 μg) and active proteasome (2.7 μg) was electrophoresed under nondenaturing conditions (5% gel) and stained with CBBR-250, or immunostaining with anti-GC4/5. Lanes L and A correspond to the latent and active proteasome. B) Latent (10 μg) and active proteasomes (27 μg) were electrophoresed under denaturing conditions (12.5% gel) and stained with CBBR-250, or immunostaining with three kinds of monoclonal antibodies (anti-GC4/5, GC3α and, GC3β). C) Protein constituents of two bands in electrophoresis under nondenaturing conditions of active proteasome were extracted from the gel and electrophoresed under denaturing conditions (12.5% gel). Protein bands were visualized by silver-stain. Lanes 1 and 2 correspond to the higher and lower band in A, respectively. Molecular weights of standard proteins are indicated at the left.

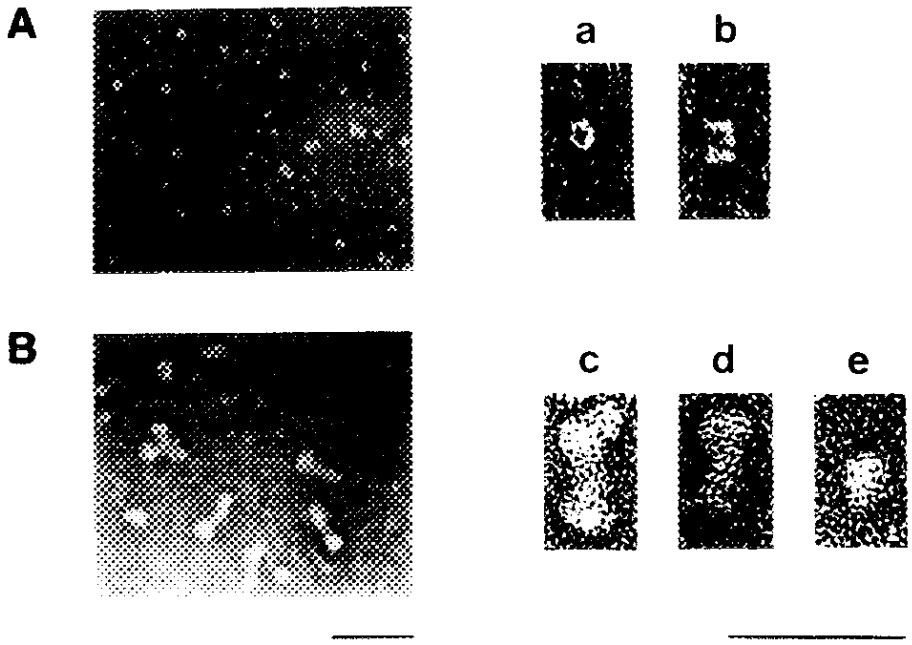


Fig. 17. Electron micrographs of latent and active proteasomes.

Latent (A, a, b) and active (B, c, d, e) proteasomes were negatively stained as described under "Materials and Methods." Typical structures were indicated in high magnification as follows: (a), ring shaped particle; (b), four stacked layer; (c), cylindrical dumbbell structure; (d), complex with only one terminal domain; (e), ball structure. Bar= 100 nm.

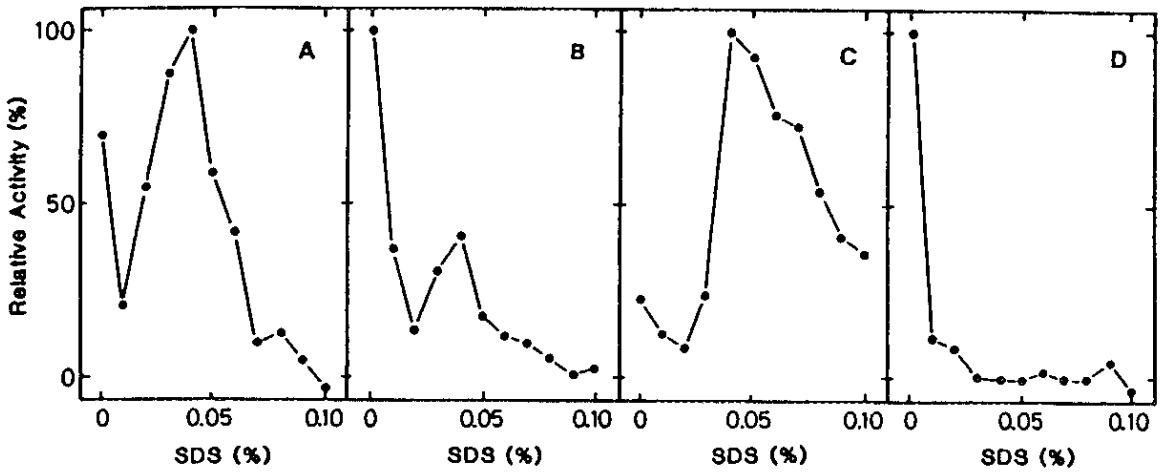


Fig. 18. Effect of SDS on peptide hydrolyzing activity.

Peptide hydrolyzing activities of active proteasome toward Suc-LLVY-MCA (A), Boc-LRR-MCA (B), Z-LLE-βNA (C), and Z-GGL-βNA (D) were measured in the presence of various concentrations of SDS (0 ~ 0.10%).

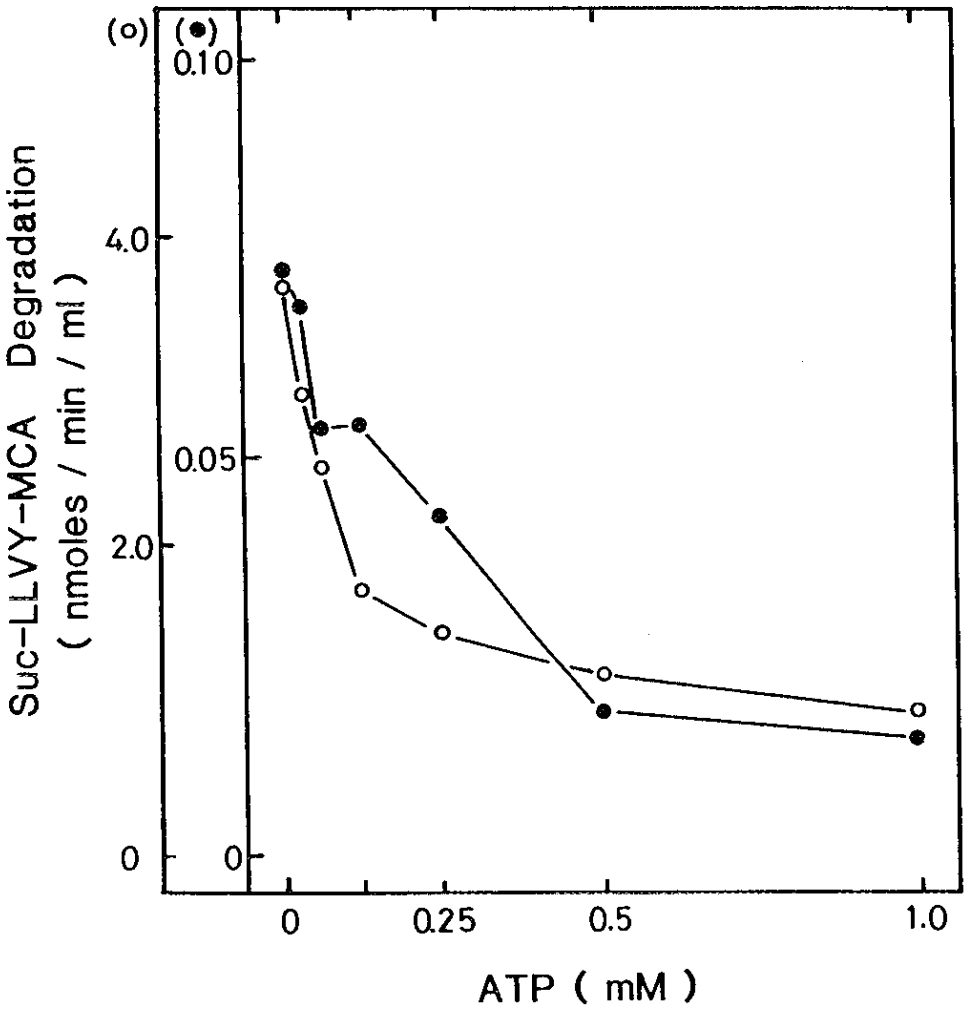


Fig. 19. Effect of ATP on Suc-LLVY-MCA hydrolyzing activity.

Suc-LLVY-MCA hydrolyzing activities of active proteasome (●) and cytosol (○) were measured in various concentrations of ATP.

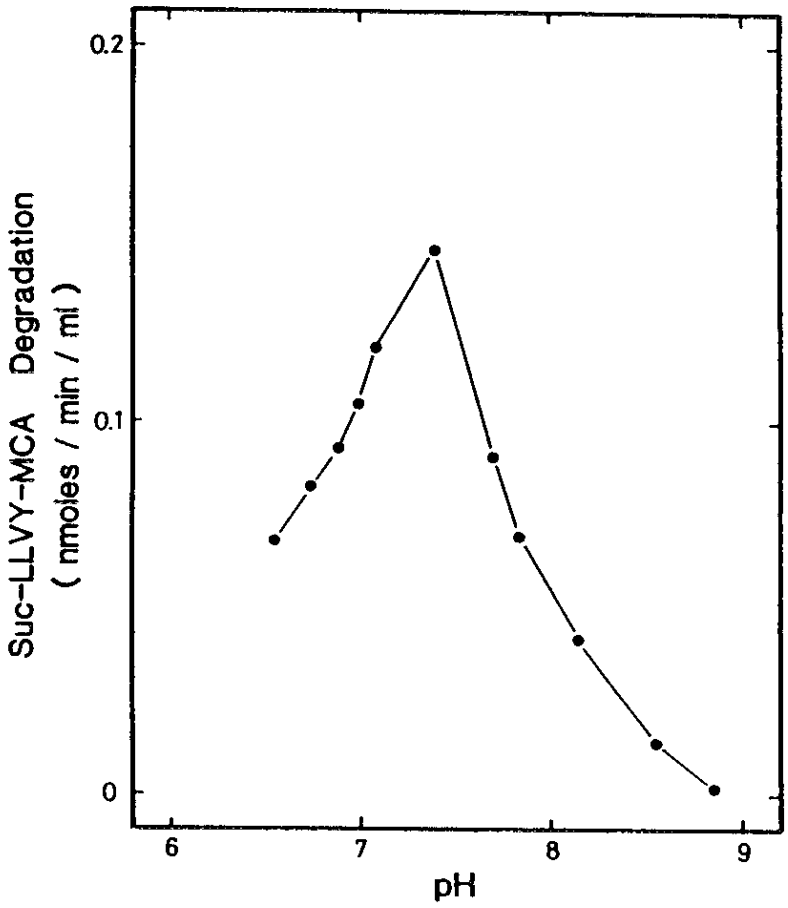


Fig. 20. Effect of pH on Suc-LLVY-MCA hydrolyzing activity.

Suc-LLVY-MCA hydrolyzing activity of active proteasome was measured at various pHs.

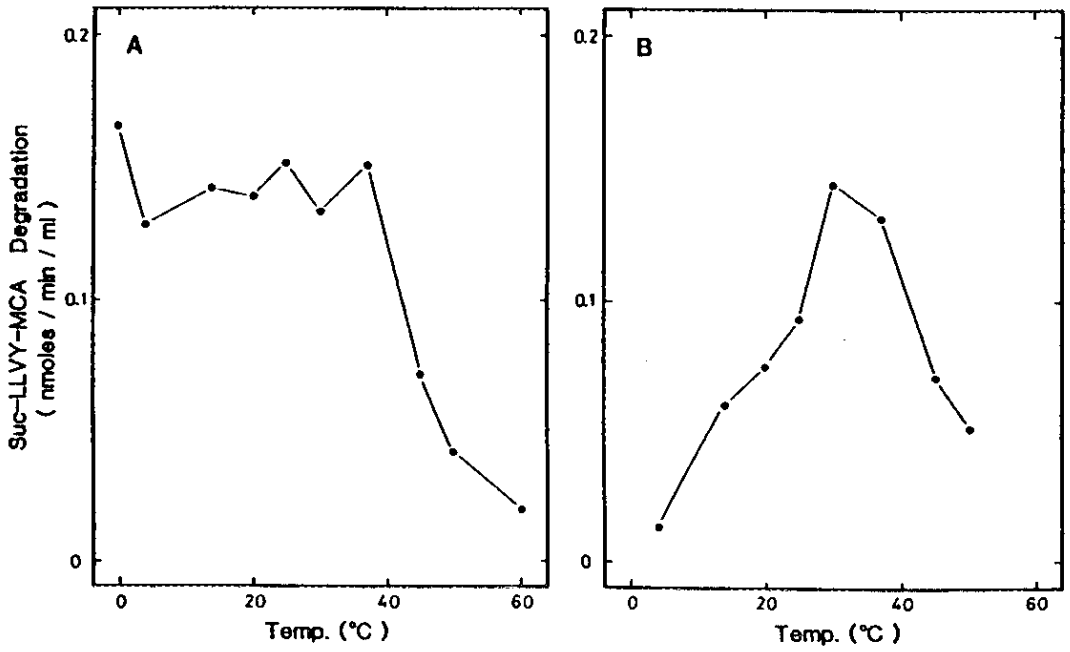


Fig. 21. Effect of temperature on Suc-LLVY-MCA hydrolyzing activity.

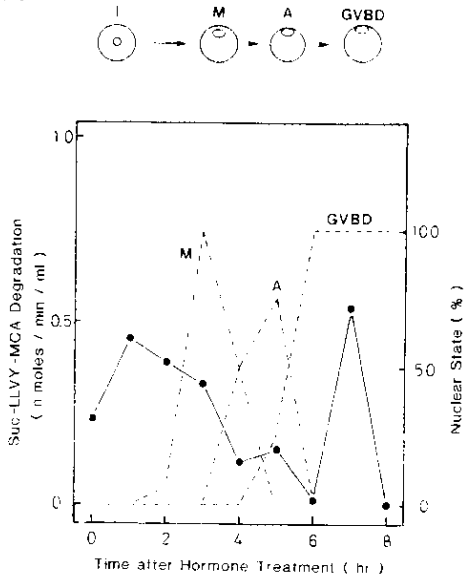
A) Active proteasome in TGMA buffer was treated for 10 min at the temperature indicated; Suc-LLVY-MCA hydrolyzing activity was then measured at 37°C. B) Suc-LLVY-MCA hydrolyzing activity of active proteasome was measured at the temperature indicated.

Fig. 22. Changes in the morphology of the oocyte (A, ---), and the activity (A, •) and protein levels (B, native PAGE; C, SDS-PAGE) during the 17α,20β-DP-induced oocyte maturation in goldfish.

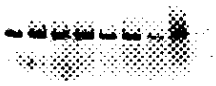
A) Schematic diagram above the figure indicates the state of the germinal vesicle: intact germinal vesicle (I), germinal vesicle during migration (M), germinal vesicle attached to the oocyte periphery (A), GVBD, germinal vesicle breakdown (see text for details). To determine the activity and protein levels of proteasome, oocyte cytosol was extracted as follows: thirty oocytes manually isolated from ovarian fragments were washed with goldfish Ringer. After excess medium was removed, 150 μl of new Ringer was added. The oocytes were crushed by ultracentrifugation at 150,000 g for 30 min at 4°C and the clear supernatant was collected. Protease activity of proteasome was measured using a fluorogenic substrate, Suc-LLVY-MCA (Peptide Institute Inc.).

B, C) Proteins were separated by polyacrylamide gel electrophoresis in either non-denaturing (native PAGE with 5% gel, B) or denaturing condition (SDS-PAGE with 12% gel, C) by the method of Laemmli (1970), and transferred to Immobilon membrane (Millipore). Membranes were blocked in 5% non-fat dry milk, and incubated with the mixture of three monoclonal antibodies against goldfish latent proteasome (GC4/5, GC3α, and GC3β) for 1 hr at room temperature. Immunocomplexes were visualized using alkaline phosphatase conjugated anti-mouse immunogloblin.

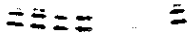
A



B



C



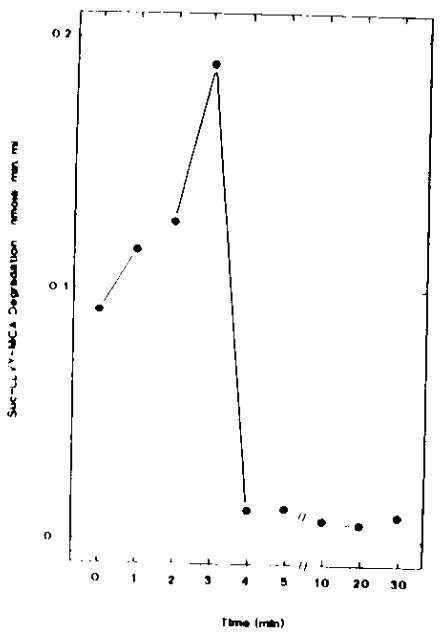
0 1 2 3 4 5 6 7 8

Time after Hormone Treatment (hr)

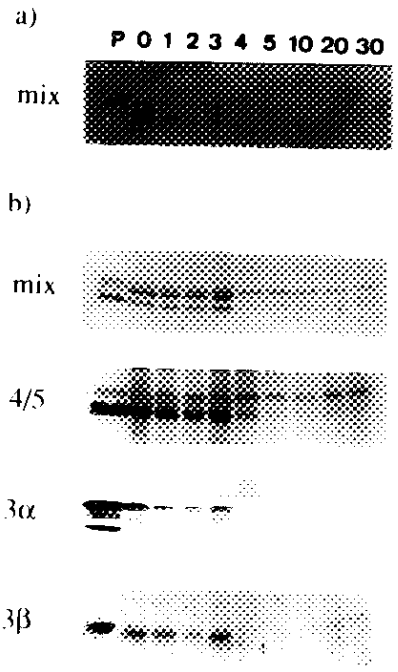
Fig. 23. Changes in the activity and protein levels of proteasome during egg activation.

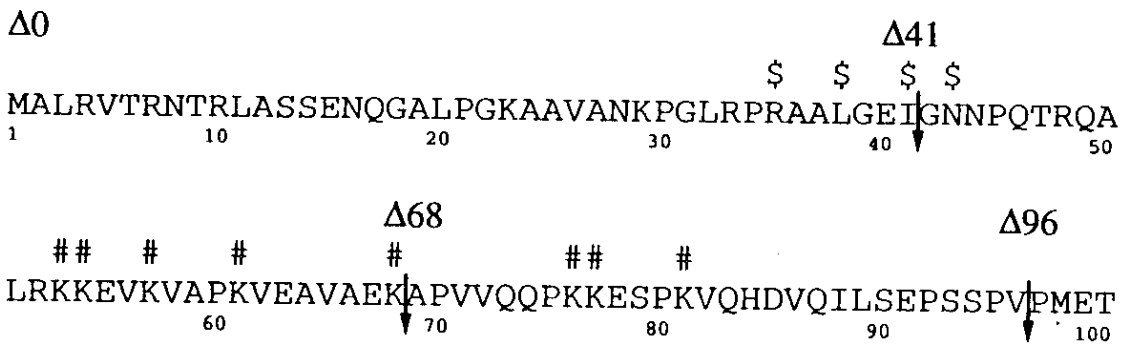
Egg extract was prepared as follows: 200 μl of ovulated eggs were combined with 300 μl of goldfish Ringer and induced activation. During incubation at room temperature, each samples were chilled on ice at the indicated time. Cytosol was extracted by centrifugation at 200,000 g for 45 min at 4 °C. Protease activity of proteasome was measured using Suc-LLVY-MCA (A). Proteins were separated by polyacrylamide gel electrophoresis in either non-denaturing (native PAGE with 5% gel, a) or denaturing condition (SDS-PAGE with 12% gel, b) by the method of Laemmli (1970), and transferred to Immobilon membrane (Millipore). Membranes were blocked in 5% non-fat dry milk, and incubated with the mixture of three monoclonal antibodies (GC4/5, GC3α, and GC3β) or each antibody against goldfish latent proteasome for 1 hr at room temperature. Immunocomplexes were visualized using alkaline phosphatase conjugated anti-mouse immunogloblin in the case of anti-proteasome.

A


0.2
0.1
0
0 1 2 3 4 5 10 20 30
Time (min)

B


a)
P 0 1 2 3 4 5 10 20 30
mix
b)
mix
4/5
3α
3β



$: destruction box
: lysine-rich strech

Fig. 24. Amino acid sequence of the N-terminal region of *E. coli*-produced goldfish cyclin B.

Amino acid sequence of N-terminal region (1 - 100) of goldfish cyclin B is indicated. Truncated sites of deletion mutants (Δ41, Δ68, and Δ96) are indicated by arrows. The destruction box is indicated by $. Lysine-rich stretch predicted to be conjugated with ubiquitin is indicated by #.

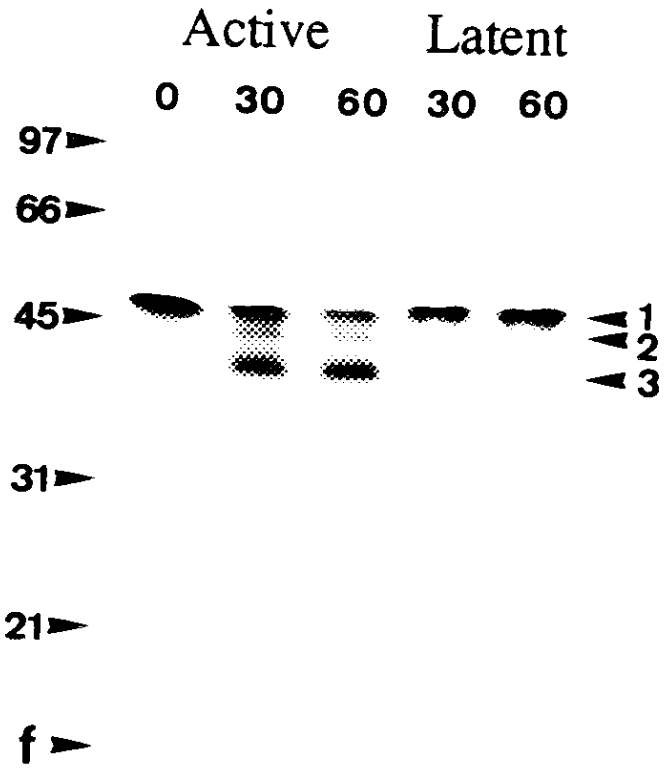


Fig. 25. Digestion of cyclin B by purified proteasomes.

Cyclin B Δ0 (final concentration; 5 μg/ml) was mixed with purified active proteasome or latent proteasome (60 μg/ml) and incubated at room temperature with agitation. Samples were combined with SDS sample buffer at the indicated times during incubation. Degradation of cyclin B was assessed by immunoblotting using anti-cyclin B (B63). Arrowheads indicate bands as follows: 1, cyclin B Δ0; 2, cyclin B digested by latent proteasome; 3, cyclin B digested by active proteasome. Molecular weights of standard proteins are indicated at the left.

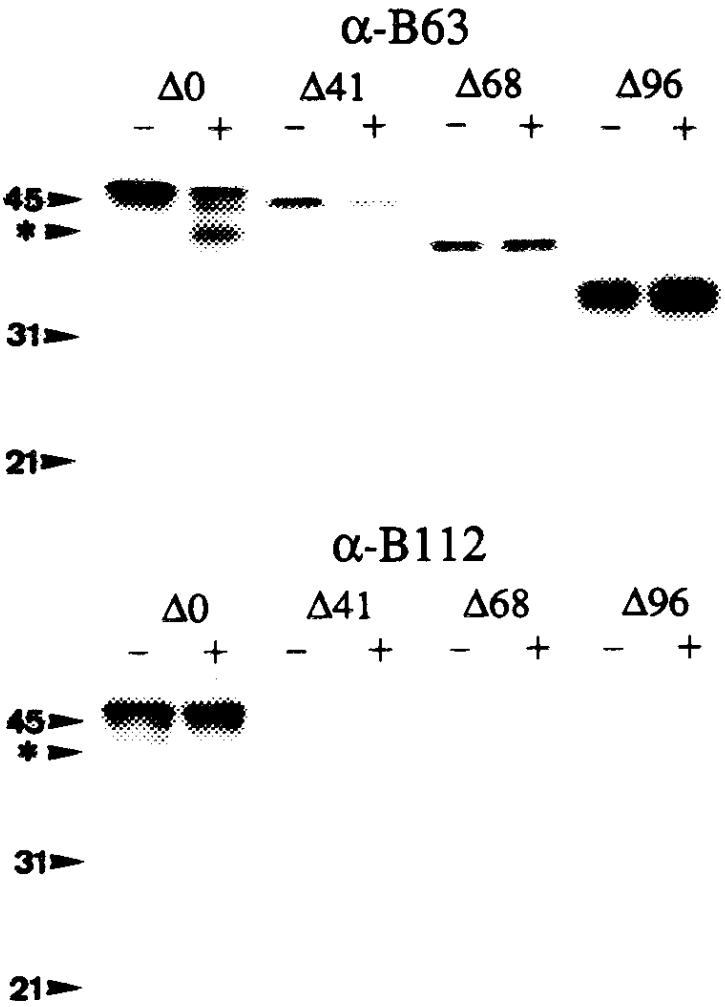


Fig. 26. Digestion of truncated cyclin Bs by active proteasome.

Intact and truncated forms of cyclin B (Δ0 , Δ41, Δ68, and Δ96) were incubated in the absence (-) or presence (+) of active proteasome for 120 min at room temperature. Cyclin degradation was assessed by immunoblotting using two kinds of anti-cyclin B (B63 and B112). The migrating position of digested cyclin B is indicated by asterisks.

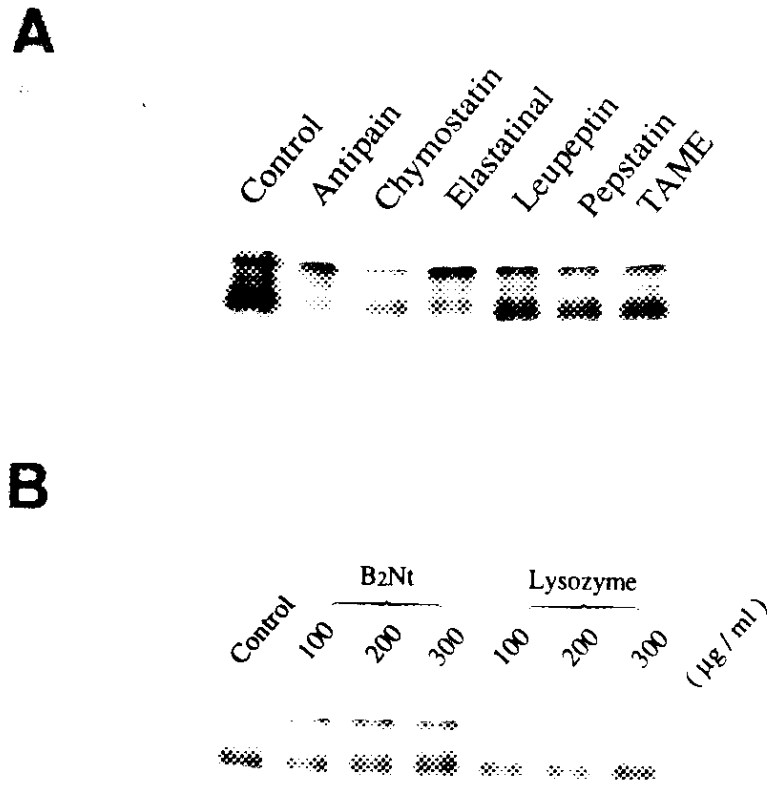


Fig. 27. Effects of various protease inhibitors on cyclin B digestion *in vitro* induced by active proteasome.

A) Cyclin B Δ0 was digested for 120 min in the absence (Control) or presence of various protease inhibitors. The final concentration of inhibitors was adjusted to 0.125 mM.

B) Cyclin B was digested in the absence (Control) or presence of various concentrations of B2Nt or lysozyme. Digestion of cyclin B was assessed by immunoblotting using anti-cyclin B (B63).

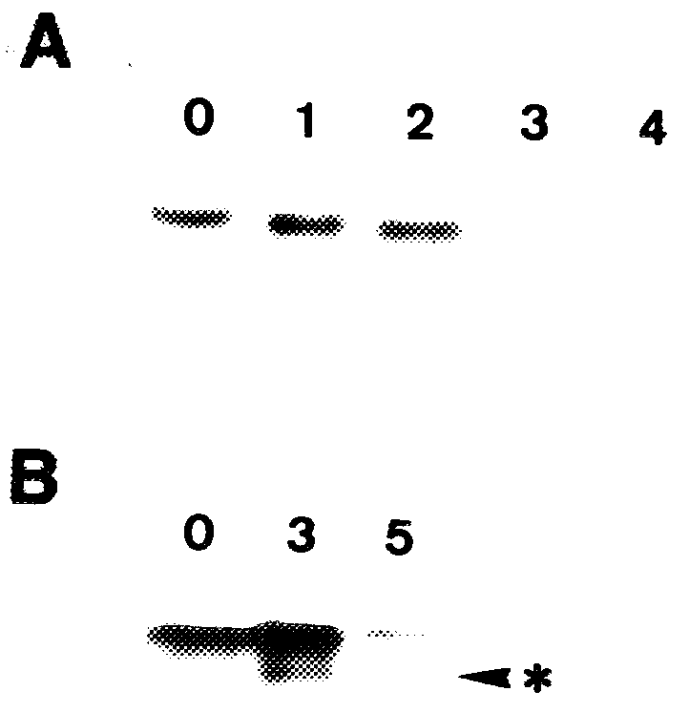


Fig. 28. Cyclin B digestion during goldfish egg activation.

A) Cytosol fraction of eggs was prepared as follows: 200 µl of ovulated eggs were combined with 300 µl of goldfish Ringer and induced activation. During incubation at room temperature, each sample was chilled on ice at the indicated times (min). Cytosol was extracted by centrifugation at 200,000 g for 45 min at 4 °C. Cyclin B in the cytosol was detected by immunoblotting using anti-cyclin B (B63).

B) Activating egg extracts were prepared as follows: ovulated eggs (2 ml) were activated with goldfish Ringer (3 ml). At the indicated times (min), activating eggs were instantly homogenized with Laemmli's SDS sample buffer (5 ml). Proteins with molecular weights ranging about 40 - 50 kDa were separated from the homogenates by SDS-PAGE (Prep Cell Model 491) and concentrated. Cyclin B in these fractions was detected by immunoblotting using anti-cyclin B (B63).

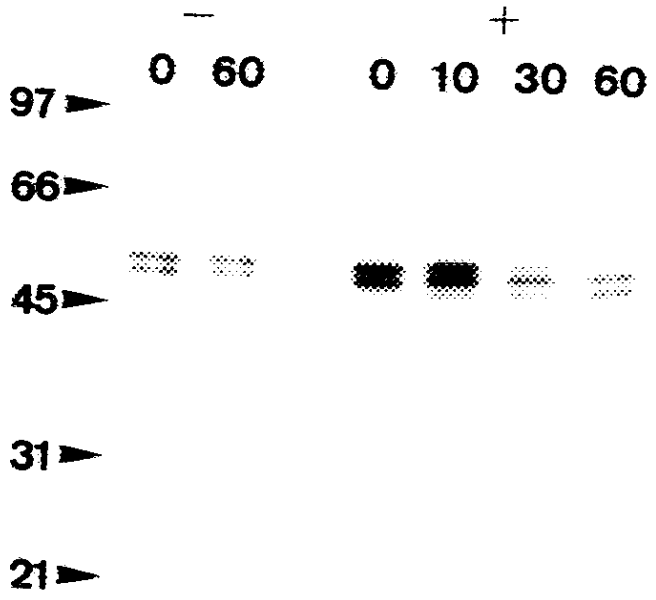


Fig. 29. Digestion of cyclin B in MPF complex by active proteasome.

MPF complex in M-phase extracts of carp was prepared by Suc-1 beads. Beads were washed with TGMA buffer and combined with active proteasome. Reaction was performed at room temperature with agitation. During incubation, samples were combined with SDS sample buffer at the indicated time. Degradation of cyclin B was assessed by immunoblotting using anti-cyclin B (B63).

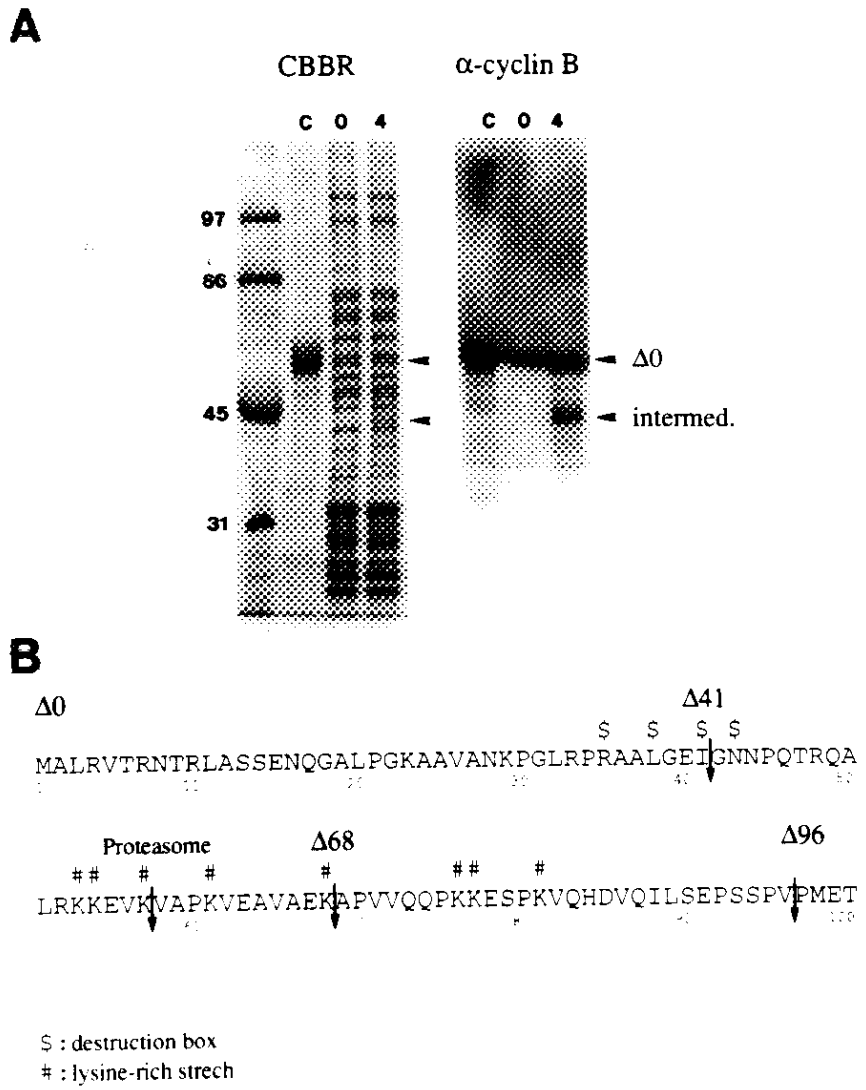


Fig. 30. Determination of the digestion site of cyclin B.

A) Cyclin B was digested in the large scale (Total amount of cyclin B; 300 μg) for 4 hours. Proteins in reaction mixtures were separated by SDS-PAGE (Multi gel 12.5, Daiichi Pure Chemicals) and visualized by CBBR staining or immunoblotting using anti-cyclin B (B63). Lanes c, 0 and 4 indicate cyclin B, reaction mixtures at time zero, and reaction mixtures at four hours, respectively. Arrowheads indicate intact cyclin B (Δ0) and intermediate (intermed.) cyclin B.

B) Amino acid sequence of goldfish cyclin B (1 - 100). The site (C-terminal of Lys 57) which is digested by active proteasome is indicated.

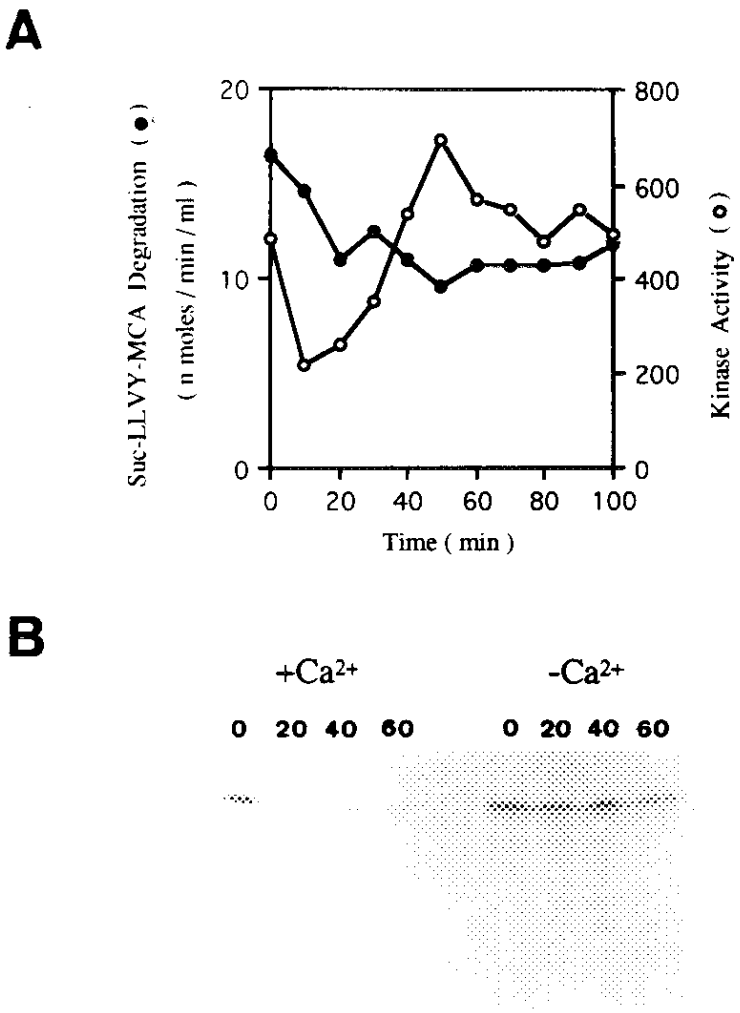


Fig. 31. Degradation of goldfish cyclin B in *Xenopus* egg extracts.

A) *Xenopus* egg extracts were prepared as described in "Materials and Methods" and activated by adding Ca^{2+}. Changes in Suc-LLVY-MCA degrading activity and kinase activity (SP peptide) in *Xenopus* egg extracts were measured during various periods of time (0 - 60 min) after activation.

B) Goldfish cyclin B was added to *Xenopus* egg extracts at a concentration of 5 µg/ml. Activation was then induced by adding Ca^{2+}. Degradation of cyclin B was assessed by immunoblotting using anti-cyclin B (B63).

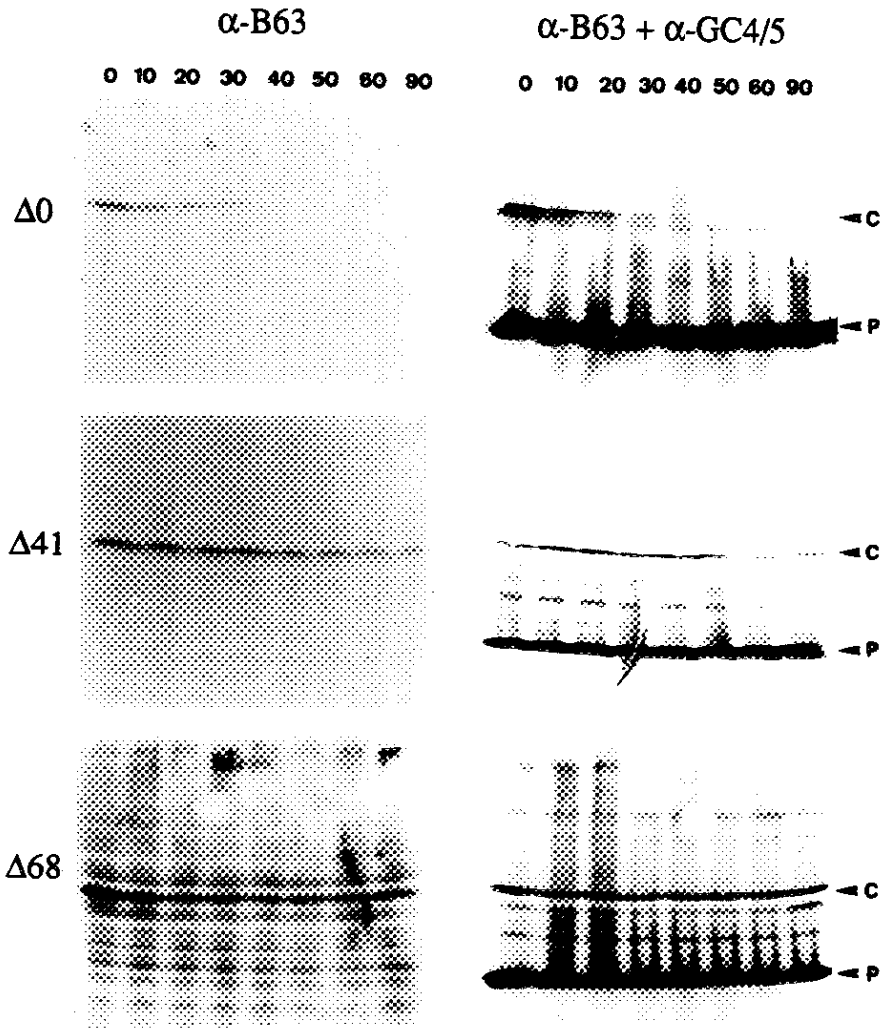


Fig. 32. Degradation of goldfish cyclin Bs in *Xenopus* egg extracts.

Intact and truncated forms of cyclin B (Δ0 , Δ41, and Δ68) were added to *Xenopus* egg extracts and then activation was induced by adding Ca^{2+}. Degradation of cyclins during various periods of time (0 - 90 min) was assessed by immunoblotting using anti-cyclin B (B63). Membranes were also stained by anti-cyclin B and anti-latent proteasome (anti-GC4/5). Arrowheads C and P indicate cyclin B and subunits of *Xenopus* proteasome which cross-reacted with anti-GC4/5.

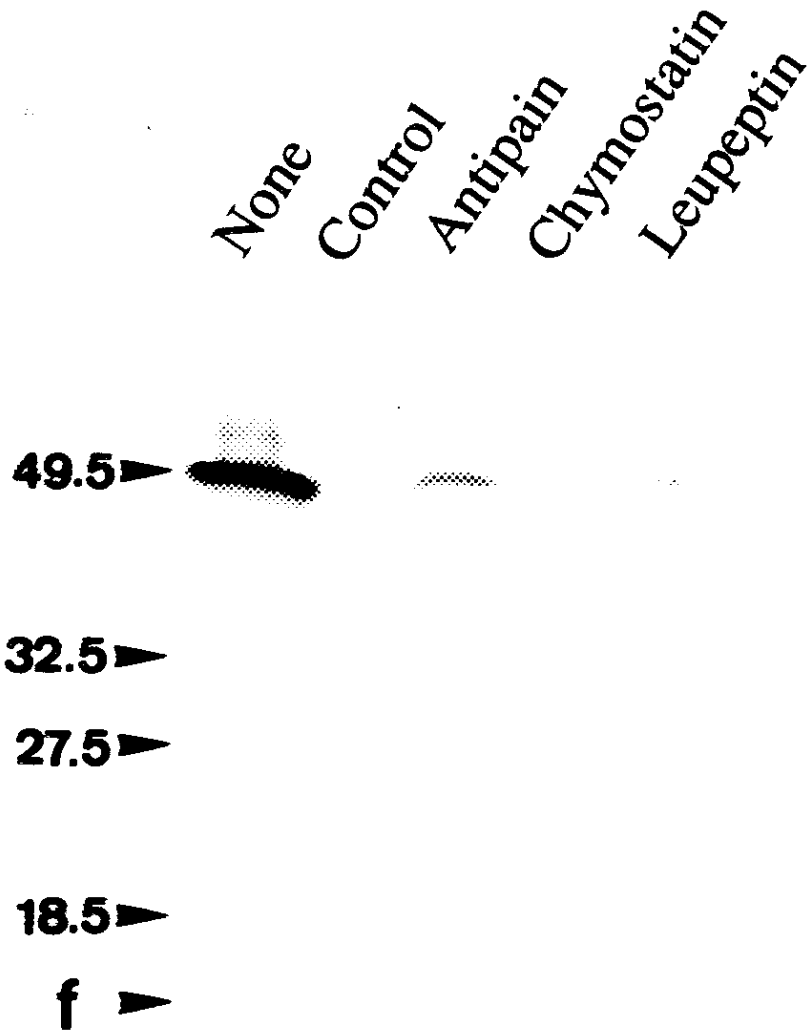


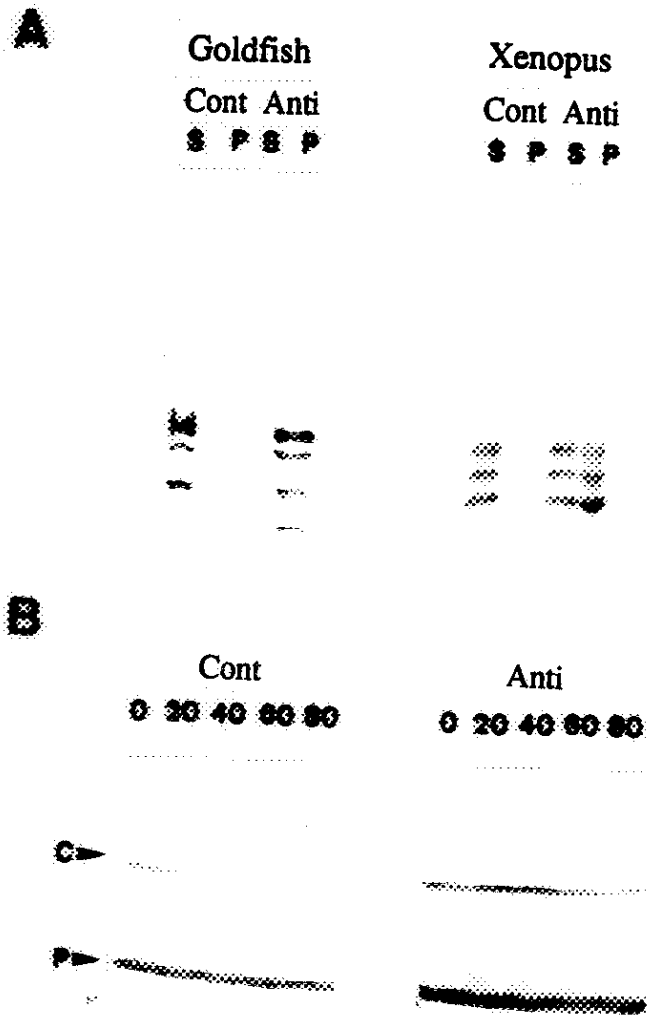
Fig. 33. Effects of various protease inhibitors on goldfish cyclin B degradation in *Xenopus* egg extracts.

Goldfish cyclin B Δ0 was incubated in the absence (Control) or presence of various protease inhibitors for 60 min at room temperature. Lane none means no reaction. Degradation of cyclin B was assessed by immunoblotting using anti-cyclin B (B63).

Fig. 34. Inhibition of cyclin B degradation in *Xenopus* egg extracts by immunodepletion of proteasome.

A) Cytosol from goldfish immature oocytes (Goldfish) and *Xenopus* egg extracts (Xenopus) were immunoprecipitated with control-IgG (Cont) or anti-goldfish proteasome polyclonal antibodies (Anti). Proteasome in the supernatant (S) or precipitate (P) was detected by immunoblotting using anti-proteasome (mixture of GC4/5, GC3α, and GC3β).

B) Cyclin BΔ0 was incubated in the immunodepleted (with control-IgG, Cont; with anti-proteasome antibodies, Anti) supernatant of *Xenopus* egg extracts for various periods of time (0 - 80 min). Cont and Anti correspond to immunodepleted with control-IgG and anti-proteasome antibodies. Degradation of cyclin was assessed by immunoblotting using anti-cyclin B (B63) and anti-proteasome (anti-GC4/5). Arrowheads C and P indicate cyclin B and subunits of *Xenopus* proteasome which cross-reacted with anti-GC4/5.


A
Goldfish
Cont Anti
S P S P
Xenopus
Cont Anti
S P S P
B
Cont
0 20 40 60 80
Anti
0 20 40 60 80
C
P

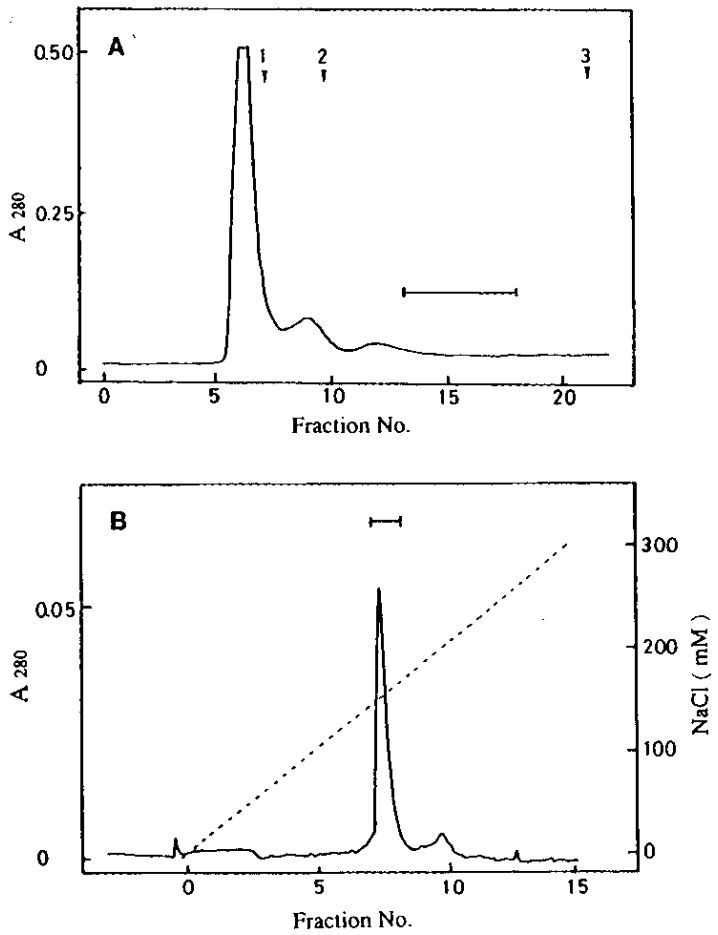


Fig. 35. Purification of ubiquitin from goldfish oocytes.

A) Bio Gel P-30 column chromatography: Fractions from CM-cellulose column chromatography were pooled and concentrated, and chromatographed on Bio Gel P-30 column (1.6x50 cm). Arrows indicate the eluted position of molecular weight standards as follow: 1, bovine serum albumin (66 kDa); 2, chymotrypsinogen (25 kDa); 3, Acetone. Pooled fractions are indicated by the horizontal line. B) Mono-S column chromatography: Fractions from Bio Gel P-30 column chromatography were pooled and chromatographed on Mono-S (HR 5/5) column. The pooled fraction is indicated by the horizontal line. Protein content was determined from absorvance at 280 nm using bovine ubiquitin as standard.

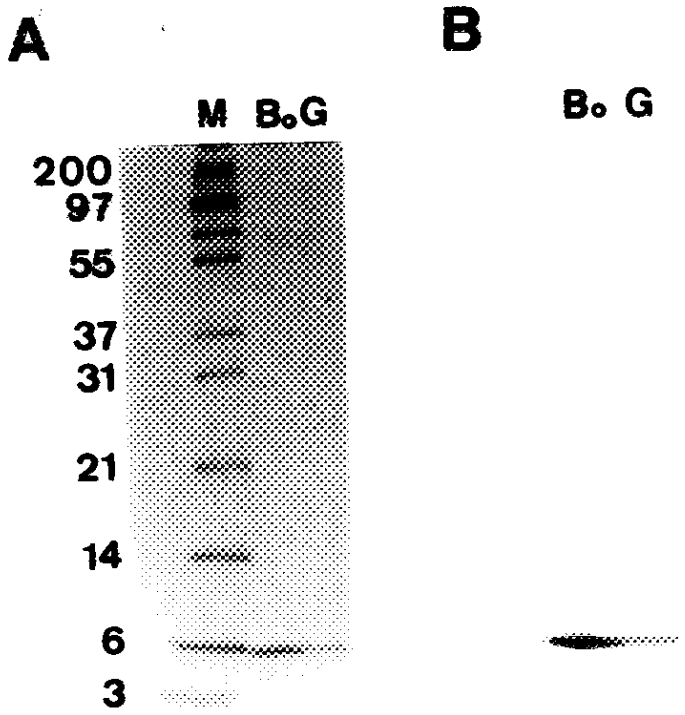


Fig. 36. SDS-PAGE and immunoblotting of purified goldfish ubiquitin.

Electrophoresis followed the method of Laemmli using 15/25% gradient gels (Daiichi pure chemicals). Proteins were then transferred to an Immobilon membrane (Millipore) using a semi-dry electroblotter (EIDO). The membrane was blocked with 5% non-fat dry milk and incubated with the anti-bovine ubiquitin anti-serum (1/100 in Tris-buffered saline) for 1 hr at room temperature. Immunocomplexes were visualized using alkaline phosphatase conjugated anti-rabbit immunoglobulin. (A) SDS-PAGE of goldfish (G) and bovine (Bo) ubiquitin stained with Coomassie blue R-250. Molecular weights of standard proteins (M) are indicated at the left.

(B) Immunoblotting analysis of goldfish ubiquitin (G) and bovine ubiquitin (Bo).

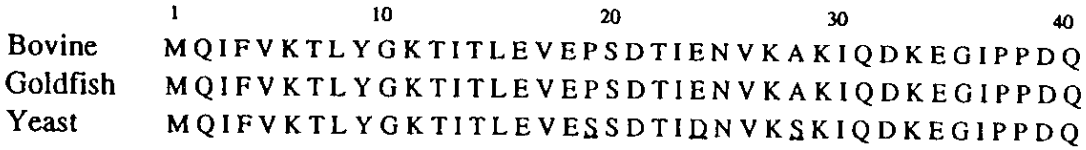


Fig. 37. Alignment of the N-terminal amino acid sequences of bovine, goldfish and yeast ubiquitin.

For the determination of the N-terminal amino acid sequence, purified ubiquitin was further purified by a reversed-phase HPLC on a μPRC C2/C18, PC3.2/3 column using a SMART system (Pharmacia). Proteins were eluted with a 0 - 100% (v/v) linear gradient of acetonitrile/water containing 0.1% (w/v) trifluoroacetic acid. The peak fraction was directly loaded onto a glass filter for sequencing the peptides using a 470A Protein Sequencer (Applied Biosystems Japan). Three amino acids of yeast ubiquitin different from those of bovine and goldfish ubiquitin are underlined.

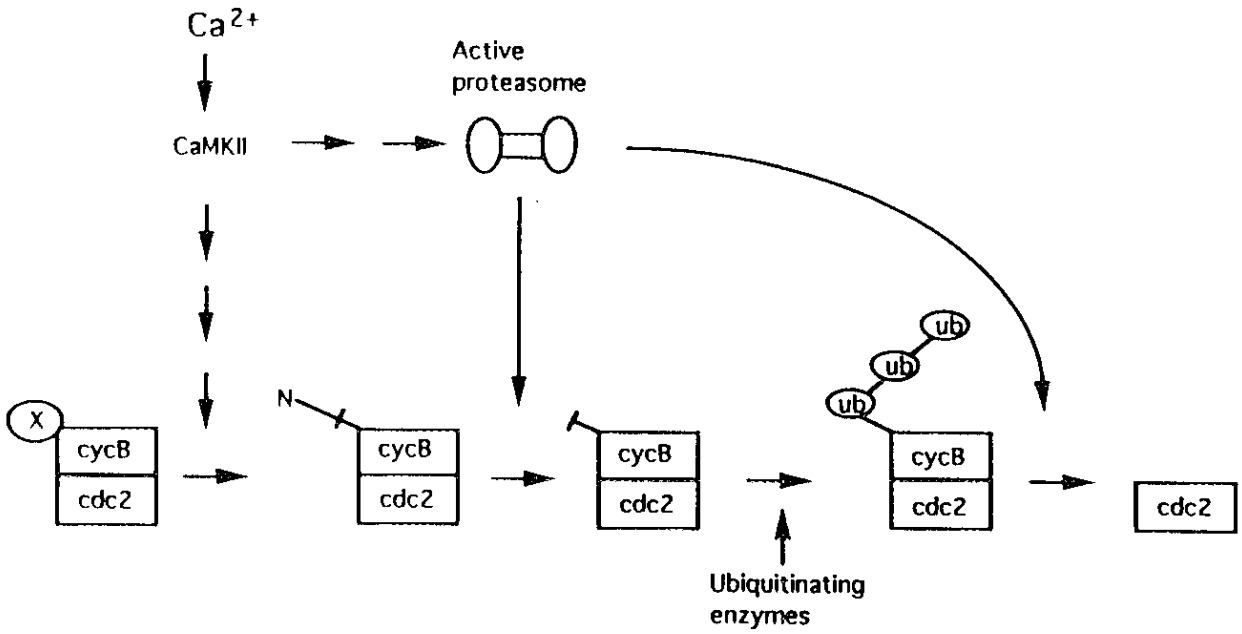


Fig. 38. A current hypothetical model for the possible participation of proteasomes in the regulation of cyclin B degradation (see the text for details).

Abbreviations used in diagram are as follows: CaMKII, calcium/calmodulin dependent protein kinase II; cycB, cyclin B; ub, ubiquitin; and X, unknown protein(s) which block the degradation of cyclin B.

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Yoshiaki Azuma, Toshinobu Tokumoto and Katsutoshi Ishikawa : High molecular weight-multicatalytic proteinases in premature and mature oocytes of *Rana pipiens*.

Molecular and Cellular Biochemistry **100**: 171–181, 1991.

High molecular weight-multicatalytic proteinases in premature and mature oocytes of *Rana pipiens*

Yoshiaki Azuma, Toshinobu Tokumoto and Katsutoshi Ishikawa
Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422, Japan

Key words: proteinase, oocyte, germinal vesicle breakdown, *Rana pipiens*

Abstract

High molecular weight, multicatalytic proteinases (named proteasomes) have been for the first time found, on the basis of different protein patterns, in the cytoplasmic soluble fractions of both non-hormone-treated (premature) and progesterone-treated (mature) oocytes of a frog (*Rana pipiens*).

These enzymes, pooled separately as two fractions sedimenting between around 19S and the bottom (over 27S) on glycerol density gradient centrifugation, were composed of several molecular forms with apparent high molecular weights ranging from over 700 kDa, as judged on Sepharose 6B gel filtration. In addition, both the fractions hydrolyzed distinctly a Tyr-containing substrate in the presence of SDS as an activator, and exhibited higher activities toward Arg-containing substrates in the absence of SDS, and activity toward a Glu-containing substrate in the presence and absence of SDS. Immunological experiments using antibodies against proteasomes purified from ovaries of *Xenopus laevis* clearly revealed characteristic cross-reactivity with both the fractions found in *Rana*.

These data suggest that these enzymes in the two fractions from the respective oocytes in *Rana* are very similar or identical to the proteasomes of *Xenopus*. The enzymes in premature oocytes eluted at 0.15–0.18M NaCl on a DEAE-cellulose column disappeared on treatment with TPCK, a well-known chymotrypsin inhibitor, suggesting that the 0.15–0.18M NaCl-eluate contained chymotrypsin-like proteinases probably latent *in ovo*. The enzymes in mature oocytes had not similar chromatographical patterns to those in premature oocytes.

These results suggest that the enzymes already present in premature oocytes may be involved through conformational alterations as to the protein pattern in oocyte maturation following induction by progesterone.

Abbreviations: AMC-7-Amino-4-methylcoumarin, Boc-*t*-Butyloxycarbonyl, Cbz-Carbobenzoxy, GVBD-Germinal Vesicle Breakdown, MCA-4-Methylcoumaryl-7-Amide, MPF-Maturation Promoting Factor, NA-2-Naphthylamide, SDS-Sodium Dodecyl Sulfate, Suc-Succinyl, TPCK-Nα-Tosyl-L-Phenylalanine Chloromethyl Ketone

Introduction

Oocyte maturation in amphibians *in vitro* is induced by progesterone [1, 2]. Following exposure of the oocyte surface to progesterone [3–7], activity of the proteinaceous cytoplasmic maturation promoting factor (MPF) appears due to the decrease in the amount of cAMP and protein synthesis, and, in turn, causes germinal vesicle breakdown (GVBD) [1, 8–10].

During the past years, evidence has accumulated that some proteinases may be involved in progesterone-induced GVBD in oocytes of amphibians with the use of various proteinase inhibitors [11–13]. In addition, we showed recently that GVBD was triggered in non-hormone-treated frog (*Rana pipiens*) oocytes following transient exposure to the synthetic chymotrypsin inhibitor, Nα-tosyl-L-phenylalanine chloromethyl ketone (TPCK) [14]. GVBD was not induced in oocytes on continuous exposure to a higher dose of TPCK in the presence or absence of progesterone. These results suggest that some enzymes, including a chymotrypsin-like proteinase, play a role in the physiological regulation of the oocyte maturation process. However, it remains to be determined whether enzyme activation is specifically required for oocyte maturation. Detailed biochemical studies of the proteinases will be required to answer these questions. Until now, there has been little information on the chemical entities of proteinases in the oocytes of amphibians. As some biochemical evidences, Slaughter and Triplett [15–17] demonstrated that ovaries and/or oocytes from *Rana pipiens* contain both chymotrypsin- and trypsin-like proteinases. More recently, non-lysosomal multicatalytic proteinases of unusually large size, such as about 20S particles named proteasomes, were found in the ovaries and/or oocytes of *Xenopus laevis* [18, 19], which were found to be similar to proteasomes from eukaryotic cells [19, 20]. However, the physiological function of these proteinases found in amphibian ovaries and/or oocytes is completely unknown.

In the course of studying the role of proteinases in oocyte maturation, using *Rana pipiens*, proteasomes from non-hormone-treated (premature) and progesterone-treated (mature) oocytes were for the first time separated and partially characterized in this study. The results show that the proteasomes in premature oocytes had activities with nearly identical characteristics to those in mature oocytes, but the protein patterns were not identical.

Considering the results together, it will be discussed that these high molecular weight, multicatalytic proteinases which are latent in premature oocytes may undergo some alterations following oocyte maturation (designated as GVBD) induced by progesterone.

Materials and methods

Animals

Gravid female leopard frogs (*Rana pipiens*) were purchased from a commercial supplier (Nasco, USA). The animals were maintained in 10% amphibian Ringer's solution at 4° C, as previously described [14].

Chemicals

Suc-Leu-Leu-Val-Tyr-MCA, Boc-Phe-Ser-Arg-MCA and Boc-Gln-Arg-Arg-MCA were purchased from Peptide Institute Inc. (Osaka, Japan). Cbz-Leu-Leu-Glu-NA was a gift from Dr. K. Tanaka of Tokushima University. DEAE-cellulose (DE-52) was from Whatman (UK), Sepharose 6B from Pharmacia (Sweden), and progesterone and TPCK from Sigma (USA). All other chemicals were of special grade.

Preparation of oocytes

Animals were sacrificed, and their ovaries were removed and placed in freshly prepared amphibian Ringer's solution. Fully grown, intact follicles were isolated from ovaries under magnification using watchmaker's forceps. Oocytes were used without removing the adhering follicle cells. Oocytes of a particular female were pooled and checked carefully under high magnification for damage.

In vitro *oocyte culture*

All incubations were carried out in amphibian Ringer's solution (AR) (112.92mM Nacl, 2.01mM KCl and 1.35mM $CaCl_2$ with 10 mg streptomycin sulfate and 100,000IU potassium penicillin G per liter) buffered with 2.4mM $NaHCO_3$ and adjusted to pH 7.4 with 1N HCl. Incubations were carried out in multiwell dishes (24 wells/dish, Costar 3524; USA). Routinely, 20 oocytes were incubated per well in 2 ml of AR at 22 ± 2° C for 24 hours without shaking. According to the following procedure, oocyte maturation induced by progesterone was

assessed after 24 hour culture, after the oocytes had been heat-fixed by boiling. Individual oocytes were ruptured under a dissecting microscope to check for the presence of the germinal vesicle. Germinal vesicle breakdown (GVBD) was used as an indicator of oocyte maturation.

Progesterone and TPCK

Progesterone was dissolved in ethanol at 2×10^{-2}M, as a stock solution, 5 μl of which was added to the test wells after dilution. Following the addition of progesterone or the vehicle, oocytes were checked for GVBD at 24 hours. In addition, when it was used, TPCK was dissolved in dimethylsulfoxide at 10 mg per ml, as a stock solution, 5 μl of which was added to the test wells. After 1 hour culture, the incubation medium was removed by suction and 2 ml of TPCK-free fresh medium was added for a 5 minute-period. The oocytes were washed twice more in the same manner and then cultured with progesterone as described above.

Preparation of the supernatant fraction from oocytes

Four hundred oocytes (premature ones cultured with the vehicle and mature ones treated with progesterone, respectively) were homogenized in 5 ml of Buffer A (0.25M sucrose, 10mM 2-mercaptoethanol, 25mM Tris-HCl, pH 7.5) with a motor-driven Teflon-pestle glass homogenizer (300 rpm, 5 strokes). The homogenates were centrifuged at 35,000 rpm (105,000xg) for 65 minutes at 4° C in a Beckman 50.3Ti rotor. The clear supernatant fractions between the lipid cap and packed yolk-pigments were retained as extracts.

Separation of the enzyme active fractions by glycerol density gradient centrifugation

An aliquot (1.5 ml) of each extract was loaded onto 20 ml of a 10–30% (v/v) glycerol density gradient in Buffer A plus 4 ml of Buffer A as an overlay, followed by centrifugation at 35,000 rpm (105,000xg) for 12 hours at 2° C in a Beckman 70Ti rotor. Fractions of 20 drops were collected, from the top to the bottom, using an automatic liquid charger, at 4° C, and an aliquot (10 μl) of each was assayed for enzyme activity. As marker proteins for sedimentation coefficient determination, thyroglobulin (19S), catalase (11.3S) and bovine serum albumin (4.5S) were used.

Separation of the enzyme active fractions by DEAE-cellulose column chromatography

An aliquot (0.5 ml) of an extract was applied to a column (0.6 × 3 cm) of DEAE-cellulose which had been previously equilibrated at 4° C with Buffer B [25mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol and 10mM 2-mercaptoethanol], and then the column was washed with the same buffer and eluted at the flow rate of 15 ml/hour with 20 ml of a 0–0.4M linear gradient of NaCl in the same buffer. Fractions of 0.5 ml were collected and aliquots (10 and 40 μl) were assayed for enzyme activity and protein content, respectively.

Estimation of molecular weights of the enzyme active fractions by gel filtration

The enzyme active fractions (5–10 ml) obtained after glycerol density gradient centrifugation were dialyzed against glycerol-free Buffer A and then concentrated seven to ten times by immersion of solid sucrose. Then, the concentrated samples (0.3–0.8 ml) were applied to a Sepharose 6B column (0.9 × 90 cm) equilibrated at 4° C with Buffer B and eluted with the same buffer at the flow rate of 2.5 ml/hour, fractions of 1 ml being collected. Aliquots (10 and 100 μl) were assayed for enzyme activity and protein content, respectively. As molecular weight marker proteins, thyroglobulin (Mr = 669 kDa), ferritin (440 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa) were used.

Enzyme assay

Eighty μl of 125mM Tris-HCl (pH 8.5) containing 125μM substrate (Suc-Leu-Leu-Val-Tyr-MCA, Boc-Phe-Ser-Arg-MCA, Boc-Gln-Arg-Arg-MCA or Cbz-Leu-Leu-Glu-NA) was first preincubated at 37° C with 10 μl of 0.5% SDS. After 10 minutes, an aliquot (10 μl) of the enzyme solution was added, followed by incubation at 37° C. After 1 hour, the reaction was stopped by adding 100 μl of 10% SDS and 2 ml of 100mM Tris-HCl (pH 9.0), and

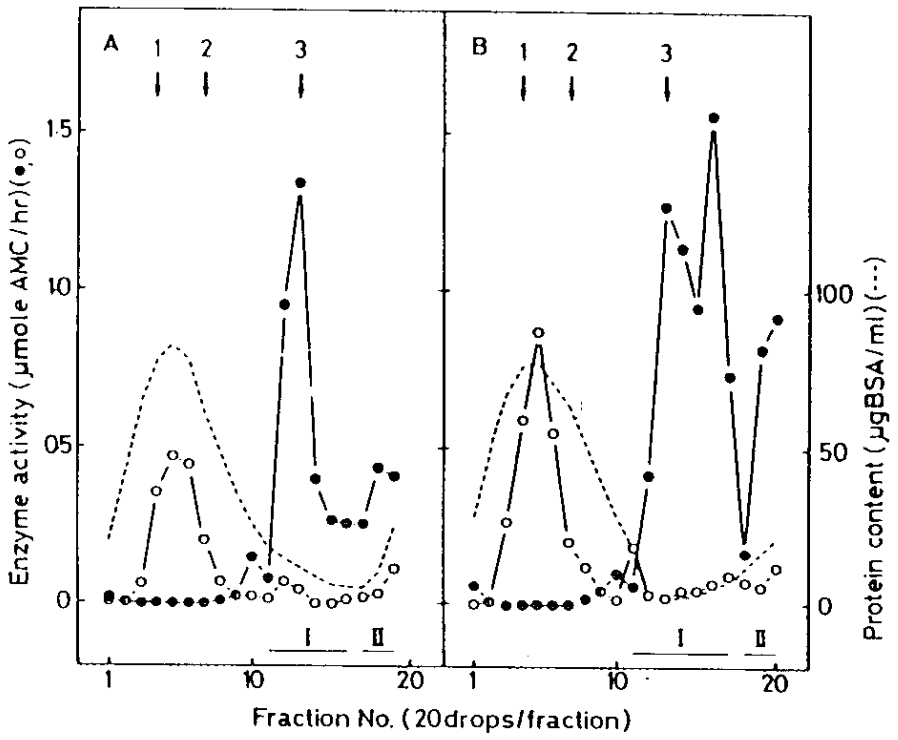

Fig. 1. Separation of enzyme active fractions from extracts of oocytes by glycerol density gradient centrifugation. The extract (0.5 ml) of premature oocytes (panel A) or mature oocytes (panel B) was applied to a 10 to 30% (v/v) glycerol density gradient, followed by centrifugation at 105,000xg for 12 hours at 2° C, as described under 'Materials and Methods'. An aliquot (10 μl) of each 20-drop-fraction was assayed for Suc-Leu-Leu-Val-Tyr-MCA (with SDS) (●—●) and Boc-Phe-Ser-Arg-MCA (without SDS) (○—○) hydrolyzing activities, respectively. An aliquot (100 μl) was used for protein content determination (- - -) by Bradford's method [21]. The arrows indicate the S values of the protein markers: 1: bovine serum albumin (4.5S), 2: catalase (11.3S) and 3: thyroglobulin (19S), applied to a parallel gradient. I and II at the bottom of the graph denote the pooled fractions.

then the amount of 7-amino-4-methylcoumarin (AMC) was measured fluorometrically (excitation at 360 nm, emission at 460 nm) with a Hitachi fluorescence spectrophotometer (type F-3000).

Immunological analysis

Fifty μl of the enzyme solution was incubated with 50 μl of rabbit anti-IgC (a gift from Dr. K. Tanaka of Tokushima University) directed against the purified proteasomes of *Xenopus laevis* (diluted from 50 μg protein/ml with Buffer B) for 1 hour at 37° C. Thirty μl of a *Staphylococcus aureus* cell suspension was added, followed by incubation with the antigen-antibody solution for 30 minutes at 37° C. Immunoprecipitates were removed by centrifugation and the supernatant was analyzed for enzyme activity using Suc-Leu-Leu-Val-Tyr-MCA with SDS, as described in the previous section.

Protein concentration

The protein content was estimated according to Bradford's method [21] with bovine serum albumin (BSA) as a standard.

Results

Separation of the enzyme active fractions from extracts of premature and mature oocytes by glycerol density gradient centrifugation

When the extracts of premature and mature oocytes in *Rana pipiens* were separated by glycerol

density gradient centrifugation, we examined both Suc-Leu-Leu-Val-Tyr-MCA and Boc-Phe-Ser-Arg-MCA hydrolyzing activities, as shown in Fig. 1. To ensure the Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity, the test was performed in the presence of SDS, which increased the activity. This figure shows that strong activities were found, as two peaks centered between around 19S and over 27S (the bottom) (as estimated from the calibration curve), in fraction Nos. 11-bottom, in premature oocytes. However, a different profile was obtained for mature oocytes (designated as 90% GVBD), i.e., three peaks centered at 19S, 24S and over 27S (the bottom), in the same fractions when compared to in the case of premature oocytes.

Furthermore, the Boc-Phe-Ser-Arg-MCA hydrolyzing activity in the absence of SDS was found in similar fractions of the extracts of premature and mature oocytes, despite the enzyme activity being low. The enzyme in the peak centered at 19S were suggested to be high molecular weight, multicatalytic proteinases (named proteasomes), as judged from the known characteristics [18–20], but peaks with S values larger than 19 were also found. In addition, a peak exhibiting strong Boc-Phe-Ser-Arg-MCA hydrolyzing activity was found at fraction Nos. 3–8, centered at around 7S, but no Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity was found, in the extracts of both premature and mature oocytes. These enzymes with low S values, were not analyzed further in the present study.

In this work, to clarify and compare the additive characteristics of proteinases with larger S values found in both types of oocytes, fractions containing enzyme activities were separately combined as I and II, corresponding to fraction Nos. 11–16 (or 17) and 17 (or 18)-bottom, constituting the distinct Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activities obtained on gradient elution (see Fig. 1), and then analyzed.

Identity of enzymes found in oocytes from Rana pipiens with proteasomes from ovaries of Xenopus laevis, as judged on immunological analysis

To identify these enzymes as proteasomes, we first used a rabbit- antibody against the purified proteasomes obtained from ovaries of *Xenopus laevis*, which were characterized previously by Tanaka *et al.* [19], and examined the immunological cross-reactivities of these enzymes (Fig. 2). Immunotitration analysis revealed that when Suc-Leu-Leu-Val-Tyr-MCA was used to detect the enzyme active fraction including the proteasomes, complete inhibition of the residual enzyme activities in the two fractions from premature oocytes was achieved by the addition of 50 μg of IgG per 2 ml. However, in those from mature oocytes, the addition of 100 μg of IgG per 2 ml was necessary for complete inhibition. These results show that the enzymes found in premature and mature oocytes from *Rana pipiens* were more or less different, but suggested to be identical or very similar to proteasomes by the immunological cross-reactivities with proteasomes from ovaries of *Xenopus*, despite the difference in species.

Estimation of the apparent molecular weights of fractions I and II from premature and mature oocytes by gel filtration on Sepharose 6B

In order to estimate the apparent molecular weights of the enzymes found in oocytes, fractions I and II, from the extracts of premature and mature oocytes, respectively, were loaded on a Sepharose 6B column (0.9 × 90 cm). The eluation profiles of the Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity are shown in Fig. 3. In premature oocytes, as shown in Fig. 3A, fraction I was eluted as a major peak centered at 700 kDa (as estimated from the calibration curve), and fraction II was eluted as double peaks centered at 700 and 1000 kDa. On the other hand, in mature oocytes, as shown in Fig. 3B, fraction I gave a major peak centered at 700 kDa with a shoulder peak centered at 440 kDa, and fraction II was eluted as double peaks centered at 700 and 1000 kDa. Although the different patterns showed the heterogeneity between premature and mature oocytes, as expected from the results of glycerol density gradient centrifugation, most of these enzymes were termed high molecular weight proteinases (over 700 kDa). The molecular mass of around 700 kDa, corresponding to 19S, was similar to that of 830 kDa estimated for proteasomes of *Xenopus* [19].

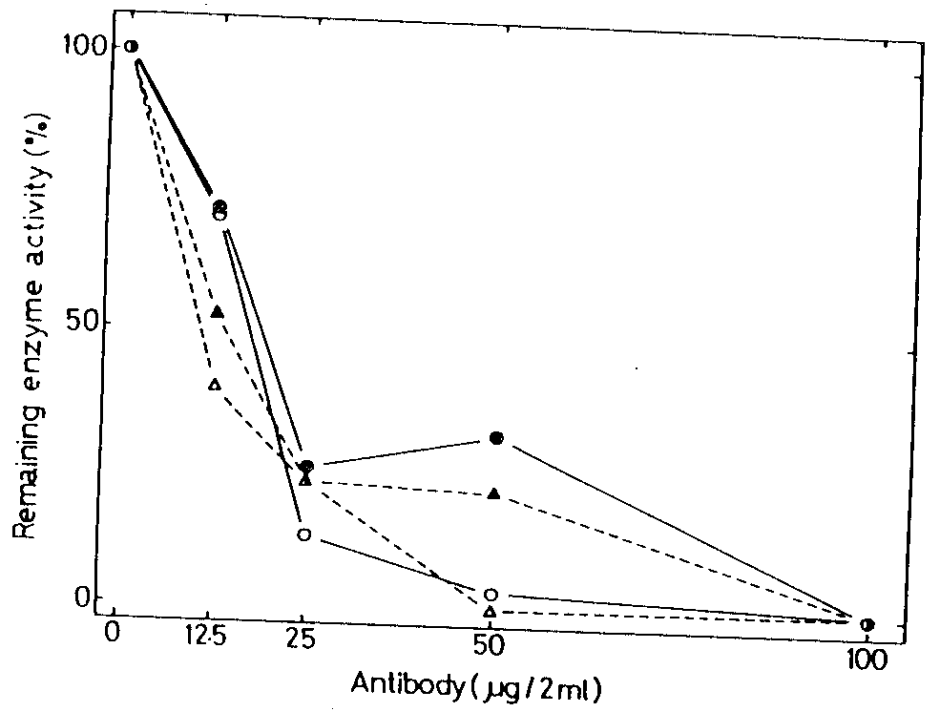

Fig. 2. Immunotitration of enzyme active fractions with proteasomes from ovaries of *Xenopus laevis*. Fractions I and II from extracts of premature and mature oocytes, respectively, as shown in Fig. 1, were incubated with rabbit anti-(*Xenopus* proteasome) IgG for 1 hour at 37° C, as described under 'Materials and Methods'. Each mixture was then incubated with *Staphylococcus aureus* for 30 minutes at 37° C. Immunoprecipitates were removed by centrifugation. The supernatant was assayed for residual Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity with SDS. The activities are given as percentages of those of controls containing the enzyme solution and the IgG dilution buffer. Fractions I (○—○) and II (△---△) from premature oocytes; Fractions I (●—●) and II (▲---▲) from mature oocytes.

Comparative enzyme activities of fractions I and II

As can be seen in Table 1, the hydrolyzing activities of the two fractions from premature and mature oocytes, respectively, with the use of four fluorogenic synthetic substrates, were examined with regard to the effect of SDS. All the substrates tested were originally designed for assaying the activities of proteasomes [19, 20]. The Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activities in all fractions were increased 5–23 fold by SDS, as expected. The enzymes in fraction II from premature oocytes were most responsible for this increase due to SDS, when compared with other fractions. In addition, the optimal concentration of SDS was 0.05% (data not shown). Fraction II of premature oocytes showed an increase in Cbz-Leu-Leu-Glu-NA hydrolyzing activity of 7 fold in the presence of SDS, while fraction I and both fractions of mature oocytes showed no enhancement with SDS. On the contrary, Arg-peptides such as Boc-Gln-Arg-Arg-MCA and Boc-Phe-Ser-Arg-MCA were scarcely or not hydrolyzed at all in the presence of SDS, irrespective of the fraction, and Boc-Gln-Arg-Arg-MCA was a more or less responsible substrate than another one in the absence of SDS.

As shown by these observations, the effect of SDS on the substrate hydrolyzing activities of each fraction was more or less different. Thus, several general conclusions could be drawn; i.e., that in the presence of SDS, Suc-Leu-Leu-Val-Tyr-MCA was degraded at a measurable rate and Cbz-Leu-Leu-Glu-NA was degraded at a more or less measurable rate. Boc-Gln-Arg-Arg-MCA was scarcely degraded, but Boc-Phe-Ser-Arg-MCA was not degraded at all.

On the basis of these findings, the substrate hy-

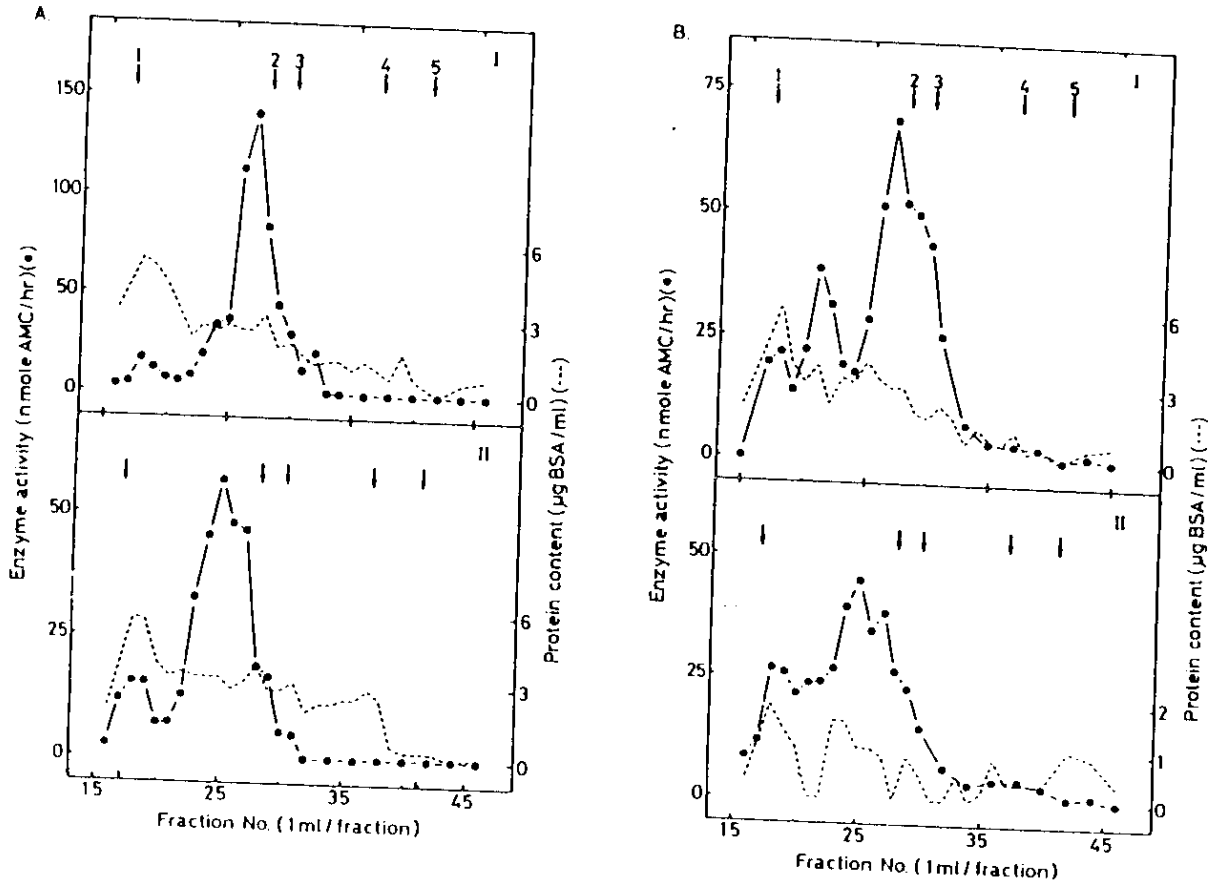

Fig. 3. Gel filtration of enzyme active fractions by glycerol density gradient centrifugation on a Sepharose 6B column. Fractions I (I) and II (II) (0.3–0.8 ml) from extracts of premature oocytes (panel A) and mature oocytes (panel B), respectively, as shown in Fig. 1, were applied on the column (0.9 × 90 cm) equilibrated at 4°C with the elution buffer [20% (v/v) glycerol-10 mM 2-mercaptoethanol-25 mM Tris-HCl, pH 7.5] at the flow rate of 2.5 ml/hour, as described under 'Materials and Methods'. Aliquots (10 and 100 μl) of 1 ml fractions were assayed for Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity with SDS (●—●) and protein content (---), respectively. The arrows indicate the elution positions of the MW markers. The markers used were 1: Blue dextran, 2: thyroglobulin (Mr = 670 kDa), 3: ferritin (440 kDa), 4: aldolase (158 kDa) and 5: bovine serum albumin (67 kDa).

drolyzing activities may have subtly different properties in fractions II from premature and mature oocytes, but all of these enzymes were termed multicatalytic proteinases [19, 20].

Separation of the enzyme active fractions from extracts of premature and mature oocytes by DEAE-cellulose column chromatography

To gain further information on the enzymes found, the remaining parts of the same extracts of the respective oocytes that were subjected to the glycerol density gradient, were subjected to DEAE-cellulose column chromatography and Suc-Leu-Leu-Val-Tyr-MCA and Boc-Phe-Ser-Arg-MCA hydrolyzing activities were assayed (Fig. 4). The prominent Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity was recovered as a broad peak centered at 0.18M NaCl, with a shoulder peak centered at 0.25M NaCl, for the extract of premature oocytes (Fig. 4A). In addition, the Boc-Phe-Ser-Arg-MCA hydrolyzing activity was found as a peak centered at 0.15M NaCl, but it overlapped with the prominent Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity. On the other hand, the prominent Suc-Leu-

Leu-Val-Tyr-MCA hydrolyzing activity in the mature oocytes was recovered as a more symmetrical peak centered at 0.18M NaCl, and the Boc-Phe-Ser-Arg-MCA hydrolyzing activity was found as a broad peak in a similar range (Fig. 4B), although a difference in these profiles between premature and mature oocytes was found. These binding behaviors on the DEAE-cellulose column can be correlated with the proteasomes found in rat liver [22]. In addition, both enzyme active fractions I and II obtained from the extracts of premature and mature oocytes on glycerol density gradient centrifugation were recovered as the same peaks, 0.15–0.18M NaCl-eluate, on the same DEAE-cellulose column (data not shown). These results also confirmed that the premature and mature oocytes themselves possess at least two different proteinases showing Suc-Leu-Leu-Val-Tyr-MCA and Boc-Phe-Ser-Arg-MCA hydrolyzing activities, as shown in Fig. 1.

Since a higher dose of TPCK has been found to interfere with progesterone-induced GVBD [14], apparent involvement of a chymotrypsin-like proteinase has been suggested in a certain step of oocyte maturation. Accordingly, as the following experiment, the extract obtained from premature oocytes exposed previously to a higher dose of TPCK was applied to the same DEAE-cellulose column as used above, but Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity was not found in any fractions (Fig. 4a). In another experiment, no activity was also detectable in any fraction, even with the extract from oocytes whose maturation was suppressed when treated with progesterone following previous exposure of the oocytes to TPCK (designated as 40% GVBD) (Fig. 4b). These results show that proteasomes, which are present in premature oocytes and not synthesized after progesterone treatment, may consist of a TPCK-sensitive proteinase, a probably chymotrypsin-like proteinase.

Discussion

We showed for the first time that supernatant fractions derived from full-grown premature oocytes as well as mature oocytes of *Rana pipiens* treated with progesterone contain high molecular weight, multicatalytic proteinases, despite being suggested to be different entities by glycerol density gradient centrifugation (Fig. 1).

These enzymes, found through the use of oocytes enclosed with follicle cells, seem to be derived

Table 1. Comparison of the enzyme activities of fractions I and II separated by glycerol density gradient centrifugation from extracts of premature and mature oocytes toward several fluorogenic substrates (μmole AMC/hr/mg protein)

Substrate	SDS	Premature oocytes		Mature oocytes	
		I	II	I	II
Suc-Leu-Leu-Val-Tyr-MCA	–	369	334	546	383
	+	2844	7779	2848	2551
		(7.7)	(23.3)	(5.2)	(7.7)
Boc-Gln-Arg-Arg-MCA	–	215	287	339	423
	+	9	38	51	1
		(0.0)	(0.1)	(0.2)	(0.0)
Boc-Phe-Ser-Arg-MCA	–	51	31	119	211
	+	0	0	0	21
		(N.E.)	(N.E.)	(N.E.)	(0.1)
Cbz-Leu-Leu-Glu-NA	–	255	209	898	485
	+	350	1457	1025	523
		(1.4)	(7.0)	(1.1)	(1.1)

Fractions I and II from premature and mature oocytes were assayed for enzyme activities as described under 'Materials and Methods'. Values in parentheses show the rate acceleration (-fold) on the addition of SDS (0.05%). N.E., not estimated.

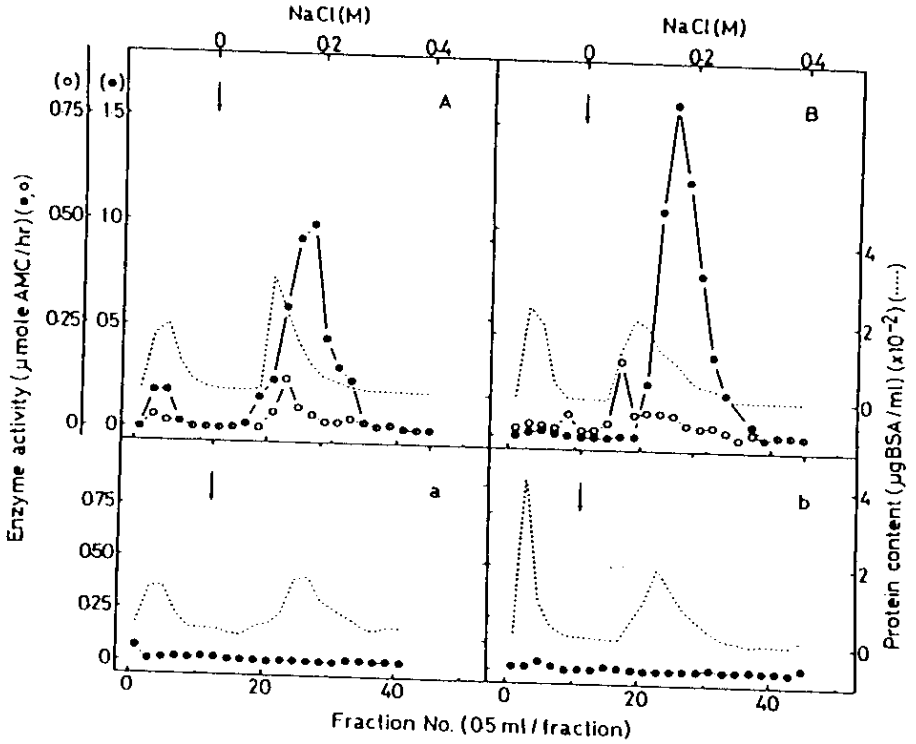

Fig. 4. Separation of enzyme active fractions in extracts of oocytes on a DEAE-cellulose column. An aliquot (0.5 ml) of the extract from premature (panel A) or mature oocytes (panel B) was chromatographed on a DEAE-cellulose column (0.6 × 3 cm) at 4° C, with elution with a linear gradient (0–0.4 M NaCl) at a flow rate of 15 ml/hour, as described under 'Materials and Methods'. The arrows show the start of the salt gradient. Aliquots (10 μl) of 0.5 ml-fractions were assayed for Suc-Leu-Leu-Val-Tyr-MCA (with SDS) (●—●) and Boc-Phe-Ser-Arg-MCA (without SDS) (O—O) hydrolyzing activities, respectively. An aliquot (40 μl) was subjected to protein content determination (- - -) by Bradford's method [21]. In parallel, aliquots (0.5 ml) of extracts from oocytes pretreated with TPCK (panel a) and ones treated with progesterone following TPCK-pretreatment (panel b), respectively, were chromatographed as described above.

not from the follicle cells but from the oocytes, and have similar properties to those of proteasomes from eukaryotic somatic and germ cells [19, 20, 23], with respect to sedimentation coefficient (Fig. 1), molecular mass range (Fig. 3), immunological cross-reactivity (Fig. 2), and hydrolyzing acitivities toward different substrates and their SDS sensitivity (Table 1). In addition, it is unclear whether or not the components with larger S values than 19S and higher molecular weights (over 700 kDa) were derived through the association of proteasomes with some components. Here, these enzymes with larger S values may have similar properties to those of ubiquitin-dependent degrading enzyme found in somatic cells [24, 25]. We also demonstrated that these proteinases were not newly synthesized following progesterone treatment, but present in premature oocytes (Fig. 4).

In the cases of all the oocyte enzymes, the Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity, corresponding to chymotrypsin-like activity, as a sensitive indicator, was appreciably enhanced by SDS, and peptidyl-glutamyl peptide bond hydrolyzing activity was also found, but Arg-containing peptide bond hydrolyzing activity was not enhanced by SDS (Table 1). The concentration of SDS (0.05%) required to activate the oocyte enzyme was also the same order of magnitude as that required for activation of mammalian proteasomes [26, 27]. Although the enzymes found in mature oocytes as well as premature oocytes have been shown to be converted from latent forms to activated ones by

SDS, it is known that fatty acids, in place of SDS, also activate the proteasomes [23, 26, 27], but we did not examine this in this study. The incorporation of arachidonic acid, a polyunsaturated fatty acid, into phosphatidyl choline has been followed and its release on induction of oocytes of *Rana pipiens* with progesterone has been reported [28]. Therefore, it may regulate the activity of the enzyme *in ovo*. In addition, it may be necessary to clarify the optimal pH and temperature, and the action of various proteinase inhibitors for the different enzyme fractions.

Until now, the named proteasome has been assumed to be homologous or identical to the 20S cylinder particle from *Xenopus* oocytes [18], the cytoplasmic 19S ribonucleoprotein from *Drosophila* [29], and the 19S 'prosome' particle from mammalian cells [30]. Thus, all of them appear to exist as large molecules composed of different proteins and RNA components often found to be associated with large particles, but we have not demonstrated a subunit composition characteristic for proteasomes in this study.

When combined with the above observations, one possible reason for the different patterns of premature and mature oocytes on glycerol density gradient centrifugation and gel filtration seems to be the different subunit components, as suggested in *Drosophila* [31], including that the alteration seems to be a post-translational modification (phosphorylation) of the polypeptides.

On the other hand, it was demonstrated that oocyte maturation (designated as GVBD) induced by progesterone was inhibited following continuous exposure to higher doses of TPCK [14; this paper]. Furthermore, as has been reported for other proteinase inhibitors (chymostatin, leupeptin and antipain) [11, 13], which were ineffective following the injection of MPF. These demonstrations suggest that the possible multicatalytic nature of the enzyme in premature oocytes may be involved before and after the MPF formation in oocyte maturation. A TPCK-sensitive proteinase, probably proteasome, may be suggested to be at least one component of the proteinases involved in oocyte maturation (Fig. 4).

Furthermore, it has been recently reported that a protein named cyclin is the only newly synthesized protein required for the induction of oocyte maturation through the activation of MPF in *Xenopus* [32]. Succeedingly, the activity of this protein is thought to be controlled by means of proteolysis. Thus, this protein is thought to be one of the essential components for cells to enter meiosis. Such a protein might be an endogenous target for proteasomes of oocytes.

Further studies are necessary to clarify more chemical properties of this oocyte enzyme and to find natural substrates of a protein nature for this enzyme, and to define its physiological function in oocyte maturation in amphibians.

Acknowledgements

The authors wish to thank Dr. K. Tanaka of Tokushima University for the excellent experimental help throughout the present study. This work was supported in part by the Special Funds for Education and Research of Shizuoka University to K.I.

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Address for offprints: K. Ishikawa, Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422, Japan

Publication

Developmental Biology, 1992, 152, 113 - 120.

Toshiaki Hirai, Masakane Yamashita, Michiyasu Yoshikuni, Toshinobu Tokumoto, Hiroko Kajiura, Noriyoshi Sakai and Yoshitaka Nagahama : Isolation and characterization of goldfish cdk2, a cognate variant of the cell cycle regulator cdc2.

DEVELOPMENTAL BIOLOGY 152, 113-120 (1992)

Isolation and Characterization of Goldfish cdk2, a Cognate Variant of the Cell Cycle Regulator cdc2

T. HIRAI,[1] M. YAMASHITA, M. YOSHIKUNI, T. TOKUMOTO, H. KAJIURA, N. SAKAI, AND Y. NAGAHAMA

Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan

Accepted March 17, 1992

This paper reports the nucleotide and predicted amino acid sequences of the goldfish cdk2, a cognate variant of the cell cycle regulator cdc2. The predicted protein sequence shows strong homology to the other known cdk2 (88% for *Xenopus* and 90% for human). A monoclonal antibody against the C-terminal sequence of goldfish cdk2 recognized a 34-kDa protein in extracts from various goldfish tissues. The protein level was high in such tissues as testis and ovary containing actively dividing cells. Protein cdk2 binds to $p13^{suc1}$, the fission yeast *suc1*$^+$ gene product, but not to cyclin B, with which cdc2 forms a complex. The kinase activity of cdk2 increased 30-fold when oocytes matured, although its protein level did not remarkably change. Anti-cdk2 immunoprecipitates from ^{32}P-labeled mature oocyte extracts contained a 47-kDa protein, which was not recognized by either anti-cyclin A or anti-cyclin B antibody, indicating complex formation of cdk2 with a protein other than cyclins A or B.

INTRODUCTION

Oocyte maturation is induced by maturation-promoting factor (MPF) under the influence of maturation-inducing hormone secreted from the follicle cells surrounding the oocyte (Nagahama, 1987; Smith, 1989). MPF was first described as an activity present in mature oocyte cytoplasm, which induces maturation under protein synthesis inhibition when microinjected into full-grown immature oocytes (Masui and Markert, 1971; Smith and Ecker, 1971). It is now known to be a universal regulator of the G2 to M phase transition in eukaryotes (Kishimoto, 1988; Hunt, 1989; Dorée, 1990; Nurse, 1990; Maller, 1991). MPF has been purified from mature oocytes of *Xenopus* (Lohka *et al.*, 1988), starfish (Labbé *et al.*, 1989a,b), and carp (Yamashita *et al.*, 1992a,b), and it consists of two subunits: the catalytic subunit is a serine/threonine protein kinase, which is a homolog of the product of the *cdc2*$^+$ gene of fission yeast (*Schizosaccharomyces pombe*), referred to as cdc2, and the regulatory subunit is cyclin B (Gautier *et al.*, 1988, 1990; Labbé *et al.*, 1988, 1989a,b; Yamashita *et al.*, 1992a,b; for review, see Hunt, 1989; Dorée, 1990; Maller, 1991).

The following characteristics are noted for cdc2 (Nurse, 1990): (1) it contains a specific 16-amino-acid sequence, the PSTAIR sequence (EGVPSTAIREISLLKE); (2) it binds to $p13^{suc1}$, the product of *suc1*$^+$ gene of fission yeast, and (3) it binds to cyclin B at M phase. Recently, however, *cdc2*$^+$-like genes have been isolated from the toad *Xenopus* (Paris *et al.*, 1991), the fruit fly *Drosophila* (Lehner and O'Farrell, 1990), human (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Tsai *et al.*, 1991), maize (Colasanti *et al.*, 1991), and the flowering plant *Arabidopsis* (Hirayama *et al.*, 1991). Cell division kinase or cyclin-dependent kinase, referred to as cdk2 (formerly Eg-1, in *Xenopus*), is a cdc2-like protein (Solomon, 1990; Elledge and Spottswood, 1991; Fang and Newport, 1991; Ninomiya-Tsuji *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991). It shares all the characteristics of cdc2 listed above, except for the ability to form a complex with cyclin B (Solomon *et al.*, 1990; Fang and Newport, 1991). In yeast, cdc2 is involved in both G2/M and G1/S transitions (Broek *et al.*, 1991). In vertebrates, however, cdc2 and cdk2 apparently play different roles. Experiments in a *Xenopus* cell-free system have suggested that cdc2 and cdk2 are involved in G2/M and G1/S transitions, respectively (Fang and Newport, 1991). It is likely that during evolution cdc2 differentiates into several variants including cdk2, each of which functions at different phases of the cell cycle. Therefore, isolation of *cdc2*$^+$-like genes and characterization of their products are of great importance for understanding the regulatory mechanisms of the cell cycle in higher eukaryotes.

From a cDNA library constructed from mature goldfish oocytes, we isolated a cDNA clone encoding the PSTAIR sequence, the hallmark of cdc2 family proteins. In this study, we sequenced the cDNA, predicted its amino acid sequence, and identified this clone as goldfish cdk2 cDNA. We also characterized its product with monoclonal antibodies against the C-terminal sequence of the cdk2 protein, the PSTAIR sequence (Yamashita *et al.*, 1991) and *Escherichia coli*-produced goldfish cyclins

[1] Current address: Department of Biological Sciences, The Nishi Tokyo University, 2525 Uenohara, Yamanashi 409-01, Japan.

0012-1606/92 $5.00

A and B (Yamashita *et al.*, 1992a; Hirai *et al.*, 1992). Changes in the activity and protein level of goldfish cdk2 were also examined during oocyte maturation.

MATERIALS AND METHODS

Animals and Oocytes

Gravid female goldfish were raised at 15°C until use. Mature oocytes (ovulated eggs) were obtained by injecting 200 IU of human chorionic gonadotropin (Puberogen, Sankyo Zoki; Yamashita *et al.*, 1990). Full-grown immature oocytes were isolated from the ovary by pipetting. *In vitro* oocyte maturation was induced by incubating full-grown immature oocytes with 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one, a natural maturation-inducing hormone in fish (Nagahama and Adachi, 1985), as described previously (Yamashita *et al.*, 1992a). Maturational processes were assessed by microscopic examination of the commencement of germinal vesicle breakdown (GVBD), after soaking the oocytes in a clearing solution (Lessman and Kavumpurath, 1984).

Cloning and Sequencing of cDNAs

A cDNA library from poly(A)$^+$ RNA isolated from mature goldfish oocytes was constructed in λgt10 using the cDNA synthesis system plus and the cDNA cloning system λgt10 (Amersham).

For screening cDNAs encoding cdc2 family, an oligonucleotide (35 mer; GAAATTTC(A/T)CG(A/G)AT(T/G)GCAGTACT(T/G)GGAAC(T/A)CCTTC), which corresponds to part of the PSTAIR sequence (EGVPSTAIREIS), was synthesized on an Applied Biosystems Model 391 DNA synthesizer. The oligonucleotide was 5′ end labeled with [γ-^{32}P]ATP (111 TBq/mmol; NEN), and used for screening approximately 1×10^6 plaques. Plaques transferred onto nylon membranes were hybridized at 50°C overnight with a 1×10^7 cpm probe in 30 ml of hybridization solution containing 6× SSC (1× SSC: 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS), and 0.02% each of Ficoll, bovine serum albumin (BSA, fraction V), and polyvinylpyrrolidone. Membranes were washed twice at 50°C for 1 hr with 6 × SSC containing 0.1% SDS.

The cDNA inserts of isolated clones were subcloned into the plasmid vector pBluescript II KS M13$^+$ (Stratagene). Serial deletion mutants of subclones were made according to Yanisch-Perron *et al.* (1985). Nucleotide sequences were determined with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical) using [α-^{32}P]dCTP and analyzed on GENETYX software (SDC).

Extraction of Tissues

One hundred micrograms of tissues (ovary, testis, intestine, liver, and muscle) was dissected from goldfish and homogenized in 200 μl of an extraction buffer (EB: 100 m*M* β-glycerophosphate, 20 m*M* Hepes, 15 m*M* $MgCl_2$, 5 m*M* EGTA, 100 μ*M* *p*-amidinophenyl methanesulfonyl fluoride, 3 μg/ml leupeptin, pH 7.5). After centrifugation at 15,000*g* for 5 min at 4°C, 200 μl of the supernatant was mixed with 200 μl of 2× SDS sample buffer and then boiled for 2 min. The samples were electrophoresed and immunoblotted, as described below.

Immature and mature oocytes were extracted by crushing in EB by ultracentrifugation (100,000*g*, 1 hr, 4°C), as described previously (Yamashita *et al.*, 1992a). A portion of the extracts was mixed with SDS sample buffer immediately after extraction, and the remainder was stored at −80°C until use.

Oocytes in the process of maturation were extracted as follows: At 1-hr intervals, 50 oocytes were washed in EB. After excess EB was removed with filter paper, 50 μl of fresh EB was added. The oocytes were homogenized and centrifuged at 15,000*g* for 10 min at 4°C. The supernatant was mixed with SDS sample buffer and immunoblotted, as described below.

Production of Monoclonal Antibodies against the C-terminal Sequence of cdk2

A peptide (cdk2-C1: CRFFRDVTMPVPPLRL), which corresponds to the C-terminal sequence of goldfish cdk2 (Fig. 1) and has an additional cysteine in the N-terminus, was synthesized using the solid-phase method (using F-moc protocol) on an Applied Biosystems Model 431A peptide synthesizer. The peptide was purified by reverse-phase HPLC and coupled to BSA and keyhole limpet hemocyanin (KLH) through its N-terminal cysteine by *N*-(ε-maleimidocaproyloxy)succinimide (Dojin). The peptide coupled to BSA and KLH was emulsified with Freund's complete adjuvant and injected into 7-week-old female BALB/c mice. Incomplete adjuvant was used for subsequent injections. After a total of four injections at 2-week intervals, hybridomas were produced by fusion of the spleen cells isolated from the immunized mice with mouse myeloma cells (P3-X63-Ag8-U1) using 50% polyethyleneglycol 4000 (Merck). The hybridomas were first screened by ELISA with a carrier-free peptide as an antigen, and the positive cells were cloned by limiting dilution, as described previously (Yamashita *et al.*, 1991). The hybridomas were finally screened by immunoblotting. One clone (C1-42) was used to detect cdk2 by immunoblotting. The isotype of this antibody was IgG_1 containing κ light chains. This antibody could not precipitate cdk2 efficiently from oo-

cyte extracts; therefore a polyclonal antibody (serum obtained from an immunized mouse) was used for immunoprecipitation.

Electrophoresis and Immunoblotting

Proteins were separated on a 12.5% SDS-polyacrylamide gel (Laemmli, 1970) and then transferred to an Immobilon membrane (Millipore) by semidry electroblotting. Following blocking in 5% nonfat dry milk, the membrane was incubated for 1 hr with culture supernatant containing either anti-PSTAIR (Yamashita *et al.*, 1991) or anti-cdk2 antibodies. Immunocomplexes were detected using alkaline phosphatase-conjugated anti-mouse immunoglobulins (Zymed), as described previously (Yamashita *et al.*, 1991).

Precipitation with p13^{suc1}

The fission yeast *suc1*$^+$ gene product, p13^{suc1}, was used to isolate cdc2 family proteins from oocyte extracts (Dunphy and Newport, 1989). The protein was coupled with CNBr-activated Sepharose beads according to the manufacturer's instructions. Oocyte extracts (50 μl) were mixed with 10 μl of p13^{suc1} Sepharose beads and incubated overnight at 4°C under continuous agitation. The beads were washed in EB containing 0.2% Tween 20, mixed with 10 μl of 2× SDS sample buffer, and boiled for 2 min. After a brief centrifugation, 10 μl of the supernatant was separated by SDS-PAGE, followed by anti-cdk2 immunoblotting.

Immunoprecipitation

Oocyte extracts (50 μl) were mixed with 10 μl of protein A-Sepharose beads and incubated for 1 hr at 4°C. After centrifugation at 15,000*g* for 5 min, the supernatant was mixed with 1 μl of either ascites fluid containing anti-cyclin B monoclonal antibody (B13: Hirai *et al.*, 1992) or anti-cdk2 serum. After incubation for 1 hr at 4°C, 10 μl of protein A-Sepharose beads was added and incubated for 1 hr at 4°C under continuous agitation. The beads were washed in EB containing 0.2% Tween 20, mixed with 2× SDS sample buffer, boiled for 2 min, then separated by SDS-PAGE, followed by immunoblotting with anti-PSTAIR, anti-cdk2, anti-cyclin A (Yamashita *et al.*, 1992a) or anti-cyclin B monoclonal antibody (Hirai *et al.*, 1992). To detect cyclins in the immunoprecipitates, biotinylated anti-cyclin antibodies and avidin-conjugated biotinylated alkaline phosphatase (ABC kit; Vector Lab.) were used to avoid visualization of the antibody used for immunoprecipitation, since portions of them migrated to positions similar to those of cyclins on SDS-PAGE (cf. Fig. 7).

Kinase Assay

Immature and mature oocyte extracts (50 μl) were immunoprecipitated with anti-cyclin B and anti-cdk2 antibody, as described above. Kinase activities of the immunoprecipitates were measured using histone H1 or a synthetic peptide (SP-peptide, KKAAKSPKKAKK; Yamashita *et al.*, 1992b) containing the consensus sequence phosphorylated by cdc2 (Moreno and Nurse, 1990), according to the procedure of Yamashita *et al.* (1992b), except for a 10-min reaction.

Labeling of Oocyte Extracts with ^{32}P

Mature goldfish oocyte extracts (40 μl) were incubated with 5 mCi/ml of [γ-^{32}P]ATP (222 TBq/mmol; NEN) for 1 hr at 25°C. After centrifugation at 15,000*g* for 5 min at 4°C, the supernatant was divided into two. One was precipitated with preimmune serum and the other was precipitated with anti-cdk2 serum, as described above. The precipitates were analyzed by autoradiography following anti-PSTAIR immunoblotting.

RESULTS AND DISCUSSION

Isolation of Goldfish cdk2 cDNA

Six clones were isolated with an oligonucleotide corresponding to the PSTAIR sequence. The longest clone had an insert of 1205 bp containing a poly(A)$^+$ tail and an open reading frame encoding 298 amino acids (Fig. 1). It encodes a putative protein kinase containing the PSTAIR sequence and four tryptophan residues conserved in the cdc2 family (Figs. 1 and 2). The predicted molecular weight of the protein encoded by this gene is 33,998. The amino acid sequence of this clone had a significant homology (64-69%) with the cdc2 of other species, but much higher homology was found with the cdk2 of *Xenopus* (88%) and human (90%) (Fig. 2). Therefore, we identified this clone as goldfish cdk2 cDNA, which was also confirmed by several immunological studies as described below. The remaining five clones isolated with the PSTAIR-oligonucleotide also encoded cdk2 (data not shown). These results suggest that in mature goldfish oocytes the levels of cdk2 mRNA are more abundant than those of cdc2 mRNA. Proteins cdk2 and cdc2 differed by 15 amino acids (Fig. 2). These should be useful in distinguishing cdk2 from cdc2, and should be key residues of cdk2, if the two proteins have different functions.

Detection of cdk2 with a Monoclonal Antibody against its C-terminal Sequence

Anti-PSTAIR antibody recognized 33- to 35-kDa bands in extracts from various goldfish tissues (Fig.

FIG. 1. The nucleotide and predicted amino acid sequence of the goldfish cdk2. The PSTAIR sequence is underlined. The residues conserved in kinases (Hanks *et al.*, 1988) are indicated by closed circles. The tentative phosphorylation sites and the four tryptophan residues conserved in cdc2 family are shown by = and #, respectively.

3A). Since the PSTAIR sequence is common to the cdc2 family proteins including cdk2 (Fig. 2), the anti-PSTAIR reactive bands should contain cdk2. To determine which band was cdk2, we raised a monoclonal antibody against the C-terminal sequence of goldfish cdk2. The antibody recognized the anti-PSTAIR reactive 34-kDa band (Fig. 3B), though cdc2 was also located in the same band. Therefore, we concluded that the anti-PSTAIR reactive 34-kDa band detected in crude extracts contains both cdc2 and cdk2. The cdk2 content was highest in the testis (Fig. 3B). Although the cdk2 level in the ovary appeared to be less than that in the testis, the content per cell should be much higher, since the number of cells in the same weight of tissues should be much less in ovary than in other tissues, because the oocytes are large. It is likely that tissues undergoing frequent cell division contain high levels of cdk2. Similar results have been reported for cdc2 in the chick (Krek and Nigg, 1989), wheat (John *et al.*, 1990), and maize (Colasanti *et al.*, 1991). A decrease in the amount of cdc2 family proteins between periods of cell division and subsequent cell growth may be a key element in the switch from cell division to cell differentiation.

The anti-goldfish cdk2 monoclonal antibody also recognized carp cdk2 (Fig. 4D), but not catfish, medaka, or eel cdk2 (data not shown), indicating that the C-terminal sequences vary from species to species.

Characterization of cdk2

As described above, the anti-cdk2 monoclonal antibody recognized a 34-kDa protein in extracts from various goldfish tissues (Fig. 3). However, part of the 34-kDa cdk2 was modified into a 33-kDa form in oocyte

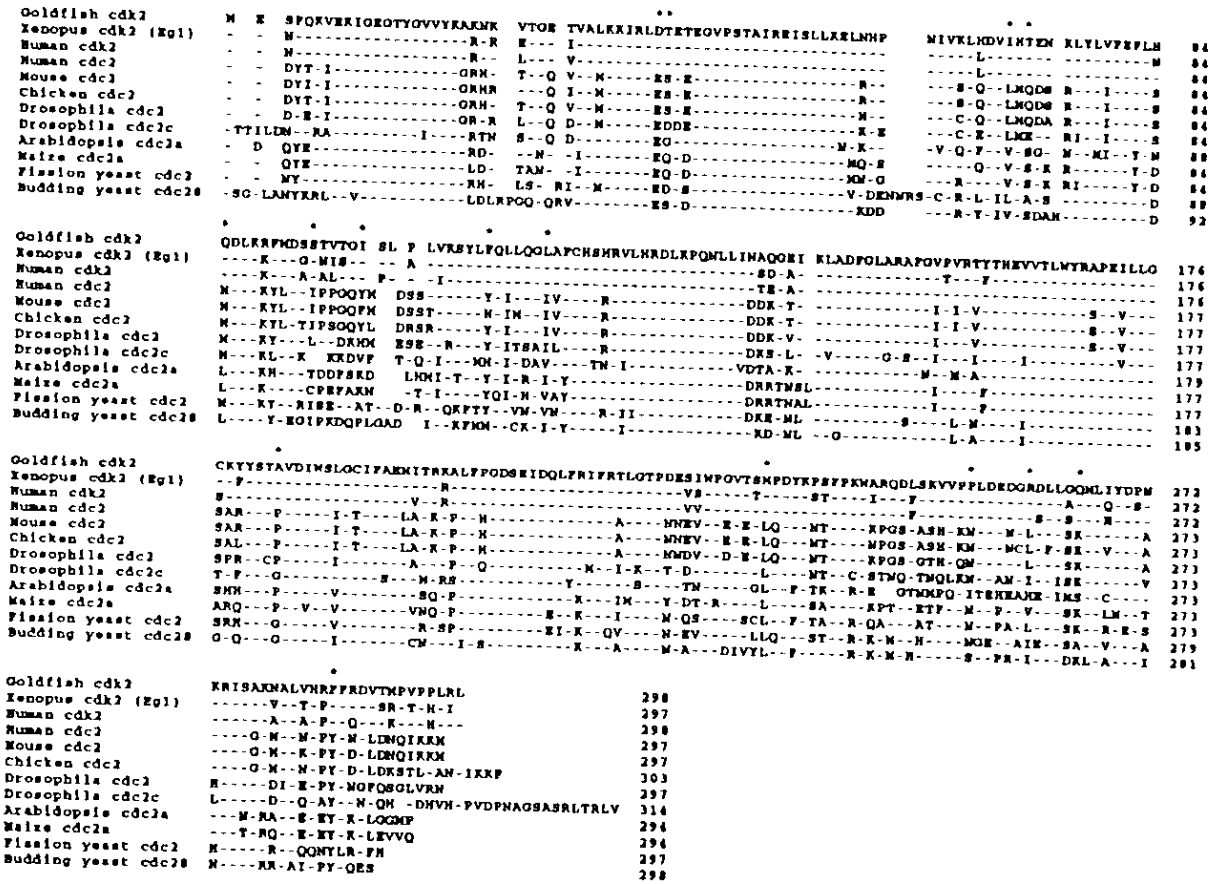

FIG. 2. Comparison of the amino acid sequences of the goldfish cdk2 with cdk2 homologs from toad (*Xenopus*, Paris *et al.*, 1991) and human (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Tsai *et al.*, 1991) and with cdc2 homologs from human (Lee and Nurse, 1987), mouse (Cisek and Corden, 1989), chicken (Krek and Nigg, 1989), fruit fly (*Drosophila*, Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990), flowering plant (*Arabidopsis*, Hirayama *et al.*, 1991), maize (Colasanti *et al.*, 1991), fission yeast (Hindley and Phear, 1984), and budding yeast (Lörincz and Reed *et al.*, 1984). Identical residues are shown by dashes. The residues that differ between cdk2 and cdc2 are indicated by asterisks.

extracts after freeze-thawing (Fig. 4A, lanes 1 and 2). The modification was more prominent in mature than in immature oocyte extracts (Fig. 4A, lanes 3 and 4). The 34-kDa cdc2 protein isolated with anticyclin B antibody from mature oocyte extracts stored at −80°C was not remarkably modified (Fig. 4B, lane 8), indicating that this modification is specific to cdk2. These results suggest that cdk2 and cdc2 have different protein characteristics. The chemical nature of this modification remains to be characterized.

Previous studies have reported that cdk2 binds to $p13^{suc1}$ but, unlike cdc2, it does not form a complex with cyclin B (Solomon *et al.*, 1990; Fang and Newport, 1991). To confirm this, we precipitated oocyte extracts with $p13^{suc1}$-conjugated beads and anti-cyclin B antibody. The $p13^{suc1}$ precipitates from goldfish oocyte extracts contained cdk2 (Fig. 4A, lanes 3 and 4), confirming the binding of cdk2 to $p13^{suc1}$. The anti-cyclin B immunoprecipitate from mature goldfish oocyte extracts did not contain anti-cdk2 reactive proteins (Fig. 4B, lane 6), whereas it contained an anti-PSTAIR reactive 34-kDa protein, which is probably cdc2 (Fig. 4B, lane 8). We also tried to examine the absence of cyclin B in anti-cdk2 immunoprecipitates. However, the monoclonal antibody could not precipitate cdk2 efficiently. Therefore, we used a polyclonal antibody against the C-terminal sequence of goldfish cdk2. The immunoprecipitates from mature oocyte extracts stored at −80°C contained 33- and 34-kDa cdk2s (Fig. 4B, lane 5), but not cyclin B (Fig. 4C, lane 9). These results clearly demonstrate that cdk2 does not form a complex with cyclin B.

MPF consists of cdc2 forming a complex with cyclin B (see Maller, 1991, for review). The absence of the binding of cdk2 to cyclin B suggests that cdk2 is not a component of MPF. To confirm this, MPF highly purified from mature carp oocytes (Yamashita *et al.*, 1992a) was stained

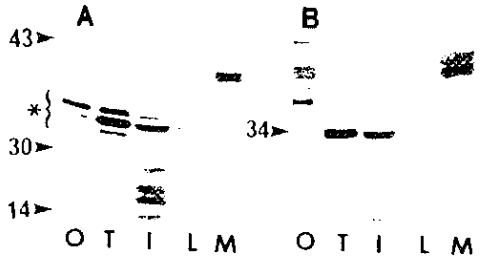

FIG. 3. Immunoblots of goldfish ovary (O), testis (T), intestine (I), liver (L), and muscle (M) extracts, as revealed by anti-PSTAIR (A) and anti-cdk2 (B) antibodies. Anti-PSTAIR reactive 33- to 35-kDa proteins are indicated by the asterisk. Figures show molecular weight in kDa.

with the anti-cdk2 antibody. The purified MPF contained anti-PSTAIR reactive 33- and 34-kDa proteins (Fig. 4D, lane 12). The anti-cdk2 antibody recognized the 33-kDa protein, but not the 34-kDa protein (Fig. 4D, lane 11). The 34-kDa protein, which is probably cdc2, corresponded to the MPF activity, but the 33-kDa protein did not (Yamashita *et al.*, 1992a). Therefore, it is likely that cdk2 is not a component of MPF. However, it is also evident that cdk2 was found in the highly purified MPF (Fig. 4D) and activated in mature oocytes (see below, Fig. 5). Further studies are required to determine the relationship between cdk2 and MPF. As described above, the 33-kDa cdk2 is a modified cdk2, since MPF was purified from extracts stored at −80°C.

Changes in cdk2 Activity and Protein Level during Goldfish Oocyte Maturation

As predicted from the cDNA, cdk2 should be a protein kinase (Fig. 1). Since cdc2 prefers histone H1 as an exogenous substrate, it is also probable that cdk2 has histone H1 kinase activity. We examined the kinase activity of cdk2 isolated from immature and mature goldfish oocyte extracts using the anti-cdk2 polyclonal antibody. Similar to cdc2 isolated with anti-cyclin B antibody, cdk2 had histone H1 kinase activity. The activity of cdk2, as well as of cdc2, increased threefold when oocytes matured ($P < 0.01$, Student's t test) (Fig. 5A). A more prominent (20- to 30-fold) increase in the kinase activity of cdk2 and cdc2 was obtained when a specific substrate for cdc2 (SP-peptide) was used as the exogenous substrate (Fig. 5B). It is unlikely that the kinase activity detected in the anti-cdk2 immunoprecipitates is due to contamination of cdc2, since it did not contain cyclin B that binds to active cdc2 (Fig. 4C). These results indicate that the activity of cdk2 actually increases during oocyte maturation and that its substrate specificity is similar to that of cdc2.

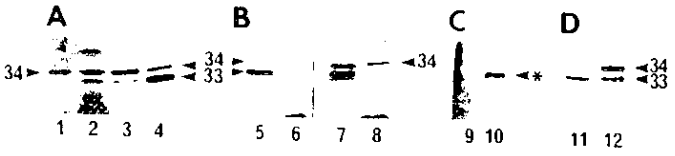

FIG. 4. Immunological characterization of cdk2. (A) Appearance of 33-kDa cdk2 in extracts stored at −80°C, as revealed by anti-cdk2 immunoblots of mature oocyte extracts prepared freshly (1) and stored at −80°C (2), and of $p13^{suc1}$ precipitates from immature (3) and mature (4) oocyte extracts stored at −80°C. (B) Absence of anti-cdk2 reactive proteins in anti-cyclin B immunoprecipitate from mature oocyte extract, as revealed by anti-cdk2 immunoblots of anti-cdk2 (5) and anti-cyclin B (6) immunoprecipitates. Anti-PSTAIR immunoblots of anti-cdk2 (7) and anti-cyclin B (8) immunoprecipitates demonstrate 34-kDa cdc2 in the anti-cyclin B immunoprecipitate. (C) Absence of cyclin B (asterisk) in anti-cdk2 immunoprecipitates from mature oocyte extract, as revealed by anti-cyclin B immunoblots of anti-cdk2 (9) and anti-cyclin B (10) immunoprecipitates. (D) Detection by anti-cdk2 antibody of anti-PSTAIR reactive 33- but not 34-kDa protein, found in MPF purified from mature carp oocytes, as revealed by anti-cdk2 (11) and anti-PSTAIR (12) immunoblotting.

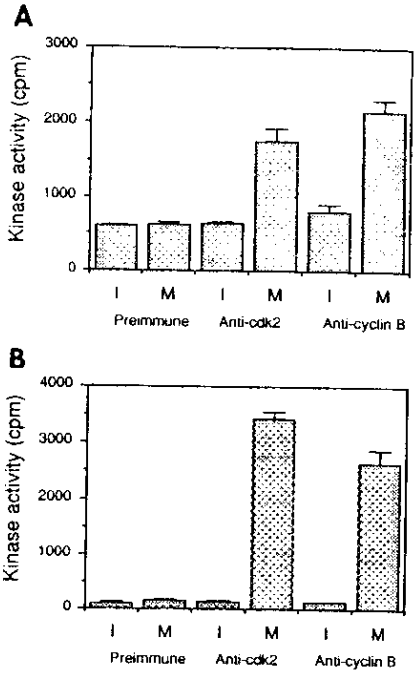

FIG. 5. Kinase activities of the immunoprecipitates from immature (I) and mature (M) goldfish oocyte extracts with preimmune serum, anti-cdk2 serum, and anti-cyclin B ascites fluid, as measured using histone H1 (A) and SP-peptide (B). Mean ± standard deviation ($n = 2$).

We also examined changes in cdk2 protein level during oocyte maturation. Despite the increased activity, the cdk2 protein level did not remarkably change during oocyte maturation (Fig. 6), as reported for cdc2 (Simanis and Nurse, 1986; Labbé *et al.*, 1988; Hirai *et al.*, 1992). It is likely that the activity of cdk2 is not controlled by its level but by chemical modifications such as phosphorylation and interactions with other proteins including

cyclins, as proposed for cdc2 (see Maller, 1991, for review). In fact, the four phosphorylation sites in cdc2 (Krek and Nigg, 1991), each of which is believed to have a crucial role in regulating cdc2 activity, are also conserved in cdk2 (Figs. 1 and 2).

Association of cdk2 with Other Proteins

The results described above confirm that cdk2 does not bind to cyclin B, which is a regulatory unit and an endogenous cdc2 substrate (Labbé *et al.*, 1989a; Gautier *et al.*, 1990; Izumi and Maller, 1991; Yamashita *et al.*, 1992a). To determine the regulatory unit for cdk2, we examined the association of cdk2 with other proteins by precipitating ^{32}P-labeled oocyte extracts with anti-cdk2 polyclonal antibody, then autoradiographing them. The anti-cdk2 immunoprecipitates contained a 47-kDa protein (Fig. 7). Although its electrophoretic mobility on SDS-PAGE was similar to those of cyclins A and B (Hirai *et al.*, 1992), the 47-kDa protein is not cyclin A or B, since neither anti-cyclin A nor anti-cyclin B antibody recognized this protein (Fig. 4C, data for cyclin A not shown). Fang and Newport (1991) have reported that *Xenopus* cdk2 is associated with a 54-kDa protein doublet and a small amount of cyclin A. The 54-kDa protein was not yet been characterized, but it may be a cyclin, since its level fluctuates according to the cell cycle (Fang and Newport, 1991). Therefore, in homology with the control mechanisms of cdc2, the activity of cdk2 may also be controlled by cyclins other than cyclins A and B.

The role of cdk2 in cell cycle control is not fully understood. Inhibition of DNA replication by depleting cdk2 from *Xenopus* egg extracts suggests an important role in G1/S transition (Fang and Newport, 1991). However, we demonstrated the increase in cdk2 kinase activity during oocyte maturation, suggesting that it also plays a role in this process. Further studies are required to fully understand the role of cdk2.

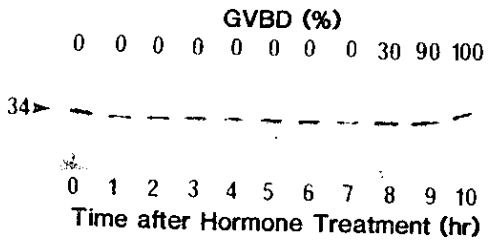

FIG. 6. Anti-cdk2 immunoblot of goldfish oocytes during maturation, showing that the 34-kDa cdk2 protein does not remarkably change during oocyte maturation. Time after hormone treatment and the percentage of germinal vesicle breakdown (GVBD) are shown below and above each lane, respectively.

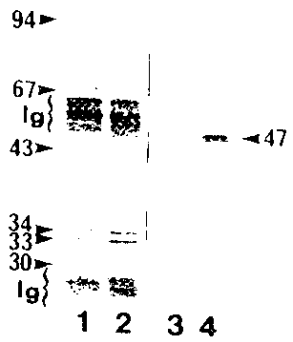

FIG. 7. Anti-PSTAIR immunoblot (1, 2) and corresponding autoradiograph (3, 4) of immunoprecipitates from ^{32}P-labeled mature goldfish oocyte extracts with preimmune (1, 3) and anti-cdk2 serum (2, 4). Note the ^{32}P-labeled 47-kDa protein in the anti-cdk2 immunoprecipitate (4), in which 33- and 34-kDa cdk2s are also present (2), since the extract stored at −80°C was used in this experiment. Immunoglobulins (Ig) used for immunoprecipitation are also shown, since anti-Ig antibody was used for visualizing the antibody-antigen complex on immunoblots.

We thank Professor P. Nurse for providing the p13^{suc1} overexpression strain of *E. coli*. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (0120210 to Y.N.), the Naito Foundation, and the Japan Health Sciences Foundation. This study was carried out under the NIBB Cooperative Research Program (91-124).

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Publication

Journal of Biochemistry, 1993, 113, 225 - 228.

Takayuki Takahashi, Toshinobu Tokumoto, Katsutoshi Ishikawa and Kenji Takahashi : Cleavage specificity and inhibition profile of proteasome isolated from the cytosol of *Xenopus* oocyte.

J. Biochem. 113, 225-228 (1993)

Cleavage Specificity and Inhibition Profile of Proteasome Isolated from the Cytosol of *Xenopus* Oocyte[1]

Takayuki Takahashi,* Toshinobu Tokumoto,** Katsutoshi Ishikawa,** and Kenji Takahashi*

*Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113; and **Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422

Received for publication, August 28, 1992

The specificity of action of the proteasome purified from the cytosol of *Xenopus* oocyte was investigated using oxidized insulin B chain as the substrate. HPLC analyses of the produced peptides followed by amino acid analyses showed that it cleaved four peptide bonds, Leu[6]-Cya[7], Glu[13]-Ala[14], Leu[15]-Tyr[16], and Leu[17]-Val[18], of the peptide. Cleavage at Leu[6]-Cya[7] was found to be specific to the *Xenopus* enzyme. The enzyme did not cleave Gln[4]-His[5] and Cya[19]-Gly[20], which are commonly hydrolyzed by proteasomes from rat and mouse liver, and human erythrocyte. In contrast to previous results obtained with the mammalian proteasome, the cleavage by the *Xenopus* enzyme was inhibited selectively by chymostatin. These results demonstrate distinct species difference in cleavage specificity and inhibition profile among proteasomes of different origins.

The 20S proteasomes are non-lysosomal, high-molecular-weight proteinase complexes that exhibit three distinct endopeptidase activities, *i.e.*, hydrolysis of peptide bonds on the carboxyl sides of the hydrophobic, acidic, and basic amino acid residues, corresponding to chymotrypsin-like, peptidylglutamyl-peptide hydrolyzing, and trypsin-like activities, respectively (*1-8*). The complexes are known to exist ubiquitously in eucaryotic cells, and those purified so far from various sources are much alike in their properties (*5*). They are generally thought to be involved in cytoplasmic protein degradation (*5, 9, 10*), but have recently been suggested to be implicated also in antigen presentation (*11*).

Recent studies by Tanaka *et al.* (*12*) and Kleinschmidt *et al.* (*13*) have shown the presence of proteasomes in cytosol and nucleus of *Xenopus laevis* oocyte. The finding is particularly interesting in connection with the maturation of amphibian oocytes. Progesterone is known to induce oocyte maturation *in vitro* (*14-16*) and the induction is blocked by proteinase inhibitors such as leupeptin, antipain, chymostatin, and N^{α}-tosyl-L-phenylalanine chloromethyl ketone, suggesting the involvement of several proteolytic enzymes in this process (*17 19*). However, little information is available at present on the chemical identities of the proteinases involved. Considering the multicatalytic nature of proteasome and its occurrence in the oocyte, it is worthwhile examining the possibility of its involvement in oocyte maturation. In order to approach this problem, it is necessary to understand the substrate specificity of *Xenopus* proteasome. To our knowledge, only a few synthetic substrates (*12, 13*), but no natural peptide substrate, have been tested for the *Xenopus* enzyme. In the present study, we investigated the specificity of action of *Xenopus* proteasome toward a peptide substrate, oxidized bovine insulin B chain. This substrate was chosen so that the results of this work could be compared directly with the available data for mammalian proteasomes (*20, 21*). We found that the proteasome selectively hydrolyzed the peptide bonds of Leu[6]-Cya[7], Glu[13]-Ala[14], Leu[15]-Tyr[16], and Leu[17]-Val[18], and the activities were inhibited by chymostatin but not by leupeptin or antipain. Thus, the *Xenopus* enzyme shows significant differences in specificity and inhibition profile as compared with the mammalian enzymes, which do not hydrolyze the Leu[6]-Cya[7] bond in the oxidized insulin B chain and are insensitive to chymostatin.

MATERIALS AND METHODS

Materials—The B chain of oxidized bovine insulin was purchased from Sigma. Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA were obtained from Peptide Institute (Osaka). Leupeptin, antipain, and chymostatin were kindly supplied by Dr. Takaaki Aoyagi (Institute of Microbial Chemistry). Other reagents used were of the highest grade available.

Xenopus Oocyte 20S Proteasome—Cytosol of ovaries from female gravid African clawed frog (*X. laevis*) was prepared through one-step ultracentrifugation without homogenization of ovarian fragments (*22*). The 20S proteasome was purified from the cytosol according to the procedure established by us. The details will be described elsewhere. In brief, the cytosol fraction was applied to a DEAE-cellulose column in 25 mM Tris-HCl buffer (pH 7.5) containing 20% glycerol and 10 mM 2-mercaptoethanol (TGM buffer). The retained proteins including proteasome were eluted with TGM buffer containing 0.2 M NaCl. The

[1] This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan, and the Saito-Chion Foundation, Shizuoka.

Abbreviations: AMC, 7-amino-4-methylcoumarin; Boc, *t*-butyloxycarbonyl; Cya, cysteic acid; HPLC, high-performance liquid chromatography; MCA, 4-methylcoumaryl-7-amide; Suc, succinyl; TGM, 25 mM Tris-HCl (pH 7.5) containing 20% glycerol and 10 mM 2-mercaptoethanol.

active fractions were pooled, concentrated through a small DEAE-cellulose column, and fractionated on a Sepharose CL-6B column in the same buffer. The active fractions were pooled and again concentrated on a DEAE-cellulose column, and chromatographed on a Phenyl-Sepharose CL-4B column in 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. The flow-through fractions containing enzyme activity were collected, diluted 10-fold with TGM buffer and applied to a DEAE-cellulose column. The column was eluted with a linear gradient of NaCl (0-0.4 M) in the TGM buffer, and the active fractions were pooled. The sample thus purified was active and had specific activities of 8.2 and 1.6 μmol/min/mg protein toward Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA, respectively. This enzyme preparation was used in the present study without any treatment for further activation. We presumed that the enzyme was activated during purification.

Enzyme and Protein Assay—Enzyme activities toward peptide substrates containing MCA were assayed at 37°C as described (*23*), essentially based on the Barrett method (*24*). The release of the fluorophore AMC was measured by a spectrofluorometer using an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Protein concentrations were measured by the method of Bradford (*25*) with bovine serum albumin as a standard, and 700 μg of protein was assumed to represent 1 nmol of enzyme.

Digestion of Oxidized Insulin B Chain and Analysis of the Peptides—In analytical runs, the B chain of oxidized insulin (1 nmol) was digested at 37°C with proteasome (2.8 pmol, 2 μg) in 50 μl of 50 mM sodium phosphate (pH 5 and 6), 50 mM Tris-HCl (pH 7 and 8), or 50 mM sodium borate (pH 9 and 10). Aliquots of 25 μl were removed at appropriate time intervals and subjected to HPLC using an Applied Biosystems 130A Separation System on a column (2.1×30 mm) of Spheri-5-RP-18 (Applied Biosystems). The elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid from 0 to 70% in 20 min at a flow rate of 0.5 ml/min. The eluate was monitored by measuring the absorbance at 220 nm. In preparative runs, the amounts of both substrate and proteasome were increased 5 times and the digest was analyzed in a Hitachi 655A-11 system on a column (0.46×25 cm) of TSKgel ODS-120T (Tosoh). The peptides were eluted with a gradient of acetonitrile (0-50% in 30 min) in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The eluate was monitored by measuring the absorbance at 215 nm. The peak fractions collected were hydrolyzed, and an aliquot of each hydrolysate dissolved in water was analyzed for amino acid composition using an Applied Biosystems automated derivatizer-analyzer (420A/130A).

RESULTS

Figure 1 shows the changes in HPLC patterns of oxidized insulin B chain digested with *Xenopus* proteasome at pH 8.0 and 37°C for various time periods. Even when the incubation was prolonged, the pattern was qualitatively the same: the height of each peak produced apparently increased with a concomitant decrease in that of the original peptide, and no additional peaks appeared. Furthermore, the produced peaks appeared to be resistant to further proteolysis by the proteasome. These results indicate that the hydrolysis takes place at rather restricted peptide bonds of the substrate.

The cleavage sites and the extents of hydrolysis were determined with the 2-h digest of the peptide. A typical HPLC pattern is shown in Fig. 2, and the peaks were analyzed for amino acid composition. As summarized in Fig. 3, the proteasome cleaved the peptide mainly at four sites, although other bonds could also be cleaved to very limited extents as suggested by many smaller, unidentified peaks observed in Fig. 2. The extents of cleavage at pH 8.0 of Leu^{6}-Cya^{7}, Glu^{13}-Ala^{14}, Leu^{15}-Tyr^{16}, and Leu^{17}-Val^{18} were estimated to be 34, 23, 21, and 16%, respectively. The relative rates of cleavage at the individual peptide bonds

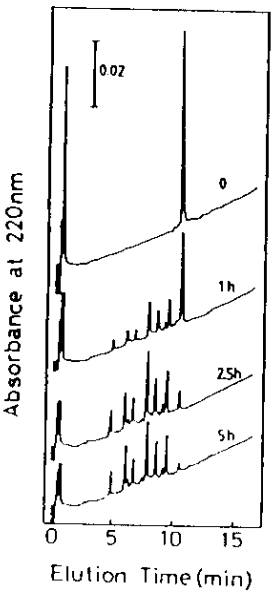

Fig. 1. Time-course of hydrolysis of oxidized insulin B chain by *Xenopus* proteasome. The B chain of oxidized insulin (1 nmol) was incubated at 37°C with the proteasome (2.8 pmol) in 50 μl of 50 mM Tris-HCl (pH 8). After incubation for the indicated time period, a half of the mixture was analyzed by HPLC using a Spheri-5-RP-18 column.

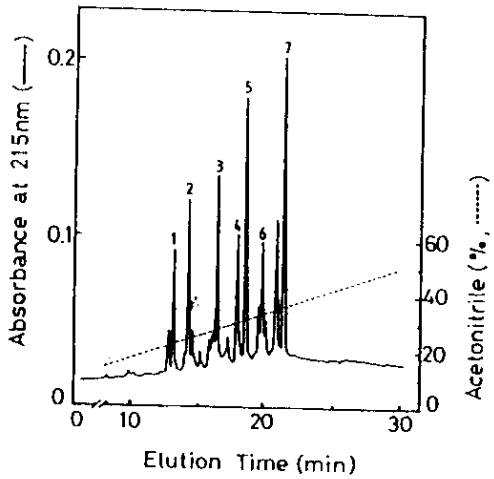

Fig. 2. HPLC analysis of oxidized insulin B chain digested with *Xenopus* proteasome. The 2-h digest at pH 8.0 was analyzed by HPLC using a TSKgel ODS-120T column. All peak fractions were collected, hydrolyzed, and analyzed for amino acid composition. The peptide peaks identified by the analysis have been given numbers which correspond to those shown in Fig. 3. Peak 7 was the original unhydrolyzed peptide, and a peak eluted at 21 min contained no significant amount of amino acids.

Fig. 3. **Positions of cleavage of oxidized insulin B chain by *Xenopus* proteasome.** Arrows below the sequence indicate the location of the peptide bonds that are cleaved at pH 8.0 in 2 h. The peptides produced by the digestion are indicated by horizontal bars with numbers which correspond to those shown in Fig. 2.

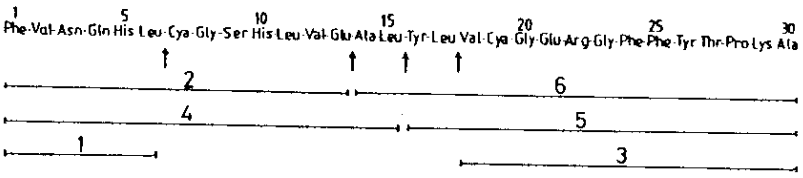

TABLE I. **Effects of inhibitors on the enzyme activity.** The B chain of oxidized insulin was digested at pH 8.0 and 37°C for 2 h with *Xenopus* proteasome at a molar ratio of 357 : 1. The digests were analyzed by HPLC as described in "MATERIALS AND METHODS." Activities toward Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA were assayed at 37°C and a final substrate concentration of 0.1 mM.

Inhibitor		Activity remaining (%)		
		Insulin B chain	Suc-Leu-Leu-Val-Tyr-MCA	Boc-Leu-Arg-Arg-MCA
Leupeptin	10 μM	108	103	3
	100 μM	106	97	1
Antipain	10 μM	102	99	2
	100 μM	96	93	1
Chymostatin	10 μM	83	58	66
	100 μM	42	19	32

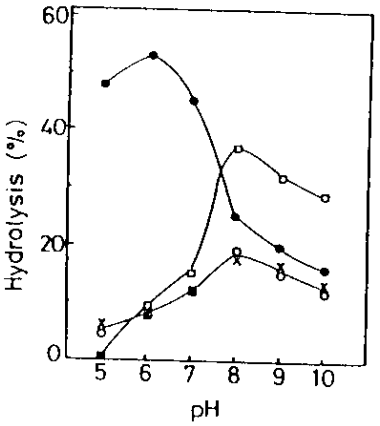

Fig. 4. **pH-dependence of cleavage at the individual peptide bonds.** The B chain of oxidized insulin digested with *Xenopus* proteasome at various pHs for 2 h was analyzed by HPLC. Percent hydrolysis at the four different cleavage sites was estimated from the amounts of the peptides produced. Cleavage sites are: □, Leu^{6}-Cya^{7}; ●, Glu^{13}-Ala^{14}; ○, Leu^{15}-Tyr^{16}; and ×, Leu^{17}-Val^{18}.

were further examined at various pH values ranging from 5 to 10 (Fig. 4). The Glu^{13}-Ala^{14} bond was cleaved optimally at pH 6 while the maximum hydrolysis was commonly seen at pH 8 for the other three peptide bonds.

Table I summarizes the effects of some proteinase inhibitors on the hydrolysis of oxidized insulin B chain by the proteasome. The effects on the activities toward two synthetic substrates, Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA, are also included for comparison. Of the three inhibitors, only chymostatin suppressed the degradation of oxidized insulin B chain. It was found that in the presence of the inhibitor, all peaks resulting from the cleavages at the four different sites were apparently reduced to similar extents (data not shown). The concentration of chymostatin required to produce 50% inhibition of the inhibitor-free, control level was approximately 60 μM. Chymostatin also inhibited the enzyme activities toward the synthetic substrates. In contrast, leupeptin and antipain were more potent inhibitors than chymostatin for the Boc-Leu-Arg-Arg-MCA hydrolyzing activity of the proteasome. The results are consistent with the finding by Tanaka *et al.* (*12*) that leupeptin and chymostatin preferentially inhibited the cleavages of *N*-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy-2-naphthylamide and Suc-Leu-Leu-Val-Tyr-MCA, respectively.

DISCUSSION

In the present study, we investigated the cleavage specificity of 20S *Xenopus* proteasome toward oxidized bovine insulin B chain. Four peptide bonds in the peptide were mainly hydrolyzed. Three of them (Glu^{13}-Ala^{14}, Leu^{15}-Tyr^{16}, and Leu^{17}-Val^{18}) are also reported to be the major cleavage sites by human erythrocyte (*21*) and rat and mouse liver (*20*) proteasomes. However, these mammalian proteasomes showed no or little cleavage at the Leu^{6}-Cya^{7} bond, which was hydrolyzed most rapidly by the *Xenopus* proteasome. Another difference is that Gln^{4}-His^{5} and Cya^{19}-Gly^{20} were rapidly cleaved by the mammalian proteasomes (*20, 21*), whereas these bonds were resistant to the *Xenopus* enzyme.

Interestingly, studies on the effect of various pH on the proteolytic activities responsible for the respective cleavages revealed that the Glu^{13}-Ala^{14} bond was optimally hydrolyzed at pH 6, which is 2 pH units lower than the others. However, all four hydrolyzing activities were inhibited only by chymostatin. Thus, it is reasonable to conclude that the catalytic center(s) that degrades oxidized insulin B chain is probably not the one associated with trypsin-like activity. Although more detailed studies are required to determine which catalytic center(s) is responsible for each cleavage, two different subunits with chymotrypsin-like activity and peptidylglutamyl-peptide hydrolyzing activity might be involved. Alternatively, only a single subunit with chymotrypsin-like activity could cleave all the peptide bonds, assuming that its substrate-binding subsite (S_1) accepts the side chain of leucine as well as that of glutamic acid.

It should be noted that cleavages at Glu^{13}-Ala^{14}, Leu^{15}-Tyr^{16}, and Leu^{17}-Val^{18} bonds found with human erythrocyte proteasome were not suppressed in the presence of chymostatin. In addition, in contrast to virtually no effect of

leupeptin on the cleavages at these sites with *Xenopus* proteasome, the same inhibitor enhances the rate of these cleavages catalyzed by the human proteasome (*21*).

Tanaka *et al.* (*12*) showed that proteasomes from various eucaryotic sources resemble each other in many respects. In the present work we demonstrated that *Xenopus* proteasome has unique cleavage specificity toward the Leu^{6}-Cya^{7} bond in addition to the specificity similar to the mammalian counterparts so far reported (*20, 21*). The results demonstrate the existence of species difference in specificity as well as the general importance of proteasomes for proteolysis. To examine the involvement of *Xenopus* proteasome in oocyte maturation, detailed analysis of protein degradation associated with the phenomenon will be necessary. Identification of a physiological substrate(s) of the proteasome in the oocyte is particularly important, and this is being attempted at present.

We thank Dr. Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo) for the generous gift of leupeptin, antipain, and chymostatin, and Mrs. Yasuko Sakurai for her assistance in amino acid analysis.

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Publication

Biochemical and Biophysical Research Communications, 1993, 192, 1106 - 1114.

Toshinobu Tokumoto and Katsutoshi Ishikawa : A novel "active" form of proteasomes from *Xenopus laevis* ovary cytosol.

Vol. 192, No. 3, 1993
May 14, 1993

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 1106-1114

A Novel "Active" Form of Proteasomes from *Xenopus laevis* Ovary Cytosol

Toshinobu Tokumoto and Katsutoshi Ishikawa[1]

Department of Biology, Faculty of Science, Shizuoka University,
Ohya 836, Shizuoka 422, Japan

Received March 29, 1993

SUMMARY: The cytosol fraction prepared from *Xenopus laevis* ovaries by one-step ultracentrifugation catalyzed the hydrolysis of succinyl-leucyl-leucyl-valyl-tyrosine-4-methylcoumaryl-7-amide with or without SDS, an activator of 20S latent proteasomes. Both activities were lost on immunodepletion with antibodies against 20S proteasome. Storage of the cytosol at 4℃ led to abolition of the SDS-independent activity, but not the SDS-dependent activity. Upon DEAE-cellulose chromatography, the enzyme catalyzing the SDS-independent activity could be separated from that responsible for the SDS-dependent activity. These results indicate that the ovary cytosol contains a large proportion of a novel, "active" form of proteasomes which does not require SDS but is unstable and is readily converted to a latent form which requires SDS. ATP is known to stabilize 26S protease complex, but ATP reduced SDS-independent activity. It seems that the "active" form of proteasomes (molecular weight : about 1000-kDa) is different from 26S protease complex.

Proteasomes are non-lysosomal high molecular weight multicatalytic protease complexes that exist widely in eukaryotic cells in a latent state [1-16]. The latent form can be activated by such agents as SDS, polylysine and fatty acids [1,4,7,10-16]. Although their physiological functions are not yet fully understood, recent studies have suggested that proteasomes are involved in oocyte maturation in the leopard frog, *Rana pipiens* [14]. The occurrence of proteasomes in the cytosol and nucleus of *Xenopus laevis* oocytes has also been reported [11,16-18], but their possible involvement in maturation has not yet been examined. In the meiotic maturation of amphibian oocytes, which is induced by progesterone *in vitro* [19], progesterone is thought to interact with the oocyte surface and thus trigger the breakdown of germinal vesicles, followed by the syntheses of the c-*mos* protein product (Mos) and cyclin and activation of a maturation promoting factor [20-22]. Our study is to elucidate the regulatory mechanism underlying oocyte maturation, particularly on the role of proteasomes in this process. To this end, it is necessary to

[1]To whom correspondence should be addressed.

Abbreviations: SDS, sodium dodecyl sulfate; Suc-LLVY-MCA, succinyl-leucyl-leucyl-valyl-tyrosine-4-methylcoumaryl-7-amide.

0006-291X/93 $4.00

study the nature of proteasomes *in ovo*. In this study, we prepared a cytosol fraction from *Xenopus laevis* ovaries by one-step ultracentrifugation, which does not involve homogenization with a buffer, and found that it catalyzed the hydrolysis of a tyrosine-containing peptide without SDS. From this and other findings, we postulate that the ovary cytosol contains a novel, "active" form of proteasomes, with previously unrecognized properties, that is distinct from the known 20S latent form[11,16,23]. In addition, the "active" form seems not to be identical with the known ATP-dependent 26S protease complex.

MATERIALS AND METHODS

Cytosol preparation from ovaries — Ovaries were isolated from sacrificed female gravid frogs (*Xenopus laevis*) (Hamamatsu Seibutsu Kyozai Corp.) and placed in freshly prepared Barth's medium (88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 7.5 mM Tris-HCl, pH 7.6, 10 mg/liter streptomycin sulfate and 100,000 IU/liter potassium penicillin G). Ovarian fragments were allowed to settle in a 4-ml centrifuge tube, the excess medium being removed. The settled fragments were then subjected to one-step ultracentrifugation for 1 hr at 105,000 x g and 4 ℃ (50.3Ti rotor; Beckman Co.). The clear supernatant fraction between the lipid cap and the packed precipitate was carefully collected with a Pasteur pipet and used as the cytosol (≒ 20 mg protein/ml).

Enzyme assay — The hydrolytic activity toward a fluorogenic peptide substrate, Suc-LLVY-MCA (Peptide Institute Inc.), was determined by measuring the fluorescence of groups liberated from this peptide as previously described [14].

Immunological analysis — The enzyme solution (300μl/60μl) was incubated at 37℃ for 1 hr with 100μl/20μl of rabbit non-immune serum IgG (Vector Laboratories Inc.) or rabbit anti-proteasome IgG (antibodies raised against 20S latent proteasomes from *Xenopus laevis* ovaries [11], purified from serum on a protein A-Sepharose CL-4B (Sigma Co.) column with Buffer A, *i.e.*, 25 mM Tris-HCl-20% glycerol-10 mM 2-mercaptoethanol, pH 7.5). The antigen-antibody solution was incubated for 30 min at 37 ℃ with protein A-Sepharose CL-4B (60μl/12μl) suspended in Buffer A. The supernatant was used for the enzyme assay after centrifugation for 5 min at 10,000 rpm.

DEAE-cellulose column chromatography of the cytosol — An aliquot (3.0 ml) of cytosol was applied to a DE-52 (Whatman Co.) column (1.2 x 4.0 cm) equilibrated at 4℃ with Buffer A, the column then being washed with the same buffer, and eluted with 150, 200 and 400 mM NaCl in Buffer A at the flow rate of 25 ml/hr. Aliquots (20 μl/40μl) of 1.0-ml-fractions were used for measuring the enzyme activity and protein content.

Sepharose 6B gel filtration of the cytosol — An aliquot (0.5 ml) of cytosol was applied to a Sepharose 6B (Pharmacia) column (0.9 x 90 cm) equilibrated at 4℃ with Buffer A, and eluted with the same buffer at the flow rate of 2.5 ml/hr. Aliquots (20 μl/40μl) of 1.0-ml-fractions were used for measuring the enzyme activity and protein content.

Protein content — The protein content was determined according to Bradford's method [24] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The *Xenopus laevis* ovary cytosol prepared by one-step ultracentrifugation exhibited significant Suc-LLVY-MCA hydrolyzing activity without SDS (Fig. 1A). This activity

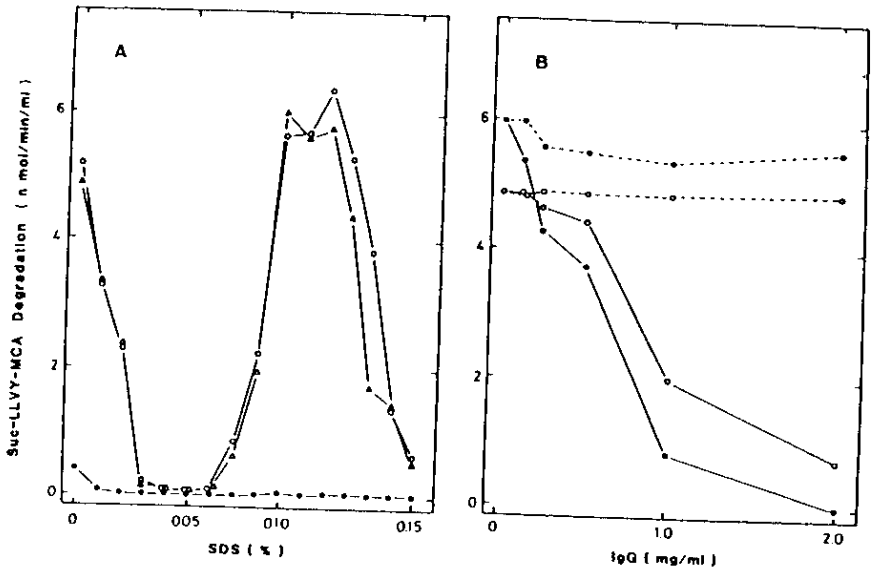

Fig. 1. Effects of SDS and anti-proteasome IgG on enzyme activity in the cytosol. (A) Aliquots of cytosol were incubated for 1 hr at 37℃ with rabbit anti-proteasome IgG (final, 1 mg protein/ml)(●), non-immune IgG (final, 1 mg protein/ml)(○) or buffer A (△). Immunoprecipitates with protein A-Sepharose CL-4B were removed by centrifugation and then the residual Suc-LLVY-MCA (10μM) hydrolyzing activity in the supernatant was measured with various SDS concentrations after 10 min at 37℃. (B) Aliquots of cytosol were incubated under the conditions given for panel (A) except that different concentrations of rabbit anti-proteasome and non-immune IgGs were added. Then the enzyme activity was measured as described for panel (A), except without or with 0.10% SDS. The symbols are as follows: rabbit anti-proteasome IgG: without (—○—)/with (—●—) SDS; non-immune IgG: without (- -○- -)/with (- -●- -) SDS.

was drastically reduced with 0.03-0.06% SDS. With a further increase in the SDS concentration, however, the activity increased again, reaching a maximum at about 0.10-0.12% SDS. Immunodepletion of proteasomes from the cytosol with anti-proteasome IgG led to the almost complete loss of the activities without and with SDS. Non-immune IgG did not affect either activity. In addition, both activities were similarly and dose-dependently inhibited by anti-proteasome IgG (Fig. 1B). These observations indicate that the ovary cytosol prepared in this study contained a large percentage of a form of proteasomes that can hydrolyze the peptide without SDS, which is called the "active" form. The above results suggest that low concentrations of SDS caused the conversion of the "active" form into the latent form, which has been shown to require a certain concentration of SDS and is known as 20S proteasomes [1-16]. This conversion also seemed to take place when freshly prepared cytosol was stored at 4℃ (Fig. 2). The activity without SDS decreased: 64, 82 and 88% of the initial level being lost after storage for 1, 4 and 8 days, respectively. However, the SDS-dependent activity remained unchanged for at least 8 days.

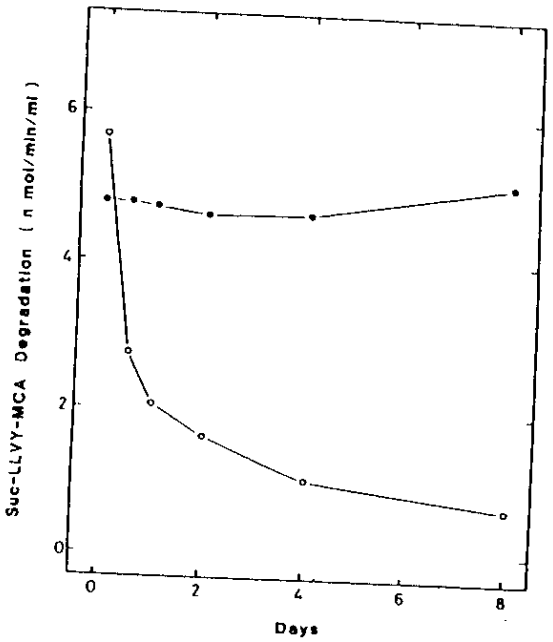

Fig. 2. Enzyme activity after keeping the cytosol at 4℃. Fresh cytosol was kept at 4℃ for various days. Then the peptide (10μM) hydrolyzing activity in an aliquot of cytosol was measured without (○) or with (●) 0.12% SDS, as described in Fig. 1.

In addition, the "active" form in the intact cytosol is partly converted to the latent form during column chromatography. The two forms can be separated from each other by DEAE-cellulose column chromatography (Fig. 3A). The large peak material (fraction 28) showed considerable activity without SDS, but it also exhibited comparable activity with 0.06% SDS, which may be due to the "active" form (Fig. 3B, left-hand panel). The SDS concentration (0.06%) giving the maximal activity determined here was considerably lower than that for the unfractionated cytosol (0.10-0.12%, see Fig. 1). A possible reason for this difference is the removal of an unknown cytosolic factor(s) that modulates the activity of the latent form of proteasomes. The small peak material (fraction 47) (Fig. 3A), on the other hand, showed only very low enzyme activity, but this activity increased 16-fold with 0.06% SDS, which may be due to the latent form (Fig. 3B, right-hand panel). The activities of both fractions were strongly inhibited by anti-proteasome IgG (Fig. 3C), indicating that the activities without and with SDS were catalyzed by two distinct forms of proteasomes. It seems that fraction 47 contained proteasomes that had already been converted to the latent form because of their instability during chromatography. In addition, Sepharose 6B gel filtration experiments indicated that the apparent molecular mass of the "active" form was estimated to be about 1000-kDa (Fig. 4), which was higher by about 300-kDa than that of the latent form (670-kDa) [11,16,23]. Thus, it is also suggested that the latent form may be formed through the dissociation of

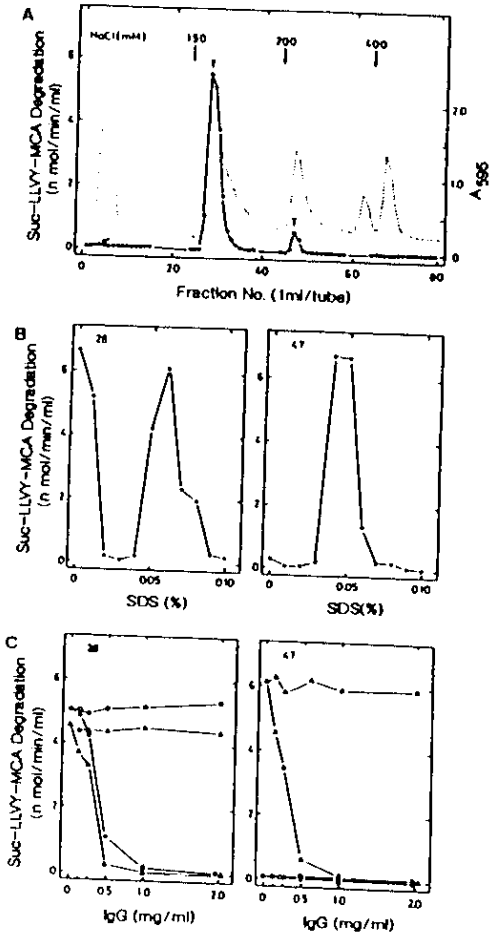

Fig. 3. DEAE-cellulose column chromatography of the cytosol. Chromatography was performed as described under MATERIALS AND METHODS. (A) Aliquots (20μl/40μl) of 1.0-ml-fractions were assayed for both peptide hydrolyzing activity without SDS (●) and protein content (····). (B) Aliquots (10μl) of fractions 28 and 47 were assayed for enzyme activity with various SDS concentrations. (C) Aliquots (10μl) of the same fractions were incubated at 37℃ for 1 hr with different amounts of anti-proteasome and non-immune IgGs. The residual activity in the supernatant was assayed without/with 0.06% SDS (fraction 28) or 0.05% SDS (fraction 47). The SDS concentrations were obtained from (B). Non-immune IgG: without (○)/with (△) SDS; anti-proteasome IgG: without (●)/with (▲) SDS.

several subunits from the "active" form. It has recently been reported that the known mammalian 26S protease complex (apparent molecular mass: 1500-kDa), which is formed through the association of 20S latent proteasomes with two ubiquitin-conjugate-degrading factors in an ATP-requiring process, may be involved in ubiquitin-dependent proteolysis [15,25-29]. To clarify the properties of the "active" form found in the ovary cytosol, the effect of ATP on the Suc-LLVY-MCA hydrolyzing activities with various concentrations of SDS was examined. 1 mM ATP-addition caused a reduction in the

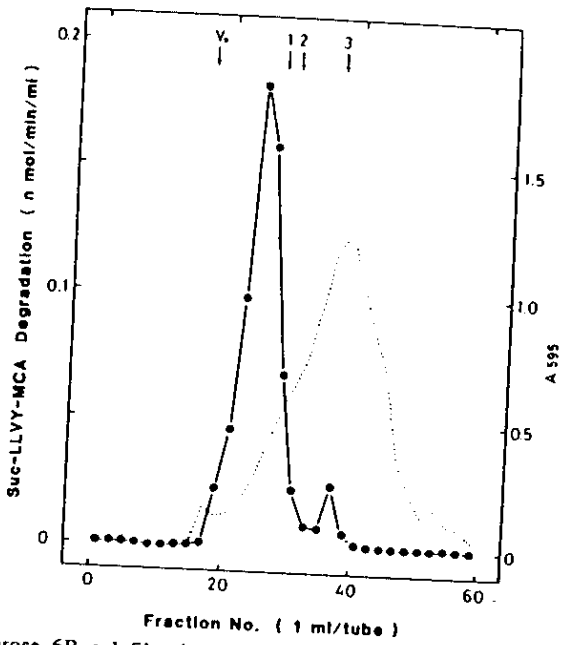

Fig. 4. Sepharose 6B gel filtration of the cytosol. Gel filtration was performed as described under MATERIALS AND METHODS. Aliquots (20μl/40μl) of 1.0-ml-fractions were assayed for both peptide hydrolyzing activity without SDS (●) and protein content (· · · · ·). The arrows indicate the elution position of the molecular weight markers: V_0: blue dextran 2000, 1: thyroglobulin (670-kDa), 2: ferritin (440-kDa) and 3: aldolase (158-kDa).

activity without SDS to 30% of the initial level, but did not affect either activity in the presence of SDS (Fig. 5A). In addition, the dose-dependency as to ATP of the SDS-independent activity was studied (Fig. 5B). The activity decreased with increasing concentrations of ATP, but the reduced activity remained unchanged (30-40% of the initial level) with more than 1 mM ATP. On the other hand, we measured the SDS-independent activity after storage of the cytosol at 4℃ for a given time in the presence of ATP (Fig. 6). The reduced activity in the ATP-supplemented cytosol was found to be restored to the initial level after a given time. The full restoration was attained at 1 hr with 1.25 mM ATP, but at 3 hr with 5 mM ATP. Thus, the activity was rapidly restored with the low concentration of ATP when compared with the case of the high concentration of ATP. With subsequent storage, both activities gradually decreased. Mg^{2+} added as a cofactor had no effect on the time course. In addition, the activity level on storage with Mg^{2+} alone without ATP decreased almost linearly, as in the case of the control (see Fig. 2). These observations suggested that a given amount of ATP added to the cytosol appears to alter the conformation of the "active" form and subsequently the accompanying activiy. Thus, ATP-addition may cause conversion of the "active" form to some ATP-dependent enzyme complex, probable like the 26S protease complex, which may reflect the reduction of the Suc-LLVY-MCA hydrolyzing activity level without SDS. Therefore,

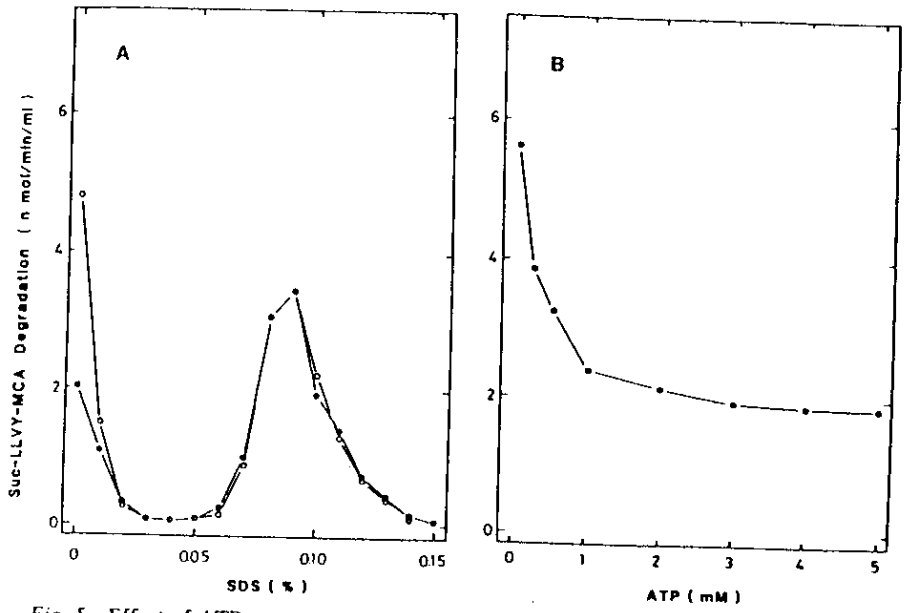

Fig. 5. Effect of ATP on enzyme activity in the cytosol. (A) Suc-LLVY-MCA (10 μM) hydrolyzing activity was measured in the presence (●) or absence (○) of 1 mM ATP with various SDS concentrations after 10 min at 37℃. (B) Increasing amounts of ATP were added to aliquots of cytosol, followed by assaying for enzyme activity without SDS.

the reactivation following subsequent storage may be due to the "active" form being reformed following the ATP-consuming step due to the action of a probable endogenous ATPase. Since on further storage the "active" form was unstable, it may be converted to the latent form such as shown in Fig. 2. From the above observations, a large proportion of the "active form" in the ovary cytosol is suggested to be different from the probable ATP-dependent 26S protease complex, judging from not only molecular mass but also the requirement of ATP for activity (Figs. 4 and 5). In addition, the endogenous inhibitor of proteasome has been very recently found in the extracts of rabbit reticulocytes, which may be one component (250-kDa) of ATP-dependent 26S protease complex [30]. If so, the "active" form found in this study may be due to the lack of the inhibitor from ATP-dependent 26S protease complex. However, the biochemical mechanism(s) by which ATP-addition to the cytosol causes fluctuation of the enzyme's conformation and activity is unclear. It has been reported that 26S-like protease complexes (in the presence of ATP) with a core of 20S latent forms have been found under near-physiological conditions in the *Xenopus* ovary cytosol [18]. However, we cannot discuss them in relation to the "active" form since their enzyme activities were not examined. In previous work performed to detect proteasomal activity, tissues were first homogenized with a buffer, a procedure leading to dilution and being time-consuming, and, therefore, conversion of the "active" form to the latent form [14]. In this study we prepared the cytosol by one-step ultracentrifugation and thus obtained an undiluted cytosol fraction. It can be concluded

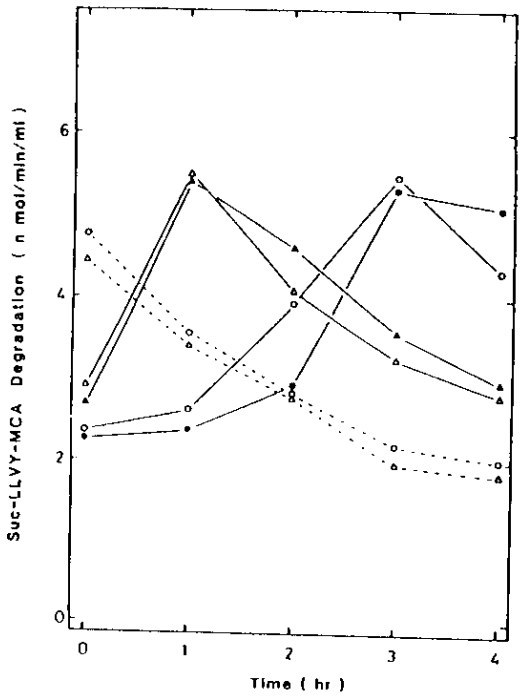

Fig. 6. Time-dependent fluctuation in enzyme activity after keeping the cytosol with ATP. Aliquots of cytosol were kept with 1.25 mM ATP (—△—), 1.25 mM ATP + 10 mM Mg^{2+} (—▲—), 5 mM ATP (—○—), 5 mM ATP + 10 mM Mg^{2+} (—●—), 10 mM Mg^{2+} (- -△- -) or DDW (- -○- -) on ice. At the indicated times, the peptide (10 μM) hydrolyzing activity in an aliquot of cytosol was measured as described under MATERIALS AND METHODS.

that the cytosol fraction of *Xenopus laevis* ovaries contains a hitherto unknown "active" form of proteasomes. Thus, its instability accounts for the fact that this form has previously eluded detection. In other words, the "active" form seems to represent the physiological native state of proteasomes. In addition, it has recently been reported that the rapid degradations of both Mos and cyclin proteins are the crucial events during meiotic maturation in *Xenopus* oocytes and recognition of both proteins has been suggested to be triggered by the action of proteasomes [22,29]. However, before a final conclusion can be reached, further work is needed on the biochemical and physiological properties of the "active" form of proteasomes, and its relationships with the 20S latent proteasomes and the 26S protease complex as to the amount of ATP.

ACKNOWLEDGMENTS

We wish to thank Dr. K. Tanaka of Tokushima University for the gift of the proteasome antibody. A part of this study was supported by grants (#01304029 and special research) from the Ministry of Education, Science and Culture of Japan, and the Saito-Chion Foundation, Shizuoka, to K.I.

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Publication

Biomedical Research, 1993, 14, 305 - 308.

Toshinobu Tokumoto, Masakane Yamashita, Michiyasu Yoshikuni and Yoshitaka Nagahama : Changes in the activity and protein levels of proteasomes during oocyte maturation in goldfish (*Carassius auratus*).

Biomedical Research 14 (4) 305-308, 1993

Changes in the activity and protein levels of proteasomes during oocyte maturation in goldfish (*Carassius auratus*)

TOSHINOBU TOKUMOTO[1,2], MASAKANE YAMASHITA[2], MICHIYASU YOSHIKUNI[2] and YOSHITAKA NAGAHAMA[2]
[1]Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki 444, and [2]Laboratory of Reproductive Biology, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444, Japan

ABSTRACT

Changes in the activity and protein levels of proteasomes were determined in goldfish oocytes during meiotic maturation induced *in vitro* by 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), a naturally occurring maturation-inducing hormone in this species. Both the activity and protein levels of proteasome exhibited two peaks during the 17α,20β-DP-induced oocyte maturation with a similar pattern of fluctuation. The first peak (a 2-fold increase) occurred before the migration of germinal vesicle (GV) (within 1 h after 17α,20β-DP treatment), followed by a gradual decrease towards GV breakdown (GVBD), reaching the lowest level at 6 h. The second peak occurred immediately after GVBD (7 h after the treatment), followed by a sharp decrease at 8 h. The time of the second peak appears to correspond to the time of the first polar body extrusion, a time when maturation-promoting factor (MPF) activity was shown to decrease transiently in goldfish oocytes during the 17α,20β-DP-induced meiotic maturation. Thus, it is suggested that proteasomes are involved in the decrease in MPF activity during the first polar body extrusion.

Meiotic maturation of fish oocytes is triggered by maturation-inducing hormone (MIH), which acts on the oocyte surface and induces the activation of maturation-promoting factor (MPF) in the oocyte cytoplasm (11). Previous studies using various protease inhibitors suggest that proteases are involved in the MIH-induced meiotic maturation of frog (3, 5) and starfish (6, 14) oocytes. Recently, a non-lysosomal multicatalytic protease named proteasome has been identified in various eukaryotic sources (13, 15). Proteasome possesses three distinct proteolytic activities (chymotrypsin-like, trypsin-like and peptidylglutamyl peptidase activities), and are sensitive to serine and thiol protease inhibitors (4, 13). In frog oocytes, serine protease inhibitors have been reported to prevent both the proteasome activity and oocyte maturation (1). It is thus suggested that proteasome is one of the most likely candidates for proteases responsible for the MIH-induced oocyte maturation. In this study, we examined changes in the activity and protein levels of proteasomes during the 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP, a natural MIH in fish)-induced oocyte maturation *in vitro* in goldfish (12).

Oocyte maturation was induced *in vitro* by incubating fragments of ovaries (each contains 20-40 oocytes) in goldfish Ringer's solution (125 mM NaCl, 2.4 mM KCl, 0.28 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2 mM HEPES, 5.6 mM glucose, 100,000 IU/l penicillin, 0.2 g/l streptomycin, pH 7.5) containing 1 μg/ml of 17α,20β-DP, as described previously (17). Maturation processes were assessed by immersing the oocytes in a clearing solution (9), enabling easy microscopic examination for the presence or absence of a germinal vesicle.

In goldfish, like in other vertebrates, the fully grown oocyte possesses a large nucleus (germinal vesicle) in meiotic prophase. The germinal vesicle of this stage is located centrally. The first visible event associated with 17α,20β-DP-induced final maturation is the migration of the germinal vesicle to the animal pole where the micropile is situated; at this stage their germinal vesicle becomes visible

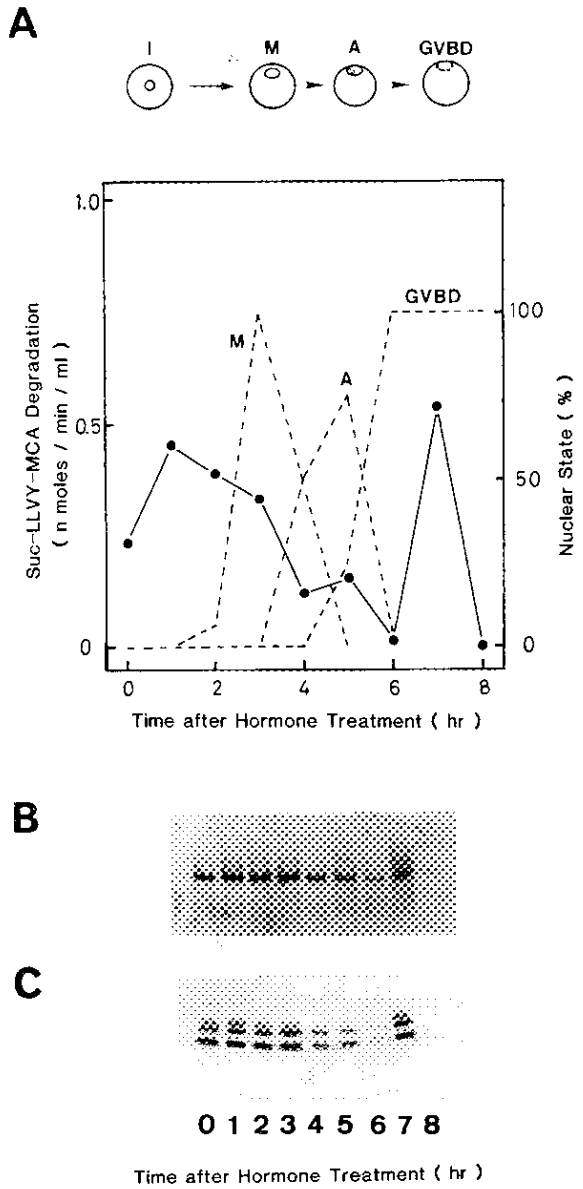

Fig. 1 Changes in the morphology of the oocyte (A, ---), and the activity (A, ●) and protein levels (B, native PAGE; C, SDS-PAGE) during the 17α,20β-DP-induced oocyte maturation in goldfish. Schematic diagram above the figure indicates the state of the germinal vesicle: intact germinal vesicle (I), germinal vesicle during migration (M), germinal vesicle attached to the oocyte periphery (A), GVBD, germinal vesicle breakdown (see text for details). To determine the activity and protein levels of proteasome, oocyte cytosol was extracted as follows: thirty oocytes manually isolated from ovarian fragments were washed with goldfish Ringer. After excess medium was removed, 150 μl of new Ringer was added. The oocytes were crushed by ultracentrifugation at 150,000 *g* for 30 min at 4°C and the clear supernatant was collected. Protease activity of proteasome was measured using a fluorogenic substrate, Suc-LLVY-MCA (Peptide Institute). Reaction was initiated by adding 10 μl of the sample to 100 μl of the reaction mixture consisting of 100 mM Tris-HCl and 10 μM Suc-LLVY-MCA (pH 7.6) which was preincubated for 10 min at 37°C. After 10-min incubation, the reaction was terminated by the addition of 100 μl of 10% SDS and 2 ml of 0.1 M Tris-HCl (pH 9.0). The concentration of the released MCA was determined on fluorescence spectrophotometer (F-5000, Shimazu) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. One unit of the activity corresponds to 1 nmol MCA liberated/min/ml. Proteins were separated by polyacrylamide gel electrophoresis in either non-denaturing (native PAGE with 5% gel) or denaturing condition (SDS-PAGE with 12% gel) by the method of Laemmli (8), and transferred to Immobilon membrane (Millipore). Membranes were blocked in 5% non-fat dry milk, and incubated with the mixture of three monoclonal antibodies against goldfish proteasome (GC4/5, GC3α and GC3β; Tokumoto *et al.*, unpublished) for 1 h at room temperature. Immunocomplexes were visualized using alkaline phosphatase conjugated anti-mouse immunogloblin, as described previously (18).

under the dissecting microscope. The membrane of the germinal vesicle then breaks down (germinal vesicle breakdown) (Fig. 1A).

It was shown previously that the proteasome activity could be detected only in the presence of a low concentration of sodium dodecyl sulfate (SDS) in the reaction mixture. However, in this study the 150,000 *g* supernatant of crushed goldfish oocytes induced to mature *in vitro* by 17α,20β-DP exhibited the proteasome activity regardless of the addition of SDS to the reaction mixture. Therefore, in this study the proteasome activity was determined in the absence of SDS. The proteasome activity in oocyte extracts prepared during oocyte maturation was measured with a synthetic peptide substrate specific to proteasomes, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA). Since the proteolytic activity measured with this substrate was completely precipitated with antibodies against proteasome (16, Tokumoto *et al.*, unpublished), the obtained activity can be considered to be solely derived from active proteasome.

Upon stimulation of oocytes with 17α,20β-DP, there was a 2-fold increase in the proteasome activity within 1 h that gradually decreased to the lowest level by 6 h post 17α,20β-DP. The proteasome activity elevated again at 7 h after 17α,20β-DP treatment, followed by a sharp drop at 8 h (Fig.

1A). The data presented in Fig. 1A are derived from a representative experiment utilizing oocytes from single gravid female goldfish. Similar experiments were repeated several times with oocytes from different females to confirm the reproducibility of the data. Although the absolute values varied between experiments, the relative pattern of changes was consistent between experiments.

Changes in protein levels of proteasomes were examined using three different monoclonal antibodies (GC4/5, GC3α and GC3β) against purified goldfish proteasome (Tokumoto *et al.* unpublished). An analysis by immunoblots of native PAGE revealed that all of the three antibodies recognized a single species of protein. The density of the band was proportional to the proteasome activity throughout the maturational processes (Fig. 1B). On immunoblots of SDS-PAGE, each of these three antibodies recognized three separate components of subunits of goldfish 20S proteasome. Again, their density fluctuated in harmony with the changes in the activity (Fig. 1C). Changes in the density of the three bands were synchronous, showing no specific changes in a specific band.

The present study demonstrates for the first time the changes in the activity and protein levels of proteasome during oocyte maturation. Both the activity and protein levels exhibited a similar pattern of fluctuation during the 17α,20β-DP-induced oocyte maturation in goldfish. These results suggest that the activity of proteasome is dependent on the amount of the protein. There were two peaks of the activity and protein levels of proteasome during oocyte maturation: the first peak immediately prior to the migration of germinal vesicle and the second one just after the completion of germinal vesicle breakdown. At present there is no experimental data to explain the functional significance of the first peak. However, some discussion could be made on the second peak. It is of great interest to note that the time of the second peak appears to correspond to the time of the first polar body extrusion (between the first and second meiotic metaphase). At present less is known about exit from mitosis than entry into mitosis. In *Xenopus* oocytes, it was found that MPF activity decreased rapidly after fertilization. More recently, it has been suggested that this rapid inactivation of MPF is induced by the degradation of cyclin B, the regulatory subunit of MPF (2, 7, 10). In fact, we previously reported that in goldfish oocytes H1 histone kinase (MPF) activity decreased transiently when the first polar body was eliminated (18). Thus, it is possible that proteasomes are responsible for the decrease in the MPF activity, probably by inducing the degradation of the regulatory subunit of MPF, cyclin B.

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (02102010 and 04044177 to Y. N.), the Naito Foundation, and the Japan Health Sciences Foundation.

Received 17 May 1993; and accepted 2 June 1993

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Publication

Biomedical Research, 1993, 14, 309 - 312.

Toshinobu Tokumoto, Hiroko kajiura, Michiyasu Yoshikuni, Masakane Yamashita and Yoshitaka Nagahama : Purification of ubiquitin from goldfish (*Carassius auratus*) oocyte cytosol.

Biomedical Research 14 (4) 309-312, 1993

Purification of ubiquitin from goldfish (*Carassius auratus*) oocyte cytosol

TOSHINOBU TOKUMOTO[1,2], HIROKO KAJIURA[2], MICHIYASU YOSHIKUNI[2], MASAKANE YAMASHITA[2] and YOSHITAKA NAGAHAMA[2]

[1]Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki 444, and [2]Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan

ABSTRACT

Ubiquitin was purified from the cytosol fraction (150,000 *g* supernatant) of goldfish ovaries containing full-grown postvitellogenic oocytes using four steps of column chromatography. The purified goldfish ubiquitin gave a single band with a molecular weight of 5.5 kDa on denaturing polyacrylamide gel electrophoresis and reacted with an anti-bovine ubiquitin antibody on Western blot. The first 40 amino acid residues of the N-terminal sequence of goldfish ubiquitin are identical with those of ubiquitins in other higher eukaryotes. These results indicate that ubiquitin exists and occurs as a free polypeptide in immature oocytes of goldfish.

Ubiquitin is an 8,600 dalton heat stable protein widely distributed in eukaryotic cells and its amino acid sequence exhibits a high degree of evolutionary conservation (3, 5, 13, 14, 17). Within the cells ubiquitin occurs either free or as a covalent adduct to various target proteins (2), with the linkage occurring between the carboxyl terminal of ubiquitin and the lysyl ε-amino groups of the target protein (8). Although there are numerous studies describing the mechanisms of ubiquitin conjugate formation, the physiological role of this post-ribosomal modification is still unclear. A widely accepted candidate for the role of ubiquitin conjugation is ubiquitin-dependent proteolysis. In the cytoplasm, ubiquitin conjugation marks the target proteins for degradation by the ubiquitin-dependent proteolytic system (6).

Mitosis is induced by the activation of maturation-promoting factor (MPF), which consists of two components, cdc2 kinase ($p34^{cdc2}$), a catalytic subunit, and cyclin B, a regulatory subunit (10). Cyclin B degradation is a critical part of the eukaryotic mitotic cell cycle (11). Recently it has been suggested that this cyclin degradation is induced by the ubiquitin-dependent proteolysis (4). However, the involvement of the ubiquitin-dependent proteolysis in meiotic cell cycle remains unknown. As a first step to answer this question, we purified ubiquitin from goldfish oocyte cytosol and determined its amino acid residues of the N-terminal sequence.

In this study, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was used to assay ubiquitin, since ubiquitin is known to migrate as a 5.5 kDa band (7); bovine ubiquitin was used as a standard. Immunoblotting using an anti-ubiquitin antibody was also used for assaying ubiquitin. Ubiquitin was purified from the 150,000 *g* supernatant prepared from goldfish ovaries containing full-grown postvitellogenic oocytes. Four steps of column chromatography (DEAE-cellulose, CM-cellulose, Bio Gel P-30 and Mono-S) were employed. On Bio Gel P-30 gel chromatography, ubiquitin was eluted at the position as the free form (Fig. 1A). In the final step of purification on Mono-S column chromatography, goldfish ubiquitin was eluted at 0.15 M NaCl with a symmetrical peak (Fig. 1B). By this procedure, 1.2 mg of ubiquitin was purified from 80 g goldfish ovary. Purified ubiquitin gave a single band with a molecular weight of 5.5 kDa (Fig. 2A). This 5.5 kDa band reacted with the anti-bovine ubiquitin antibody (Fig. 2B).

The primary structure of ubiquitin has been reported in bovine (14), human (5), trout (17), insect (3) and yeast (13). The amino acid sequence of these ubiquitins is completely identical, except

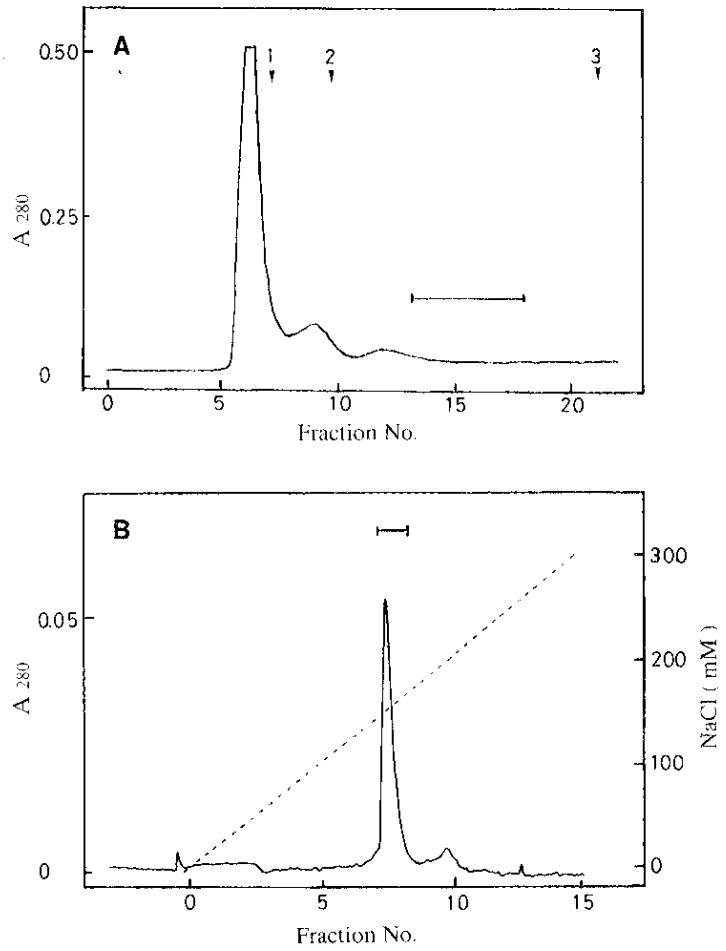

Fig. 1 Purification of ubiquitin from goldfish oocytes. Goldfish ovaries were dissected into fragments of 50–100 oocytes in goldfish Ringer's solution (125 mM NaCl, 2.4 mM KCl, 0.28 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2 mM HEPES, 5.6 mM glucose, 100,000 IU/l penicillin, 0.2 g/l streptomysin, pH 7.5) and washed with the Ringer's solution three times. Oocytes were homogenized with 4 vol of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.25 M sucrose and the homogenate was centrifuged at 150,000 *g* for 1 h. The supernatant was applied to a DEAE-cellulose column (DF52, 2.6× 12.0 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 10 mM 2-mercaptoethanol. Flow-through and first one column volume eluate were collected and used for ubiquitin purification. After pH was adjusted to 4.5 with HCl, the DEAE flow-through fraction was applied to a CM-cellulose column (CM52, 2.6× 18.8 cm) equilibrated with 25 mM ammonium acetate at pH 4.5 (buffer A, pH adjusted at room temperature). The column was washed with five column volumes of buffer A and bound materials were eluted with 50 mM ammonium acetate at pH 5.5 at room temperature, and 20 ml fractions were collected. Fractions were assessed by SDS-PAGE or immunoblotting with anti-bovine ubiquitin anti-serum (Sigma) and the fractions containing ubiquitin were collected and concentrated with a CM-cellulose column as following; pH of fraction was adjusted to 4.5 and applied to column (1.0×6.4 cm), then proteins were eluted with buffer A containing 500 mM NaCl. The concentrated fraction was applied to Bio Gel P-30 column (Bio Rad, 1.6 × 50.0 cm) equilibrated with buffer A, and 5 ml fractions were collected. Fractions containing ubiquitin were pooled and applied to a Mono-S column (Pharmacia, HR 5/5) equilibrated with buffer A. Proteins were eluted using a linear gradient (total volume: 30 ml) of 0–0.3 M NaCl in buffer A (fraction volume: 2 ml). The peak fraction at a concentration of 0.15 M NaCl was collected as purified ubiquitin. All procedures were performed at 4°C. A: Bio Gel P-30 column chromatography: Fractions from CM-cellulose column chromatography were pooled and concentrated, and chromatographed on Bio Gel P-30 column (1.6× 50 cm). Arrows indicate the eluted position of molecular weight standards as follows: 1, bovine serum albumin (66 kDa); 2, chymotrypsinogen (25 kDa); 3, acetone. Pooled fractions are indicated by the horizontal line. B: Mono-S column chromatography: Fractions from Bio Gel P-30 column chromatography were pooled and chromatographed on Mono-S (HR 5/5) column. The pooled fraction is indicated by the horizontal line. Protein content was determined from absorbance at 280 nm using bovine ubiquitin as standard.

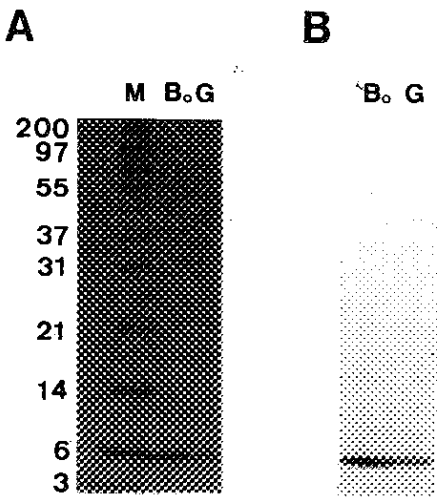

Fig. 2 SDS-PAGE and immunoblotting of purified goldfish ubiquitin. Electrophoresis followed the method of Laemmli (9) using 15/25% gradient gels (Daiichi Pure Chemicals). Proteins were then transferred to an Immobilon membrane (Millipore) using a semidry electroblotter (EIDO). The membrane was blocked with 5% non-fat dry milk and incubated with the anti-bovine ubiquitin anti-serum (1/100 in Tris-buffered saline) for 1 h at room temperature. Immunocomplexes were visualized using alkaline phosphatase conjugated anti-rabbit immunoglobulin, as described previously (16). A: SDS-PAGE of goldfish (G) and bovine (Bo) ubiquitin stained with Coomassie blue R-250. Molecular weights of standard proteins (M) are indicated at the left. B: Immunoblotting analysis of goldfish ubiquitin (G) and bovine ubiquitin (Bo)

for that of yeast ubiquitin which differs in only three of 76 residues from that of the animal ubiquitins. We determined the first 40 amino acid residues of the N-terminal sequence of the purified goldfish ubiquitin. To this end the purified ubiquitin was further purified by a reverse-phase HPLC. As shown in Fig. 3, the N-terminal 40 amino acid sequence of goldfish ubiquitin is identical with that of the animal ubiquitins and differs in only three amino acid residues from that of yeast ubiquitin.

Evidence is now accumulating to suggest that proteolysis plays an essential role in the progression of the eukaryotic cell cycle. Recently, nonlysosomal high molecular weight multicatalytic protease complexes, named proteasome, have been identified in various eukaryotic sources (12). In frog oocytes, serine protease inhibitors prevent both the proteasome activity and meiotic maturation of oocytes (1). It is thus suggested that proteasome is one of the most likely candidates for protease responsible for meiotic cell cycle. Recently, we have purified and characterized 20 S (latent) proteasome and active proteasome (26S proteolytic complex) from full-grown ovaries of goldfish (Tokumoto *et al.*, unpublished). In the accompanying paper (15), we have suggested that the proteasome is involved in the degradation of cyclin B during the first polar body extrusion in goldfish oocytes. The present study clearly indicates that a large amount of ubiquitin occurs as a free polypeptide in immature oocytes of goldfish. We have also shown that approximately the same amount of proteasome is present in immature oocytes of goldfish (Tokumoto *et al.*, unpublished). It is possible that both ubiquitin and proteasome are involved in the degradation of cyclin B during meiotic cell cycle of goldfish oocytes, which in turn leads to the inactivation of MPF.

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (02102010 and 04044177 to Y.N.), the Naito Foundation, and the Japan Health Sciences Foundation.

	1	10	20	30	40
Bovine	M Q I F V K T L Y G K T I T L E V E P S D T I E N V K A K I Q D K E G I P P D Q				
Goldfish	M Q I F V K T L Y G K T I T L E V E P S D T I E N V K A K I Q D K E G I P P D Q				
Yeast	M Q I F V K T L Y G K T I T L E V E S S D T I D N V K S K I Q D K E G I P P D Q				

Fig. 3 Alignment of the N-terminal amino acid sequences of bovine, goldfish and yeast ubiquitin. For the determination of the N-terminal amino acid sequence, purified ubiquitin was further purified by a reversed-phase HPLC on a μPRC C2/C18, PC3.2/3 column using a SMART system (Pharmacia). Proteins were eluted with a 0–100% (v/v) linear gradient of acetonitrile/water containing 0.1% (w/v) trifluoroacetic acid. The peak fraction was directly loaded onto a glass filter for sequencing the peptides using a 470A Protein Sequencer (Applied Biosystems Japan). Three amino acids of yeast ubiquitin different from those of bovine and goldfish ubiquitin are underlined.

Received 8 June 1993; and accepted 18 June 1993

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Publication

Biomedical Research, 1993, in press.

Toshinobu Tokumoto and Katsutoshi Ishikawa : 20S latent proteasomes isolated from the cytosol of *Xenopus* oocytes: Purification and partial characterization.

Biomedical Research 14 (6) 391-401, 1993

20S LATENT PROTEASOMES ISOLATED FROM THE CYTOSOL OF *XENOPUS* OOCYTES: PURIFICATION AND PARTIAL CHARACTERIZATION

TOSHINOBU TOKUMOTO[1,2] and KATSUTOSHI ISHIKAWA[1,3]
[1]Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422, Japan

ABSTRACT

20S latent proteasomes (multicatalytic protease complexes) have been purified from the cytosol of *Xenopus laevis* oocytes by means of conventional chromatographic techniques. The enzyme thus obtained showed a molecular weight of approximately 670 kDa as estimated on gel filtration. Polyacrylamide gel electrophoresis of the enzyme gave a single band under non-denaturing conditions, and at least 9 bands in the range of 25–33 kDa under denaturing conditions. The enzyme hydrolyzed some peptide 4-methylcoumaryl-7-amide (MCA) substrates, with a special preference for Tyr-MCA and Arg-MCA bonds. Further, the enzyme was shown to significantly cleave the Tyr-MCA bond in the presence of SDS, which is known as an activator of 20S latent proteasomes, but under the same conditions, the Arg-MCA bond was resistant to the enzyme. Degradation of the Tyr-MCA bond by the enzyme showed a pH optimum in the slightly alkaline range. Immunological experiments using antibodies against *Xenopus* 20S proteasomes clearly revealed cross-reactivity with the purified enzyme. The properties examined are similar to those of 20S proteasomes isolated from various eukaryotic cells.

The multicatalytic protease complexes named proteasomes are thought to play a main role in the non-lysosomal proteolytic pathway in eukaryotes (17). It was recently shown that there are different forms, named mainly 20S and 26S proteasomes. Among them, 20S proteasomes may be the principal core of ubiquitin/ATP-dependent 26S proteasomes from various sources (3, 7, 14, 15, 18, 19, 21, 23, 25–27), and are called latent (inactive) forms since they can be activated by some agents such as SDS, polylysine and fatty acids (3, 15, 21, 25). Recent studies by Tanaka *et al.* (23) and Kleinschmidt *et al.* (10) have shown the presence of proteasomes in the cytoplasm and the nucleus of amphibian (*Xenopus laevis*) oocyte. This finding is interesting in connection with the maturation of amphibian oocytes, which is known to be induced by progesterone (8, 12, 13). Here, it is suggested that some proteolysis may be involved in the *in vitro* oocyte maturation in amphibians (6). Recently, we also reported that some protease inhibitors, e.g., *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), specific for chymotrypsin, and diisopropyl fluorophosphate (DFP), specific for serine-protease, inhibited the oocyte maturation in *Rana pipiens* (1, 9) and *Bufo japonicus* (Takahashi, Tokumoto and Ishikawa, in preparation), respectively. In addition, the proteasomes in premature oocytes were found to be sensitive to TPCK and DFP. These observations suggest for the first time that some proteolysis in oocyte maturation in frogs may be due to the action of proteasomes.

On the other hand, it was recently suggested that the c-*mos* product (Mos protein), and cyclin protein, a specific component of maturation promoting factor, which may be synthesized following

[2]Present address: Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan
[3]To whom correspondence should be addressed

induction by progesterone, may be degraded specifically by probable ubiquitin/ATP-dependent 26S proteasomes during oocyte maturation in *Xenopus laevis* (4, 16). Thus, it is very important that the involvement of proteolysis may be analyzed by focusing on the proteasomes. However, the forms of proteasomes in the physiological state and their activation mechanism remain unclear. The final goal of our study is to clarify the involvement of proteasomes in the oocyte maturation in amphibians.

Very recently, we found for the first time the presence of a novel proteasome that can hydrolyze succinyl(Suc)-Leu-Leu-Val-Tyr-MCA, as a typical peptide substrate for proteasomes, without SDS, in the cytosol of *Xenopus* oocytes (24). We proposed to call it the 'active' form of proteasome. The 'active' form may be easily dissociated into a very stable 20S latent form and regulatory factors like 26S proteasomes, but different from 26S proteasomes (24). Thus, the latent form of proteasome may be the core of the 'active' form. Among proteasomes, the 'active' form itself may play a critical role in the physiological state in the oocyte maturation process. In order to clarify the nature of the 'active' form, it is necessary, as a first step, to completely elucidate the nature of the stable 20S latent proteasome, because it is easy to isolate and purify.

In the present paper, we report a procedure for purifying 20S latent proteasomes from cytosol prepared through one-step ultracentrifugation from *Xenopus laevis* oocytes, involving conventional column-chromatographic techniques, and characterized some properties of the purified enzyme. In addition, the partial specific characteristics of the same 20S proteasome were previously reported by us (20).

MATERIALS AND METHODS

Animals

Female gravid African clawed frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu Kyozai Corporation (Hamamatsu, Japan).

Chemicals

Chemicals were obtained from the sources indicated. 4-Methylcoumaryl-7-amide (MCA) substrates, i.e., Suc-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), Suc-Ala-Ala-Pro-Phe-MCA (Suc-AAPF-MCA), Boc-Leu-Arg-Arg-MCA (Boc-LRR-MCA) and Boc-Phe-Ser-Arg-MCA (Boc-FSR-MCA) from Peptide Institute (Osaka, Japan); sodium dodecyl sulfate (SDS) from Nacalai Tesque (Kyoto, Japan); DEAE-cellulose (DE-52) from Whatman (U.K.); Sepharose CL-6B, Phenyl-Sepharose CL-4B and Protein A-Sepharose CL-4B from Pharmacia (Sweden); hydroxylapatite (Bio-Gel HTP) from Bio-Rad (U.S.A.); normal rabbit serum and ABC-AP kit from Vector Lab. (U.S.A.); and a nitrocellulose membrane (Immobilon) from Millipore (U.S.A.). All other reagents were of the highest grade available.

Preparation of Cytosol from Ovarian Oocytes

Ovaries were isolated from sacrificed animals and placed in freshly prepared Barth's medium [88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 7.5 mM Tris-HCl, pH 7.6, with 10 mg streptomycin sulfate and 100,000 IU potassium penicillin G per liter]. Ovarian fragments were washed three times with cold Barth's medium and then allowed to settle in a centrifuge tube, and then excess medium was removed. The clear supernatant (cytosol) between the lipid cap and the packed components was obtained by one-step ultracentrifugation at 105,000 *g* for 1 h (50.3 Ti rotor; Beckman) without homogenization of ovarian fragments. Cytosol (≈ 20 mg protein/ml) was carefully withdrawn using a Pasteur pipet.

Enzyme Assay

The substrate solution (10 μl of substrate dissolved in dimethylsulfoxide with 20 μl of 0.5 M Tris-HCl, pH 8.5, and 60 μl of different SDS concentrations in distilled water) was first preincubated for 10 min at 37°C, and then an aliquot (10 μl) of the enzyme solution was added (final concentration of each substrate, 10 μM), followed by incubation at 37°C for a given period. The reaction was stopped by adding 100 μl of 10% SDS and 2 ml of 0.1 M Tris-HCl, pH 9.0. The enzyme activity was determined by measuring the fluorescence (excitation at 360 nm and emission at 460 nm) of 7-amino-4-methylcoumarin (AMC) with a fluorescence spectrophotometer (F-3000; Hitachi, Japan). One unit of activity was defined as the amount of the enzyme that liberated 1 μmol of AMC per minute.

Purification of the Enzyme

20S proteasomes were purified from cytosol by

sequential column chromatographies on DEAE-cellulose, hydroxylapatite, Sepharose CL-6B, Sepharose CL-6B again and then DEAE-cellulose again. All procedures were performed at 4°C.

Cytosol was applied to a column (2.74 × 10 cm) of DEAE-cellulose equilibrated with 25 mM Tris-HCl, pH 7.5, containing 20% glycerol and 10 mM 2-mercaptoethanol (TGM buffer). After the column had been washed with the same buffer, the enzyme was eluted at a flow rate of 25 ml/h with a linear gradient of NaCl (0–0.4 M; total, 500 ml). Fractions were collected at 5 ml/tube. The enzyme activity fraction obtained was pooled and then dialyzed against 25 mM sodium phosphate, pH 6.8, containing 20% glycerol and 10 mM 2-mercaptoethanol (PGM buffer). The enzyme activity fraction thus obtained was applied to a column (2.75 × 10 cm) of hydroxylapatite equilibrated with PGM buffer. The column was washed thoroughly with the same buffer, and then the enzyme was eluted at a flow rate of 15 ml/h with a linear gradient of sodium phosphate (25–300 mM; total, 500 ml). Fractions were collected at 5 ml/tube. The enzyme activity fraction obtained was pooled and then concentrated to about 10 ml on a column (1.25 × 4.1 cm) of DEAE-cellulose, and then chromatographed on a column of Sepharose CL-6B in TGM buffer at a flow rate of 20 ml/h. Fractions were collected at 5 ml/tube. The enzyme activity fraction obtained was rechromatographed on a Sepharose CL-6B column in the same manner as above. The pooled enzyme activity fraction from a column of Sepharose CL-6B was again applied to a column (0.95 × 4.3 cm) of DEAE-cellulose equilibrated with TGM buffer, and then eluted with a linear gradient of NaCl (0–0.4 M; total, 100 ml). Fractions were collected at 2 ml/tube. The enzyme activity fraction eluted at 0.16–0.18 M NaCl was pooled as the purified enzyme and then stored at 4 or −20°C for subsequent studies.

Gel Electrophoresis

Protein samples were electrophoretically separated, with or without SDS, according to the protocol of Laemmli (11).

Immunological Analysis

For immunoprecipitation, anti-(*Xenopus* proteasome) IgG and control IgG were purified from rabbit anti-*Xenopus* anti-serum and normal rabbit serum, respectively, with Protein A-Sepharose CL-4B. Samples were incubated with these IgGs in TGM buffer for 1 h at 2 or 37°C, and then Protein A-Sepharose CL-4B suspended in TGM buffer was added, followed by further incubation (30 min at 2 or 37°C). Immuno-complexes were precipitated by centrifugation and residual enzyme activity in the supernatant was determined.

For immunoblotting, samples separated by SDS-polyacrylamide gel electrophoresis (PAGE) were electrophoretically transferred to nitrocellulose membrane. The membrane was blocked with 3% gelatin and then incubated with anti-(*Xenopus* proteasome) IgG. The antigen-antibody complex was visualized with the ABC-AP kit.

Protein Assay

The protein content was estimated from the absorbance at 595 nm according to Bradford's method using bovine serum albumin as a standard (2).

RESULTS

Effect of SDS on the Enzyme Activity in the Cytosol

As shown in Fig. 1A, the *Xenopus laevis* oocyte cytosol prepared by one-step ultracentrifugation exhibited significant Suc-LLVY-MCA-hydrolyzing activity in the absence of SDS. This activity was drastically reduced at 0.04–0.07% SDS. With a further increase in the SDS concentration, however, the activity increased again, reaching a maximum at about 0.12% SDS. Both the SDS-dependent and -independent hydrolyses reached a plateau after about 30 min (Fig. 1B). Immunodepletion of proteasomes from the cytosol with anti-proteasome IgG led to an almost complete loss of the activity in both the absence and presence of SDS (Table 1). The activities with and without SDS have been confirmed to be due to proteasomes, which may be 20S latent and 'active' proteasomes, respectively (24). In addition, Table 1 shows the relative rates of hydrolysis of several fluorogenic peptide substrates by the fresh cytosol in the absence of SDS. Suc-LLVY-MCA was by far the best substrate, whereas Suc-AAPF-MCA was the poorest. Among the Arg-containing peptides tested, Boc-LRR-MCA was hydrolyzed well, but Boc-FSR-MCA was poorly attacked. These results suggest that 'active' proteasomes contain chymotrypsin-like and trypsin-like enzymes as components, as expected (24).

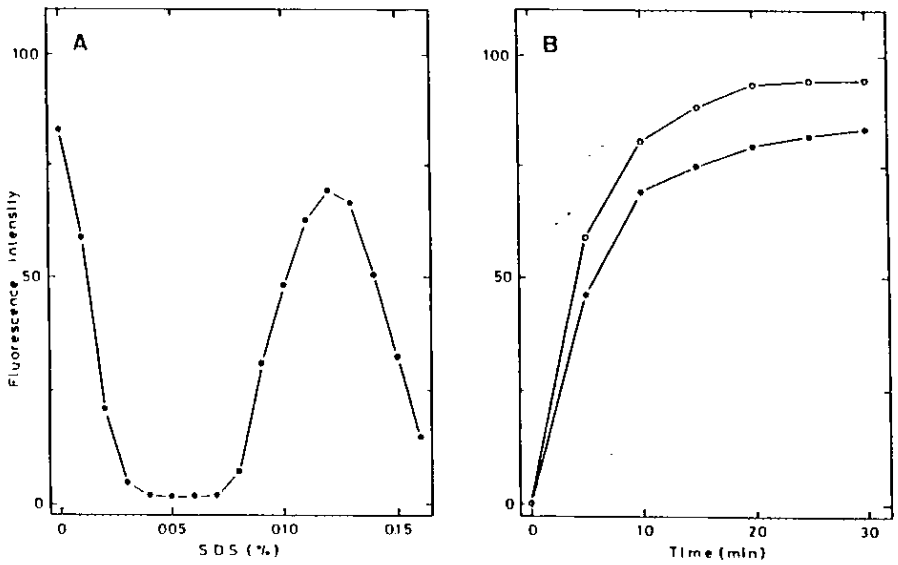

Fig. 1 Effect of SDS on the enzyme activity in cytosol. (A) Suc-LLVY-MCA-hydrolyzing activity was measured for various SDS concentrations after 10 min at 37°C. (B) The same activity was also measured after various times at 37°C without (○) and with (●) 0.12% SDS.

Table 1 *Effect of Immunotitration of the Enzyme in Cytosol with Anti-Proteasome IgG*

Substrate	SDS (0.12%)	Remaining activity (m units/ml)		Inhibition (%)
		Control-IgG	Anti-IgG	
Suc-LLVY-MCA	–	28.6	1.9	93
	+	27.1	0.3	99
Suc-AAPF-MCA	–	5.7	6.6	0
Boc-LRR-MCA	–	26.1	8.9	66
Boc-FSR-MCA	–	9.8	6.2	37

Cytosol fractions were incubated with rabbit anti-(*Xenopus* proteasome) IgG (anti-IgG) or control-IgG, and then Protein A-Sepharose CL-4B as described under Materials and Methods. Immunoprecipitates were removed by centrifugation. The supernatant was assayed for residual peptide hydrolyzing activity.

Purification of 20S Proteasomes

The results of purification of 20S latent proteasomes, which can be activated by SDS (Figs. 2–5), are summarized in Table 2. Here, we measured Suc-LLVY-MCA-hydrolyzing activity in both the presence and absence of SDS during purification. In addition, the optimal SDS concentration for stimulation of the enzyme in cytosol was 0.11–0.12%, as shown in Fig. 1, but chromatography caused a decrease in the SDS concentration (0.04–0.05%) giving the maximal SDS-dependent activity. The enzyme gave a single activity peak upon gel filtration on a Sepharose CL-6B column, and its molecular weight was estimated to be approximately 670 kDa, as shown in Fig. 4. From 61 g of *Xenopus* ovaries, 0.32 mg of proteasomes was obtained, with 123-fold purification and a yield of 5.3%, with SDS.

Analyses of Enzyme Purity and Subunit Composition

We conducted non-denaturing and denaturing

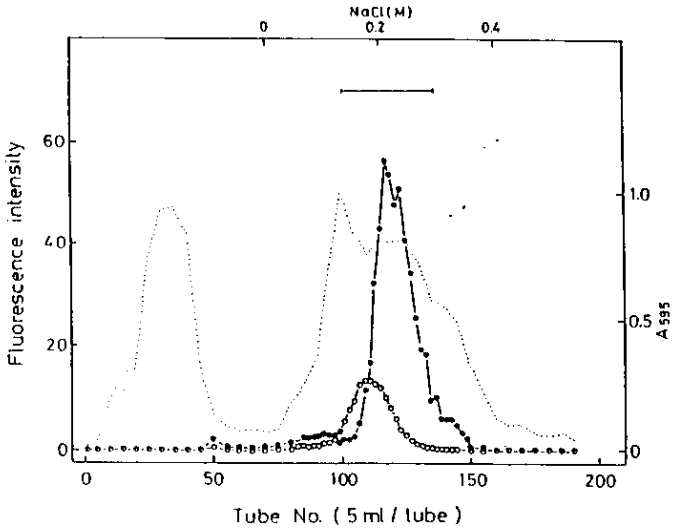

Fig. 2 DEAE-cellulose column chromatography. The details of the procedure are given under Materials and Methods. The absorbance at 595 nm (----) and the activity toward Suc-LLVY-MCA without (○) and with (●) 0.05% SDS were measured. The horizontal line at the top indicates the fractions pooled.

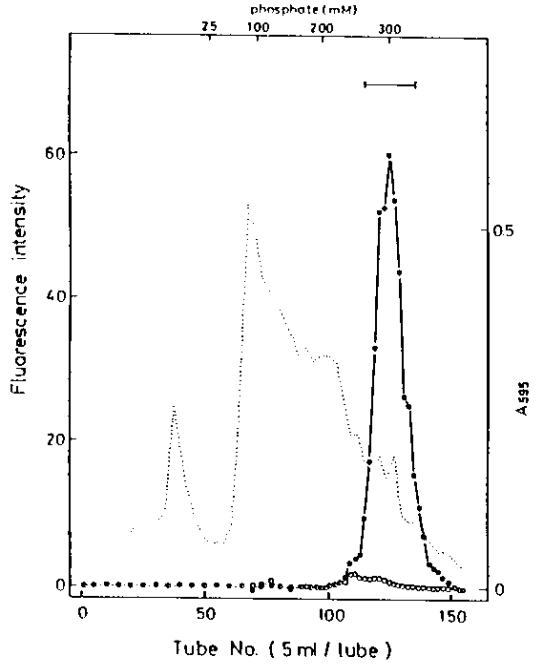

Fig. 3 Hydroxylapatite column chromatography. The details of the procedure are given under Materials and Methods. The absorbance at 595 nm (----) and the activity toward Suc-LLVY-MCA without (○) and with (●) 0.05% SDS were measured. The horizontal line at the top indicates the fractions pooled.

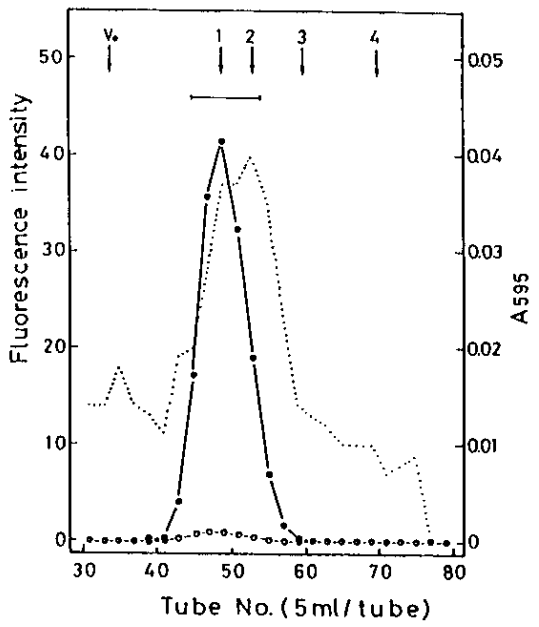

Fig. 4 Sepharose CL-6B column chromatography. The details of the procedure are given under Materials and Methods. The absorbance at 595 nm (----) and the activity toward Suc-LLVY-MCA without (○) and with (●) 0.05% SDS were measured. The horizontal line at the top indicates the fractions pooled. The column was calibrated with proteins of known molecular weight: V_o, blue dextran; 1, thyroglobulin (669 kDa); 2, apoferritin (440 kDa); 3, catalase (240 kDa); 4, alcohol dehydrogenase (150 kDa)

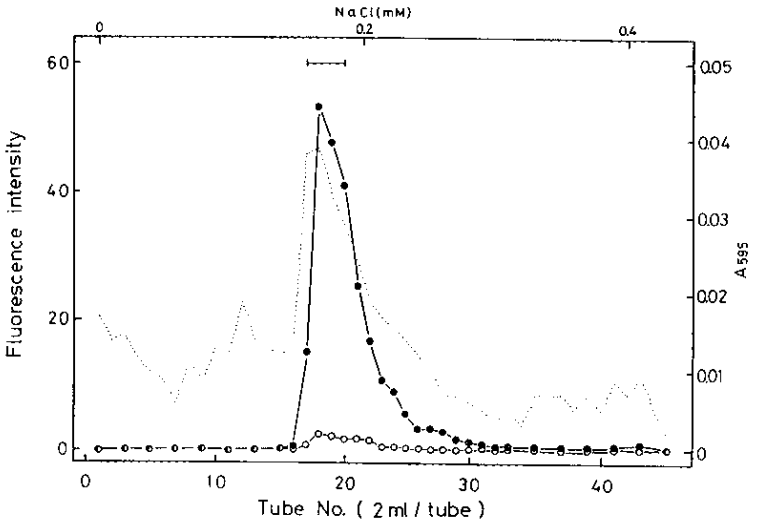

Fig. 5 Second DEAE-cellulose column chromatography. The details of the procedure are described under Materials and Methods. The absorbance at 595 nm (----) and the activity toward Suc-LLVY-MCA without (○) and with (●) 0.05% SDS were measured. The horizontal line at the top indicates the fractions pooled.

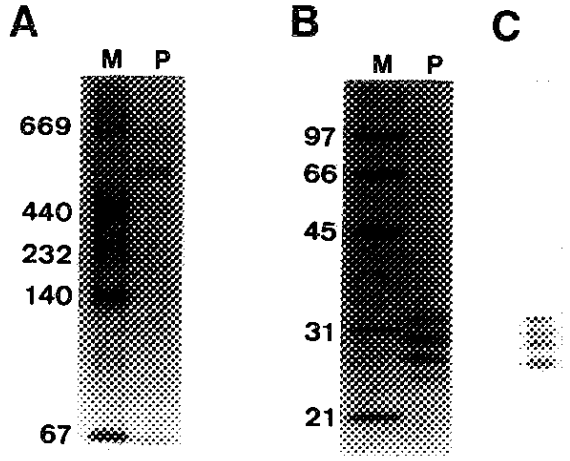

Fig. 6 Polyacrylamide gel electrophoresis (PAGE) of the purified enzyme. The purified enzyme (1.2 μg) was electrophoresed under non-denaturing conditions (panel A; lane P, 5% gel), and 5.3 μg was electrophoresed under denaturing conditions (panel B; lane P, 12% gel). The gels were stained with Coomassie Brilliant Blue R-250. The molecular weights (kDa) of the standard proteins are indicated at the left side (lane M). Panel C shows the immunoblotting analysis of the purified enzyme separated by SDS-PAGE in 12% gel using polyclonal antibodies against 20S proteasomes, as described under Materials and Methods.

PAGEs to assess the purity and subunit composition of the final enzyme preparation. Non-denaturing PAGE gave one major band (Fig. 6A). Suc-LLVY-MCA-hydrolyzing activity in the presence of SDS was detected in the same band (data not shown). This enzyme seems to be homogeneous. SDS-PAGE resolved the enzyme into at least nine protein components in the molecular weight range of 25–33 kDa, which were distinct subunits of 20S latent proteasomes (23) (Fig. 6B).

Immunological Analysis of the Enzyme

Immunological cross-reactivity of the purified enzyme was examined by using polyclonal antibodies raised against 20S proteasomes from *Xenopus* ovaries (23) (Fig. 6C). Four of the subunit compositions reacted significantly with the antibodies. In addition, the purified enzyme was immunoprecipitated with anti-proteasome IgG, but not with control IgG (Fig. 7).

Substrate Specificity of the Enzyme

The activity of the enzyme toward several MCA-substrates is shown in Table 3. Among those tested, Suc-LLVY-MCA with SDS was the best substrate, as expected. Suc-LLVY-MCA, Boc-LRR-MCA, Boc-QRR-MCA and Boc-FSR-MCA were hydro-

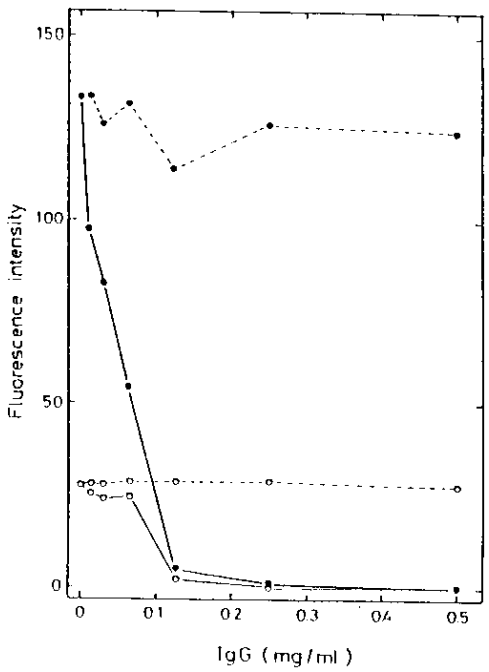

Fig. 7 Immunoprecipitation of the purified enzyme with anti-proteasome IgG. The enzyme (final concentration, 30 μg/ml) treated with the indicated amounts of control IgG or anti-proteasome IgG and immuno-complexes were precipitated with Protein A-Sepharose CL-4B. Then, the remaining activity in the supernatant was determined. The solid lines and broken lines indicate treatment with anti-IgG and control IgG, respectively. The closed circles and open circles indicate the Suc-LLVY-MCA-hydrolyzing activity without (○) and with (●) 0.05% SDS, respectively.

lyzed without SDS to some extent. Suc-AAPF-MCA was not hydrolyzed at all. Thus, the enzyme showed latent Suc-LLVY-MCA activity.

Effect of SDS on the Enzyme Activity

Fig. 8 shows the SDS-dependence of the activity of the purified enzyme toward Suc-LLVY-MCA (panel A) and not that of that toward Boc-LRR-MCA (panel B). The optimum SDS concentration for Suc-LLVY-MCA-hydrolyzing activity is 0.05% SDS, and this activity is 24.3-fold when compared with that without SDS.

Effect of pH on the Enzyme Activity

Fig. 9 shows the pH dependence of the activity toward Suc-LLVY-MCA of the purified enzyme. The optimum pH value lies at pH 8.1 and 7.5, with and without SDS, respectively. Thus, it is a neutral or slightly alkaline protease.

Effects of Protease Inhibitors on the Enzyme Activity

Table 4 shows the effects of some protease inhibitors on the peptide-hydrolyzing activity of the purified enzyme. Antipain and DFP (10 mM) strongly inhibited the Suc-LLVY-MCA-hydrolyzing activity of the enzyme with SDS under the conditions used. Further, the enzyme was somewhat inhibited by chymostatin. Leupeptin, TPCK and DFP (1 mM) had little effect on the activity. However, a higher concentration (2 mM) of leupeptin had an inhibitory effect (data not shown).

On the other hand, inhibitory effects of the same inhibitors on the hydrolyses of Suc-LLVY-MCA and Boc-LRR-MCA without SDS are also shown. The effects are a little different between the two activities. Thus, Suc-LLVY-MCA-hydrolyzing activity was inhibited by DFP (10 mM) and chymostatin. Boc-LRR-MCA-hydrolyzing activity was strongly inhibited by leupeptin and antipain. TPCK had little effect on either activity.

DISCUSSION

In the present paper, we report for the first time in more detail the purification of 20S latent (inactive) proteasomes from cytosol obtained on one-step ultracentrifugation of *Xenopus* oocytes through conventional column chromatographies, and characterized some of their properties. These enzymes, found through the use of oocytes enclosed with follicle cells, seem to be derived not from the follicle cells but from the oocytes. In addition, the 'active' proteasome, which is found in cytosol and can hydrolyze Suc-LLVY-MCA, a typical peptide substrate for proteasomes, without SDS, may be dissociated into a 20S proteasome and other regulatory factors through the repeated purification procedures, because the amount of the 'active' form with the Suc-LLVY-MCA-hydrolyzing activity without SDS decreased gradually (Table 2), as described previously (24). On the other hand, it has been reported by Tanaka *et al.* (23) and Kleinschmidt *et al.* (10) that 20S proteasomes were purified from the same source and that their partial properties were characterized, but the 'active' proteasomes were not examined.

The enzyme which was purified in the present

Table 2 *Summary of Purification of 20S Proteasome from* Xenopus laevis *Ovary*

Purification step	Total protein (mg)	SDS	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Cytosol fraction	733.5	–	1,958	2.7	1.0	100
		+	2,744	3.7	1.0	100
				(1.4)		
DEAE-cellulose (1)	265.8	–	754	2.8	1.1	38.5
		+	2,379	9.0	2.4	86.7
				(3.2)		
Hydroxylapatite	16.3	–	87	5.3	2.0	4.4
		+	1,780	109.0	29.2	64.9
				(20.6)		
Sepharose CL-6B (1)	6.46	–	63	9.8	3.6	3.2
		+	955	147.9	39.7	34.8
				(15.1)		
Sepharose CL-6B (2)	2.19	–	66	30.0	11.2	3.4
		+	684	312.2	83.8	24.9
				(10.4)		
DEAE-cellulose (2)	0.32	–	6	18.6	6.9	0.3
		+	146	457.7	122.8	5.3
				(24.6)		

Activities of each fraction toward Suc-LLVY-MCA were determined in the presence (+) and absence (–) of 0.05% SDS (0.12% SDS in cytosol fraction alone) as described under Materials and Methods. Values in parentheses show the rate acceleration (-fold) on the addition of SDS.

Table 3 *Activities of the Purified Enzyme toward Several MCA Substrates*

Substrate	SDS	Specific activity (units/mg protein)	Relative activity[a] (%)
Suc-LLVY-MCA	–	18.6	100
	+	457.7	2,461
Suc-AAPF-MCA	–	0.0	0
Boc-LRR-MCA	–	17.1	92
Boc-QRR-MCA	–	8.2	44
Boc-FSR-MCA	–	7.3	39

Activities toward several peptide substrates were determined as described under Materials and Methods. [a]The activity without 0.05% SDS toward Suc-LLVY-MCA was taken as 100%.

study was shown to cross-react immunologically with antibodies against 20S proteasomes purified from *Xenopus* ovaries by Tanaka *et al.* (23) (Figs. 6 and 7). In addition, it showed similar properties to those from various eukaryotic cells including *Xenopus* oocytes (see below). Therefore, it has been confirmed that the purified enzyme may be a typical 20S proteasome. 1) The molecular mass is estimated to be about 670 kDa (Fig. 4), which is similar to that of 675 kDa estimated by Kleinschmidt *et al.* (10). However, this value is less than the 840 kDa estimated physicochemically by Tanaka *et al.* (23). 2) The enzyme consists of about nine protein subunit components in the molecular weight range of 25 to 33 kDa (Fig. 6). These results indicate that the purified enzyme giving a single band in non-denaturing PAGE is similar to that reported previously for proteasomes purified by Tanaka *et al.*

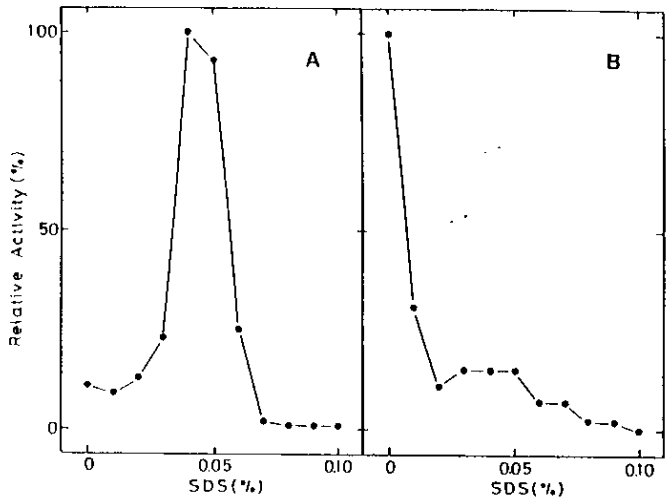

Fig. 8 Effect of SDS on the enzyme activity. The Suc-LLVY-MCA (panel A) and Boc-LRR-MCA (panel B) hydrolyzing activities of the purified enzyme (0.1 μg) were measured in the presence of various SDS concentrations. The activities are expressed as percentages of the maximum ones.

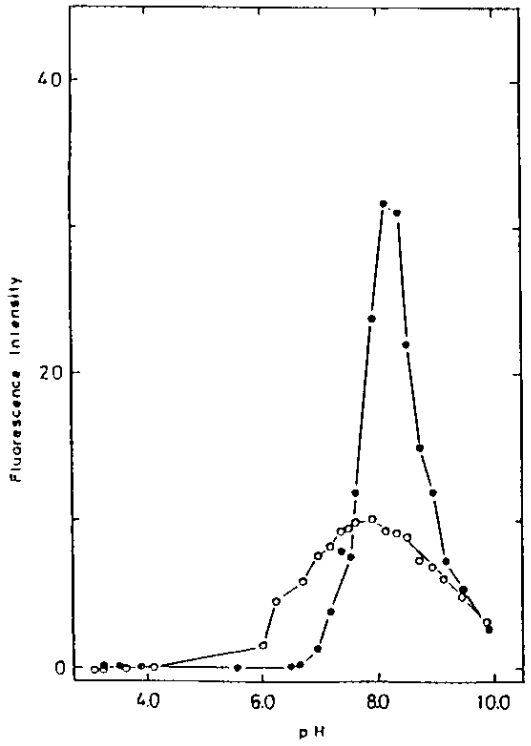

Fig. 9 Effect of pH on the enzyme activity. The Suc-LLVY-MCA-hydrolyzing activity of the purified enzyme (0.1 μg) was measured after 5 min at 37°C at various pHs without (○) or with (●) 0.05% SDS.

(23), and Kleinschmidt *et al.* (10). Immunological analysis with anti-proteasome IgG gave bands for four of the protein components recognized by the antibodies previously reported (23). 3) The enzyme has at least chymotryptic and tryptic activities (Table 3 and Fig. 8). In addition, the enzyme was found to have additional peptidyl glutamyl peptidase activity by Tanaka *et al.* (23). 4) Suc-LLVY-MCA-hydrolyzing activity is significantly enhanced by the addition of low concentrations of SDS (Table 3 and Fig. 8A), whereas Boc-LRR-MCA-hydrolyzing activity is not enhanced by the addition of SDS (Fig. 8B). Thus, this enzyme showed latent Tyr-containing peptide hydrolyzing activity, but not latent Arg-containing peptide hydrolyzing activity. This observation is similar to that of Tanaka *et al.* (23). 5) The optimal pH of Suc-LLVY-MCA-hydrolyzing activity may be a little dependent on the presence or absence of SDS, but it is a neutral or slightly alkaline pH value (Fig. 9). In addition, the optimal pH of the enzyme with SDS reported by Tanaka *et al.* (23) was 8.0–8.5.

On the other hand, among protease inhibitors tested, Suc-LLVY-MCA-hydrolyzing activity with SDS was inhibited by chymostatin, but not by antipain (Table 4). These observations were not inconsistent with the previous ones (20). Boc-LRR-MCA-hydrolyzing activity was inhibited by leupeptin and antipain, but not by chymostatin. These

Table 4 *Effects of Some Protease Inhibitors on the Activity of the Purified Enzyme*

Inhibitor	Final concentration (mM)	Inhibition (%)		
		Suc-LLVY-MCA		Boc-LRR-MCA
		(−)	(+)	(−)
None		0	0	0
Antipain	0.2	5	72	70
Chymostatin	0.1	61	46	6
Leupeptin	0.3	8	6	74
DFP	1.0	31	12	0
	10.0	77	71	3
TPCK	0.4	7	10	5

Purified enzyme (0.1 μg) was used for assay. Activities were measured using Suc-LLVY-MCA and Boc-LRR-MCA as the substrates in the presence (+) or absence (−) of 0.05% SDS as described under Materials and Methods.

lower inhibitory effects were also similar to the previous observations (20). In addition, Suc-LLVY-MCA-hydrolyzing activity stimulated by low concentrations of SDS may be due to degradation by the enzyme through the probable interaction of the substrate with SDS, as described by Tanaka *et al.* (22). As the enzyme is inactivated on preincubation with SDS (data not shown), the enzyme activity was determined after preincubation of the substrate with SDS in the present study. Thus, some conformational alteration may affect the catalytic sites of the enzyme complex.

From the above observations, it was confirmed that 20S proteasomes purified from the cytosol of *Xenopus* oocytes may be similar to those found in eukaryotic cells. However, as described previously (17, 20), there may be some species difference in specificity as well as the general importance of proteasomes for proteolysis.

Here, it has been reported that not 20S but 26S proteasomes may be involved in the ubiquitin/ATP-dependent non-lysosomal proteolytic pathway of various eukaryotic cells (5). It is known that 26S proteasomes consist of 20S proteasomes and several regulatory factors, such as the 'active' proteasome found in *Xenopus* oocytes (24). In addition, we suggest that the 'active' proteasomes found in *Xenopus* oocytes may be converted to the probable 26S proteasomes on the addition of ATP (24). Therefore, the degradation of Mos and cyclin proteins following ubiquitination involved in the oocyte maturation in *Xenopus* may be due to 26S proteasomes or 'active' proteasomes.

As the next step, it will be necessary to clarify the biochemical properties of 'active' proteasomes found in *Xenopus* oocytes and to look for any endogenous substrates. In addition, the physiological role of the 20S proteasome may be insignificant, but since this enzyme can cleave some kinds of peptide bonds without SDS (20), we may not be able to rule out some involvement of 20S proteasomes in the regulation of intracellular proteolysis during the amphibian oocyte maturation process.

We wish to thank Dr Keiji Tanaka of Tokushima University for the gift of the anti-(*Xenopus* proteasome) polyclonal antibodies. A part of this study was supported by grants (No. 01304029 and special research) from the Ministry of Education, Science and Culture of Japan, and the Saito-Chion Foundation, Shizuoka, to K.I.

Received 29 September 1993; and accepted 13 October 1993

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Publication

Molecular Reproduction and Development, 1993, in press.

Mika Takahashi, Toshinobu Tokumoto and Katsutoshi Ishikawa : DFP-sensitive multicatalytic protease complexes (proteasome) in the control of oocyte maturation in the toad, *Bufo japonicus*.

DFP-sensitive Multicatalytic Protease Complexes (Proteasomes) in the Control of Oocyte Maturation in the Toad, *Bufo japonicus*

Mika Takahashi,* Toshinobu Tokumoto,* and Katsutoshi Ishikawa[1]

Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422, Japan

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Correspondence to: Katsutoshi Ishikawa, Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422, Japan; Telephone: (054) 237-1111 ext. 5707; Fax: (054) 238-0986

Running title: *Bufo* proteasomes in oocyte maturation

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* Present address: Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan

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[1] To whom correspondence should be addressed.

ABSTRACT

The inhibition by diisopropylfluorophosphate (DFP), a typical serine protease inhibitor, of progesterone-induced oocyte maturation was investigated in oocytes of the Japanese toad *Bufo japonicus* for the first time. Oocytes with DFP applied externally did not undergo germinal vesicle breakdown (GVBD), which is an early signal of oocyte maturation, in response to progesterone. The most inhibitory period was found to be 0 – 0.5 $GVBD_{50}$ (when 50%GVBD was taken as 1.0), *i.e.*, before the beginning of GVBD. DFP-sensitive proteases, which seem to be multifunctional non-lysosomal protease complexes (proteasomes) were suggested to be already present in the cytosol of premature oocytes. Peptide hydrolyzing activity, as reflected by proteasome activity, was found to be regulated before and after GVBD. In addition, immunoblotting experiments regarding the native electrophoretic protein pattern of the proteasomes throughout the maturational process demonstrated that the proteasomes undergo alterations in their mobility dependent on the maturational process.

These data raise the possibility that the activities of some endogenous DFP-sensitive proteasomes among proteases play distinct, essential roles in oocyte maturation triggered by progesterone in *Bufo*. In addition, the effect of sodium dodecyl sulfate (SDS), which is known as an activator of proteasomes, on enzyme activity will be discussed.

Key words: Protease, Oocyte, Germinal vesicle breakdown, Amphibian

INTRODUCTION

It is known that meiotic maturation of amphibian oocytes is arrested at the first prophase and reinitiated by progesterone (Masui and Clarke, 1979; Schuetz, 1985). Progesterone acts on the oocyte surface (Ishikawa *et al.*, 1977; Godeau *et al.*, 1978) to produce a cytoplasmic maturation promoting factor (MPF) and thus germinal vesicle breakdown (GVBD), as the first easily observable event of meiotic reinitiation, is caused. Here, the synthesis of the c-*mos* protein product (Mos) occurs soon after hormonal stimulation and this synthesis is required for both activation of MPF and GVBD (Sagata *et al.*, 1989). In addition, it seems that Mos proteins are rapidly degraded by the ubiquitin-dependent proteolytic pathway (Nishizawa *et al.*, 1992). MPF is composed of proteins exhibiting histone H1 kinase activity whose principal subunit is $p34^{cdc2}$, and cyclin, a regulatory subunit whose abundance fluctuates throughout the cell cycle (Maller, 1990). The transition following the cession of GVBD is induced by the degradation of cyclin, which in turn leads to the inactivation of MPF. In addition, it has been reported that oocytes treated with leupeptin, antipain and chymostatin, protease inhibitors of microbial origin, do not undergo progesterone-induced GVBD in amphibian (*Xenopus laevis*) (Guerrier *et al.*, 1977; Picard *et al.*, 1987). We showed that a higher concentration of N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a synthetic inhibitor of chymotrypsin, inhibited progesterone-induced GVBD (Ishikawa *et al.*, 1989).

These results suggest that proteolysis plays a critical part in the oocyte maturation process. However, until now, there has been little information on the nature and mode of action of the endogenous proteases in amphibian oocytes. More recently, non-lysosomal multicatalytic protease complexes of unusually large size, such as about 20S particles named proteasomes, were found in the ovaries and/or oocytes of *Xenopus laevis* (Kleinschmidt *et al.*, 1988; Tanaka *et al.*, 1988; Takahashi *et al.*, 1993; Tokumoto and Ishikawa, 1993), which were found to be similar to proteasomes from eukaryotic cells (Orlowski, 1990; Goldberg, 1992). Proteasomes have been proposed to be chymotrypsin-like, trypsin-like and

peptidylglutamyl peptidases. We showed that in an amphibian (*Rana pipiens*), proteasomes showed predominantly cytoplasmic localization in non-hormone treated (premature) and mature oocytes (Azuma *et al.*, 1991). The results show that the protein patterns of proteasomes in premature oocytes are not identical to those in mature oocytes.

No studies have yet been reported on clarification of the relationship between the inhibition of GVBD by protease inhibitors and the protease (proteasome) entity. Taking these points into consideration using the Japanese toad (*Bufo japonicus*) in the present study, we report for the first time the results of investigations on the inhibition by diisopropylfluorophosphate (DFP), an irreversible synthetic inhibitor of a serine protease (Powers and Harper, 1986), of meiotic maturation, and analysis of the dynamics of the nature or action of DFP-sensitive proteasomes throughout oocyte maturation. The results indicate that a DFP-sensitive protease with at least two different catalytic sites which is present in premature oocytes and identical or similar to proteasomes may be involved in GVBD. This observation that proteasomes can be found in close association with a DFP-effect raises the possibility that the toad proteasomes may be involved in oocyte maturation.

In addition, the toad (*Bufo japonicus*) has several advantageous characteristics compared to popular *Xenopus* oocytes: (a) the size of the oocyte is larger, therefore the cytosol can be easily obtained in sufficient amounts for biochemical analysis; (b) many homogeneous populations of oocytes can be easily manually obtained from one animal; and (c) if isolated oocytes are kept under cold air, an experiment can take up to five days.

MATERIALS AND METHODS

Animals

Adult female toads (*Bufo japonicus*) captured in October were purchased from a commercial dealer in Saitama (Japan). The animals were kept in the open air until the next January, and then moved to an indoor cold room and maintained at 4°C.

Chemicals

The chemicals were obtained as follows: fluorogenic peptide substrates, *i.e.*, Succinyl (Suc)-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) (LLVY), Suc-Ala-Ala-Pro-Phe-MCA (AAPF), *t*-Butyloxycarbonyl (Boc)-Phe-Ser-Arg-MCA (FSR), Boc-Leu-Arg-Arg-MCA (LRR) and Boc-Gln-Arg-Arg-MCA (QRR) from Peptide Institute Inc. (Japan), progesterone, DFP, cycloheximide and protein A-Sepharose CL-4B from Sigma Co. (USA), sodium dodecyl sulfate (SDS) from Nacalai Tesque Co. (Japan), and non-immune rabbit serum and a Vectastain ABC-alkaline phosphatase kit from Vector Laboratories Inc. (USA). Polyclonal rabbit antibodies raised against 20S proteasomes from *Xenopus laevis* ovaries were a gift from Dr. K. Tanaka (Tokushima University, Japan) (Tanaka *et al.*, 1988). All other chemicals were of special grade and commercially available.

Isolation of Intact Follicles

Fully grown, intact follicles were manually removed from ovarian tissues, isolated from the sacrificed animals, under magnification, using watchmaker's forceps, and placed in freshly prepared De Boar's medium (110 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.4) (Yamasaki *et al.*, 1988) with 20 mg penicillin G potassium and 20 mg streptomycin sulfate per liter.

***In Vitro* Culture of Follicles**

All incubations were carried out in De Boar's medium. Incubations were carried out in multiple-well dishes (12 wells/dish, Costar 3524; USA). Routinely, 20 follicles were incubated per well in 2 ml of De Boar's medium at 20±1°C for 12 ~ 15 hr. Each treatment was performed in duplicate, unless otherwise noted. After the follicles had been heat-fixed by boiling, breakdown of the germinal vesicle (GVBD) as an indication of oocyte maturation was assessed as following; follicles were checked carefully for damage and cytolysed oocytes were removed and individual oocytes were ruptured under a dissecting microscope to check for the presence of the germinal vesicle.

Hormones and Test Reagents

Progesterone was dissolved in ethanol at 40 mM as a stock solution, and 5 or 10 µl was

added to the test wells after dilution. DFP was diluted with propyleneglycol and 5 µl was added to the test wells. Cycloheximide was dissolved in De Boar's medium and 5 µl was added to test wells. If necessary, after designated culture periods, the incubation medium was removed by suction and 2 ml of agent-free fresh medium was added for a 5-min period. The follicles were washed twice more in the same manner. After 12 ~ 15-hr additional incubation, the follicles were fixed and checked for GVBD.

Cytosol Preparation

Intact follicles were allowed to settle in a 4-ml centrifuge tube, the excess media being removed. The cytosol was obtained as the clear supernatant fraction between the lipid cap and packed components obtained on one-step ultracentrifugation for 1 hr at 35,000 rpm (105,000xg) and 4°C (50.3 Ti rotor; Beckman Co.). The cytosol (≈ 8 mg protein/ml) was then carefully withdrawn for the enzyme assay using a Pasteur pipet.

Enzyme Assay

The substrate solution (10 µl of substrate dissolved in dimethyl sulfoxide (DMSO) with 20 µl of 0.5 M Tris-HCl, pH 8.5, and 60 µl of distilled water or different concentrations of SDS) was first preincubated for 10 min at 37°C, and then an aliquot (10 µl) of the enzyme solution was added, followed by incubation at 37°C in a total volume of 100 µl. After 10 min, the reaction was stopped by adding 100 µl of 10% SDS and 2 ml of 0.1 M Tris-HCl (pH 9.0), the enzyme activity then being determined by measuring the 7-amino-4-methylcoumarin (AMC) fluorescence (excitation: 360nm, emission: 460 nm) with a fluorescence spectrophotometer (F-3000; Hitachi Co.). One unit of enzyme activity is defined as that releasing n mole of AMC min^{-1} ml^{-1} under the conditions specified.

Immunological Analysis

The enzyme solution (300 µl) was incubated at 37°C for 1 hr with 100 µl of non-immune IgG/anti-proteasome IgG (purified from serum on a protein A-Sepharose CL-4B column with Buffer A, *i.e.*, 25 mM Tris-HCl-20% glycerol-10 mM 2-mercaptoethanol, pH 7.5). Protein A-Sepharose CL-4B (60 µl) suspended in Buffer A was then added, followed by 30-

min incubation at 37°C with the antigen-antibody solution. The supernatant was used for the enzyme assay after centrifugation for 5 min at 10,000 rpm.

Western Blotting Analysis

Polyacrylamide gel electrophoresis under non-denaturing conditions using the method of Laemmli (1970) was accomplished with 5% (wt/vol) acrylamide gels, and proteins were transferred to nitrocellulose membrane sheets (Immobilon from Millipore, USA). The membranes were blocked with 3% (w/v) gelatin in 500 mM NaCl-20 mM Tris-HCl (pH 7.4) at 37°C and then incubated with anti-proteasome IgG for 1 hr at room temperature. The membranes were then washed and antigen-antibody complexes were visualized with the Vectastain ABC-alkaline phosphatase kit.

Protein Content

The protein content was estimated according to Bradford's method (1976) with bovine serum albumin as a standard.

RESULTS

Inhibition of Progesterone-Induced GVBD by DFP

After intact follicles had been initially incubated for specified periods (0.5, 1 or 2 hr) with two different doses of DFP, different doses of progesterone were added to the culture medium and GVBD was assessed (Fig. 1). With the concentration of 1.36 mM DFP for a 2-hr period, GVBD induced by progesterone (1 μM) was significantly prevented. Whereas GVBD in the presence of 1 μM progesterone was completely inhibited with 2.72 mM DFP for a 0.5-hr period, 70% inhibition of GVBD occurring when the concentration of progesterone was increased to 10 μM. These results suggest that DFP-sensitive proteases are present in premature oocytes and involved in GVBD induced by progesterone. In addition, some variations were found to occur in groups of oocytes derived from different females.

(Fig. 1)

To determine whether or not DFP-sensitive proteases act in any step before GVBD occurs, DFP was administered every 1 hr before and after progesterone addition, and GVBD was assessed following the additive culture (Fig.2). The inhibitory periods for GVBD with DFP were found to be until about 0.52 $GVBD_{50}$ when 50% GVBD was taken as 1.0. On the other hand, it is known that GVBD is either prevented or occurs, depending upon the time of cycloheximide, an inhibitor of protein synthesis, addition to progesterone-treated oocytes (Ishikawa *et al.*, 1989). This inhibitory periods were found to be until about 0.69 $GVBD_{50}$ which was later than that with DFP. Thus, the results suggest that DFP-sensitive proteases may involve the preceding step of cycloheximide sensitivity.

(Fig. 2)

In addition, DFP was pulse-administered every 1 hr before and after progesterone addition, and GVBD was assessed following the additive culture (Fig. 3). The most inhibitory periods for GVBD with DFP were found to be 0 ~ 0.1, 0.2 ~ 0.3 and 0.35 ~ 0.45 $GVBD_{50}$, respectively, after progesterone addition. These results indicate that DFP-sensitive proteases in premature oocytes may act primarily in the period (on average, 0 ~ 0.5 $GVBD_{50}$, in spite of the difference between animals) prior to the protein synthesis period before GVBD occurs.

(Fig. 3)

Enzyme Activity in the Cytosol

To analyze the nature of possible DFP-sensitive proteases in premature oocytes, some typical peptide substrate hydrolyzing activities of proteasomes were measured with different concentrations of SDS, an activator of latent 20S proteasomes (Orlowski, 1990; Goldberg, 1992), utilizing cytosol obtained through one-step ultracentrifugation (Fig. 4).

(Fig. 4)

It was found that cytosol from premature oocytes showed significant LLVY-hydrolyzing activity even in the absence of SDS. The activity was profoundly reduced in the presence of 0.02 ~ 0.07% SDS. However, 74% of the activity appeared again as the

SDS concentration was further increased, the maximum being reached at about 0.10% SDS, but it decreased with a further increase in the SDS concentration. It should be noted that the activity without SDS was at least 1.2-fold that at 0.10% SDS. LLVY-hydrolyzing activities with and without SDS were inhibited to 60 % and 80 %, respectively, by the continuous presence of 1.36 mM DFP. When the oocytes were treated with 1.36 mM DFP for 1 hr before progesterone addition (as shown in Fig. 1 or 3), LLVY-hydrolyzing activities with and without SDS were inhibited to 50 % (data not shown). In addition, LRR and QRR, as peptide substrates containing Arg, were hydrolyzed without SDS. The activity was reduced with 0.04 ~ 0.05% SDS. When SDS was gradually increased, the maximum activity was observed at a SDS concentration of 0.06 ~ 0.07%, but the activity with SDS recovered to 12 to 31% without SDS. On the other hand, cytosol from mature oocytes showed the same LLVY-hydrolyzing activity profile, but the optimal SDS concentration was lower, being 0.09 ~ 0.10%. The results presented indicate that the enzyme may be present in probable DFP-sensitive proteasomes, which do not require SDS and which has two different catalytic (chymotryptic and tryptic) sites.

To confirm that cytosol contains proteasomes, we used rabbit-antibodies against proteasomes purified from ovaries of *Xenopus laevis*, which were characterized previously by Tanaka *et al.* (1988), and estimated the residual enzyme activity through the addition of antibodies, with the use of five fluorogenic synthetic substrates (Table 1). Immunotitration analysis revealed that 78% and 98% of the enzyme activity against LLVY without and with SDS, respectively, found in the cytosol may be due to an enzyme identical or similar to proteasomes from ovaries of *Xenopus*, despite the difference in species. Sixty percent of the enzyme activities against QRR and LRR without SDS may be proteasome activity. However, the enzyme activity against FSR or AAPF were less or not found. These results show that as long as the specified substrates, *e.g.*, LLVY, QRR and LRR, are concerned (as shown in Fig. 4), the enzyme in the cytosol has two different catalytic (chymotryptic and tryptic) sites, as expected if it is identical or very similar to proteasomes.

(Table 1)

Enzyme Activity Fluctuates During the Oocyte Maturation Process

In order to clarify the physiological role of proteasomes, as Fig. 5 illustrates, the time course changes in enzyme activities toward two substrates (LLVY and LRR) due to proteasomes in the oocyte maturation process in the cytosol from oocytes treated with progesterone only (GVBD after 24-hr culture: 100%) were compared with those in the case of follicles treated with ethanol only (GVBD after 24-hr culture: 0%) or progesterone- and DFP-treatment (GVBD after 24-hr culture: 0%).

(Fig. 5)

Fluctuation of the LLVY-hydrolyzing activity of proteasomes, which do not require SDS, was observed for the experimental group treated with progesterone only, showing increases at 0.3 and 0.9 ~ 1.5 $GVBD_{50}$. In addition, the time course changes of activity with SDS were similar to that without SDS, except for the increased activity at 0.3 $GVBD_{50}$, but the activities in the DFP-treated group were lower than those in the control group. Fluctuation of the LRR-hydrolyzing activity without SDS was observed for the experimental group treated with progesterone only, showing increases at 0.3 and 1.2 ~ 1.5 $GVBD_{50}$. This profile was similar to that of the LLVY-hydrolyzing activity, which does not require SDS, but the activity in the DFP-treated group was not different from that in the control group.

These results show that significant LLVY- and LRR- hydrolyzing activities, which do not require SDS, were seen at 0.3 $GVBD_{50}$ after progesterone addition, but the LLVY-hydrolyzing activity, which requires SDS, was not significant. When GVBD began, all activities increased significantly. The period of the transient increase in enzyme activity before initiation of GVBD may correspond to the inhibitory period (0 ~ 0.5 $GVBD_{50}$) for GVBD on pulse application of DFP, as shown in Fig. 3. This suggestion was confirmed when the changes of enzyme activities in the cytosol, along with the inhibitory effect of pulse exposure to DFP on GVBD, were analyzed in the same animal (Fig. 6).

(Fig. 6)

On the other hand, in order to determine the change in the molecular form of proteasomes during oocyte maturation, the cytosol was electrophoresed on a polyacrylamide gel under non-denaturing conditions and the bands were detected by Western blotting analysis using anti-proteasome antibodies (Fig. 7). When treated with progesterone, the bands migrated more or less at 0.6 $GVBD_{50}$ and slightly at 1.2 $GVBD_{50}$ to the anode, whereas the bands in the case of the control cytosol remained invariant during the 24-hr culture. This observation also provides key support for that proteasome activity may fluctuate during oocyte maturation.

(Fig. 7)

DISCUSSION

The present study has for the first time demonstrated that during oocyte maturation induced by progesterone in *Bufo japonicus*, the apparent peptide hydrolyzing activity in the absence of SDS, an activator of latent 20S proteasomes, of multicatalytic protease complexes (Orlowski, 1990; Azuma *et al.*, 1991; Goldberg, 1992) found in the cytosol from premature oocytes increases in two different steps: preceding and after completion of GVBD (0.3 and 0.9 – 1.5 $GVBD_{50}$ when 50%GVBD was taken as 1.0) (Figs. 5 and 6). Furthermore, the increase in LLVY-hydrolyzing activity in the presence of SDS was not observed before the beginning of GVBD, but only in the step following completion of GVBD (Fig. 5). These enzymes, found through the use of oocytes enclosed in follicle cells, seem to be derived not from the follicle cells but from the oocytes, and are clearly both chymotrypsin- and trypsin-like proteases, as expected (Figs. 4-6 and Table 1). In addition, the enzyme activity did not increase in oocytes treated with progesterone following DFP-treatment or control oocytes during the culture (Figs. 5 and 6). Therefore, it is suggested that DFP-sensitive proteases may be involved in oocyte maturation. One of these results shows that when the GVBD assay was indicated DFP inhibited at 0 – 0.5 $GVBD_{50}$ before the beginning of GVBD following progesterone addition, corresponding to the increasing period of LLVY-hydrolyzing activity due to proteasomes, which do not require SDS (Figs.

1-3, 5 and 6).

Here, microinjection of MPF from mature oocytes of *Xenopus laevis* into DFP-treated *Bufo* oocytes led to GVBD (data not shown). Thus, we consider that the protease highly susceptible to DFP must participate somewhere between progesterone binding and MPF formation/activation. This is consistent with the previous report that microbial protease inhibitors (leupeptin, antipain and chymostatin) prevented *Xenopus laevis* oocyte maturation induced by progesterone, but did not inhibit the maturation induced by MPF injection (Guerrier *et al.*, 1977). In addition, it was reported by us that the probable involvement of TPCK (a known inhibitor of chymotrypsin)-sensitive proteasomes before initiation of GVBD was also observed in a system of oocytes from *Rana pipiens* (Azuma *et al.*, 1991).

It is highly likely that the alterations in the enzyme activity during oocyte maturation may be due to one of the net charge of proteasomes existing *in vivo* through the electrophoretic pattern (Figs. 5-7). Post-translational modification, such as protein phosphorylation, has been suggested to be responsible for the developmental-regulational alterations in their subunit patterns of proteasomes from *Drosophila* (Haass and Kloetzel, 1989) and chick (Ahn *et al*, 1991). Any modification that brings the changes in the electrophoretic pattern should alter the position of the bands of the proteasomes (Fig. 7). Therefore, the changes in the subunit pattern may be attributed to the maturation-specific alterations in the expression of the proteasomes in oocytes.

In this study it is shown that the oocyte cytosol contained a form of proteasomes that can hydrolyze LLVY in the absence of SDS (Fig. 4). The similar observation is indicated in the cytosol from *Xenopus laevis* ovaries (Tokumoto and Ishikawa, 1993). We proposed to call it the active form of proteasomes. The above results also suggest that low concentrations of SDS converted the active form into a known 20S latent form, which has been shown to require a certain concentration of SDS (Orlowski, 1990; Azuma *et al.*, 1991; Goldberg, 1992). The latent form of proteasomes may be the core of the active form of proteasomes, since the LLVY-hydrolyzing activity with or without SDS was precipitated with anti-

proteasome IgG of *Xenopus laevis* (Table 1).

Recently, it was reported that ATP-dependent 26S multicatalytic protease complexes were found in the physiological state in the presence of ATP in mammalian cells and converted to 20S proteasomes through the release of regulatory components from them in the absence of ATP (Armon *et al.*. 1990; Driscoll and Goldberg, 1990; Orino *et al.*, 1991; Tsukahara *et al.*, 1991). If the active form of proteasomes is identical with these complexes, then the possibility arises that the former plays a critical role in the oocyte maturation process. These recent reports are very helpful for explanation of our data. However, at present, we have no data indicating that the active form may regulate ATP-dependent activity.

Proteasomes existing in premature oocytes from *Bufo* were inhibited by DFP, but the general inhibitory effect on proteasomes of DFP may be dependent on the substrates used and the source of proteasomes (Orlowski, 1990). Thus, DFP inhibited a chymotrypsin-like enzyme alone in one proteasome specimen, but chymotrypsin, trypsin and peptidylglutamyl-peptide hydrolyzing activities in another specimen. In addition, proteasomes contain a serine-like protease, such as chymotrypsin and trypsin, but it is not a typical serine protease. The amino acid sequence of some subunit component of proteasomes from rat shows no homology with the sequence of a known protease (Orlowski, 1990; Goldberg, 1992).

On the other hand, the increases in LLVY- and LRR-hydrolyzing activities due to proteasomes in the periods (0.3 and 1.0 $GVBD_{50}$) during oocyte maturation (Figs. 5 and 6) may correspond to the periods when the degradations of Mos and cyclin proteins are triggered by ubiquitin-dependent proteolysis (Gloetzer *et al.*, 1991; Nishizawa *et al.*, 1992). Thus, a ubiquitinated conjugated protein may be degraded by ATP-dependent 26S protease complexes. Afterwards, endogenous protein substrates should be clarified.

According to the above observations, during oocyte maturation in *Bufo*, DFP-sensitive proteolysis may be related to the activation and inactivation, through a dissociation-association cycle, of the 26S protease complexes or the active form of proteasomes. These results prompt us to consider that oocyte maturation is a good model with which to study the physiological role of proteasomes. In addition, the cytosol exhibits FSR- and AAPF-

hydrolyzing activities, but these activities may not be due to proteasomes (Table 1). When the role of proteolysis in oocyte maturation is discussed, attention should be paid to some proteases other than proteasomes.

Acknowledgements

We wish to thank Dr. M. Yamashita of National Institute for Basic Biology for the excellent experimental help in microinjection. We also wish to thank Dr. K. Tanaka of Tokushima University for the gift of anti-proteasome antibodies. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (to K.I.), and from the Saito-Chion Foundation, Shizuoka (to K.I.).

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Table 1

Enzyme activities toward several peptide substrates after immunodepletion of proteasomes in the cytosol.

Substrate	SDS	Activity(U/ml)		Inhibition (%)
		control-IgG	anti-IgG	
LLVY	-	4758	1068	77.5
	+	9969	162	98.4
QRR	-	1446	558	61.5
LRR	-	3004	1188	60.5
FSR	-	3506	2873	18.1
AAPF	-	3289	3329	0.0

Aliquots of cytosol from intact follicles were incubated for 1 hr at 37℃ with rabbit anti-proteasome IgG (anti-IgG: finally, 2 mg/ml) or non-immune IgG (control-IgG: finally, 2 mg/ml). Immunoprecipitates obtained with protein A-Sepharose CL-4B were removed by centrifugation, and then the residual substrate (10mM)-hydrolyzing activities in the supernatant were assayed, against several substrates, with or without 0.10% SDS, as described under "MATERIALS AND METHODS". Values are expressed as U(unit) of remaining activity as to p mole of AMC min^{-1}.

Figure legends

Fig. 1. Effect of DFP on progesterone-induced GVBD. Intact follicles were preincubated at 20°C in 2 ml medium containing different concentrations of DFP {0 (propyleneglycol) (○), 1.36 (●) and 2.72 mM (△)} (20 follicles/well; duplicate wells per treatment). After various periods {0.5 (A), 1 (B) or 2 hr (C)} of culture at 20°C, different doses of ethanol or progesterone were added. GVBD in all oocytes was assessed after 15-hr culture.

Fig. 2. Comparison of effects of DFP and cycloheximide on progesterone-induced GVBD. Different groups of intact follicles from the same animal were incubated with 1 μM progesterone at 20°C (20 follicles/well; duplicate wells per treatment). One group was fixed at regular intervals in order to establish the occurrence and timing of GVBD (---). Separate groups were treated with DFP (finally, 1.36 mM) (●) or cycloheximide (finally, 28.4 μM) (○) added at designated times relative to time zero (hormone addition). Such DFP- or cycloheximide-treated follicles were cultured for a further 15 hr after time zero and then examined for GVBD. In addition, the period from time zero to 50% GVBD has been normalized to a scale of 0 ~ 1.0 $GVBD_{50}$ to facilitate comparison with other data.

Fig. 3. Effect of pulse application of DFP on progesterone-induced GVBD. Different groups of intact follicles from three animals (A ~ C) were incubated with 1 μM progesterone at 20°C (20 follicles/well; duplicate wells per treatment). After 1 hr, the follicles were washed with 2 ml medium as described under "MATERIALS AND METHODS". One group was then fixed at regular intervals in order to establish the occurrence and timing of GVBD (---). In separate groups, DFP (finally, 1.36 mM) was added at 1 hr intervals at designated times relative to time zero (hormone addition), and after a further 1 hr the follicles were washed as described above. All such DFP-treated follicles were cultured for a further 14 hr after time zero and then examined for GVBD.

The effects of the timing of DFP addition on GVBD are shown as bars (⊢⊣) at the times of DFP addition. In addition, the period after hormone addition has been normalized (as described in Fig. 2) (a ~ c).

Fig. 4. Effect of SDS on enzyme activities in the cytosol. Peptide substrate hydrolyzing activities in the cytosol were measured with various concentrations of SDS after 10 min at 37°C, as described under "MATERIALS AND METHODS". The enzyme sources were as follows: ethanol-treated follicles (GVBD after 12-hr culture: 0%): LLVY (–●–), LRR (–○–) and QRR (–△–); progesterone (1 μM)-treated follicles (GVBD after 12-hr culture: 100%): LLVY (--○-); progesterone (1 μM)-and DFP (1.36 mM)-treated follicles (GVBD after 12-hr culture: 5%): LLVY (–▲–).

Fig. 5. Enzyme activities in the cytosol during oocyte maturation induced by progesterone. Intact follicles from the same animal were randomly distributed into culture wells containing different media (2 ml) (20 follicles/well). Three different groups of follicles were cultured with progesterone (1 μM) (without,–●–, or with,–△–, 1.36 mM DFP) or ethanol (--○-). GVBD at each point after hormone treatment was assessed using 40 oocytes (- - -). Cytosol specimens were collected from 440 follicles at every 2 hr after hormone addition, as described under "MATERIALS AND METHODS". Aliquots of each cytosol specimen were assayed for enzyme activities using various peptide substrates: A, LLVY (without SDS); B, LLVY (with 0.10% SDS); C, LRR (without SDS). In addition, the period after hormone addition has been normalized on the upper line.

Fig. 6. Enzyme activities in the cytosol and inhibitory effect of pulse exposure to DFP during oocyte maturation induced by progesterone. Intact follicles from the same animal were randomly distributed into culture wells (20 follicles/well) containing different media

(2 ml). A group of progesterone (1 μM)-treated follicles was fixed at regular intervals and GVBD was assayed until 10 hr. In separate groups of progesterone-treated follicles, DFP (finally, 1.36 mM) was added as described in Fig. 2. After 1-hr exposure, all such DFP-treated follicles were cultured for a further 12 hr after time zero, and examined for GVBD as shown in Fig. 2. On the other hand, cytosol specimens were collected from 440 follicles at the designated times and assayed for enzyme activities without SDS against two substrates (LLVY, ● ; LRR, ○). In addition, the period after hormone addition has been normalized on the upper line.

Fig. 7. Western blotting analysis of proteasomes during oocyte maturation induced by progesterone. At various times after the addition of progesterone (1 μM) (●) or ethanol (○), GVBD was assessed (A). The period after hormone addition has been normalized on the upper line. Aliquots of the cytosol collected from the follicles during oocyte maturation (as shown in Figs. 5 and 6) were electrophoresed under non-denaturing conditions in 5% polyacrylamide gel and Western blotted with anti-proteasome IgG as described under "MATERIALS AND METHODS"(B). The eight lanes (from left to right) correspond to the following times: 0, 2, 4, 6, 8, 10 and 12 hr (0, 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 $GVBD_{50}$, respectively), and 24 hr following progesterone (1) or ethanol (2) treatment. Only the region of the blot corresponding to typical proteasomes is shown.

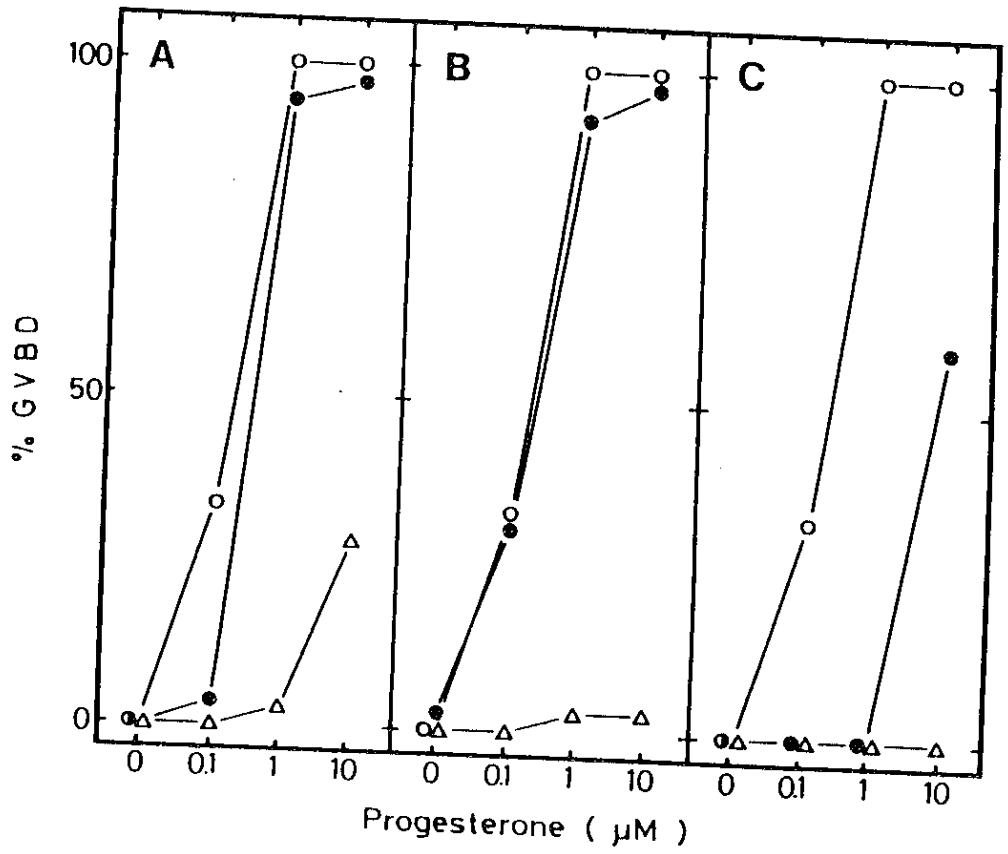
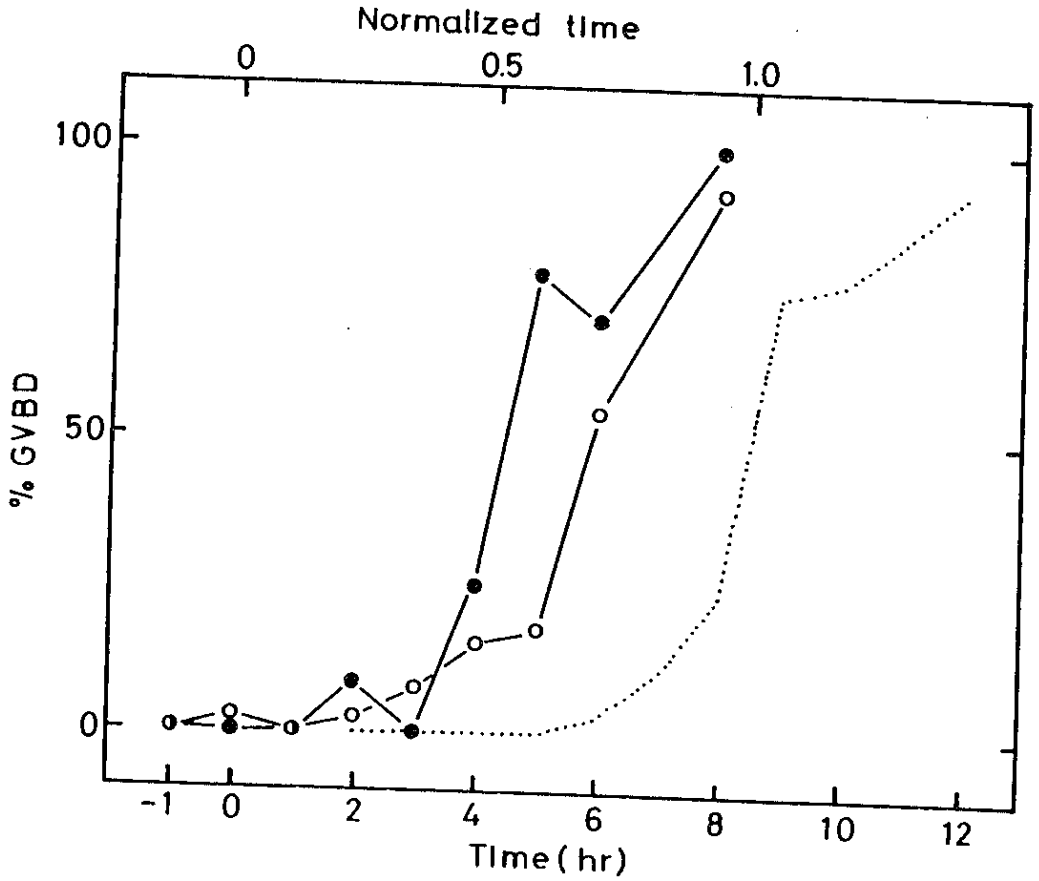


Fig. 1 Takahashi et al.



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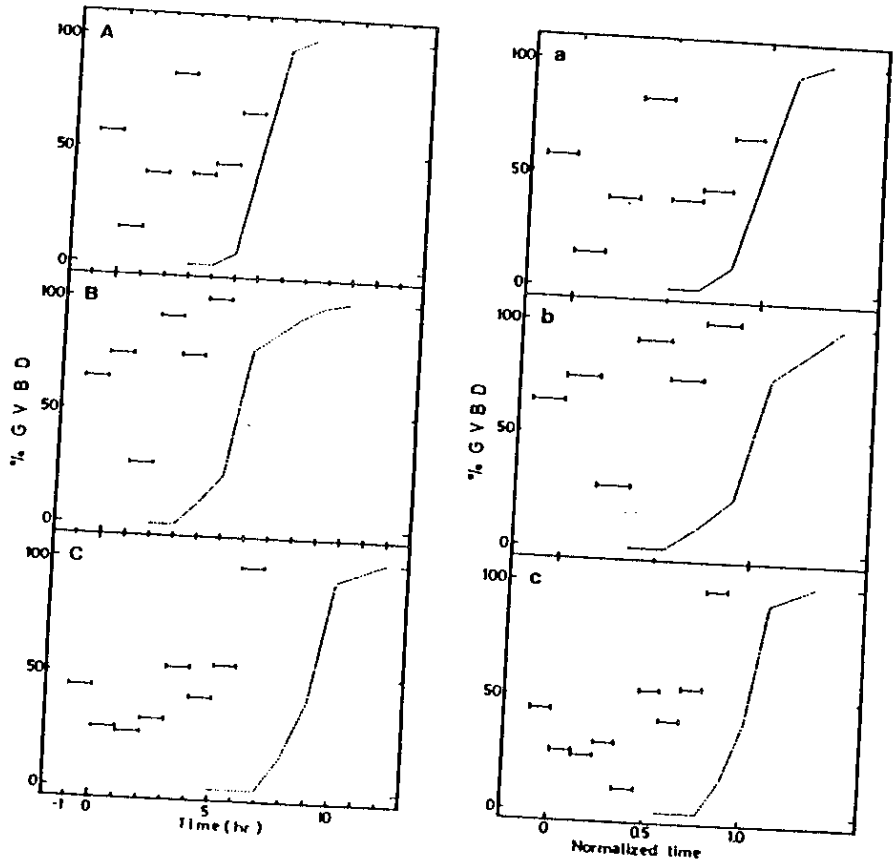

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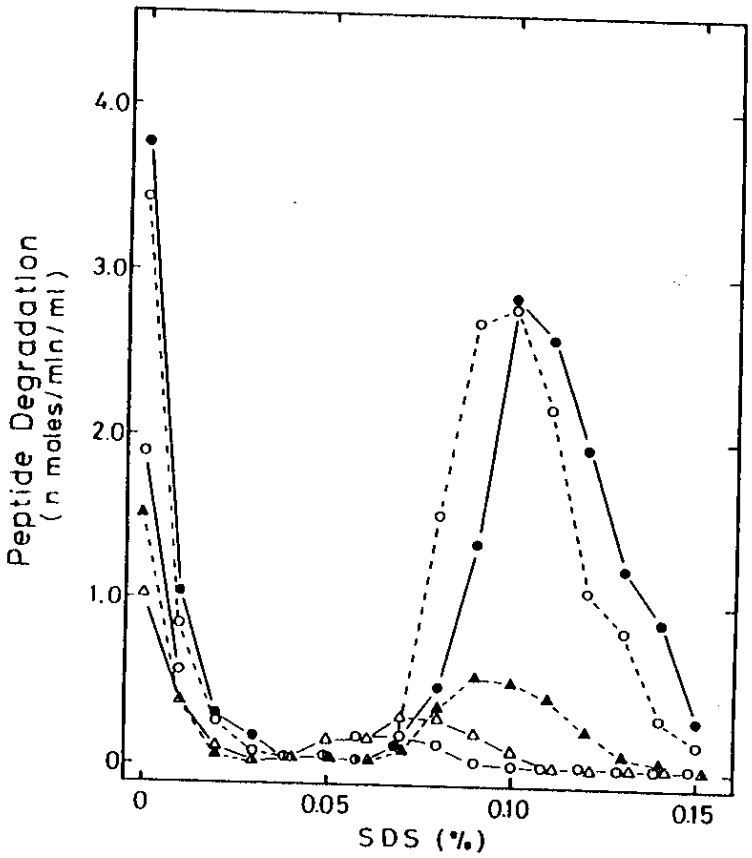

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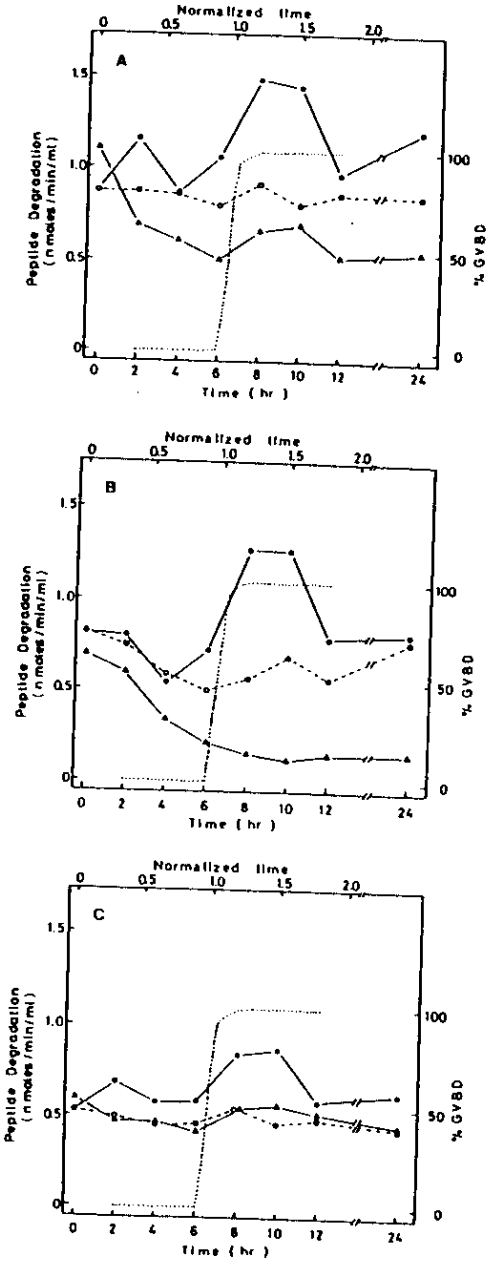

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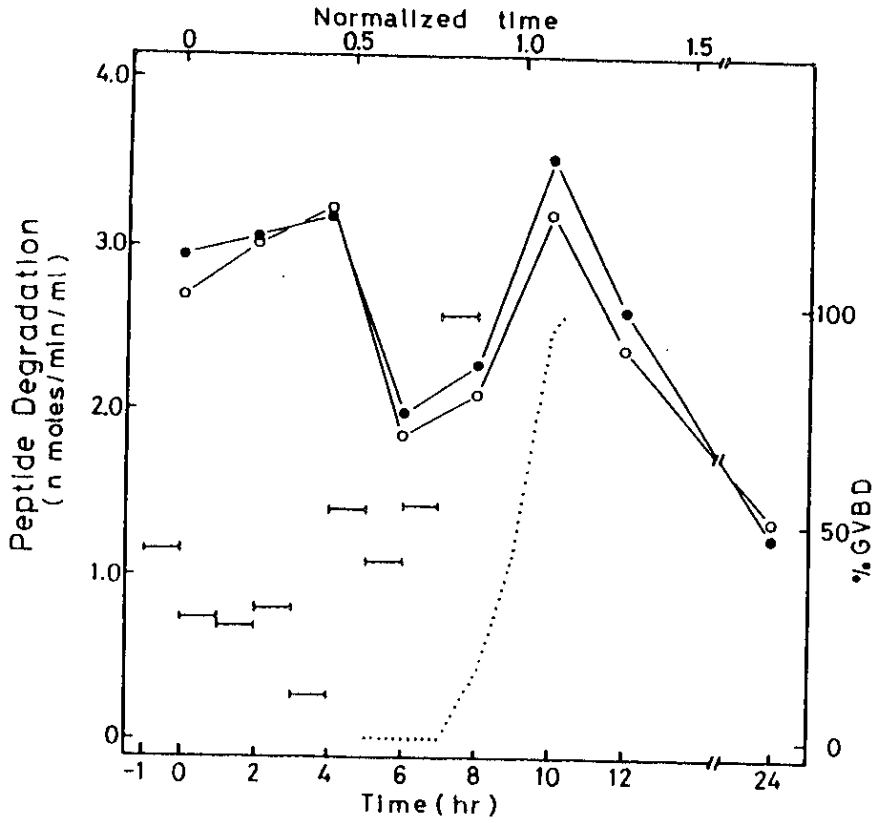

Fig. 6 Takahashi et al.

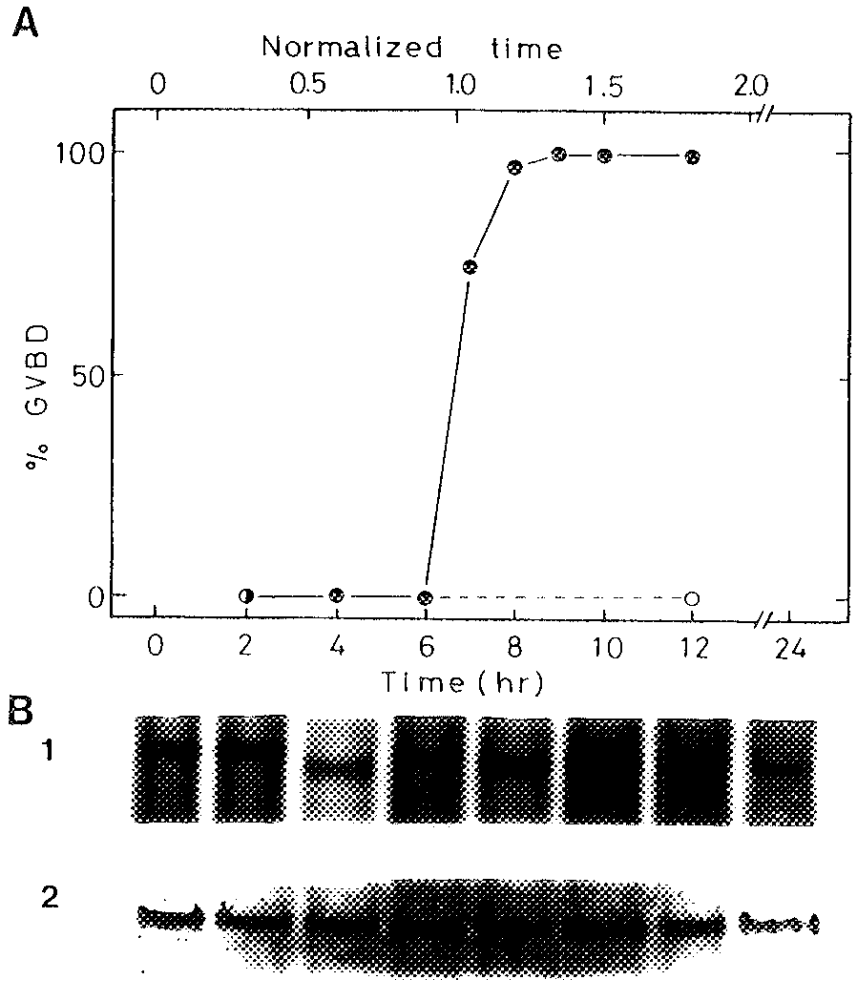

Fig. 7 Takahashi et al.

Publication

実験医学, 1993, 11, 73-79.

高橋美佳、徳元俊伸、山下正兼、長濱嘉孝： 魚卵 — 卵成熟の実験モデル.

実験医学 増刊
Vol.11 No.12 1993
EXPERIMENTAL MEDICINE

別　　刷

株式会社 羊土社

〒101　東京都千代田区神田多町2－4
第2滝ビル　TEL03(3295)4550(代)

魚卵-卵成熟の実験モデル

高橋美佳　徳元俊伸　山下正兼　長濱嘉孝

卵成熟の研究にはこれまで，哺乳類，カエル，魚類，ヒトデなどの卵がよく使われてきた．なかでも魚卵では，卵成熟を制御する3種の要因（生殖腺刺激ホルモン，卵成熟誘起ホルモン，卵成熟促進因子）のすべてが化学的に同定され，卵成熟制御のカスケードが内分泌学と発生生物学の両面からバランスよく研究されている．

はじめに

卵成熟とは，卵が発生能を獲得し精子を受け入れるようになることであり，有性生殖を営む生物種の存続には必須な過程である．卵や精子は生殖細胞と呼ばれ，生物個体を構成する多くの体細胞とはいろいろな点で異なる．生殖細胞の特徴の1つは，卵や精子が形成される過程で染色体数が半減することであり，これを保障する仕組みが減数分裂（meiosis）と呼ばれる生殖細胞のみに見られる独特な細胞分裂様式である[1]．卵巣内で卵は第一減数分裂を開始してすぐその分裂を前期の状態で停止する．多くの動物卵ではこの時期に多量の卵黄を蓄積するので，この時期は成長期とも呼ばれる．卵成熟の過程は，第一減数分裂の前期の状態で停止していた卵がホルモン刺激により分裂を再開して，第一減数分裂を終え，さらに第二減数分裂の中期まで達することである．従って，卵成熟はヒトデでは1時間以内，脊椎動物でも長くて1～2日以内に完了する短期間内に起こる現象であるが，前述したごとく卵の染色体が半減するという生物種の存続を保障する重要な過程なのである．

これまで卵成熟の仕組みは，哺乳類，カエル，魚類，ヒトデなどを実験材料として活発になされてきた．現在では，これらの動物卵の中でも魚類卵を用いた研究が，卵成熟の仕組みを総合的に理解するという観点からみると最も進んでいるといえる．ではなぜ，魚卵を材料とした卵成熟の研究がそのような進展を遂げることができたのか．その答えは次に述べるように魚卵は卵成熟研究のための実験材料として哺乳類やカエル卵にはない，いくつかの利点があったからにほかならない．動物卵の卵成熟研究が活発になされるようになったのは1960年代からであり，その引き金となったのはカエルやヒトデ卵で，*in vitro* でホルモンを添加することにより卵成熟を誘起させる実験系が確立されたことである．魚類卵でこのような研究は，メダカ（*Oryzias latipes*）やインド産ナマズ（*Heteropneustes fossilis*）卵を用いて1970年代に入ってからなされた．その後，サケ科魚類やキンギョ，その他の魚卵を用いた研究が次々になされた．

生殖生物学の研究で魚類を用いることの利点の1つは，この仲間にはいろいろな生殖周期・様式を示すものがみられるということである[2]．もちろん魚類では卵生が主流で，これらの種は水中で体外受精を行う．

[キーワード＆略語]
魚類，卵成熟，脳下垂体，濾胞細胞，卵母細胞
GTH：gonadotropin（生殖腺刺激ホルモン）
MIH：maturation-inducing hormone（卵成熟誘起ホルモン）
MPF：maturation-promoting factor（卵成熟促進因子）

The fish oocyte-An experimental model for the study of oocyte maturation

Mika Takahashi/Toshinobu Tokumoto/Masakane Yamashita/Yoshitaka Nagahama, 岡崎国立共同研究機構基礎生物学研究所生殖研究部門

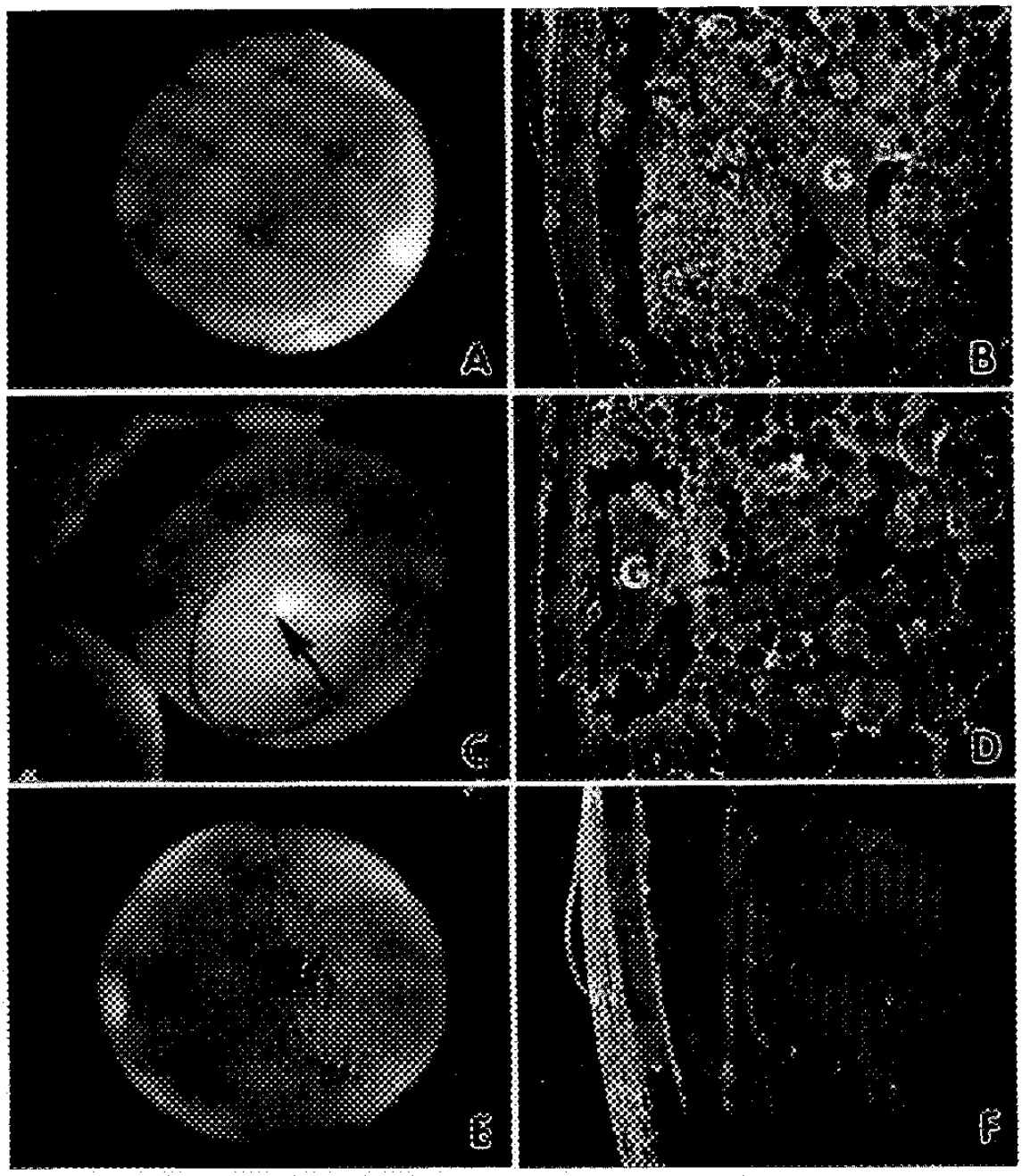

図1　ニジマスの卵成熟過程
卵成熟開始前には，卵核胞（G）は動物極から離れて位置するため実体顕微鏡下では観察できない（A，B）．しかし，ホルモン処理により卵核胞が動物極に移動すると実体顕微鏡でも容易に観察できるようになり（C, D），さらに卵成熟が進むと卵核胞が崩壊する（E, F）．B, D, F は走査顕微鏡写真

なかにはグッピー（*Poecilia reticulata*）などのように哺乳類とよく似た胎生を示すものもみられるし，またその中間ともいえる卵胎生と呼ばれる種も少なくない．卵成熟との関連では，サケ科魚類のように数年の生活史の終わりに一度しか卵成熟・産卵しないものもあるし，またメダカのように光周期や水温を調節することにより毎日決まった時刻に卵成熟・産卵を繰り返す種もある．卵成熟研究の実験材料としての前者の利点は，一生に一度しか産卵しないことから生殖腺の中の生殖細胞の発達や成熟の過程がほぼ完全に同調していることである．このことは複雑なホルモン現象を個体レベルで解明するためにはきわめて大切なことであるばかりでなく，実験に必要な同調卵を一度に多量に得ることができるという細胞・分子レベルの研究にとっても欠かすことができない大きな利点である．また，このサケ科魚の卵は大型であるため，いろいろな

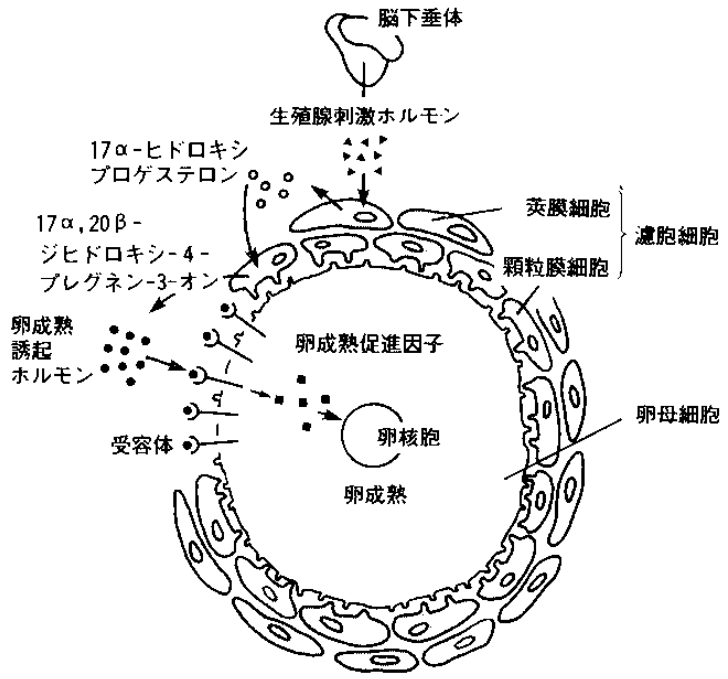

図2　サケ科魚類の卵成熟制御機構

外科手術が実体顕微鏡のもとで可能である．この点については後で触れる．一方，メダカでは飼育環境条件を調節することにより，卵黄形成から卵成熟に至る過程を2～3日間で完了させることができるので，卵の経時的な変動を調べる目的には貴重な材料となる[3]．最近のいろいろな生化学的・分子生物学的手法の急速な進展により，約1 mmの直径をもつメダカ卵を一個ずつ解析することが可能になりつつあり，毎日数十個の卵を産むメダカを実験材料にしての生化学・分子生物学的アプローチが十分に可能となってきた．さらに，キンギョも卵成熟研究のとても優れた実験材料である．この魚は春に産卵するが，12～14℃の低温で飼育すると卵黄形成はゆっくりと進行するものの，卵成熟は起こらない．この方法を利用すれば，卵成熟実験を少なくとも6～7カ月は継続することができる[4]．*in vitro*の実験系が確立されたことが卵成熟研究の進展を促進したことは述べたが，これには正確で簡便な生物検定法も必要である．多くの魚類の未成熟卵は不透明であるが，成熟すると透明または半透明となるので成熟の有無を比較的簡単に判別できる．また，卵成熟の過程で卵核胞（germinal vesicle：GV）は動物極に移行した後に崩壊（GV break down）する．この動物極付近にあるGVは実体顕微鏡下で観察することができる（図1）．多くの海産魚の卵は成熟後に完全に透明化するものもある．

以下に述べるように，サケ科魚類，キンギョ，メダカなどを用いて得られた卵成熟に関する知見が，ほかの魚類にもあてはまるばかりでなく，ほかの脊椎動物や無脊椎動物とも驚くほどよく似ている[5]．実験動物モデルとしてそこから得られる結果がいろいろな生物に普遍的であることは，理想的なことである．本稿では，いろいろの魚類を用いた研究を概説しながら，ほかの動物における知見とも比較し，卵成熟機構の普遍性についても言及したい．

1．卵の構造

卵成熟の仕組みを説明する前に，卵の構造を述べておく必要がある．われわれが一般に卵（例えばサケの筋子の1つ1つ）と呼んでいるのは，実は単一な構造ではなく，1個の卵（正確には卵母細胞という）を体細胞性の濾胞細胞層が取り囲んでおり，この構造単位を卵胞と呼んでいる（図2）．脊椎動物の卵は例外なく2層の濾胞細胞（外側の莢膜細胞層と内側の顆粒膜細胞層）でおおわれているが，それぞれの層は単層から多層まで脊椎動物で一定していない．魚類では顆粒膜細胞層は一層であるが[6]，哺乳類では多層である．サケ科魚類ではこの卵胞が数百～数千個卵巣に存在するのである．

2．卵成熟の第一次制御因子―生殖腺刺激ホルモン（Gonadotropin：GTH）

卵巣や精巣の活動を制御する最も重要なホルモンは脳下垂体から分泌されるGTHである．脊椎動物のGTHには，濾胞刺激ホルモン（FSH）と黄体形成ホルモン（LH）の2種類があるのが一般的であるが，最近の研究から魚類でもLH様とFSH様の2種類のGTHが存在することが定説となりつつある[7]．

このうちLH様GTHは魚類ではGTH II（maturational GTH）と呼ばれ，卵成熟に重要な働きをするGTHである．一方，魚類のFSH様GTHと考えられているのはGTH Iと呼ばれるもので，これは最近の研究から卵黄形成期や精子形成期に脳下垂体で多く生産され，分泌されることが明らかになり，生殖腺の発達に重要な働きをすると考えられている．

ヒトから魚類まで，おそらくすべての脊椎動物において，卵成熟は上述のLH様GTHが脳下垂体から一

過性に多量分泌されることにより誘起されると推察される．従って，脊椎動物の卵成熟の第一次制御因子はLH様GTHである．しかし，脳下垂体の活動はその上位にある視床下部の働きによって調節されているので，LH様GTHの多量分泌に先だち視床下部からのGTH放出因子などの刺激が必要であることはいうまでもない．魚類では光や温度の日周変動がこのGTH放出因子の働きを介してGTH分泌に強く影響を与える．その顕著な例が，すでに述べたキンギョの低温処理（12～14℃）による卵成熟阻止であり，これは，キンギョではこの水温では脳下垂体からのLH様GTHの多量一過性分泌が起こらないためである．

LH様GTHは確かに卵成熟の第一次制御因子であるが，このホルモンが卵に直接働いて卵成熟を誘起させるのではない．ここでGTH作用を仲介するのはすでに述べた卵を囲む体細胞性の2層の濾胞細胞である．このことは，これらの濾胞細胞層を外科的に除去したいわゆる裸の卵を，GTHとともに試験管内で培養しても卵成熟は起こらないことから証明される．もちろん，同じ条件下で濾胞細胞が付着したままの卵，すなわち卵胞を培養すると卵成熟は起こる．この場合の濾胞細胞の役割はGTHの刺激に反応して，ステロイド性の卵成熟誘起ホルモンを生成することにある[8]（図2）．

3．卵成熟の第二次制御因子—卵成熟誘起ホルモン（maturation-inducing hormone：MIH）

LH様GTHの働きで脊椎動物の卵濾胞細胞で生成されるMIHが最初に単離・同定されたのはサケ科魚のアマゴ（*Oncorhynchus rhodurus*）の17α，20β-ジヒドロキシ-4-プレグネン-3-オン（17α，20β-DP）であり，1985年のことである[9]．その後の研究で17α，20β-DPはサケ科魚類はもちろん，ほかの多くの魚類でもMIHとして作用していることが明らかにされた．また，ある種の海産魚で17α，20β-DPに類似した17α，20β，21-トリヒドロキシ-4-プレグネン-3-オンがMIHと推察されている[10]．現在でも，脊椎動物でMIHが生化学的に同定されたのは魚類のみであるが，カエルでは以前からプロゲステロンがMIHと考えられてきた．棘皮動物のヒトデのMIHは1969年に金谷らにより1-メチルアデニンと同定された[11]．われわれは，鳥類から魚類までの脊椎動物のMIHはおそらくプロゲステロンもしくはその類似ステロイドであろうと推察している．ここでは詳しく述べないが，哺乳類ではMIHの存在を示す強い実験証拠はいまのところなく，むしろLHの刺激後にそれまで卵胞中に存在し，卵成熟の進行を阻止する物質，すなわち卵成熟抑制因子（oocyte maturation inhibitor：OMI）が解除されるために卵成熟が誘起されるという考えが支配的である[5]．

GTHの刺激により卵濾胞細胞でMIHが生成される仕組みが詳しく調べられているのは，サケ科魚類の濾胞細胞での17α，20β-DPについてのみである．前述したように，サケ科魚の卵胞は大型であるため実体顕微鏡下で時計用ピンセットにより莢膜細胞層と顆粒膜細胞層に分離することができる．この利点を生かしてわれわれは，莢膜細胞層と顆粒膜細胞層の単独培養あるいは共培養を行うことにより，GTHによる17α，20β-DPの生成に関して2細胞型モデルをはじめて提唱した[12]（図3）．これによると，莢膜細胞で前駆体の17α-ヒドロキシプロゲステロンが生成され，これが顆粒膜細胞に移行してそこに局在するステロイド20β-水酸基脱水素酵素（20β-HSD）により17α，20β-DPに転換される．これより先われわれは，卵成長（卵黄形成）期の濾胞細胞ではGTHの働きにより，エストラジオール-17βがやはり莢膜細胞と顆粒膜細胞の相互作用（2細胞型モデル）で生成されることを明らかにしている[13]（図3）．すなわち，この場合は莢膜細胞で前駆体のテストステロンが生成され，これが顆粒膜細胞に局在する芳香化酵素によりエストラジオール-17βに転換される．GTHがエストラジオール-17βや17α，20β-DPの生成を促進する際にはいずれも，GTH受容体-G蛋白質-アデニル酸シクラーゼ-cAMPを介して種々のステロイド代謝酵素の活性を調節する[14]．

このように卵成熟期にかけて卵濾胞細胞でのステロイド生合成系がエストラジオール-17βから17α，20β-DPに転換する[14]．われわれはこのステロイド生合成系の転換が，卵を成長期から成熟期に移行させる重要な仕組みであると考え，これを分子・遺伝子レベルで解明したいと考えている．その第一歩としてわれわれはコレステロールからエストラジオール-17βと17α，20β-DPが生合成される際に必要とされる，すべてのステロイド代謝酵素遺伝子をクローニングする

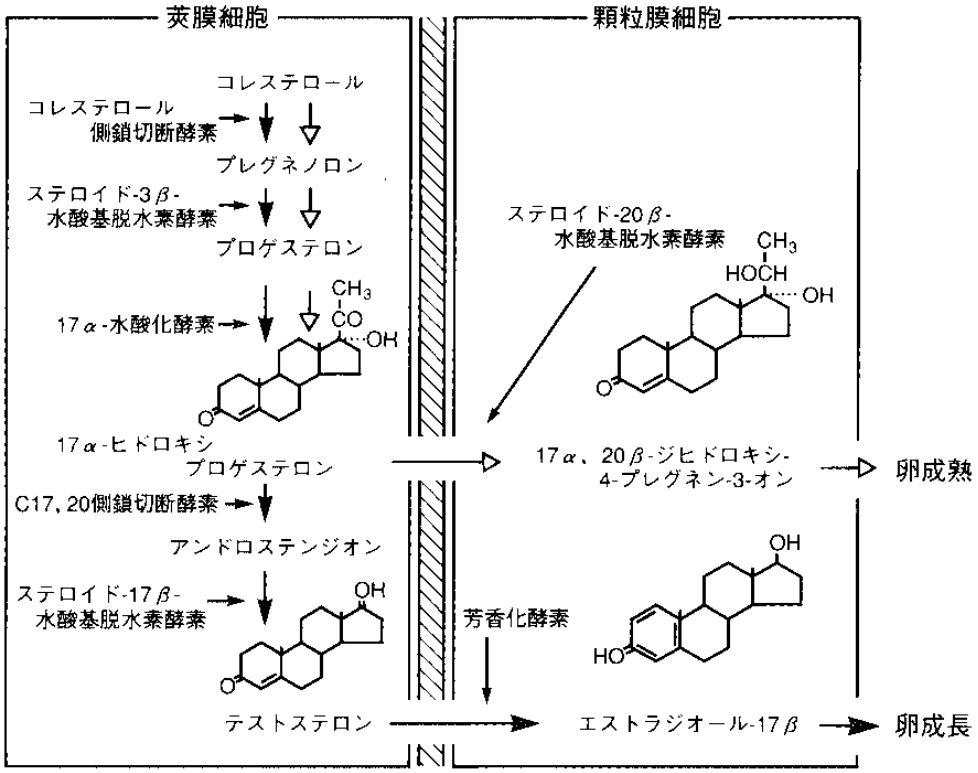

図3　サケ科魚類の卵成長（卵黄形成）期と卵成熟期のステロイドホルモンの生合成

ことを計画し，現在までにそのほとんどをニジマス（*Oncorhynchus mykiss*）とメダカから単離し，その塩基配列を決定した[15-17]．現在，それらをプローブとして卵成長期と成熟期における各種ステロイド代謝酵素のmRNA量の変動を調べている．ここでは紙面に限りがあるので詳しくは述べないが，卵成熟期直前の莢膜細胞と顆粒膜細胞でGTHなどのホルモンによりステロイド代謝酵素遺伝子の発現が抑制，促進されていることが明らかになってきた．特に顆粒膜細胞で，芳香化酵素（テストステロンをエストラジオール-17βに転換させる酵素）遺伝子の発現が急激に抑制されるなどの顕著な変化がみられる．現在，ニジマス卵巣からの20β-HSD遺伝子のクローニングを含めて，おのおののステロイド代謝酵素蛋白質の大量発現およびそれらを用いてのポリ・モノクローナル抗体の作製を行っているので，近い将来にはGTHによる顆粒膜細胞での芳香化酵素遺伝子の抑制および，20β-HSD遺伝子発現の促進などをはじめとしたGTHによる各種ステロイド代謝酵素遺伝子の発現調節機構の全貌が明らかになるものと思われる．

サケ科魚類のMIHである17α，20β-DPはステロイドホルモンである．従って，これまで得られている知見からは，その受容体は細胞質もしくは核に存在すると推察される．しかし，17α，20β-DPを十分成長したキンギョの未成熟卵に微小注射しても被注射卵は成熟しない．ところが，キンギョ未成熟卵は17α，20β-DPを含む培養液中では成熟する．これらのことから，17α，20β-DPは卵の表面で作用すると考えられる．カエルやヒトデの卵でも同じことがみられ，MIHの共通な作用機構である可能性が高い[5,18]．また，魚類の卵細胞膜に17α，20β-DP受容体が局在することが結合実験から明らかにされた[19]．さらに，最近の研究から，この17α，20β-DP受容体はGTHの作用で卵成熟直前に卵細胞膜に出現することがわかった．

4．卵成熟促進因子（maturation-promoting factor）

17α，20β-DPで成熟させたキンギョ卵の細胞質をほかの未成熟卵に注射すると，被注射卵は17α，20β-DPの処理なしに成熟する．このことは17α，20β-DPを処理された卵内に新たな因子が形成され，これが最終的に卵を成熟させることを示す．この成熟

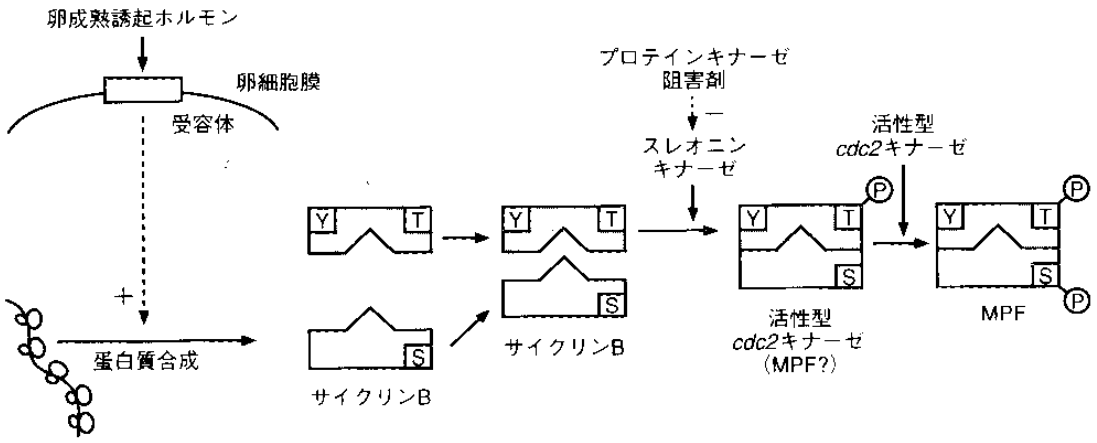

図4　キンギョ卵成熟における MPF の形成と活性化機構のモデル
卵成熟誘起ホルモンの卵表への刺激はサイクリンBの合成を誘起する．合成されたサイクリンBとすでに存在する *cdc2* キナーゼの相互作用の結果，未同定のキナーゼによりスレオニン(T)がリン酸化される．その後，*cdc2* キナーゼはサイクリンBと強く結合し，活性型となる．最後に，*cdc2*キナーゼによりサイクリンBのセリン(S)がリン酸化されて，MPF ができる．チロシン(Y)

しつつある卵内で生成される因子を MPF と呼ぶ．この MPF 活性は未成熟卵への微小注射により検定されるほかに，無細胞系で雄性前核を染色体化させることによっても検定できる．

GTH や MIH は種特異的であるが，MPF 活性には種特異性はない．例えば，キンギョ卵から得た MPF をカエル卵に注射してもカエル卵は成熟し，その逆も起こる．さらに，MPF 活性は減数分裂のみならず体細胞分裂中期でも認められる．われわれは高等植物（ユリ）の花粉母細胞でも MPF 活性を見出した[20]．従って，MPF はあらゆる種に普遍的な，減数分裂・体細胞分裂に関わらず細胞分裂中期を誘起する因子（M-phase-promoting factor：MPF）といえる．

われわれは一度に多量の卵を得ることができる（一尾のコイから1kgの卵を得ることは困難なことではない！）コイ（*Cyprinus carpio*）から MPF をほぼ純品までに精製することに成功した[21]．コイ MPF はカエルやヒトデの MPF と同様に *cdc2*キナーゼ（触媒サブユニット）とサイクリンB（調節サブユニット）の複合体である．これまでにわれわれは，これらのサブユニットに加えて，いくつかの細胞周期調節因子の cDNA をキンギョ卵の cDNA ライブラリーからクローニングし，それらの蛋白質を大腸菌で合成させた[21-23]．また，それらの合成蛋白質を抗原としていくつかのモノクローナル抗体を作製することに成功している．現在，これらのモノクローナル抗体をプローブとしてキンギョ卵における MPF の活性化・不活性化の分子機構を調べているが，ここではこれまでに得られた結論のみを簡単に述べる[24]．サイクリンBは，17α，20β-DP で処理される前のキンギョの未成熟卵にはなく，このホルモン処理の後に新しく合成される．ついで，すでに卵内に存在する *cdc2* キナーゼと複合体を形成した後に，*cdc2* キナーゼのスレオニン（161番目）のリン酸化が起こり，*cdc2* キナーゼが活性化する．最後にサイクリンBのセリンのリン酸化が起こって MPF ができる．このことはサイクリンBが未成熟卵に存在し，*cdc2* キナーゼとすでに複合体を形成しているカエルやヒトデの場合とは著しく異なる（図4）．

おわりに

魚類卵を用いての研究から，発生の出発点ともいえる卵成熟という現象が実に巧妙に調節されていることがおわかりいただけたと思う．試験管内でも10数時間そこそこで起こる卵成熟の過程に実にさまざまな仕組みが集約されていることに驚かされる．その反面，また新しい疑問がはっきりとしてきたことも事実である．卵成熟直前にみられる濾胞細胞の劇的な機能転換の分子機構，ステロイドホルモンの新しい作用機構として注目される17α，20β-DP の卵表レセプターの分子構造および，それと卵内の MPF の活性化を連結させる細胞内情報伝達システムの同定，リン酸化・脱

リン酸化を主体とするMPFの活性化・不活性化の分子機構などの解明がわれわれの当面の研究ターゲットである．しかし，残念ながらこのような研究は卵成熟はどのような仕組みで起こるかという疑問には答えを提供するものであるが，ではなぜ卵がこの過程で発生能を獲得し精子を受け入れるようになるかという，卵成熟に関する発生学上の基本的な疑問に解答を与えるものではない．また，第一減数分裂から第二減数分裂に移行の過程ではDNA合成は起こらない．そのために，卵や精子の染色体の半減が起こるのである．これは通常の体細胞でみられる細胞分裂とは根本的に異なることであり，進化の過程で有性生殖を行う生物が子孫の存続のために獲得した貴重な形質である．しかし，この仕組みもまだ解明されていない．これらの問題を解析するために必要な分子プローブの多くが整った今，適当な実験材料を選択することがますます重要になっている．魚の卵は卵成熟についてのこのような疑問に対して，これからも多くの答えを提供してくれるものと確信している．

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〈筆頭著者プロフィール〉
1993年，総合研究大学院大学博士課程修了．現在は基礎生物学研究所細胞情報部門の非常勤講師．魚卵に惹かれ，今はゼブラフィッシュを扱っている．

Summary

The fish oocyte-An experimental model for the study of oocyte maturation.

Oocyte maturation has been investigated extensively using oocytes of various fishes such as salmonids, goldfish and medaka. Three major mediators of oocyte maturation, gonadotropin, maturation-inducing hormone and maturation-promoting factor, have been purified and characterized. They function sequentially at the level of the follicle layer, the oocyte surface and the oocyte cytoplasm. Fish oocytes provide an excellent model system to investigate molecular mechanisms of oocyte maturation in multicellular animals.