コイ卵におけるカルパインおよびカルパスタチンの存在

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Detection of Calpain and Calpastatin in Carp Eggs*1

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In order to examine the participation of calpain and calpastatin in the biological process, carp eggs were chosen as the experimental material. Calpain activity was hardly detected in carp egg crude extract. However, the activities of calpain and calpain inhibitor became recognizable through the DEAE-cellulose chromatography of the crude extract. Carp egg calpain partially purified by further column chromatographies was classified as calpain II from the degree of Ca^{2+} requirement for the activity. The optimum pH was 7.1–7.2 and the activity was strongly inhibited by leupeptin, E-64 and antipain.

Carp eggs seemed to contain also calpastatin, endogenous inhibitor for calpain, since the inhibitory activity was extremely heat stable.

Calpain*4 (Ca^{2+}-dependent cysteine proteinase, E.C. 3.4.22.17) and calpastatin (specific and endogenous inhibitor for calpain) show the widespread distribution in various vertebrate tissues.[2-4] Moreover, the inhibitory effect of calpastatin on calpain from different tissues and species is well-conserved.[2-5] These facts are likely to suggest that calpain and calpastatin may concern with any biological function in the living cells. Some possible biochemical processes with which calpain and calpastatin may concern are proposed (e.g. activation of protein kinases; turnover of myofibrillar proteins, cytoskeletal proteins and neurofilamental proteins; platelet aggregation; degradation of steroid hormone receptors. See the reviews by ISHIURA[6] and MURACHI[4]). However, true biological function of calpain and calpastatin is not clearly understood.

In order to examine the participation of calpain and calpastatin in the biological phenomenon, we chose carp eggs as the experimental material, because of two following reasons. (1) It was expected that calpain and calpastatin might concern with irreversible biological process such as fertilization. (2) It was easy to obtain large quantities of eggs from carp as the purification source.

At the beginning of the study it was necessary to detect calpain and calpastatin in carp eggs, but we failed to detected calpain activity in the crude extract. However, we could show calpain activity in the fractions eluted from DEAE-cellulose chromatography of the crude extract. Carp egg calpain was partially purified by the further column chromatographies and classified as calpain II*5 from the degree of Ca^{2+} requirement for the activity.

On the other hand, calpain inhibitory activity which was eluted from DEAE-cellulose chromatography was considered to be partly due to calpastatin judging from its extreme heat stability.

Materials and Methods

Materials

Eggs were collected from mature female carp *Cyprinus carpio* which was purchased from a commercial supplier. These unfertilized eggs were washed sufficiently with distilled water and used for purification. The sources of other materials used in this work were as follows: DEAE-

*1 This study was presented at the Annual Meeting of the Japanese Society of Scientific Fisheries on October of 1984.

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*4 Calpain is a recommended name by the Nomenclature Committee of the International Union of Biochemistry.[1]

*5 Two subclasses of calpain are known to exist, namely calpain I which requires micromolar Ca^{2+} and calpain II which requires millimolar Ca^{2+} for the activity, respectively.[2]

Abbreviations: E-64, *N*-{*N*-(L-3-trans-carboxyoxyrane-2-carbonyl)-L-leucyl}agmatine; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*,*N*′-tetraacetic acid.

cellulose (DE-52) from Whatman, DEAE-Sepharose CL-6B from Pharmacia, hydroxylapatite from Bio-Rad, Ultrogel AcA 34 and AcA 44 from LKB, papain and molecular weight marker proteins from Sigma and Hammarsten-grade casein from Merck. Other reagent grade chemicals were purchased from Wako Pure Chemicals.

Carp muscle calpain (calpain II) was partially purified by DEAE-cellulose and Ultrogel AcA 34 column chromatographies according to the method of MURACHI *et al.*[2]

Assay of Calpain and Papain

Calpain activity was determined with casein as substrate according to the method of YOSHIMURA *et al.*[7] The standard reaction mixture containing 0.4% casein, 5 mM cysteine, 5 mM Ca^{2+}, 50 mM imidazole-HCl buffer, pH 7.5, and the enzyme solution in a total volume of 1 m*l* was incubated at 30°C for 30 min. The reaction was stopped by the addition of 1 m*l* of 10% trichloroacetic acid and the acid soluble products formed were colorimetrically determined by the method of Ross and SCHATZ.[8] The reaction mixture without Ca^{2+} was taken as the blank. One unit of calpain activity was defined as the amount that caused an increase in the absorbance at 750 nm of 1.0 for 30 min.

Papain activity was determined with casein as substrate as described previously.[9]

Assay of Inhibitor

Inhibitor assay was performed as described previously.[9] Briefly, an appropriate volume of inhibitor and proteinase solution was preincubated for 5 min at room temperature. The remaining proteinase activity was then assayed as described above. One unit of calpain inhibitory activity was defined as the amount that reduced 1.0 unit of carp muscle calpain II activity.

Electrophoresis

Non-denaturing polyacrylamide disc gel electrophoresis was carried out according to the method of DAVIS[10] using 12.5% polyacrylamide gel.

Protein Determination

Protein concentration was determined by the method of LOWRY *et al.*[11] using bovine serum albumin as a standard.

Results

Separation of Calpain and its Inhibitor(s) from Carp Egg Extract

All purification procedures were carried out at

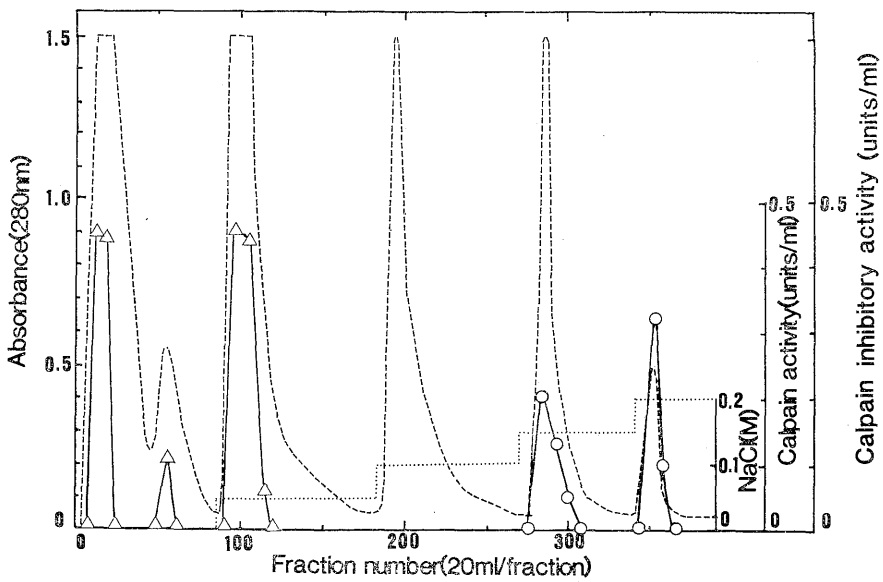

Fig. 1. Separation of calpain and calpain inhibitor(s) from carp egg extract by the chromatography of DEAE-cellulose. Carp egg extract (8,100 mg) after dialysis against buffer A was applied on a column (2.5×30 cm) of DEAE-cellulose. The stepwise elution was performed with buffer A, buffer A containing 0.05 M, 0.1 M, 0.15 M and 0.2 M NaCl at a flow rate of 40 m*l*, ---, absorbance at 280 nm; ○—○, calpain activity; △—△, calpain inhibitory activity; ···, NaCl concentration.

0–4°C. Carp eggs (90 g) were homogenized with 270 m*l* of buffer A (20 mM Tris-HCl buffer containing 1 mM EGTA and 5 mM 2-mercaptoethanol, pH 7.5) containing 0.25 M sucrose in a Waring blendor for 3 min at top speed. The homogenate was centrifuged at 10,000×g for 30 min and the obtained supernatant was further centrifuged at 105,000×g for 60 min. The final supernatant was dialyzed against buffer A overnight. Calpain activity was hardly detected in the dialysate (designated as crude extract in the present study) probably due to the co-existing inhibitor(s) for calpain.

In order to separate calpain and its inhibitor(s), DEAE-cellulose chromatography was performed. The crude extract was applied on a column (2.5×30 cm) of DEAE-cellulose equilibrated with buffer A. The column was washed out with buffer A and the stepwise elution with buffer A containing 0.05 M, 0.1 M, 0.15 M and 0.2 M NaCl was followed. As shown in Fig. 1, calpain inhibitory activity was detected in unadsorbed and 0.05 M NaCl fractions, while calpain activity was detected in 0.15 M and 0.2 M NaCl fractions, respectively. Such calpain activity was hardly detected with 0.1 mM Ca^{2+} but detected with 5 mM Ca^{2+}. Therefore, the caseinolytic activity was supposed to be due to calpain II, less-Ca^{2+}-sensitive form of calpain.[2] Calpain fractions eluted with buffer A containing 0.15 M NaCl (fraction numbers 277–320) and 0.2 M NaCl (fraction numbers 345–360) were combined, dialyzed against buffer A and used for the further purification.

On the other hand, fractions eluted from the column with buffer A containing 0.05 M NaCl that showed calpain inhibitory activity (fraction numbers 92–107) were pooled, dialyzed against buffer A and used for the further experiment.

Partial Purification of Carp Egg Calpain

The dialysate of calpain preparation as described above was applied on a column (2×23 cm) of DEAE-Sepharose CL-6B equilibrated with buffer A and the elution was performed with buffer A having 0–0.3 M NaCl gradient in a total volume of 1,000 m*l*. Calpain activity was eluted with buffer A containing 0.15 M NaCl. Calpain fractions were pooled and dialyzed against buffer B (0.001 M potassium phosphate buffer containing 5 mM 2-mercaptoethanol and 0.1 M NaCl, pH 7.5) overnight.

The dialysate was applied on a column (1×6 cm) of hydroxylapatite equilibrated with buffer B and the elution was performed with 0.001–0.2 M potassium phosphate buffer gradient containing 5 mM 2-mercaptoethanol and 0.1 M NaCl, pH 7.5, in a total volume of 200 m*l*. Calpain fractions eluted with 0.05 M potassium phosphate buffer were pooled and concentrated to 1.6 m*l* by Amicon PM-10 membrane.

The concentrate was applied on a column (1.5×100 cm) of Ultrogel AcA 34 equilibrated with buffer A containing 50 mM NaCl. Calpain fractions were pooled, concentrated to 1.0 m*l* by Amicon PM-10 membrane and rechromatographed on the same column. Calpain activity was eluted in a peak that coincided with a protein peak. Calpain fractions were pooled, dialyzed against buffer A and used for the further experiments.

The purity of the final preparation was analyzed with non-denaturing polyacrylamide disc gel electrophoresis (Fig. 2). The dense protein band that coincided with calpain activity was observed, while some other contaminated protein bands were still detected. In order to confirm that this

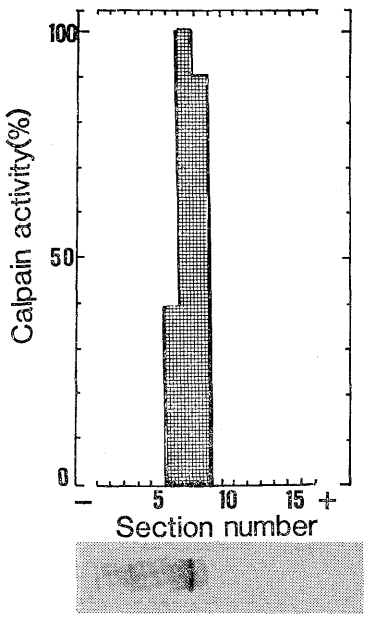

Fig. 2. Non-denaturing polyacrylamide disc gel electrophoresis of carp egg calpain preparation eluted from second Ultrogel AcA 34 chromatography. Twenty micrograms of sample was applied on two columns of gel (12.5% polyacrylamide), respectively. After electrophoresis, one gel was stained with Coomassie Brilliant Blue. The other was sliced into 16 sections and each section was homogenized with 1.0 m*l* of buffer A. Then calpain activity was measured as described in the text.

calpain preparation did not contain any other casein hydrolyzing neutral proteinase except for calpain, caseinolytic activity in the absence or presence of Ca^{2+} was measured as a function of time. As a result, no caseinolytic activity was detected in the absence of Ca^{2+}, while the increase of the absorbance at 750 nm was observed in the presence of 5 mM Ca^{2+} with the prolonged incubation time. Therefore, we considered that this preparation was enough pure to examine the properties of carp egg calpain.

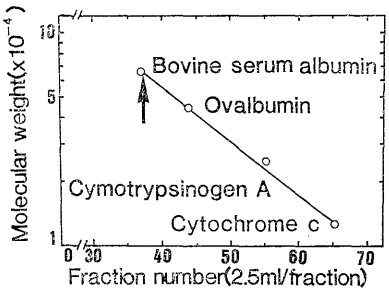

Fig. 3. Molecular weight estimation of carp egg calpain by Ultrogel AcA 44 gel filtration. Partially purified carp egg calpain was applied on a column (1,5×100 cm) of Ultrogel AcA 44. The elution was performed with buffer A containing 50 mM NaCl at a flow rate of 10 ml/h. The arrow indicates the fraction which carp egg calpain was eluted. The molecular weight marker proteins were as follows: Bovine serum albumin (67,000), ovalbumin (43,000), α-chymotrypsinogen A (25,000) and cytochrome c (12,700).

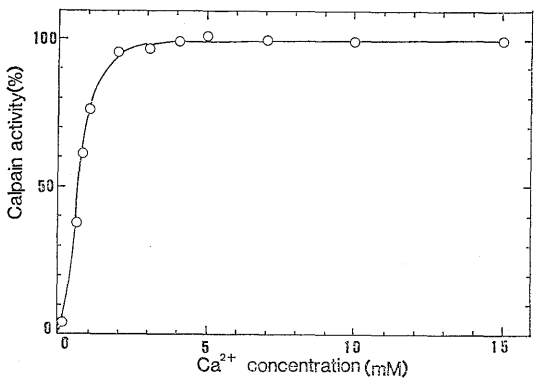

Fig. 4. Effect of Ca^{2+} concentration on the activity of carp egg calpain. The reaction mixture contained 100 μl of the enzyme solution, 5 mM cysteine, 0.4% casein, 50 mM imidazole-HCl buffer, pH 7.5, and the indicated concentration of Ca^{2+} in a total volume of 1 ml. Ca^{2+} concentration was calculated as the excess amount of EGTA which was derived from the enzyme solution.

Some Properties of Carp Egg Calpain

Molecular weight of carp egg calpain was estimated by the gel filtration of Ultrogel AcA 44 (Fig. 3). The enzyme was eluted in the same fraction as bovine serum albumin which was eluted in another run of the same column for the calibration. Therefore, the molecular weight of carp egg calpain was estimated to be 67,000.

On the other hand, carp egg calpain showed maximal activity at more than 2 mM Ca^{2+} (Fig. 4) and at pH 7.1–7.2 (Fig. 5). Additionally, leupeptin, antipain and E-64 strongly inhibited the activity of carp egg calpain (Table 1).

Heat Stability of Calpain Inhibitory Activity

As shown in Fig. 6, calpain inhibitory activity

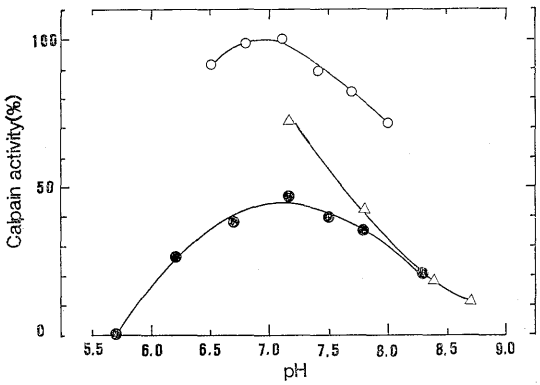

Fig. 5. Effect of pH on the activity of carp egg calpain. The reaction mixture contained 100 μl of the enzyme solution, 5 mM cysteine, 0.4% casein, 5 mM Ca^{2+} and the indicated pH of 50 mM each buffer in a total volume of 1 ml. ○—○, imidazole-HCl buffer; ●—●, Tris-maleate buffer; △—△, Tris-HCl buffer.

Table 1. Effect of inhibitors on the activity of carp egg calpain

Inhibitors	Concentration (μg/ml)	Activity (%)
None	—	100
Leupeptin	0.1	63
	1.0	33
	10	18
Antipain	0.1	81
	1.0	58
	10	26
E-64	0.1	72
	1.0	48
	10	15

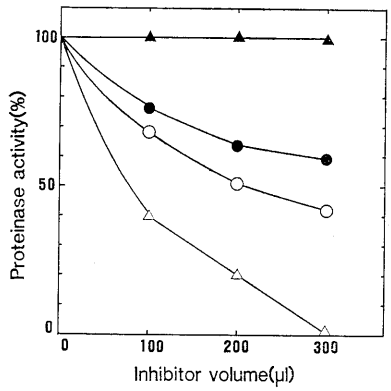

Fig. 6. Heat stability of the calpain inhibitor fractions eluted from DEAE-cellulose with buffer A containing 0.05 M NaCl (Fig. 1, fraction numbers 92–107). Dose dependent inhibitory effect of the inhibitor solution on carp muscle calpain II before (○—○) or after (●—●) heat treatment at 95°C for 10 min and on papain before (△—△) or after (▲—▲) the same heat treatment was determined.

which was eluted from DEAE-cellulose with buffer A containing 0.05 M NaCl (Fig. 1) inhibited both carp muscle calpain and papain with a dose dependent manner. Papain inhibitory activity, however, was completely lost after heat treatment at 95°C for 10 min, while calpain inhibitory activity was fairly heat stable.

Discussion

Calpain and calpastatin are known to distribute widely in various animal tissues.[2-4] However, the biological function of them is not clearly understood. In order to make an approach to this question, we chose carp eggs as the experimental material.

We could not detect any calpain activity in crude egg extract probably because of calpastatin and other proteinase inhibitor(s). However, by the chromatography of DEAE-cellulose, calpain and calpain inhibitor were separated (Fig. 1). Pooled calpain fractions were further purified by the following column chromatographies of DEAE-Sepharose CL-6B, hydroxylapatite, Ultrogel AcA 34 and Ultrogel AcA 34. Obtained calpain preparation still contained a small quantity of some other proteins (Fig. 2), but showed no caseinolytic activity in the absence of Ca^{2+}. Therefore, we considered that this preparation contained no other neutral proteinase which hydrolyzed casein except for calpain.

The molecular weight of carp egg calpain was estimated to be 67,000 by the gel filtration (Fig. 3). Generally, mammalian calpains are known to consist of two subunits, namely 80,000 and 30,000 dalton.[2-4] Different from mammalian calpains, the molecular weight of carp egg calpain was rather smaller, even if it lacked 30,000 dalton subunit.

Carp egg calpain showed maximal activity at more than 2 mM Ca^{2+} (Fig. 4). The result clearly indicated that carp egg calpain was classified as calpain II, less-Ca^{2+}-sensitive form of calpain.[2] Additionally, carp egg calpain showed maximal activity around neutral pH range (Fig. 5) and its activity was strongly inhibited by leupeptin, antipain and E-64 (Table 1). These facts also supported that the enzyme belongs to calpain family.[2-4]

On the other hand, we examined some properties of the calpain inhibitor fractions (Fig. 1, fraction numbers 92–107) to know whether these fractions contained calpastatin or not. Calpastatins from mammalian tissues are known to show specific inhibitory effect on calpain among various proteinases and to be extremely heat stable.[5] In our experiment (Fig. 6), the pooled preparation inhibited not only calpain but also papain. However, these inhibitory activities could be distinguished from each other in heat stability. After heat treatment at 95°C for 10 min, calpain inhibitory activity was slightly decreased, while papain inhibitory activity was completely lost. The result demonstrated that the preparation contained at least two types of proteinase inhibitors. One was calpastatin which was extremely heat stable and did not show the inhibitory effect on papain. The other was a group-specific proteinase inhibitor which was not so heat stable and showed the inhibitory effect on cysteine proteinases, such as calpain and papain.

In the present study, we suggested the existence of calpain and calpastatin in unfertilized carp eggs, but could not obtain homogeneous calpain preparation by 5-step column chromatographies. TANEDA *et al.*[12] purified calpain II from carp muscle by only 3-step column chromatographies including DEAE-cellulose, Ultrogel AcA 34 and DEAE-cellulose. We also purified calpain II from carp muscle by 3-step column chromatographies including DEAE-cellulose, hydroxylapatite and Ultrogel AcA 34.* The difficulty of purification of calpain from eggs was possibly

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ascribed to the relative structural complexity of egg compared with muscle.

On the other hand, Ca^{2+} is known to play a critical role at the time of fertilization (see the review by EPEL[13]). Furthermore, calpain is known to activate some protein kinases, such as phosphorylase b kinase[14-17], protein kinase C[18] and cyclic AMP dependent protein kinase,[19] irreversibly *in vitro* by the limited proteolysis. Therefore, it is attractive to speculate that such activation by calpain may occur in eggs after fertilization. We now attempt to detect calpain and calpastatin in fertilized eggs and compare their properties with those in unfertilized eggs.

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