J. Biochem. 122, 286-293 (1997)

Detection of *In Vivo* Proteasome Activity in a Starfish Oocyte Using Membrane-Impermeant Substrate[1]

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Received for publication, January 29, 1997

A method was investigated for monitoring the activity of protease(s) in cytosol of a single starfish oocyte using succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid as the substrate, which was injected into the cell. After preincubation of immature oocytes with a proteasome inhibitor, *N*-carbobenzoxy-L-leucinyl-L-leucinyl-L-norvalinal, the initial hydrolysis of the substrate was remarkably inhibited. The inhibitor blocked 1-methyladenine-triggered cyclin degradation, which is known to be mediated by proteasome. However, calpain inhibitor E-64 did not inhibit the hydrolysis of the substrate. These results suggested that the protease activity measured by this method is mainly attributable to cytoplasmic proteasome. The hydrolysis of the substrate was partially inhibited by bestatin, suggesting that the substrate was cleaved by aminopeptidase. Thus, the initial velocity of hydrolysis of the substrate (V_0) by proteasome was assayed in a living oocyte after preinjection of bestatin. The values of V_0 increased gradually after 1-methyladenine addition and reached the maximum level at the time corresponding to cyclin degradation. The calculated maximum velocity of hydrolysis by a mature oocyte was approximately three times higher than that by an immature oocyte. The Michaelis-Menten constant value was also higher in mature than immature oocytes. These results suggest that proteasome-dependent proteolysis is regulated not only by ubiquitination of substrates, as is generally believed, but also by the proteasome activity itself.

Key words: *in vivo*, oocyte maturation, protease, proteasome, starfish.

Cellular proteolysis is a highly controlled, complex process that takes place in virtually all compartments of cells. Biochemical and histochemical methods have been used to characterize and localize such proteases as cathepsins in lysosome, signal peptidases in endoplasmic reticulum and mitochondria, processing proteases in secretory granules, metallo-proteases in plasma membrane, and calpains or proteasomes in cytosol (*1*). These methods, however, have the disadvantage that protease activity has to be assayed after homogenization and fractionation, or after fixation, which may cause unexpected artifacts. It has been difficult to detect the enzyme activity in living cells in real time.

In this study, we developed a sensitive and continuous assay of cytosolic proteases in a single living cell. To quantify cytosolic protease activity in living cells, substrates for the enzyme need to be present in the cytosol throughout the assay. In other words, substrates and produced dyes should be membrane-impermeant. Although peptide amides of 7-amino-4-methylcoumarin (AMC) have been widely used as fluorogenic substrates of proteases in biochemical assays, AMC was not suitable for our purpose because it was membrane-permeant. We demonstrated in this work that a water-soluble fluorogenic amine, 7-amino-coumarin-4-methanesulfonic acid (ACMS), designed by Sato *et al.* (*2*), was membrane-impermeant and that the peptide amide of ACMS could be used as a substrate for *in vivo* cytosolic protease assays. Using this substrate, we developed a method to quantify the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) in a single living cell, termed *in vivo*, real time, cytosolic enzyme assay (ICEA).

Meiosis reinitiation in starfish is induced by the hormone 1-methyladenine (1-MA) (*3*). Receptors of 1-MA couple to the $\alpha\beta\gamma$ trimeric G protein (*4*), which is sensitive to pertussis toxin (*5-7*). Since activation of cyclin B/cdc2 kinase and germinal vesicle breakdown (GVBD) are triggered by the injection of purified $\beta\gamma$ subunit into immature oocytes, it is concluded that hormonal stimulation dissociates $\beta\gamma$ from α and that the dissociated $\beta\gamma$ mediates the

[1] This study was supported in part by grants from the Ministry of Education, Culture, Sports and Sciences of Japan to Hoshi (#05558092) and Chiba (#08275214, #08780671).

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Abbreviations: AMC, 7-amino-4-methylcoumarin; ACMS, 7-amino-coumarin-4-methanesulfonic acid; V_{max}, the maximum velocity; K_m, Michaelis-Menten constant; ICEA, *in vivo*, real time, cytosolic enzyme assay; 1-MA, 1-methyladenine; GVBD, germinal vesicle breakdown; V_0, initial velocity of hydrolysis of the substrate; Z-Leu-Leu-NVa-H, *N*-carbobenzoxy-leucyl-leucyl-norvalinal; Z-Leu-Leu-Leu-H, *N*-carbobenzoxy-leucyl-leucyl-leucinal; $[S]_0$, initial concentration of substrate; Suc-Phe-Leu-Arg-CAMS, succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid; Boc-Leu-Met-CMAC, *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin; Boc-Leu-Met-MAC-SG, Boc-Leu-Met-7-amino-4-methylcoumarin-glutathione conjugate.

signal of 1-MA (*8, 9*).

It is well documented from yeast to human that the entrance into metaphase in mitosis as well as meiosis is governed by cyclin B/cdc2 kinase. Exit from metaphase is initiated by rapid cyclin destruction in sea urchin (*10*), starfish (*11*), frog (*12*), clam (*13*), and human (*14*). In yeast, B-type cyclin destruction is necessary for exit from mitosis (*15*), supporting the hypothesis that, in eukaryotic cells, exit from metaphase is controlled by a specific protease. Indeed, the cyclin destruction that causes inactivation of cdc2 kinase is mediated by a large multi-subunit protease complex called proteasome.

Proteasome degrades many cytosolic proteins including cyclin when they are ubiquitinated (*16-21*). A cyclin-selective ubiquitin ligase in a large component is activated near the end of metaphase whereas it is inactive during interphase (*22-24*), indicating that proteolysis of cyclin is directly regulated by ubiquitination to cyclin. Using biochemical methods, Sawada *et al.* (*25*) reported that the activity of proteasome in starfish oocytes is increased more than twice under the influence of 1-MA before GVBD during maturation. Also, it is reported that the proteasome is activated during *in vivo Xenopus* egg activation (*26*) or in the ascidian cell cycle (*27*), although the activity of proteasome appears to be constant during the cell cycle in frog egg extract (*28*). The regulatory mechanism of proteasome activity is still unclear.

To determine whether the proteasome activity *in vivo* is really changed during oocyte maturation, we applied our new method, ICEA, to starfish oocytes. When we microinjected the membrane-impermeant substrate of proteasome into starfish oocytes, the initial velocity of the hydrolysis of the substrate (V_0) increased after 1-MA addition and reached the maximum level at the time of cyclin destruction. Calculated V_{max} of proteasome was approximately three times higher in mature oocytes than in immature ones. The K_m value was also higher in mature oocytes than in immature ones. These results suggest that proteasome in immature oocytes is activated by the hormonal stimulation to hydrolyze cyclin B at the end of metaphase.

MATERIALS AND METHODS

Animals and Oocytes—Starfish *Asterina pectinifera* were collected on the Pacific coast of Honshu and kept in laboratory aquaria supplied with circulating seawater at 17°C. To remove follicle cells, isolated ovaries were incubated in ice-cold calcium-free seawater and released oocytes were washed twice with calcium-free seawater. They were stored in artificial seawater at 20°C. Oocytes obtained by this procedure are at the first meiotic prophase (germinal vesicle stage) and are referred to as "immature." Oocyte maturation was induced by the addition of 1 μM 1-methyladenine. We refer to an oocyte that has undergone GVBD as a "mature oocyte."

Reagents and Antigen—ACMS and succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid were synthesized as described previously (*2*). *N*-Carbobenzoxy-L-leucinyl-L-leucinyl-L-norvalinal (Z-Leu-Leu-NVa-H), Z-Leu-Leu-leucinal (Z-Leu-Leu-Leu-H), and E-64 were purchased from Peptide Institute, Osaka. Bestatin and leupeptin were purchased from Sigma Chemical (St. Louis, MO). Rabbit anti-cyclin B antibody was kindly provided by Dr. T. Kishimoto (Tokyo Institute of Technology).

Optical Equipment and Recording Fluorescence Intensity—Fluorescence from an oocyte injected with ACMS or the substrate was collected with a 20×, 0.5 N.A. objective and focused onto a photomultiplier (Nikon, P1) mounted on an inverted fluorescence microscope TMD with a xenon lamp (Nikon, Tokyo). The photomultiplier was connected with a pen recorder Type 3066 (Tosoh, Tokyo). To measure fluorescence, an excitation filter at 380±10 nm (Nikon), a dichroic beam splitter at 400 nm (Nikon), and a 450-nm emission filter (Nikon) were used. An oocyte was illuminated for 1 s to measure fluorescence intensity at intervals of 10 s or more. Continuous illumination caused a gradual decrease of fluorescence. Fluorescent micrographs were taken on Neopan-400 film (Fuji Photo Film, Tokyo) from the microscope. Exposure time was 5 s.

Microinjection—Microinjection into an oocyte and quantitation of injection volumes were done according to the methods of Hiramoto (*29*) and Kishimoto (*30*). Oocytes were held between two coverslips separated by two pieces

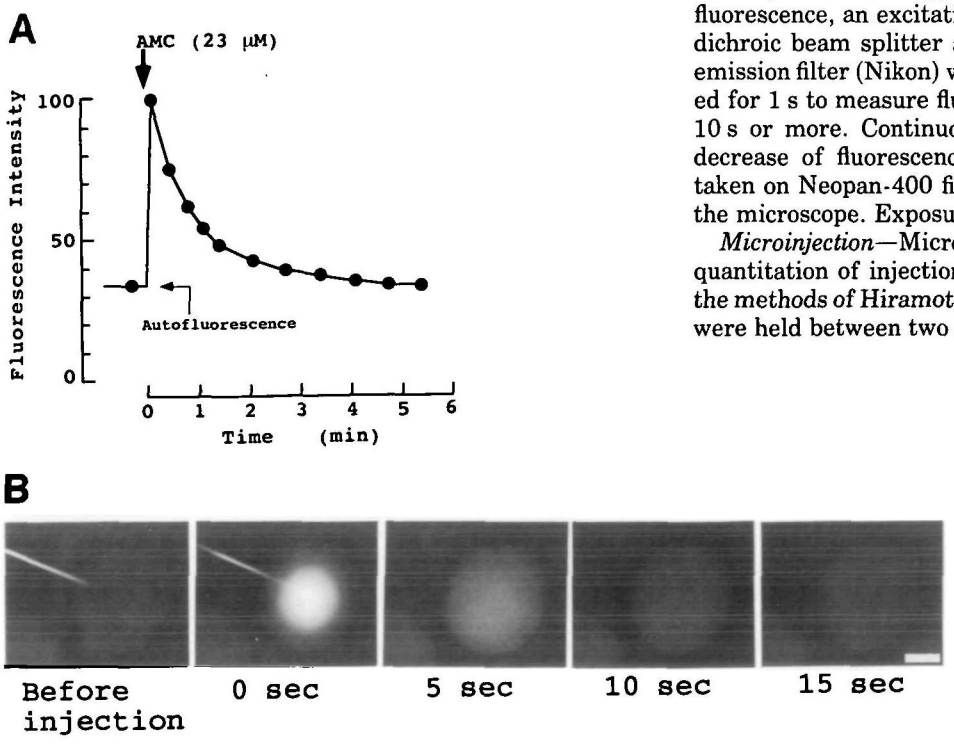

Fig. 1. **Plasma membrane is permeable to AMC.** (A) After recording autofluorescence of an immature oocyte, AMC was injected into the oocyte at the final concentration of 23 μM. (B) Fluorescent micrographs show the oocyte before and after injection of 23 μM AMC. Time after injection is indicated. Immediately after injection, fluorescence intensity of AMC decreased. The micropipet filled with AMC is visible in the photographs taken before injection and at 0 s. Bar: 50 μm.

of double-stick tape during microinjection and observation (7).

Solution—Suc-Phe-Leu-Arg-CAMS dissolved in dimethyl sulfoxide at the concentration of 50 mM was diluted with 9 volumes of 100 mM Hepes buffer, pH 7.0, to make 5 mM Suc-Phe-Leu-Arg-CAMS solution for injection. Leupeptin, bestatin, E-64, and ACMS for injection were dissolved in distilled water at the concentration of 25, 25, 10, and 1 mM, respectively. To inhibit proteasome, oocytes were incubated in seawater containing Z-Leu-Leu-NVa-H (100 μM) or Z-Leu-Leu-Leu-H (100 μM) for 2-3 h.

SDS-PAGE and Immunoblot Analysis—Oocytes treated with or without 1-MA and Z-Leu-Leu-NVa-H were analyzed by SDS-PAGE on 10% gels, and proteins were transferred to a PVDF transfer membrane (Millipore, Bedford, MA). The membrane was blocked with PBS containing 5% skim milk and incubated with anti-cyclin B antibody for 1 h at room temperature. After washing with PBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody at a dilution of 1:200 for 1 h. After washing the membrane, bound antibody was detected using an immunostaining kit HRP-1000 (Konika, Tokyo).

Determination of Kinetic Constants—Fluorescence intensity of the oocyte injected with the substrate was captured at 10 s after the injection of the substrate and then at 10 s intervals in real time continuously for a few min. Each capture required 1 s as mentioned in "*Optical Equipment and Recording Fluorescence Intensity.*" The rate of increase of ACMS was constant for at least 1 min after injection of the substrate. Thus, V_0 was calculated from the data of the first 1 min after injection. Fluorescence intensity was normalized using the data of preinjected ACMS as shown in Fig. 3. V_0 was expressed as μmol liberated ACMS/liter cytoplasm/min (μM/min).

To estimate *in vivo* values of V_{max} and K_m, oocytes were injected with the substrate at final concentrations of 20, 30, 50, 85, 100, and 170 μM in cytoplasm. These substrate concentrations were used as the initial concentrations of the substrate ($[S]_0$). V_0 obtained at each $[S]_0$ was plotted as V_0 on the ordinate and $V_0/[S]_0$ on the abscissa (Eadie-Hofstee plot). The line has an intercept of V_{max} on the V_0 axis and a slope of $-K_m$. The mean$\pm$standard error of the mean (SE) was calculated.

RESULTS

Permeability of Plasma Membrane to AMC and ACMS—Peptide amides of AMC have been used for the biochemical study of various proteases including proteasome. Since protease activity is determined by fluorescence intensity of AMC produced in the course of enzymatic hydrolysis, we microinjected AMC into a starfish oocyte to determine whether this compound was stably retained in the cell. Immediately after injection of AMC, however, fluorescence intensity decreased (Fig. 1, A and B), which suggests that the plasma membrane is permeable to AMC. On the other hand, when ACMS was injected into an oocyte, no significant decrease of fluorescence intensity was observed (Fig. 2, A and B). These results indicate that ACMS does not permeate the plasma membrane and is stable in the cell. Also, ACMS diffused to homogeneity throughout cytoplasm within 10 s after injection (Fig. 2B). Thus, we chose peptide amides of ACMS as substrates for cytosolic proteases.

Proteolytic Hydrolysis of a Peptide Amide of ACMS—Proteasomes exhibit proteolytic (tryptic) activities against peptide amides of AMC (*25, 31*). We thus predicted that the peptide amide of ACMS, succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid (Suc-Phe-Leu-Arg-CAMS), should be a suitable substrate for monitoring proteasome activity. In fact, Boc-Phe-Ser-Arg-MCA was used to detect the fractionated proteasome activity of starfish oocytes (*25*).

When Suc-Phe-Leu-Arg-CAMS was microinjected into a starfish immature oocyte, fluorescence of ACMS increased (Fig. 3A), indicating that tryptic activity in the living cell could be monitored with this substrate. To measure the enzyme activity quantitatively, we first injected ACMS and calibrated the fluorescence intensity of ACMS without autofluorescence of the oocyte (Fig. 3B). Then, we injected Suc-Phe-Leu-Arg-CAMS and monitored the increasing fluorescence intensity of released ACMS (Fig. 3B). The

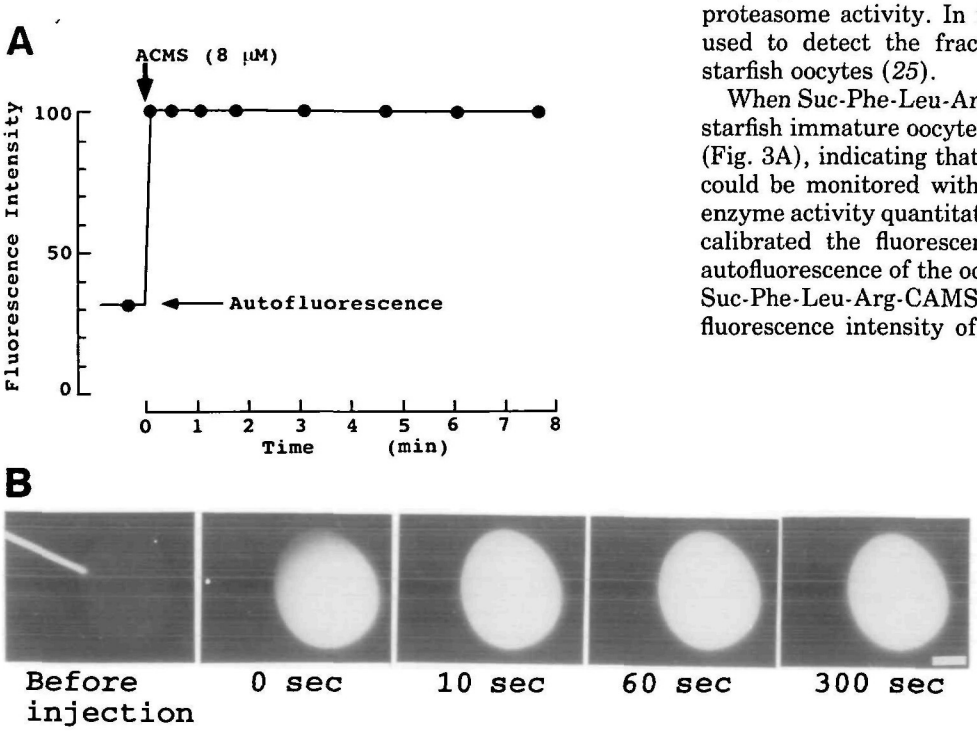

Fig. 2. **Plasma membrane is impermeable to ACMS.** (A) After recording autofluorescence of an immature oocyte, ACMS was injected into the oocyte at the final concentration of 8 μM. (B) Fluorescent micrographs show the oocyte before and after injection of 23 μM ACMS. Time after injection is indicated. Fluorescence intensity of ACMS was stable in the oocyte. The micropipet filled with ACMS is visible in the photograph taken before injection. Bar: 50 μm.

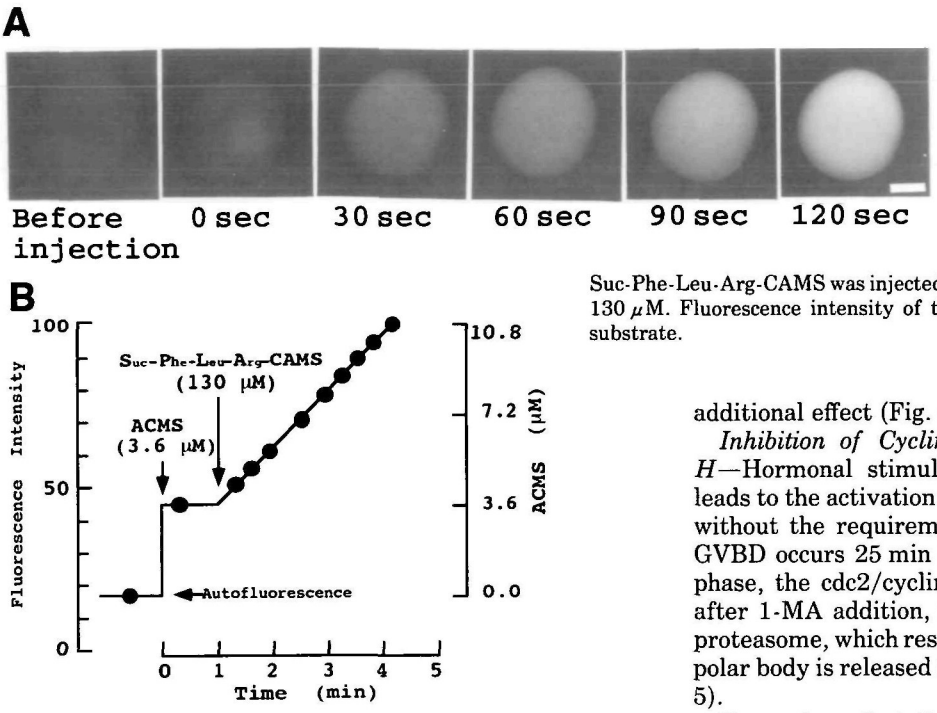

Fig. 3. **Injected Suc-Phe-Leu-Arg-CAMS in cytosol is cleaved and ACMS is released.** (A) Fluorescent micrographs show the oocyte before and after injection of Suc-Phe-Leu-Arg-CAMS at the final concentration of 120 μM. Time after injection is indicated. Bar: 50 μm. (B) After recording autofluorescence of an immature oocyte, ACMS was injected into the oocyte at the final concentration of 3.6 μM. Then Suc-Phe-Leu-Arg-CAMS was injected into the oocyte at the final concentration of 130 μM. Fluorescence intensity of the oocyte increased after injection of the substrate.

initial rate of hydrolysis was constant for at least a few min. Calculated V_0 was 2 μM/min.

Effects of Protease Inhibitors on Hydrolysis of Suc-Phe-Leu-Arg-CAMS—When leupeptin, a known inhibitor of tryptic activities, was injected into an oocyte that had been preinjected with the substrate, the rate of hydrolysis decreased (Fig. 4A). This indicates that the liberation of CAMS is, at least partly, due to tryptic activities in the cell.

N-Carbobenzoxy-leucyl-leucyl-norvalinal (Z-Leu-Leu-NVa-H) is an inhibitor of proteases such as proteasome (*31*). When immature oocytes were preincubated in seawater containing Z-Leu-Leu-NVa-H for 2 h, the initial hydrolysis of the substrate was inhibited (Fig. 4B). Similar results were obtained with *N*-carbobenzoxy-leucyl-leucyl-leucinal (Z-Leu-Leu-Leu-H), which also inhibits proteasome (*32*) (Fig. 4C). These results suggest that Suc-Phe-Leu-Arg-CAMS was mainly hydrolyzed by the proteasome. Other nonlysosomal intracellular proteases such as calpains are sensitive to E-64, while the proteasome is insensitive to it (*31*). When E-64 was injected, no decrease in the hydrolysis of the substrate was observed (Fig. 4D). Thus, it is concluded that the proteasome is responsible for the observed enzymatic activity.

Although the initial hydrolysis of the substrate was completely inhibited by two proteasome inhibitors, as shown in Fig. 4, B and C, the rate of hydrolysis increased gradually and reached maximum at 5 min after injection of the substrate. These results suggest that endopeptidases may hydrolyze the substrate, and newly formed peptidyl CAMS without the protecting group may be hydrolyzed by exopeptidases. As expected, when an inhibitor against exopeptidases, bestatin, was injected into an oocyte that had been treated with proteasome inhibitor Z-Leu-Leu-NVa-H, the rate of hydrolysis decreased (Fig. 4 E), while leupeptin, an inhibitor of endopeptidase, had no significant additional effect (Fig. 4F).

Inhibition of Cyclin Degradation by Z-Leu-Leu-NVa-H—Hormonal stimulation of 1-MA in starfish oocytes leads to the activation of cdc2/cyclin complex in cytoplasm without the requirement for new protein synthesis, and GVBD occurs 25 min after 1-MA addition. During metaphase, the cdc2/cyclin complex is stable, but 70-80 min after 1-MA addition, cyclin is suddenly degraded by the proteasome, which results in exit from metaphase. The first polar body is released 80-90 min after 1-MA addition (Fig. 5).

To confirm that Z-Leu-Leu-NVa-H inhibited starfish proteasome *in vivo*, degradation of cyclin was monitored by Western blots. Figure 5 shows that this peptide aldehyde inhibited cyclin degradation and polar body formation of 1-MA-treated oocytes, but did not inhibit GVBD. Thus, it is concluded that Z-Leu-Leu-NVa-H inhibited proteasome, proteasome-dependent cyclin degradation and exit from metaphase. This inhibitory effect was highly specific, because GVBD occurred normally (Fig. 5).

Hydrolysis of Suc-Phe-Leu-Arg-CAMS Was Enhanced after Hormonal Stimulation—The possible substrate of proteasome, Suc-Phe-Leu-Arg-CAMS, was cleaved in starfish oocytes, and this cleavage was inhibited by the proteasome inhibitors (Fig. 4, B and C). These results strongly suggest that proteasome is mainly involved in the hydrolysis of the substrate, although this substrate or its degradation product by other endoprotease was also cleaved partly by exopeptidases (Fig. 4E). To measure solely the activity of proteasome, we determined the concentration of bestatin required to eliminate the contribution of exopeptidases. Figure 6 shows that 420 μM bestatin was sufficient. In the following experiments, oocytes were preinjected with 420 μM bestatin in order to estimate the V_0 of proteasome-dependent hydrolysis of the substrate.

Figure 7 shows the V_0 values during oocyte maturation. Proteasome activity increased gradually after 1-MA treatment and reached an almost maximal level just before the first polar body formation.

To calculate V_{max}, the substrate at 20-170 μM was injected into immature or mature oocytes (70-100 min after 1-MA addition). The measured V_0 at the initial concentration of the substrate ($[S]_0$) was plotted on the ordinate against $V_0/[S]_0$ on the abscissa (Eadie-Hofstee plot), as shown in Fig. 8. The intercept of V_{max} on the ordinate was high in mature oocytes (2.3 ± 0.17 μM/min) and low in immature oocytes (0.84 ± 0.068 μM/min). The K_m value was determined from the slope of each line;

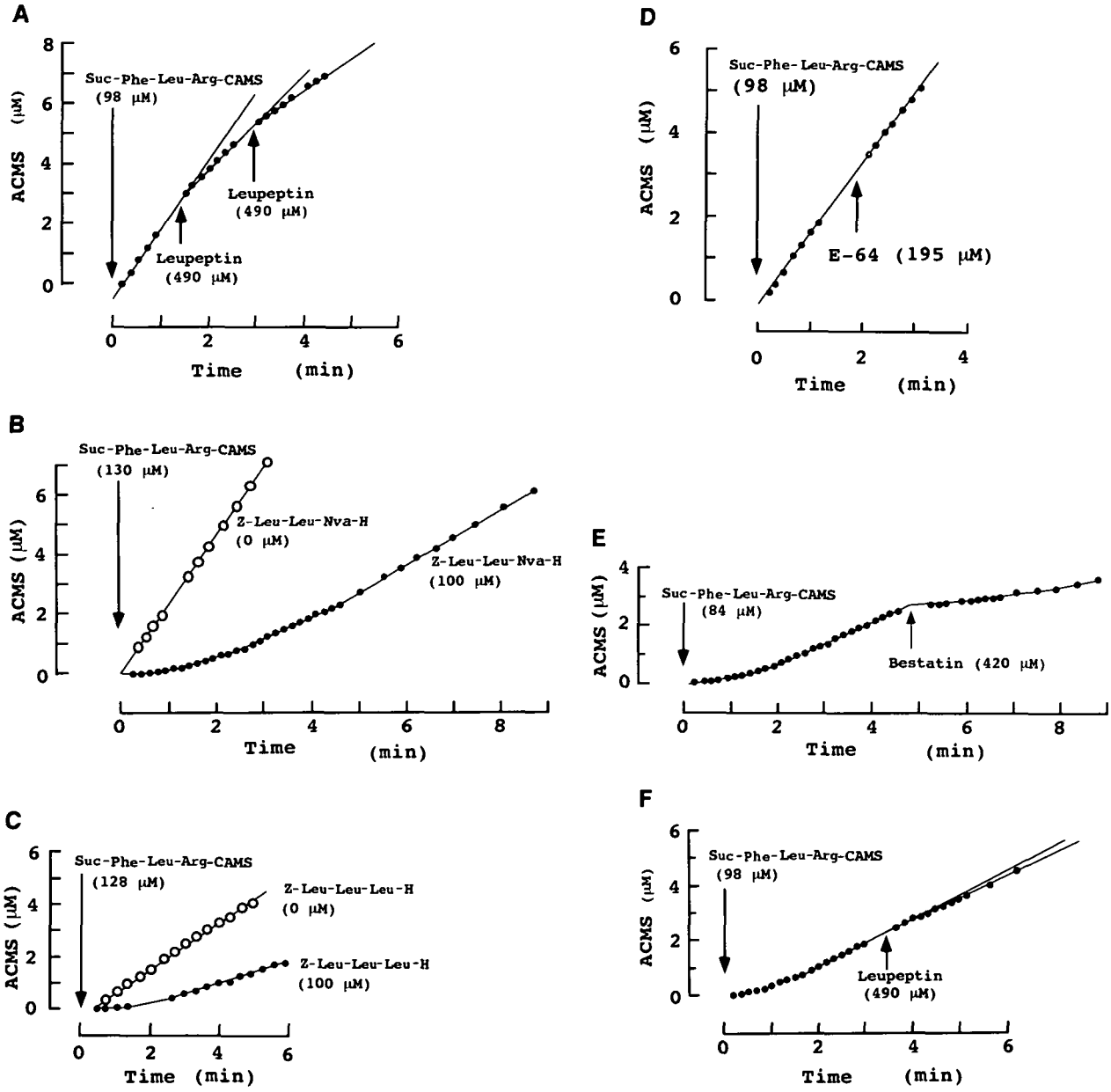

Fig. 4. **Effects of protease inhibitors on the *in vivo* hydrolysis of Suc-Phe-Leu-Arg-CAMS.** The substrate was injected into an immature oocyte at time 0. Fluorescence intesity of the oocyte was normalized by injection of ACMS as shown in Fig. 3B. The final concentrations of compounds in the oocyte or seawater are indicated in parentheses. (A) Leupeptin was injected into the oocyte. Lines are extended in order to distinguish rates of hydrolysis between the experiments before and after injection of leupeptin. (B) Oocytes were pretreated with (closed circle) or without (open circle) Z-Leu-Leu-NVa-H in seawater before injection of the substrate. (C) Oocytes were pretreated with (closed circle) or without (open circle) Z-Leu-Leu-Leu-H in seawater before injection of the substrate. (D) E-64 was injected into the oocyte. (E) An oocyte treated with Z-Leu-Leu-NVa-H in seawater was injected with the substrate, then bestatin was injected. (F) An oocytes treated with Z-Leu-Leu-NVa-H in seawater was injected with the substrate, then leupeptin was injected.

mature oocytes showed high K_m ($60\pm9.7\,\mu M$), while immature gave low K_m ($30\pm6.6\,\mu M$). Similar results were obtained from different animals, as shown in Table I.

DISCUSSION

In this study, we have developed an *in vivo*, real time, cytosolic enzyme assay, ICEA. This new method, unlike the standard biochemical or histochemical assay, measures enzyme activity of a living cell in real time without fractionation or fixation. Using ICEA, we found that the activation of proteasome in starfish oocytes was triggered by hormonal stimulation. The proteasome activity continued to increase after GVBD and reached the maximum level near the end of metaphase, when native cyclin B was hydrolyzed by the enzyme. Indeed, V_{max} was approximately three times higher in mature oocytes than in immature ones. Since *de novo* protein synthesis is not required for 1-MA-induced GVBD (*33*) and proteasome was activated immediately after 1-MA addition (Fig. 7),

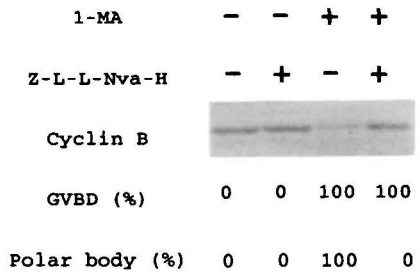

Fig. 5. **Z-Leu-Leu-NVa-H blocked cyclin destruction and polar body formation but did not inhibit 1-MA-induced GVBD.** Immature oocytes were treated with (+) or without (−) 100 μM Z-Leu-Leu-NVa-H for 2 h, then with (+) or without (−) 1 μM 1-MA. GVBD was scored 25 min after 1-MA addition. First polar body formation was scored 90 min after 1-MA addition. At least 40 oocytes were scored for each point. Also, 60 oocytes were collected before or 100 min after 1-MA addition for Western blotting analysis of cyclin B.

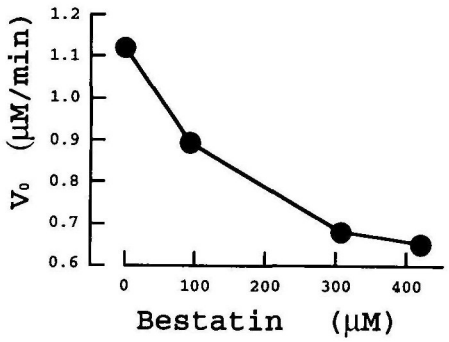

Fig. 6. **Immature oocytes were injected with bestatin, then with 84 μM Suc-Phe-Leu-Arg-CAMS.** Similar results were obtained in mature oocytes (data not shown). Velocities were calculated from the data of the first 1 min after injection.

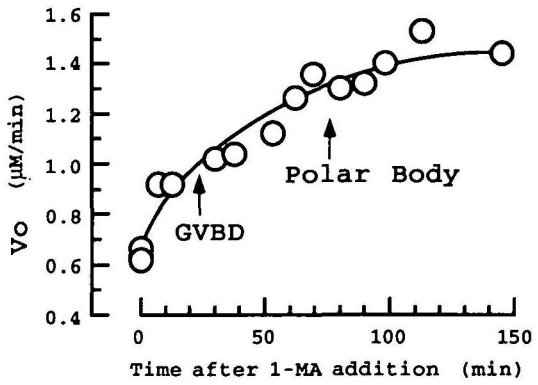

Fig. 7. **Changes in V_0 values of Suc-Phe-Leu-Arg-CAMS after 1-MA addition.** The substrate was injected into 420 μM bestatin-preinjected oocytes to a final concentration of 85 μM. GVBD and first polar body formation occurred at 25 and 75 min after 1-MA addition, respectively.

the increase in the activity is not likely to be due to translation of new proteasome. Thus, we concluded that the proteasome is rather inactive in immature oocytes, but that it is activated after hormonal stimulation to destroy cyclin at the end of metaphase. High activity of proteasome was

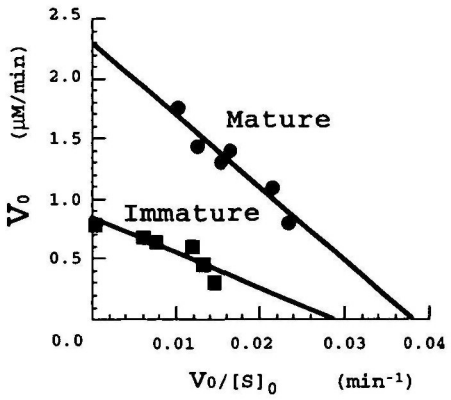

Fig. 8. **Eadie-Hofstee plot using the data of *in vivo* hydrolysis of injected Suc-Phe-Leu-Arg-CAMS.** Oocytes were preinjected with 420 μM bestatin. See text for details.

TABLE I. **Kinetic parameters, V_{max} and K_m, for proteasome as determined in immature and mature oocytes of three starfish using Suc-Phe-Leu-Arg-CAMS.**

Batch	V_{max} (μM/min)[a]		K_m (μM)[a]	
	Immature	Mature	Immature	Mature
A	0.84±0.068	2.3±0.17	30±6.6	60± 9.7
B	0.75±0.060	2.7±0.47	16±4.8	71±21
C	0.86±0.099	3.0±0.57	22±8.7	66±24

[a]Means value±SE (n=6).

maintained after metaphase or after the end of meiosis I (Fig. 7).

Our conclusion was partly supported by biochemical study: Using gel filtration, Sawada *et al.* (*25*) fractionated extracts from immature or maturing starfish oocytes and found that the protease activity in the proteasome fraction of maturing oocytes (5-30 min after 1-MA treatment) was two times higher than that of immature oocytes (before 1-MA treatment). However, after GVBD, the activity of fractionated proteasome from mature oocytes (50 min after 1-MA treatment) was reduced to the level of the immature oocytes (*25*), which was not confirmed in this study. Further study is necessary to understand the reason for this apparent discrepancy.

Proteasome activity is influenced by calcium (*34, 35*) and ATP concentration (*36*), and regulatory factors (*37-39*). Although 1-MA induces a transient calcium elevation, which is insufficient for maturation, calcium decreases rapidly to a resting level during oocyte maturation (*40*). Also, ATP levels are stable during maturation (Chiba, Nakano, and Hoshi, unpublished results). Thus, regulators may be involved in the change of the proteasome activity. The change of K_m during maturation (Table I) may support this assumption: change of K_m was demonstrated when an activator (PA28) was added to proteasome, although the activator decreased the K_m value (*38*).

Rosser *et al.* (*41*) synthesized a fluorogenic calpain substrate *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (Boc-Leu-Met-CMAC). Boc-Leu-Met-CMAC is a membrane-permeant compound. After diffusion into cells, Boc-Leu-Met-CMAC is conjugated to endogenous glutathione (GSH) by endogenous glutathione S-transferase forming Boc-Leu-Met-7-amino-4-methylcoumarin-glutathione conjugate (Boc-Leu-Met-MAC-SG). Intracellu-

lar proteolytic hydrolysis of Boc-Leu-Met-MAC-SG releases the fluorescence of MAC-SG. They measured fluorescence intensity of MAC-SG in a single, cultured rat hepatocyte using digitized video fluorescent microscopy and found calpain activity was increased by ATP-induced intracellular calcium release. However, MAC-SG was rather membrane-impermeant, although a significant portion of this compound showed diffusion from cells (*ca.* 20% in 25 min). Although they estimated relative activity of calpain, they could quantitate neither V_0 nor K_m and V_{max} value, because the initial concentration of the substrate and released MAC-SG in a cell was unknown. Also if glutathione conjugation is the rate-limiting step, this method cannot be used to detect the protease activity.

In principle, all cytoplasmic protease activities can be measured by ICEA using suitable substrates. However, a few points must be noted when ICEA is applied. First, not all peptide amides of ACMS are readily soluble in aqueous solutions at millimolar level, while ACMS is readily soluble. The solubilities of these substrates depend on their peptide sequences and protecting groups. In this study, we made several substrates like Z-Phe-Leu-Arg-CAMS, but they were hardly soluble in water (data not shown). Although Z-Phe-Leu-Arg-CAMS was readily soluble in an organic solvent such as dimethylsulfoxide, it was deposited in the cell immediately after injection of the solution. This problem was overcome by changing the protecting group to a succinyl group. The second important point is that the detected protease does not always originate from a single enzyme. More than two enzymes may be involved in the apparent activity. To clarify this possibility, the peptide sequence of the substrate should be specific for the enzyme of interest. Also, a specific inhibitor of the enzyme should be used to demonstrate that it affects the *in vivo* protease activity measured by ICEA. If the hydrolysis of the substrate is completely inhibited by the inhibitor, the enzyme of interest is likely to be involved in the substrate cleavage measured by ICEA. In this study, Z-Phe-Leu-Arg-CAMS was hydrolyzed by a two-enzyme system consisting of an endopeptidase and exopeptidase. To deactivate the exopeptidase, oocytes were preinjected with bestatin. Moreover, if the hydrolysis of a substrate is demonstrated to originate from one enzyme, *in vivo* effects of new inhibitors can be evaluated using ICEA. The last point to be noted is that the values of V_0, V_{max}, and K_d in this study are apparent, because the real volume of cytosol, excluding organelles such as endoplasmic reticulum, mitochondria, and lysosome, is unknown. Thus, real values should be higher than the apparent values obtained in this study.

ICEA will be applicable for studies of proteases involved in protein stability, apoptosis, immune response, cell cycle, fertilization, development, and differentiation. Also, this new method will be useful to confirm hypotheses presented by biochemical and histochemical methods. Using ICEA, a cell can be treated as a tiny, living test-tube.

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