STUDIES ON FISH MUSCLE PROTEASES

HARUHIKO TOYOHARA

1984

STUDIES ON FISH MUSCLE PROTEASES

HARUHIKO TOYOHARA

1984

ABBREVIATIONS

BAA,	Benzoyl-arginineamide
BANA,	Benzoyl-arginine-naphthylamide
DAN,	Diazoacetylnorleucine-methylester
Dip-F,	Diisopropyl-fluorophosphate
DTT,	Dithiothreitol
EDTA,	Ethylenediamine tetraacetic acid
EGTA,	Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*',-tetraacetic acid
FPM,	Cu-Folin positive matter
GTA,	Glycyl-tyrosineamide
NPM,	Ninhydrin positive matter
p-CMB,	*p*-Chloromercuribenzoic acid
PMSF,	Phenylmethylsulphonyl fluoride
SDS,	Sodium dodecylsulfate
TCA,	Trichloroacetic acid
TLCK,	Tosyl-lysine-chloromethylketone
TMA,	Trimethylamine
Tricine,	Tris(hydroxymethyl)methylglycine
Tris,	Tris(hydroxymethyl)aminomethane
Z,	Benzyloxycarbonyl

In this study, the author uses "protease" as a word which includes both an endopeptidase and an exopeptidase. On the other hand, he uses "proteinase" as a word which means only an endopeptidase.

CONTENTS

CHAPTER I

INTRODUCTION

It is well-known that fish meat deteriorates more rapidly than mammalian meat after death. For many years, this phenomenon has been recognized vaguely as the muscle protein degradation by muscle proteases. Sakai[1] and Ohya and Shimada[2] reported on the post-mortem protein breakdown of fish muscle in 1914 and 1923, respectively. Until 1970, much attentions were mainly paid on an acid proteinase(cathepsin D) in fish muscle as a factor which might cause post-mortem protein breakdown[3-13]. Among these situation, Makinodan *et al.*[5] revealed a unique heat stable proteinase in fish muscle. Later, they also revealed that Cathepsins A, B, C[14] and a neutral proteinase (tentatively called a sub-endopeptidase)[15] exist in fish muscle(see reviews by Makinodan[16,17]).

On the other hand, in 1950s and 1960s, there were many reports on the post-mortem protein breakdown of mammalian and chicken meat from the view of meat ageing[18-49](see reviews by Fujimaki and Okitani[50] and Okitani[51]). Furthermore, from the studies

mainly on muscle dystrophy, there have been known to exist some proteases in muscle as well as other tissues[52-56]. These earlier studies on muscle proteases, however, were confined to mainly cathepsin D, of which activity is easily detectable in crude muscle extract. In comparison with acid proteinase, studies on neutral proteinases in muscle were very few since detection of the activity was difficult in the crude extract.

Recently, chromatographic procedures for proteins have been markedly developed. These new purification technique made it possible to detect some neutral proteinase activities which had not been detected in the crude extract, by eliminating co-exsisted their inhibitors(see reviews by Laskowski, Jr. and Kato[57] and Murachi *et al.*[58]).

Generally, post-mortem pH in muscle is considered to be around 6.0-6.5 for fish muscle[59-63], while that for mammalian muscle to be below 6.0[51]. Therefore, if muscle proteases would participate in the post-mortem protein breakdown, they could show activities around pH 6.0-6.5.

In the present studies, the author re-examined autolysis of fish muscle after death(CHAPTER II) and

the distribution of some known proteinases in fish muscle(CHAPTER III). In CHAPTER IV, the author did closer examination on two acid proteases(cathepsins A and D) and discussed them in relation to autolysis. Detection and characterization of some neutral proteinases which have never been known to distribute in fish muscle will be described in CHAPTERS V and VII. In CHAPTER VI, two proteinase inhibitors in fish muscle will be also described.

CHAPTER II

AUTOLYSIS OF FISH MUSCLE

In earlier studies, Sakai[1], Ohya and Shimada[2] and Ohya *et al.*[64] reported the effects of pH, temperature and some salts on the rate of autolysis of fish muscle homogenate. Subsequent studies by Ohya and Sumi[65], Yoshimura *et al.*[66] and Fujii and Tomita[67] indicated the increase of TMA and NH_3 under sterilized condition, while Shimidu and Kiriyama[68] and Manita *et al.*[12] reported that some antiseptics considerably lowered the rate of autolysis.

In 1957, Shewan and Jones[69] showed the increase of some amino acids during autolysis. Recently, Makinodan *et al.*[62] showed the increase of amino acids and peptides during autolysis in the presence of chloramphenicol. They also indicated the changes of the SDS polyacrylamide gel electrophoresis pattern during autolysis[62]. It is noteworthy that they used blocks of fish muscle in the study.

In this chapter, the author re-examined autolysis of fish muscle both quantitatively and qualitatively, using "blocks" and "muscle homogenate".

MATERIALS AND METHODS

Autolysis of "blocks" and "muscle homogenate"

Carp(*Cyprinus carpio*) of approximately 900g was obtained from a commercial supplier. Carp was killed by decapitation, eviserated and washed by water immediately to be free of blood. Only white muscle was collected in an iced beaker. Small pieces of muscle were used as "blocks". These "blocks" were incubated at 37°C with 3 volumes of *Ringer* solution containing 3mM NaN_3 for indicated time in the figures and homogenized with a Waring blendor. "Muscle homogenate" was prepared by homogenizing 1 volume of muscle and 3 volumes of *Ringer* solution containing 3mM NaN_3 with a Waring blendor.

Quantitative analysis on autolysis

To stop the reaction, 5%(w/v) TCA was added to homogenate of "blocks" and "muscle homogenate". TCA soluble amino acids and peptides filtered through Toyo No.5c filter paper were determined by the method of Yemm and Cocking[70] and Lowry *et al.*[71], respectively. These values were expressed as the difference before and after incubation.

Qualitative analysis on autolysis

Homogenate of "blocks" was directly treated by SDS and analyzed with SDS polyacrylamide gel electrophoresis according to the method of Weber and Osborn[72]. For the molecular weight estimation, known marker proteins(bovine serum albumin, ovalbumin and cytochrome c) were run on the same gel.

Effect of protease inhibitors on autolysis

An aliquot of the solution of protease inhibitors was added to the "muscle homogenate" to get the final concentration as indicated in the table and incubated at 37°C for 24hr. Then, quantitative analysis was performed as described above.

RESULTS

1. Increase of TCA soluble peptides and amino acids during autolysis

As shown in Fig.1, the amounts of TCA soluble peptides(abbreviated as FPM in the figure) of "blocks" and "muscle homogenate" were increased as a function

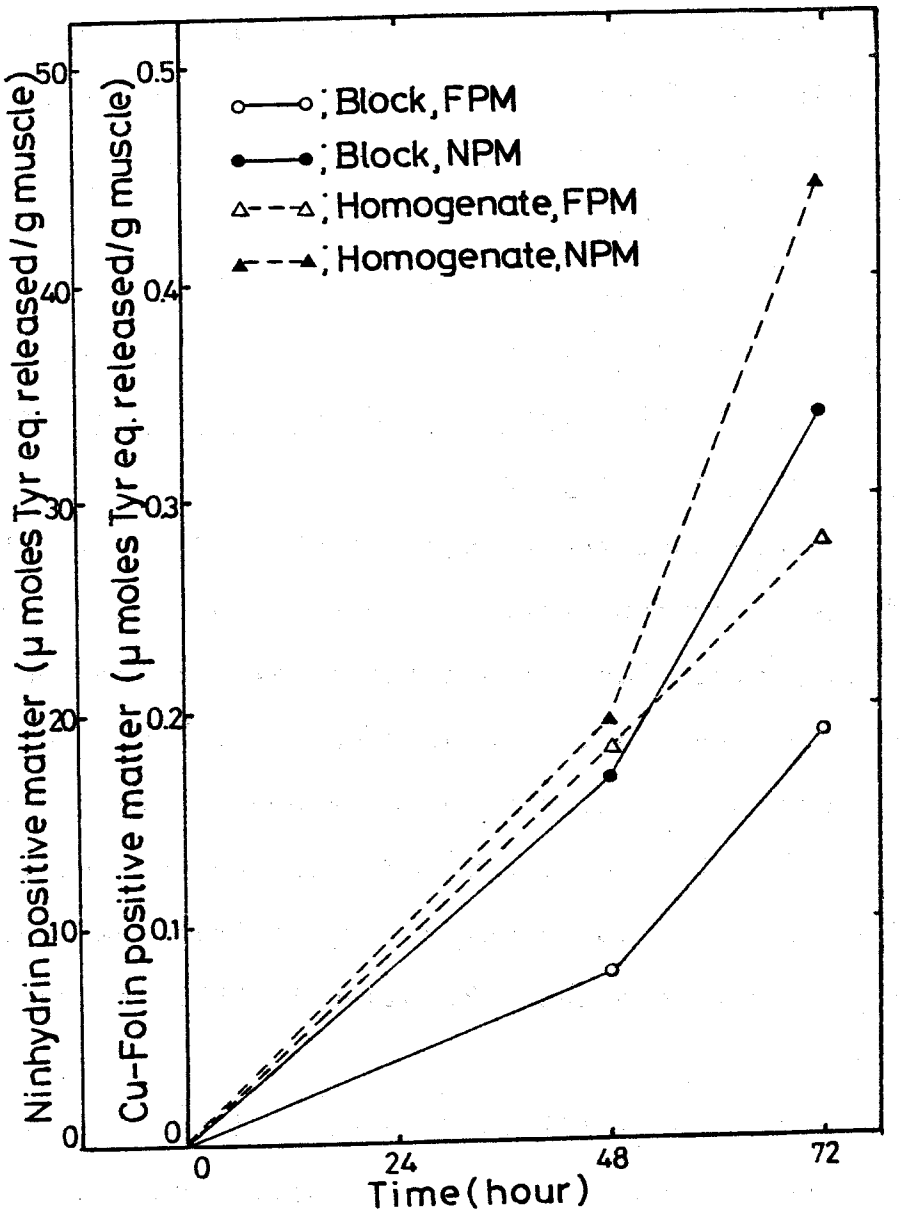

Fig.1. Increase of ninhydrin positive matter(NPM) and Cu-Folin positive matter(FPM) during incubation at 37°C as "blocks" and "muscle homogenate" in the presence of 3mM NaN_3.

of time as well as those of amino acids(abbreviated as NPM in the figure). Higher values were observed for both FPM and NPM in the case of "muscle homogenate" than "blocks". This fact suggests that some biochemical changes after death might take place more easily in "muscle homogenate" than "blocks".

2. Changes of SDS polyacrylamide gel electrophoretic pattern during autolysis

Fig.2. shows the changes of the SDS polyacrylamide gel electrophoretic pattern. The intensity of some protein bands indicated as "-" became less dense dependent on the incubation time. On the other hand, the intensity of some protein bands indicated as "+" became more dense dependent on the incubation time. Those changes suggest that some protein degradation might take place in "blocks" during autolysis.

The results obtained from Figs. 1 and 2 show that some proteolytic systems possibly concern with the post-mortem protein degradation. In order to clarify what proteolytic system might concern, autolysis of "muscle homogenate" in the presence of various protease inhibitors was quantitatively examined.

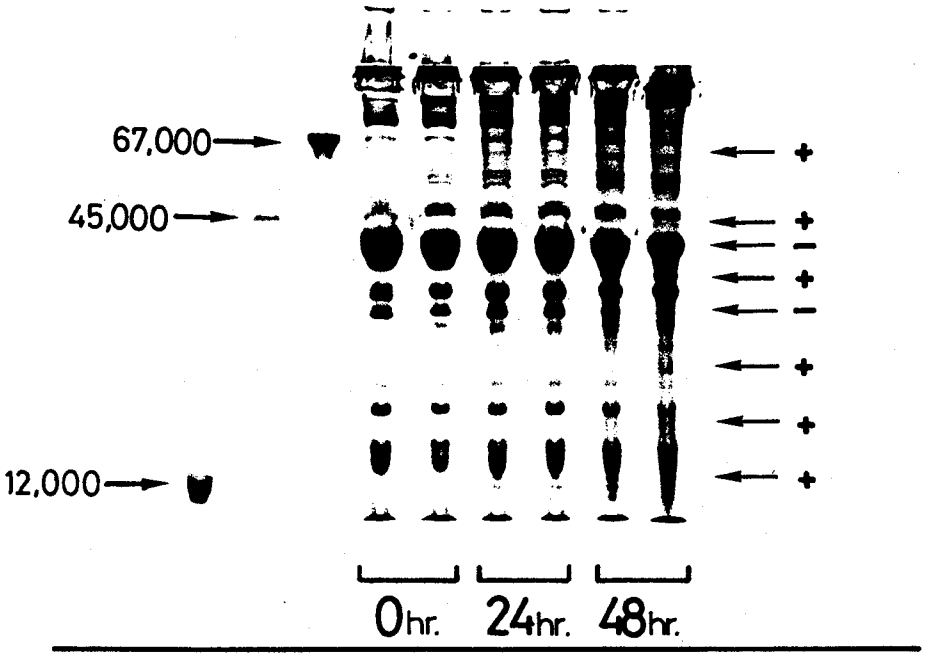

Fig.2. Changes of SDS polyacrylamide gel electrophoretic pattern of carp muscle "blocks" during incubation at 37°C in the presence of 3mM NaN_3. Electrophoresis was carried out as described in MATERIALS AND METHODS in 15% polyacrylamide slab gel. (+) shows the protein bands of which intensity gradually increased, and (-) shows the protein bands of which intensity gradually decreased. Molecular weight markers were as follows: bovine serum albumin(67,000), ovalbumin(45,000) and cytochrome c(12,000).

3. Effect of protease inhibitors on autolysis

As shown in Table 1, high concentration of EDTA and EGTA inhibited the increase of FPM and NPM. Pepstatin and leupeptin slightly inhibited those of FPM and NPM. On the other hand, iodoacetic acid greatly enhanced FPM. The reason for the increasing effect is not clear.

Table 1. Effect of protease inhibitors on autolysis.

Chemicals(final concentration)	Relative activity(%)	
	Cu-Folin	Ninhydrin
None	100	100
Pepstatin(1 μg/mℓ)	87	77
Leupeptin(2 μg/mℓ)	89	88
Dip-F(1 mM)	70	95
Soybean trypsin inhibitor(100 μg/mℓ)	113	102
DAN + Cu^{2+}(each 1 mM)	108	111
*p*CMB(0.06 mM)	89	94
Iodoacetamide(10 mM)	99	102
Iodoacetic acid(10 mM)	263	98
EDTA(1 mM)	88	52
(5 mM)	74	43
(10 mM)	56	51
EGTA(1 mM)	105	96
(5 mM)	85	53
(10 mM)	80	59

DISCUSSION

Shewan and Jones[69], Uchiyama *et al.*[59], Manita *et al.*[12] and Makinodan *et al.*[62] reported the increase of TCA soluble amino acids during autolysis of fish muscle. In particular, Makinodan *et al.*[62] showed the increase of peptides and the changes of SDS polyacrylamide gel electrophoretic pattern and suggested the participation of some proteolytic systems in fish muscle autolysis. Recently, Makinodan *et al.*[73] also showed metallo, serine and/or cysteine proteinases[74] might take part in autolysis. Results presented herein support their suggestions. Seki and Watanabe[75] reported that SDS polyacrylamide gel electrophoretic pattern of carp muscle myofibrils showed a gradual decrease in the intensity of the troponin T, α-actinin and a gradual appearance of the 15,000 dalton protein and several other protein bands in the presence of Ca^{2+} during autolysis.

The author cannot, however, eliminate the additional possibility that the post-mortem protein degradation may due to the non-enzymatical reaction, since the increase of TCA soluble amino acids and peptides were recognized to some extent even in the presence

of some protease inhibitors(Table 1). There have been many reports suggesting the protein degradation after death in mammalian and chicken muscle[18,28,36,38,44-47,76-100](see reviews[50,51,101]). On the other hand, some investigators have denied the proteolysis after death[20,25,49,102-105](see reviews[106,107]). Recently, Takahashi *et al.*[105] showed the release of a protein factor that modified the actin-myosin interaction in the post-rigor muscle. These discrepancies among the reports suggest that the process of the post-mortem protein degradation may be markedly complicated. Thus, it is very likely that there underlies any unknown mechanism in this process. The author, however, surmises that proteolysis proceeds in the post-mortem fish muscle from the results presented in this chapter. At present, it is unknown what kind(s) of proteinase may relate to the process. In CAHPTERS IV, V and VII, the author will show some possible proteinases relating to the post-mortem protein degradation.

CHAPTER III

CONFIRMATION OF AN ACID PROTEINASE(CATHEPSIN D), A NEUTRAL PROTEINASE(TENTATIVELY CALLED A SUB-ENDOPEPTIDASE)AND AN ALKALINE PROTEINASE IN THE MUSCLE OF VARIOUS FISH

As described in CHAPTER I, there has been known to distribute at least three proteinases, namely cathepsin D[3,6-12,108-120], sub-endopeptidase[15,21], and alkaline proteinase[5,10,108,122-128] in fish muscle(see reviews by Makinodan[16,17]). Recently, Makinodan *et al.*[129] showed that these proteinases exist in muscle of various species of fish including deep sea fish.

In this chapter, the author confirmed the existence of these three proteinases in muscle of various fish by examining the effect of pH on the activities and studied their distribution among various species of fish.

MATERIALS AND METHODS

Preparation of the crude enzyme solution

Carp(*Cyprinus carpio*), rainbow trout(*Salmo gairdnerii*), red sea bream(*Pagrus major*), flat fish(*Limanda herzensteini*) and black halibut(*Eopsetta grigorjewi*) were obtained alive. White croaker(*Argyrosomus argentatus*), common mackrel(*Trachurus japonicus*) and yellow tail(*Seriola quinqueradiata*) were obtained in rigor state. Ten grams of each fish muscle were homogenized with 20mℓ of 33mM phosphate buffer, pH 7.2, in a Waring blendor at top speed for a minute and stood at 4°C for 3hr. The supernatant obtained at 13,000xg for 30min was dialyzed against the same buffer to get the crude enzyme solution.

Measurement of the proteinase activities

Cathepsin D, sub-endopeptidase and alkaline proteinase activities were determined as the hydrolytic activities against hemoglobin, hemoglobin and casein, respectively, according to the method of Kunitz[130] or Anson[131].

Cathepsin D activity was assayed in a reaction mixture containing 1%(w/v) acid denatured hemoglobin, 330mM formate buffer, pH 3.0, and 0.5mℓ of the

enzyme solution in a total volume of 5mℓ. The reaction mixture was incubated at 37°C for an hour.

Sub-endopeptidase activity was assayed in a reaction mixture containing 1%(w/v) acid denatured hemoglobin, 112mM citrate-phosphate buffer, pH 7.2, and 0.5mℓ of the crude enzyme solution in a total volume of 2.5mℓ at 37°C for 2hr[15].

Alkaline proteinase activity was assayed in a reaction mixture containing 1%(w/v) heat denatured casein, phosphate buffer(100mM KH_2PO_4-50mM $Na_2B_4O_7$), pH 8.0, and 0.5mℓ of the enzyme solution in a total volume of 2.5mℓ at 63°C for an hour.

In each assay, reaction was stopped by adding 5% (w/v) TCA and TCA soluble products formed were colorimetrically determined according to the method of Lowry *et al.*[71] for cathepsin D and alkaline proteinase or Yemm and Cocking[70] for sub-endopeptidase. Each proteinase activity was expressed as nano moles of tyrosine equivalent released per mℓ of the enzyme solution per hour. In order to study the effect of pH on each proteinase activity, 100mM citrate-20mM Na_2HPO_4 buffer for cathepsin D or 100mM KH_2PO_4-50mM $Na_2B_4O_7$ buffer for sub-endopeptidase and alkaline proteinase was used.

Protein was determined according to the method of Lowry *et al.*[71] using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

1. Effect of pH on the activities of cathepsin D, sub-endopeptidase and alkaline proteinase from fish muscle

As shown in Fig.3, these three proteinase activities were detected in common mackerel, white croaker and black halibut muscle. Though the specific activities of these proteinases were different among species, almost the same pH dependencies of three proteinases were recognized.

2. Distribution of the activities of cathepsin D, sub-endopeptidase and alkaline proteinase from the muscle of various fish species

Table 2 shows the distribution of three proteinase activities in carp, red sea bream, rainbow trout, flat fish and yellow tail muscle. Each enzyme activity was detected in the muscle of all fish examined. However, each proteinase activity in rainbow trout and flat fish was relatively high,

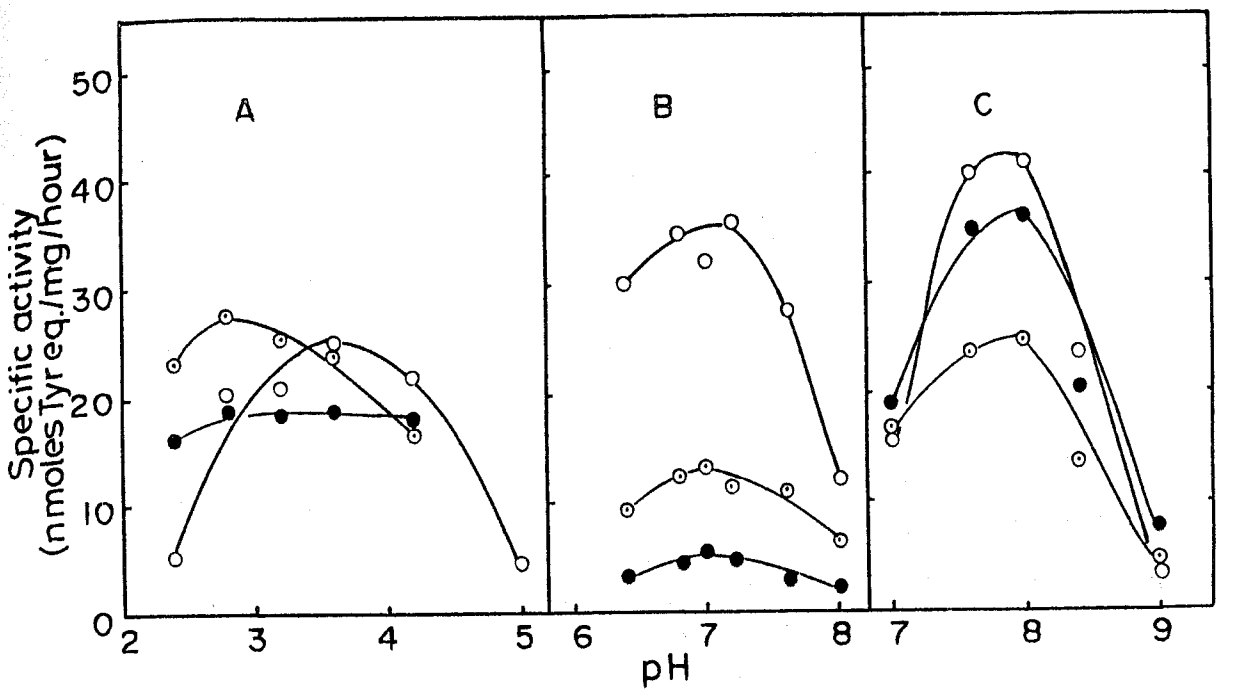

Fig.3. Effect of pH on the activities of cathepsin D (A), sub-endopeptidase(B) and alkaline proteinase(C) from the muscle of common mackerel(●—●), white croaker(⊙—⊙) and black halibut(○—○).

while that in red sea bream and yellow tail was low. Since the amounts of each muscle proteinase activity were different among species, these differences might reflect the rate of autolysis of various fish.

In earlier review by Tarr[132], the existence of the acid proteinase supposed to be cathepsin D was shown in fish muscle. Subsequent studies have

Table 2. Average values of three proteinase activities in the muscle of various species of fish.

	Cathepsin D	Neutral proteinase	Alkaline proteinase
Carp	14.5 (7.6 - 22.8)	15.8 (5.5 - 26.6)	36.5 (18.6 - 56.0)
Red sea bream	2.3 (1.5 - 3.0)	8.6 (5.0 - 12.4)	42.5 (38.0 - 51.2)
Rainbow trout	20.7 (16.5 - 25.5)	30.2 (19.3 - 40.0)	56.3 (44.9 - 69.4)
Yellowtail	5.6 (5.2 - 6.1)	11.4 (10.0 - 13.7)	33.5 (32.3 - 35.0)
Flat fish	61.3 (59.2 - 63.3)	31.2 (29.0 - 33.4)	69.7 (67.4 - 72.4)

The values of each proteinase activity were the average of 3-4 experiments.

revealed the distribution of cathepsin D in muscle of various species of fish[6-9,11,110,113,115,116], shrimp[117] and squid[118]. Muscle alkaline proteinase has been also known to distribute widely in various fish[5,10,123]. On the other hand, reports on sub-endopeptidase has been limited[15,111]. Recently, Makinodan *et al.*[129] showed that sub-endopeptidase distributes in the muscle of common mackerel, sardine, horse mackerel, white croaker, lizard fish and some deep-sea fish such as black halibut, soft eelpout, pectral rattail and thread-fin hakeling.

Data shown in this chapter confirm that these three proteinases widely distribute in the muscle of various species of fish. From the view point of the post-mortem protein degradation, it is unlikely that alkaline proteinase takes part in the process, since the proteinase requires rather high temperature around 60 °C for the activity. It is unknown, however, whether cathepsin D and/or sub-endopeptidase may participate in the process. In particular, cathepsin D has long been thought to be the only enzyme participating in autolysis. Hence, as described in the following chapter, the author purified cathepsin D from carp muscle and examined the possibility to concern with the muscle protein degradation after death.

CHAPTER IV

PURIFICATION AND PROPERTIES OF TWO ACID PROTEASES FROM CARP MUSCLE

SECTION I. CATHEPSIN D

Earlier studies on the intracellular proteases have been confined to acid proteases mainly to cathepsin D(EC.3.4.23.5). So far as muscle proteinases, cathepsin D has been thought to be the only enzyme to exist until 1960. Since Ohya and Shimada[2] showed that the optimum pH for autolysis was around pH 4.8, the existence of an acid proteinase in fish muscle has been surmised for a long time. In 1958, Siebert[108] and Saito and Samejima[3] demonstrated the enzymological properties of fish muscle cathepsin D for the first time using the crude muscle extract. Since then, many investigators have paid attentions on cathepsin D in relation to the postmortem protein degradation[7,9,11,108-120].

Siebert *et al.*[109] purified cathepsin D from cod muscle about 3,000-fold against the crude extract. Later, Bird *et al.*[8], Reddi *et al.*[114] and Ueno *et al.*[133] reported that fish muscle cathepsin D dis-

tributed in lysosomes. Reddi *et al.*[114] also showed that hydrolysis of the sarcoplasmic fraction by the muscle lysosomal fraction of winter flounder occurred at pH 3.0-7.0. Doke *et al.*[117] reported that the optimal hydrolysis of sarcoplasmic fraction by the crude muscle extract of *Tilapia mossambica* occurred at pH 5.0. However, it is unknown whether the hydrolysis is due to cathepsin D or not.

Whereas cathepsin D is physiologically considered to take part in the intracellular protein degradation in lysosomes[133-135], it is uncertain whether fish muscle cathepsin D concerns with autolysis. Therefore, the author purified cathepsin D from carp muscle as an electrophoretically homogeneous preparation and examined whether the enzyme participates in the post-mortem protein degradation.

MATERIALS AND METHODS

Measurement of the proteinase activities

Activities of cathepsins A, B, and C were measured by the hydrolysis of the following substrates, respectively: Z-Glu-Phe[136], BANA[137] and GTA[138].

Cathepsin D, sub-endopeptidase and alkaline proteinase activities were determined as described in CHAPTER III. Effect of cathepsin D on carp muscle myofibrils was examined by SDS polyacrylamide gel electrophoresis[72]. Carp muscle myofibrils were prepared according to the method of Tokiwa and Matsumiya[13].

Purification of carp muscle cathepsin D

Unless otherwise described, all operations were done at 4 °C. Carp(*Cyprinus carpio*) of approximately 900g were obtained from a commercial supplier. Carp was killed by decapitation, eviserated and washed by water immediately to be free of blood. Only white muscle of 1,000 g was collected and homogenized with 2,000mℓ of 100mM NaCl containing 1mM EDTA in a Waring blendor for 1 min at top speed. After standing for 30min, homogenate was centrifuged at 13,000 xg for 30min. The supernatant was dialyzed against 100mM NaCl containing 1mM EDTA overnight(crude solution). To the crude solution, 1M HCl was added to adjust pH 4.2 and the suspension was heated at 30 °C for 10min, and then pH of the suspension was re-adjusted to the original pH with 1M NaOH and the solution was centrifuged at 13,000xg for 10min to remove the precipitate(acid treated fraction).

Powdered ammonium sulfate was added to this fraction to get 60% saturation(w/v). After standing for 3hr, the precipitate was collected by the centrifugation at 13,000xg for 30min and dissolved in 100mM NaCl containing 1mM EDTA. This solution was dialyzed against 100mM NaCl containing 1mM EDTA overnight and centrifuged at 13,000xg for 10min to remove the precipitate(ammonium sulfate fraction). Cold acetone (-20°C) was added to this fraction to get 30%(v/v) acetone solution, stood for 2hr at -20°C and centrifuged at 13,000xg for 30min. Then, cold acetone was added to the supernatant to get 60%(v/v) acetone solution, stood for 2hr at -20°C and centrifuged at 13,000xg for 30min. The precipitate was collected and dissolved in 5mM Tris-HCl buffer, pH 8.0, containing 20mM NaCl, 1mM EDTA and 100mM sucrose(buffer A). The solution was dialyzed against buffer A overnight and centrifuged at 13,000xg for 10min to get the clear solution(acetone fraction). The acetone fraction was applied on a column(2.2x18cm) of DEAE-Sephadex A-50(from Pharmacia Fine Chemicals) equilibrated with buffer A and eluted with 0.02M, 0.12M, 0.22M and 0.52M NaCl in buffer A. The fraction eluted with 0.12M NaCl were pooled(DEAE-Sephadex

fraction). This fraction was dialyzed against 1% (w/v) glycine solution overnight, concentrated to 4mℓ with a Collodion bag and applied on a preparative electrofocusing(LKB 2117 Multiphor). The electrofocusing was performed at 8watts for 10 hr and then the proteolitically active fractions were desorbed from the gel by 50mM phosphate buffer, pH 7.0, containing 20mM NaCl, 1mM EDTA and 100mM sucrose(buffer B). Each fraction's pH was measured by suspending each gel in a small amount of distilled water. The active fractions were pooled and dialyzed against buffer B overnight(electrofocusing fraction). This fraction was concentrated to 3.6mℓ with a Collodion bag and applied on a column(1.5x90cm) of Sepharose 6B(from Pharmacia Fine Chemicals) equilibrated with buffer B. The elution was carried out with buffer B and the active fractions were pooled(Sepharose fraction).

RESULTS

1. Purification of cathepsin D from carp muscle

Fig.4 shows the elution profile of cathepsin D from a column of DEAE-Sephadex A-50. Cathepsin D was eluted with 0.12M NaCl. Cathepsin A was detected in the fractions eluted with 0.22M NaCl. Further purification and properties of cathepsin A will be described in the subsequent section. The DEAE-Sephadex fraction of cathepsin D was separated into many proteins by the electrofocusing(Fig.5). The highest activity of cathepsin D was observed in the fractions of pH 5.3-5.4. The elution profile of the electrofocusing fraction from a column of Sepharose 6B is shown in Fig.6. The enzyme was eluted as a single peak which corresponds to one obvious protein peak. Table 3 gives data on a typical purification of cathepsin D from carp muscle. Purification of the final enzyme preparation was about 2,000-fold over the crude enzyme solution with a yield of 3.6%. As shown in Fig.7, the final preparation gave a single band in disc electrophoretic analysis and the band coincided with the activity which was determined from another gel.

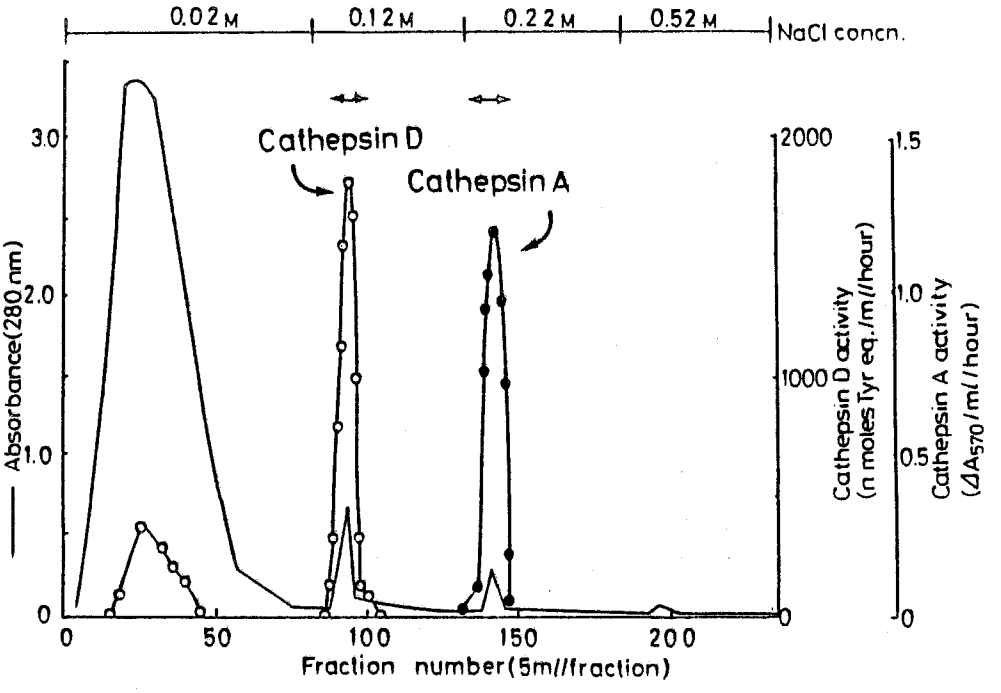

Fig.4. Elution profile of the acetone fraction of cathepsin D from DEAE-Sephadex A-50 chromatography. The acetone fraction(160mg) was applied on a column (2.2x18cm) and eluted with a stepwise manner of NaCl in buffer A at a flow rate of 40mℓ/hr. Then, fractions of 5mℓ were collected.

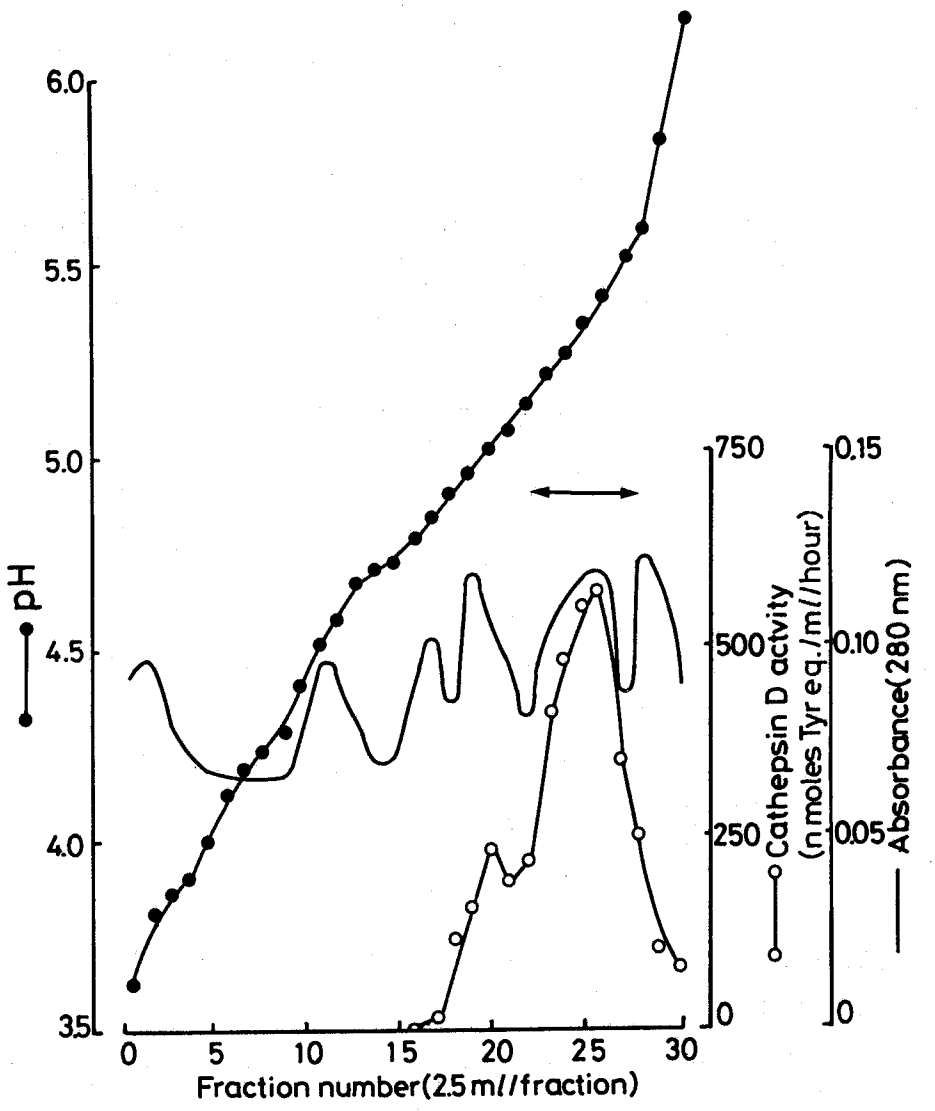

Fig.5. Elution profile of DEAE-Sephadex fraction of cathepsin D from a preparative electrofocusing of LKB 2117 Multiphor. The DEAE-Sephadex fraction (18mg) was applied on the slab gel of Ultrodex and electrophoresed. Then, fractions of 2.5mℓ were collected. Chromatographic procedures are detailed in MATERIALS AND METHODS.

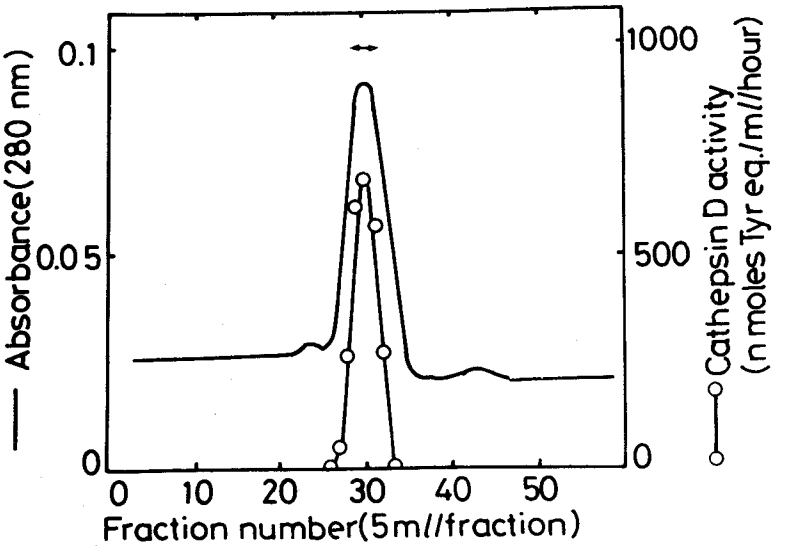

Fig.6. Elution profile of the electrofocusing fraction from Sepharose 6B gel filtration. The electrofocusing fraction(0.75mg) was applied on a column(1.5x90cm) of Sepharose 6B and eluted with buffer B at a flow rate of 4mℓ /hr. Then, fractions of 5mℓ were collected.

Table 3. Purification of cathepsin D from carp muscle.

Fraction	Volume (mℓ)	Protein (mg/mℓ)	Activity*	Purification (-fold)	Yield (%)
Crude enzyme	2720	6.54	124	1	100
Acid treatment (pH 4.2)	1570	3.80	140	1.9	65
Ammonium sulfate fractionation(0-60%)	85	10.2	1150	5.9	29
Acetone fractionation (30-60%)	50	3.50	1120	17	17
DEAE-Sephadex A-50 chromatography	40	0.200	910	240	11
Electrofocusing	17	0.075**	800	560	4.0
Sepharose 6B gel filtration	15	0.022	815	2000	3.6

*Nanomoles tyrosine equivalents per mℓ enzyme solution per hour.
**Calculated from the absorbance at 280nm.

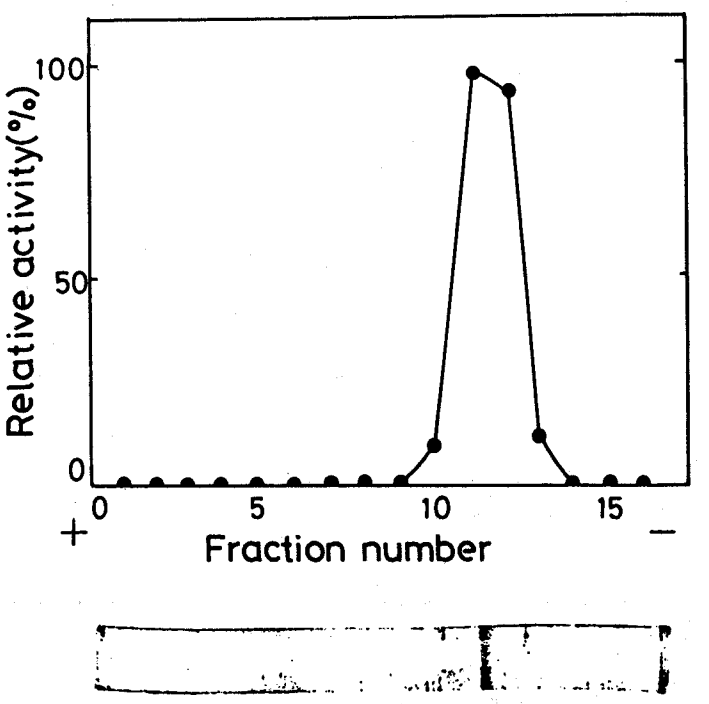

Fig.7. Disc electrophoretic analysis of the purified cathepsin D preparation. Sepharose fraction (40μg) was applied on 7.5% polyacrylamide gel and electrophoresed as described in MATERIALS AND METHODS. The gel was stained with Coomassie brilliant blue R-250. The graph demonstrates the enzyme activity determined by 3mm sections of another gel which was electrophoresed simultaneously.

Neither activity of cathepsins A, B, C nor subendopeptidase and alkaline proteinase was detected in the final enzyme preparation.

2. Some properties of carp muscle cathepsin D

Physicochemical properties

Fig.8 illustrates the estimation of the molecular weight by gel filtration. The molecular weight of carp muscle cathepsin D was estimated to be 41,000. From the position of the most active fraction in the electrofocusing(Fig.5), the isoelectric point was determined to be 5.4.

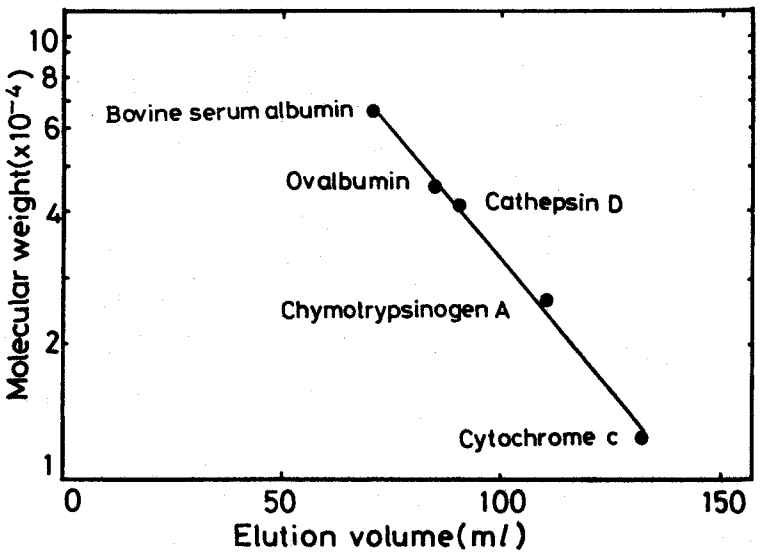

Fig.8. Molecualr weight estimation of carp muscle cathepsin D by Sephadex G-100 gel filtration. A column(1.5x90cm) was equilibrated with buffer B and eluted with the same buffer at a flow rate of 7mℓ/hr. Molecular weight markers were as follows: bovine serum albumin(67,000), ovalbumin(45,000), chymotrypsinogen A(25,000) and cytochrome c(12,400).

Enzymatic properties

(1) Effect of inhibitors on the activity

As shown in Table 4, the enzyme activity was completely inhibited by the addition of pepstatin (1μg/mℓ) and DAN+Cu^{2+}(each 1mM) which are known to inhibit an aspartic proteinase[74,140]. Dip-F and soy bean trypsin inhibitor which are known to inhibit a serine proteinase[74,140], had no effect on the activity. DTT+EDTA(each 2mM) which is known to activate a cysteine proteinase[74,140], and *p*-CMB(1mM) which is known to inhibit a cysteine proteinase[74,140], had no effect on the activity. DTT+EDTA and *o*-phenanthroline(1mM), which are known to inhibit a metallo proteinase[74,140], had no effect on the activity. Solvents such as ethanol, methanol and *iso*-propanol as well as 1mM cysteine and glutathione had no effect. But, 2-mercaptoethanol increased the activity about 20%.

(2) Effect of temperature on the activity

The optimum temperature for 1hr incubation was around 50°C(Fig.9). When the enzyme was pre-incubated in the absence of substrate in 1.0M formate buffer, pH 3.2, for 30min, it was fairly stable at 40°C, retaining about 85% of the full activity, but lost about 85% of the activity at 50°C.

Table 4. Effect of inhibitors on the activity of carp muscle cathepsin D.

Inhibitor	Concentration	Relative activity(%)
None		100
Pepstatin	1 μg/mℓ	0
DTT + EDTA	0.2 mM (each)	102
Dip-F	1 mM	107
Soy bean trypsin inhibitor	100 μg/mℓ	112
o-Phenanthroline	1 mM	100
pCMB	1 mM	90
DAN + Cu^{2+}	1 mM (each)	0

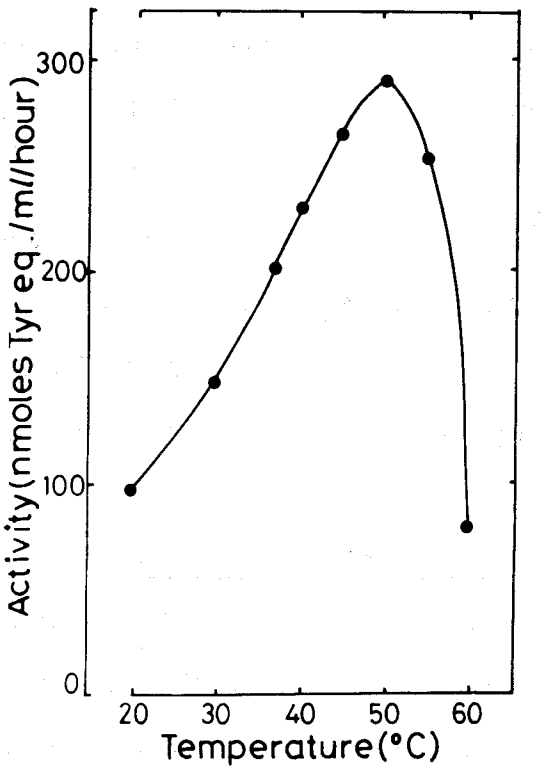

Fig.9. Effect of temperature on the activity of carp muscle cathepsin D. Incubation was carried out at the indicated temperature.

(3) Effect of pH on the activity

Effect of pH on the hydrolysis of hemoglobin by the enzyme is shown in Figs. 10 and 11. When 1.0M buffer(final concentration of 0.6M) was used, optimum pH was found to be 2.6-2.8(Fig.10). At the acidic limit, the activity fell off rapidly with pH and on the less acidic side, the curve showed a distinct shoulder near pH 4.0. The pH curve obtained with 0.2M buffer(final concentration of 0.12M) showed the optimum activity near pH 3.2 and did not form any shoulder(Fig.11). When the enzyme was pre-incubated in the absence of substrate at 37°C for 30min with 50mM glycine-HCl(pH 1.0-2.7), formate(pH 3.0-5.0), acetate(pH 5.0-6.0) and phosphate(pH 6.0-8.0) buffers, the activity was stable in the range of pH 3.0-5.0 and rather stable in the range of pH 5.0-7.0, while it was quite labile at pH 2.0.

(4) Effect of the enzyme on the hydrolysis of carp muscle myofibrils

As illustrated in Fig.12, at pH 3.2, the high molecular weight and 45,000 dalton bands vanished and many distinct bands appeared after 24 hr incubation. This phenomenon was not observed in the presence of pepstatin. At pH 6.5, the electrophoretic pattern

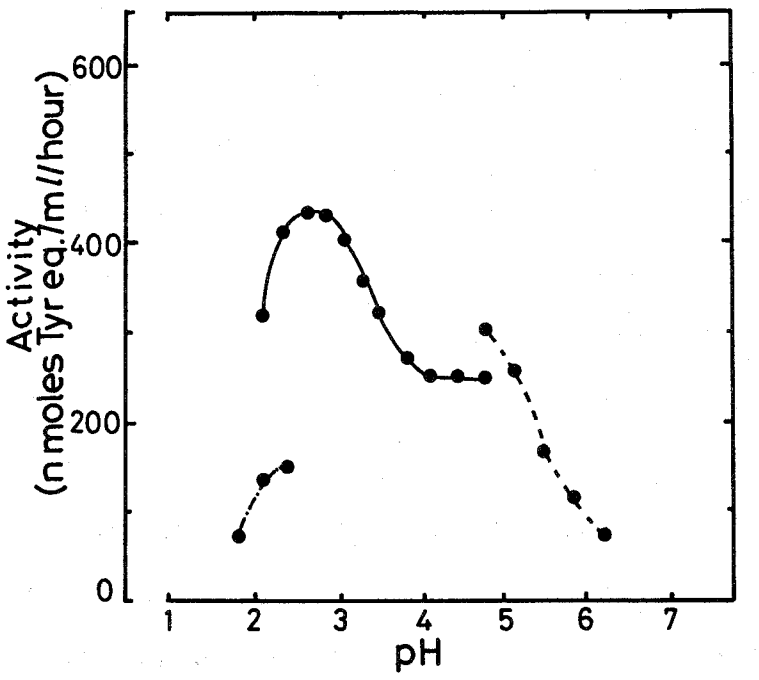

Fig.10. Effect of pH on the activity of carp muscle cathepsin D in 0.6M buffer. The buffers used were as follows: glycine-HCl(●-·-●), formate(●——●) and acetate(●---●).

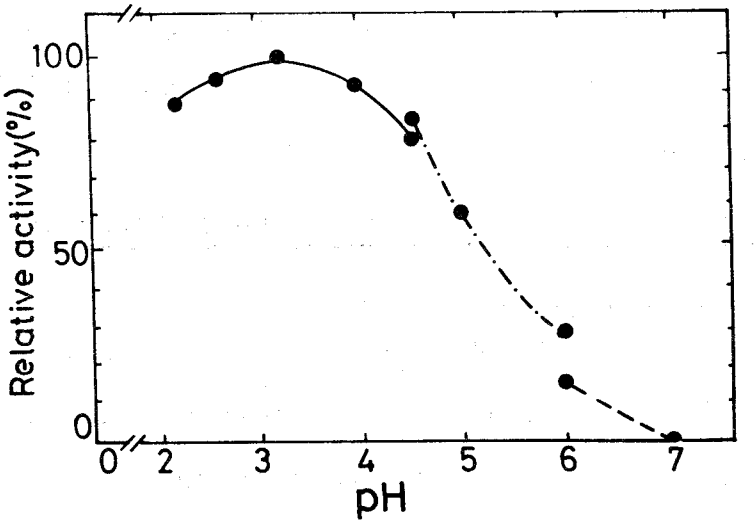

Fig.11. Effect of pH on the activity of carp muscle cathepsin D in 0.12M buffer. The buffers used were as follows: formate(●——●), acetate(●-·-●) and phosphate(●--●).

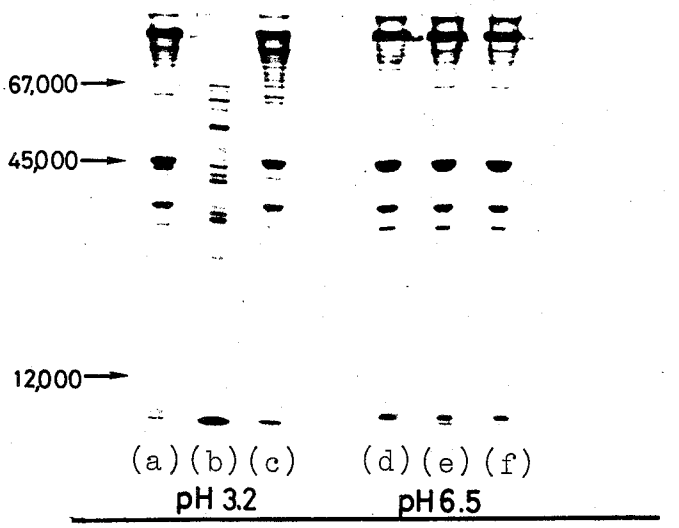

Fig.12. SDS polyacrylamide gel electrophoretic analysis on carp muscle myofibrils treated with carp muscle cathepsin D. A reaction mixture which contained 5.5μg cathepsin D, 170mM formate buffer(a-c) or phosphate buffer(d-f), 1mg myofibrils, 100ppm chloramphenicol and 1μg/mℓ pepstatin(c,f) or above mentioned buffer(a,b,d,e) in a total volume of 2.5mℓ was incubated at 37°C for 24hr. SDS polyacrylamide gel electrophoresis was carried out as described in MATERIALS AND METHODS on 15% polyacrylamide slab gel: (a); zero hour incubation at pH 3.2, (b); without pepstatin at pH 3.2, (c); with pepstatin at pH 3.2, (d); zero hour incubation at pH 6.5, (e); without pepstatin at pH 6.5, (f); with pepstatin at pH 6.5.

hardly changed after incubation either in the presence or absence of pepstatin.

Effect of pH on the hydrolysis of carp muscle myofibrils in the absence or presence of 3M urea is presented in Fig.13. The optimum pHs for the hydrolysis of myofibrils in the absence and presence of 3M urea were found to be 3.2 and 4.0, respectively. Myofibrils were not hydrolyzed above pH 6.0. The activity in the presence of 3M urea was lower than that in the absence of it at every pH examined.

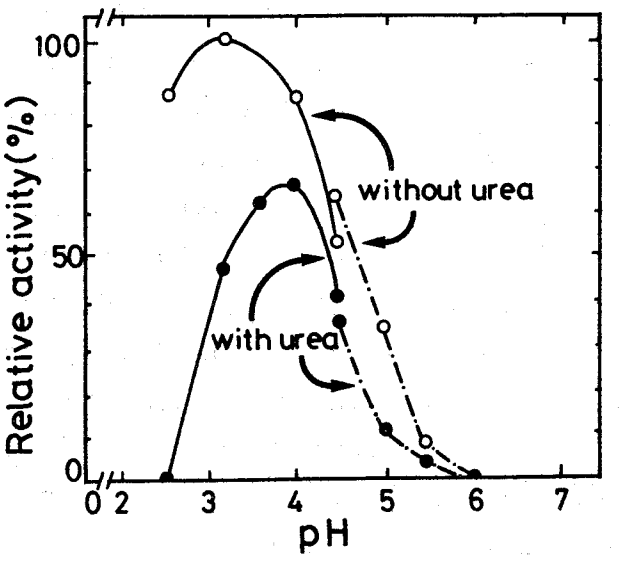

Fig.13. Effect of pH on the hydrolysis of carp muscle myofibrils by carp muscle cathepsin D in the presence (●——●) or absence(○——○) of 3M urea. The buffers used(final concentration of 0.12M) were as follows: formate(pH 2.4-4.5), acetate(pH 4.5-6.0).

3. Intracellular distribution of carp muscle cathepsin D[135)]

It is reported that fish muscle cathepsin D[8,114,133,214,215)] exists in lysosomes as well as organ cathepsin D. In order to examine the intracellular distribution of the enzyme, tissue must be disintegrated to the desired degree. In case of fish muscle, the muscle tissue cannot be destroyed by such gentle homogenization as for organ tissues[216)], abundant of lysosomal enzyme activity being remained in myofibrillar fraction. However, severe homogenization will induce more destruction of particles. In anyway, the author adopted a Waring blendor for homogenizing muscle tissue.

Carp ordinary muscle was homogenized with 4 volumes of ice cold 0.25M sucrose containing 1mM EDTA in a Waring blendor at top speed for 30sec. The homogenate was fractionated with differential centrifugation: 800×g for 10min for the myofibrillar fraction(Mf); 10,000×g for 10min for the mitochondrial fraction(Mit); 27,000×g for 30min for the lysosomal fraction(Lys); 79,000×g for 90min for the microsomal fraction(Mic) and the final supernatant fraction(Sup). Each pellet was suspended in 0.25M sucrose containing

1mM EDTA and 0.1%(v/v) Triton X-100. Appropriate Triton X-100 was also added to the supernatant fraction.

Fig.14 shows the intracellular distribution of carp muscle cathepsin D. Relative specific activity was the highest in Lys fraction, but there was no significance between the values of Lys and Mic fraction. Thus, clear uneven localization of cathepsin D in lysosomes was not shown in the present study. The cause may be attributed to the rupture of lysosomes by the homogenization, possible heterogeneous population of lysosomes[114,215], or muscle lysosomes being small size and specific gravity in comparison with those of organs[217].

DISCUSSION

Makinodan and Ikeda[11,136] reported some properties of carp muscle cathepsin D using the partially purified preparation. The results presented here almost coincide with theirs. The author also examined the effect of carp muscle cathepsin D on autolysis using the purified preparation.

Molecular weights of 32,000, 38,000 and 50,000

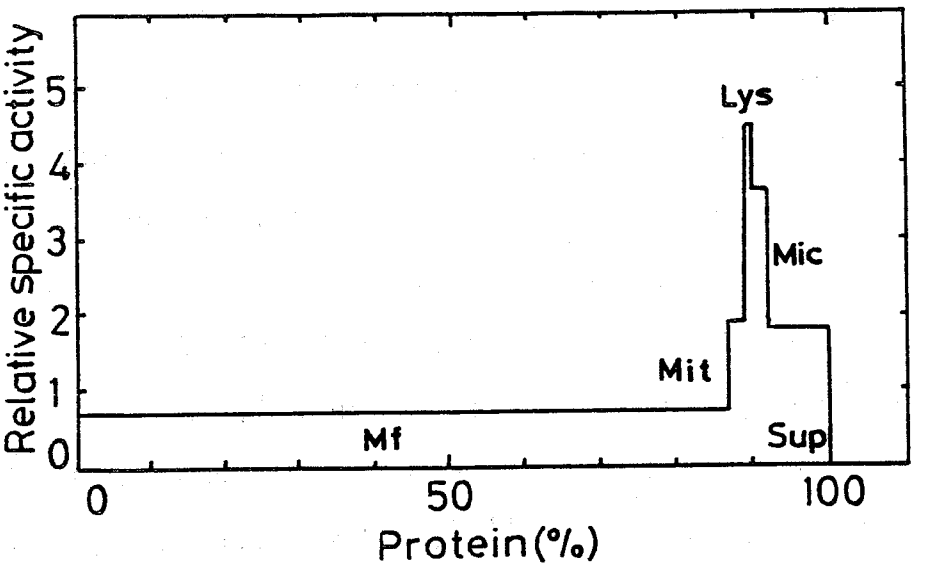

Fig.14. Intracellular distribution pattern of carp muscle cathepsin D. The pattern was represented according to the method of Duve *et al.*[216] The results are the means of 5 experiments.

are reported for the muscle cathepsin D from winter flounder[114], *Tilapia mossambica*[117] and cod[7,109], respectively. Siebert[141] stated that molecular weight should not be taken as a strong criterion for cathepsin D since that of cathepsin D showed species and tissue diversity. As for cathepsin D from rat and chicken muscle, 36,000[142], and 42,000-45,000[143] have been reported, respectively. The activity of carp muscle cathepsin D was inhibited by pepstatin and DAN+Cu^{2+}, but hardly affected by DTT+EDTA, Dip-F,

soy bean trypsin inhibitor, *o*-phenanthroline and *p*-CMB(Table 4). The molecular weight was estimated to be 41,000(Fig.8) and the iso-electric point to be pH 5.4(Fig.5). From these results, the enzyme was identified as cathepsin D under the classification by Barret[134]. Carp muscle cathepsin D was not affected by cysteine(data not shown), and this result was similar with muscle cathepsin D from chicken[143] and *Tilapia mossambica*[117]. The activity was stimulated about 40% by 2-mercaptoethanol. This result coincided with that reported by Makinodan and Ikeda[11].

The optimum pH with 1M buffer(final concentration of 0.6M) was 2.6-2.8, but with 0.2M buffer(final concentration of 0.12M) it was near 3.2. The cause of such difference of optimum pH is not clear. The optimum pH is possibly influenced by the effect of the ionic strength[144]. The pH activity curve with 1.0M buffer showed a shoulder near pH 4.0. It is well-known that cathepsin D shows a shoulder on the less acidic side of the optimum pH[134]. The optimum pH value, 3.2, was lower in comparison with 4.6 for cod muscle cathepsin D against urea denatured hemoglobin[7,109]. About 25% of the maximum activity was found around pH 6.5 on cod muscle cathepsin D[7,109],

but no activity was observed at such less acidic pH with carp muscle cathepsin D(Figs. 10 and 11). The optimum pH of *Tilapia mossambica* muscle cathepsin D with 40mM acetate buffer against acid denatured hemoglobin is 2.8 and the curve of pH dependency of the activity showed a shoulder near pH 4.0[117]. The difference of the optimum pHs among cod, carp and *Tilapia mossambica* depends possibly on the different substrates used rather than species specificity. As for cathepsin D from mammalian muscle, the optimum pHs of 4.0 have reported with 0.05M-0.25M acetate buffer against urea denatured hemoglobin[44,142].

The optimum temperature of 1hr incubation was about 50°C, but the enzyme was very labile at the same temperature in the absence of the substrate. The protective effect of the substrate against heating is generally recognized[10]. The optimum temperature of chicken muscle cathepsin D was 40°C and the activity at 50°C was very low[143]. The property is different from that of carp muscle cathepsin D(Fig.9).

The acid proteinase activity has been observed in fish muscle for a long time and it was the only enzyme known to exist in muscle before 1960. On the other hand, post-mortem pH of fish muscle does

not drop so much as mammalian muscle. Recently, Makinodan *et al.*[62] reported that the ultimate pH for post-mortem carp muscle is 6.3-6.6. This fact makes it difficult to relate muscle cathepsin D to autolysis. Carp muscle cathepsin D showed a typical endopeptidase nature, since it acted on hemoglobin and released nona or deca peptides[15], and the released products from hemoglobin were further hydrolyzed by muscle cathepsin A[136]. These facts seem to suggest that muscle cathepsin D is possibly involved in the initial stage of the post-mortem protein degradation.

The author sought the activity around neutral pH range of carp muscle cathepsin D. The optimum pH for the hydrolysis of hemoglobin was near 3.2 (Figs. 10 and 11), but that for myofibrils in the presence of 3M urea was near 4.2(Fig.13). However, considering the decrease in the maximum activity in the presence of 3M urea, this shift in optimum pH relates to the unstability of the enzyme due to lower pH conditions and the presence of urea[134,145]. Myofibrils were not hydrolyzed at pH 6.5(Fig.12). This fact denies the possibility of the participation of muscle cathepsin D in autolysis. Reddi *et al.*[114]

carried out an interesting study about cathepsin D from winter flounder muscle and recognized that muscle lysosomal fraction hydrolyzed the sarcoplasmic fraction at pH 3-7. But the lysosomal fraction might contain some protease(s) other than cathepsin D.

Kazakova and Orekhovich[146] reported that when rat liver cathepsin D was coupled to activated Sepharose, the enzyme were totally inactive in the acidic medium and exhibited the maximum activity at pH 7.0 against hemoglobin. If this is true, the relation of lysosomal-bound cathepsin D to autolysis may be possible. However, the same experiment in other laboratory could detect no change in pH optimum in comparison with the free enzyme[134], nor could the author for carp muscle cathepsin D.

From the above results, the participation of muscle cathepsin D in autolysis is very doubtful. Geist and Crawford[115] reported that fish muscle cathepsin D did not play any significant role in the post-mortem protein degradation during cold storage. Cathepsin D, however, distributes widely in tissues of various animals[134]. This fact may suggest that cathepsin D is probably a physiologically important enzyme and takes part in the

metabolism of proteins in the cell.

SECTION II. CATHEPSIN A

Since cathepsin A(EC.3.4.16.1) was first found in bovine spleen[148], it has been shown to distribute widely in mammalian tissues[149]. However, the homogeneous preparations have been only obtained from bovine spleen[56,150], rat liver[151,152], pig kidney[153,154], but not from muscle. Makinodan and Ikeda[14] clarified the existence of cathepsin A in fish muscle and investigated the properties of the enzyme. The enzyme preparation they used still contained some contaminated proteins[14]. Thus, it has been desirable to obtain a homogeneous preparation to know preciser properties and its relation to autolysis. By using preparative electrofocusing, the author purified cathepsin A from carp(*Cyprinus carpio*) muscle as an electrophoretically homogeneous preparation and reinvestigated its properties. The author also examined whether the enzyme participates in the postmortem protein degradation.

MATERIALS AND METHODS

Measurement of the proteinase activities

Cathepsin A activity was measured by the hydro-

lysis of Z-Glu-Phe according to the modified method of Makinodan and Ikeda[136], unless otherwise described. The reaction mixture contained 120mM acetate buffer, pH 5.0, 7mM Z-Glu-Phe and the enzyme solution in a total volume of 1.0mℓ was incubated at 37°C for 1hr. The reaction was stopped by 2.5% TCA, and released phenylalanine was colorimetrically determined by the method of Yemm and Cocking[70]. Activity was expressed as nanomoles of phenylalanine released or the absorbance at 570nm per mℓ of the enzyme solution per hour. Effect of cathepsin A on the muscle myofibrils prepared according to the method described in SECTION I were examined by SDS polyacrylamide gel electrophoresis[72]. Specific conditions and other additions are described in the legends. Disc electrophoresis was carried out according to the method of Davis[139]. Protein was determined according to the method of Lowry *et al.*[71] using bovine serum albumin as the standard, or the absorbance at 280nm.

Activities of cathepsins B, C, and D were determined according to the method of Barret[137], DE LA Haba *et al.*[138] and Makinodan and Ikeda[120], respectively.

Purification of carp muscle cathepsin A

Unless otherwise described, all operations were carried out at 4°C. Cathepsins A and D were separated by DEAE-Sephadex A-50 chromatography as described in SECTION I. Cathepsin A fractions eluted from DEAE-Sephadex were pooled, dialyzed overnight against 1% glycine(w/v) solution and concentrated to 4.2mℓ with a Collodion bag. The concentrated solution was applied on a preparative electrofocusing at 3-8watts for 10hr, each gel was packed into a small column and eluted with an aliquot of 0.2M acetate buffer, pH 5.0. The pH of each fraction was measured by suspending gel in small amount of distilled water. To remove Ampholine contaminated, each fraction was dialyzed against 0.2M acetate buffer, pH 5.0. The active fractions were pooled and concentrated to 3mℓ with a Collodion bag. The concentrated solution was applied on a column(1.5x 90cm) of Sepharose 6B(from Pharmacia Fine Chemicals) equilibrated with 50mM phosphate buffer, pH 7.0, containing 20mM NaCl, 1mM EDTA and 100mM sucrose (buffer B). The elution was carried out with the same buffer and proteolytically active fractions were pooled as the final preparation.

RESULTS AND DISCUSSION

1. Purification of cathepsin A from carp muscle

As shown in Fig.4 of SECTION I, cathepsin A eluted with 0.22M NaCl from DEAE-Sephadex A-50. Fig.15 shows the elution profile from the electrofocusing. The highest activity was observed around pH 4.6 fraction. The elution profile of this fraction from Sepharose 6B is shown in Fig.16. Table 5 shows a typical purification chart of cathepsin A from carp muscle. Purification of the final enzyme preparation was about 1,700-fold over the crude enzyme solution with a yield of 1.5%. As shown in Fig.17, the final preparation gave a single band in disc electrophoretic analysis and the band coincided with the activity which was determined from another gel. Neither activity of cathepsins B, C, nor D was observed in the final preparation.

2. Some properties of carp muscle cathepsin A

Physicochemical properties

The molecular weight of carp muscle cathepsin A was determined by gel filtration on Sepharose 6B (Fig.18). The molecular weight was estimated to be 36,000. Makinodan and Ikeda[136] showed 34,000

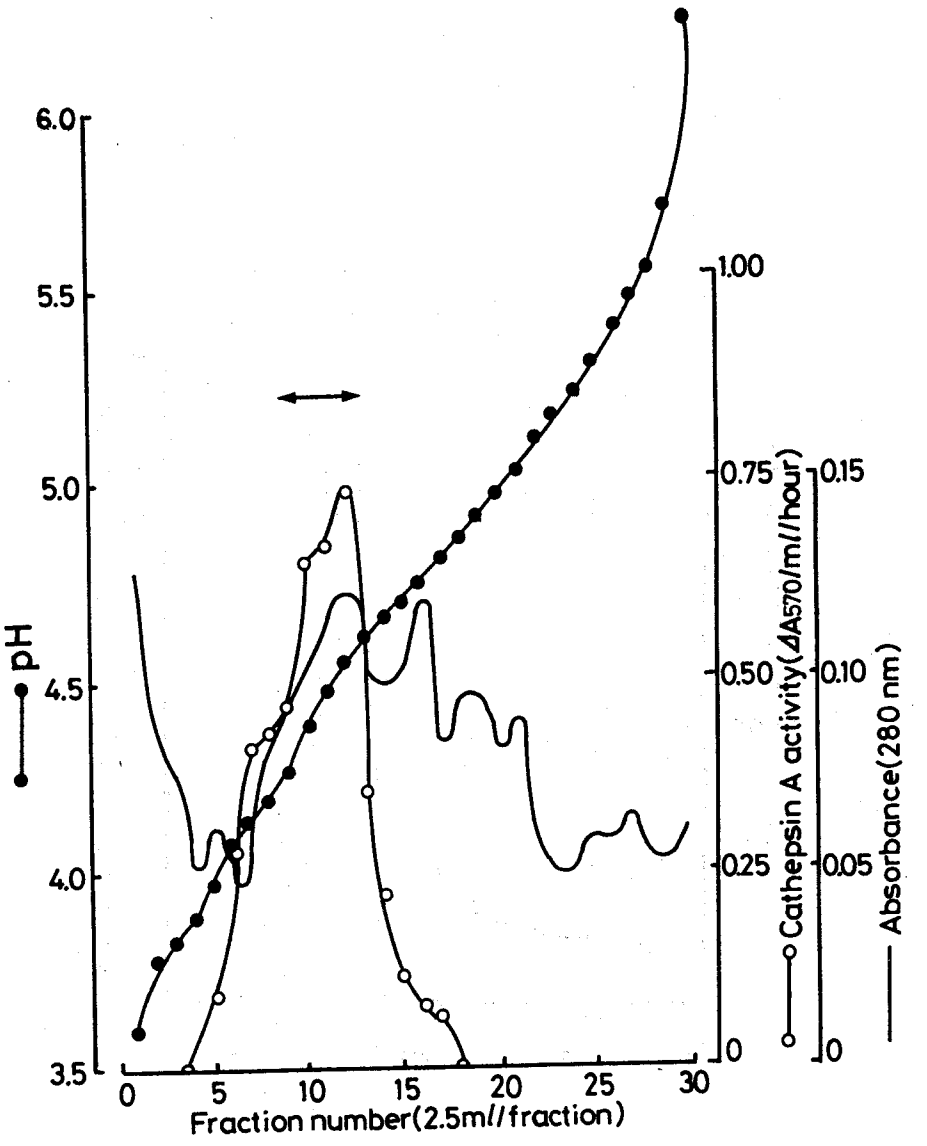

Fig.15 Elution profile of DEAE-Sephadex fraction of cathepsin A from a preparative electrofocusing on LKB 2117 Multiphor. DEAE-Sephadex fraction(14mg) was applied on the slab gel of Ultrodex and electrophoresed as described in MATERIALS AND METHODS. Then, fractions of 2.5mℓ were collected.

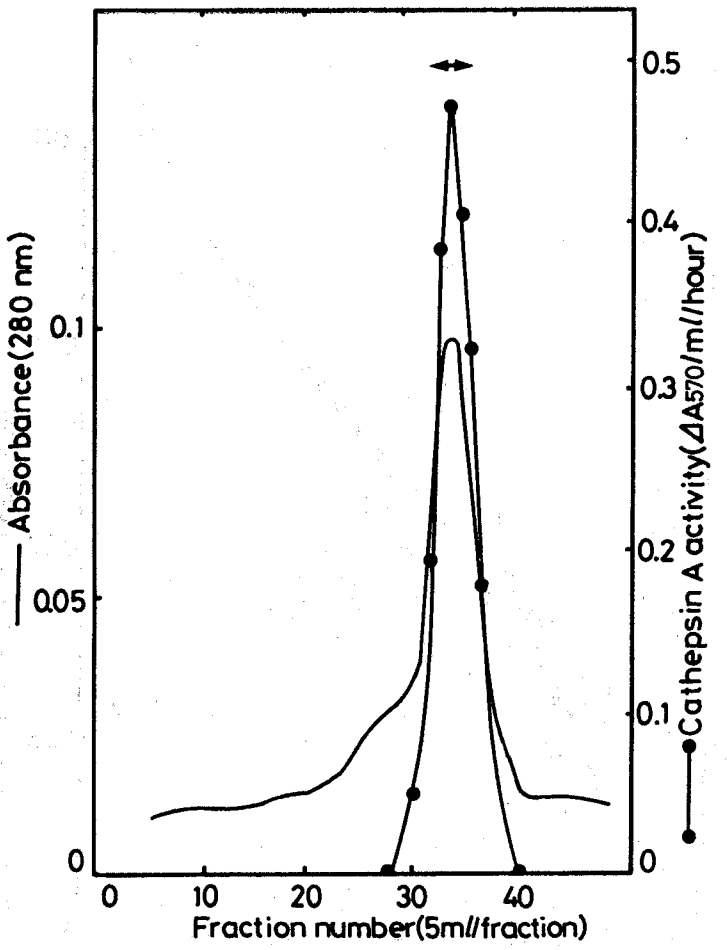

Fig.16 Elution profile of the electrofocusing fraction of cathepsin A from Sepharose 6B gel filtration. The electrofocusing fraction(0.82mg) was applied on a column (1.5x90cm) of Sepharose 6B and eluted with buffer B at a flow rate of 6mℓ/hr. Then, fractions of 5mℓ were collected.

Table 5. Purification of cathepsin A from carp muscle.

Fraction	Volume (mℓ)	Protein (mg/mℓ)	Activity*	Purification (-fold)	Yield (%)
Crude enzyme	2720	6.54	0.279	1	100
Acid treatment (pH 4.2)	1570	3.80	0.394	2.5	82
Ammonium sulfate fractionation(0-60%)	85	10.2	3.43	8	38
Acetone fractionation (30-60%)	50	3.50	4.06	28	27
DEAE-Sephadex A-50 chromatography	35	0.103	1.64	379	8.8
Electrofocusing	18	0.072**	1.92	640	4.6
Sepharose 6B gel filtration	8	0.020	1.45	1700	1.5

*Nanomoles phenylalanine equivalents per mℓ enzyme solution per hour.
**Calculated from the absorbance at 280nm.

and Iwata *et al.*[155] showed 81,000 using partially purified preparation. The reason for those discrepancies is unknown. From the position of the most active fraction in the electrofocusing(Fig.15), the isoelectric point of carp muscle cathepsin A was estimated to be pH 4.6.

Enzymatic properties

(1) Effect of the inhibitors on the activity

Table 6 shows the effect of protease inhibitors on the activity. Chelating reagents, such as EDTA EGTA or *o*-phenanthroline had no effect. However, Dip-F, PMSF, iodoacetamide, antipain and $HgCl_2$ comp-

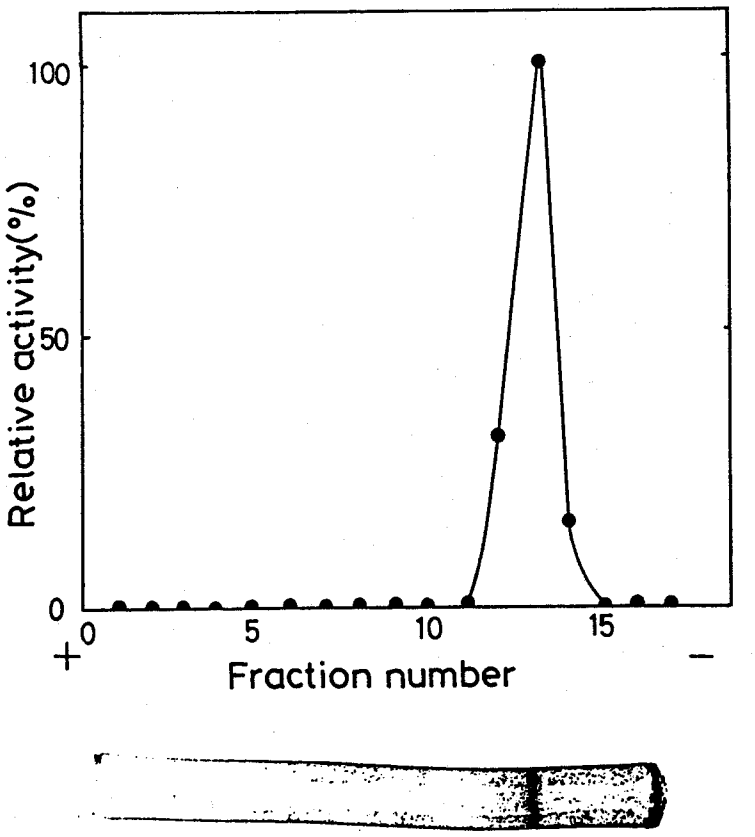

Fig.17. Disc electrophoretic analysis of the purified carp muscle cathepsin A preparation. Sample (50μg) was applied on 7.5% polyacrylamide gel and electrophoresed as described in MATERIALS AND METHODS. The gel was stained with Coomassie brilliant blue R-250. The graph demonstrates the enzyme activity determined by 3mm sections of another gel which was electrophoresed simultaneously.

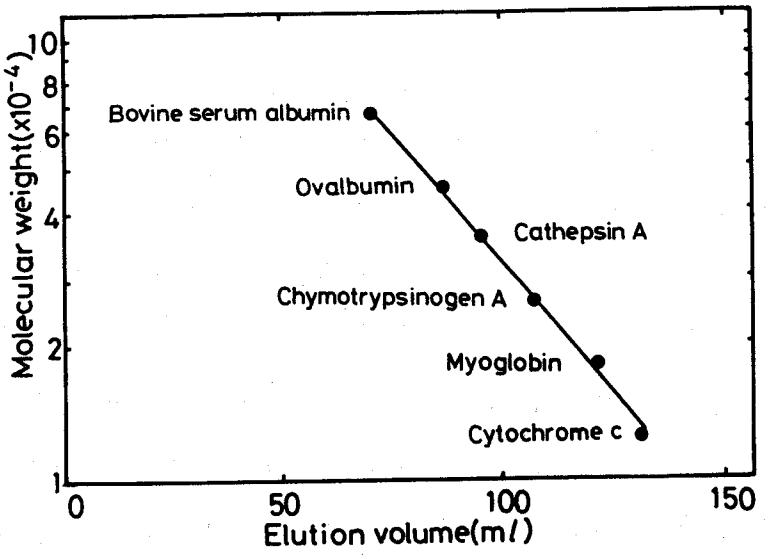

Fig.18 Molecular weight estimation of carp muscle cathepsin A by Sephadex G-100 gel filtration. A column(1.5x90) was equilibrated with buffer B and eluted with the same buffer at a flow rate of 7mℓ/hr. Molecular weight markers were as follows: bovine serum albumin(67,000), ovalbumin(45,000), chymotrypsinogen A(25,000), myoglobin(18,000), cytochrome c(12,400).

Table 6. Effect of inhibitors on the activity of carp muscle cathepsin A.

Inhibitors	Concentration	Relative activity(%)
None		100
EDTA	1 mM	100
o-Phenanthroline	1 mM	100
2-Mercaptoethanol	1 mM	142
Iodoacetamide	1 mM	0
Dip-F	0.1 mM	0
PMSF	0.1 mM	4
$HgCl_2$	0.1 mM	8
NaI	1 mM	125
NaBr	1 mM	123
Antipain	2 µg/ml	0

letely inhibited the activity. Those findings coincided with those of Makinodan and Ikeda[136]. Iwata *et al.*[156] reported that 2-mercaptoethanol reduced the activity to 71%, but as shown in Table 4, 2-mercaptoethanol, NaI and NaBr activated the activity. From the result described above, serine and cysteine residues in the enzyme molecule may be involved in the active site.

(2) Effect of temperature on the activity

Fig.19 shows the temperature dependency of the activity. The enzyme showed the maximum activity at 40°C, while it showed only 35% of the maximum activity at 50°C.

(3) Effect of pH on the activity

As shown in Fig.20, the enzyme was active between pH 3.0-6.0 with the optimum pH at 5.0. No activity was detected at pH 6.5 that was near to the post-mortem pH range[59-63]. Makinodan and Ikeda[136] and Iwata *et al.*[156] reported that the optimum pH for Z-Glu-Phe was pH 5.0.

(4) Substrate specificity

Z-Glu-Tyr has been known to be a typical substrate for cathepsin A from mammalian tissues[148]. However, carp muscle cathepsin A showed more affi-

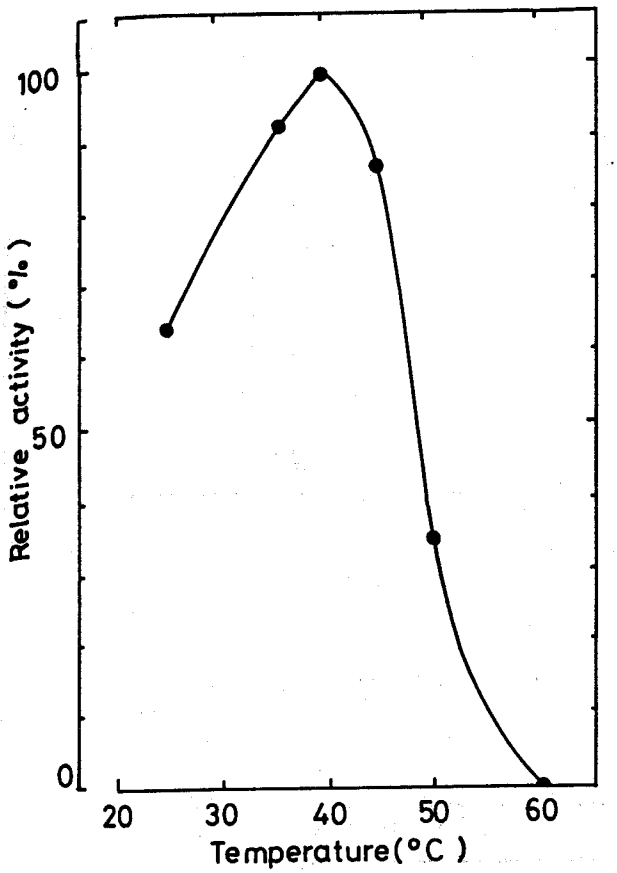

Fig.19 Effect of temperature on the activity of carp muscle cathepsin A. Incubation was carried out at the indicated temperature and the activity was determined.

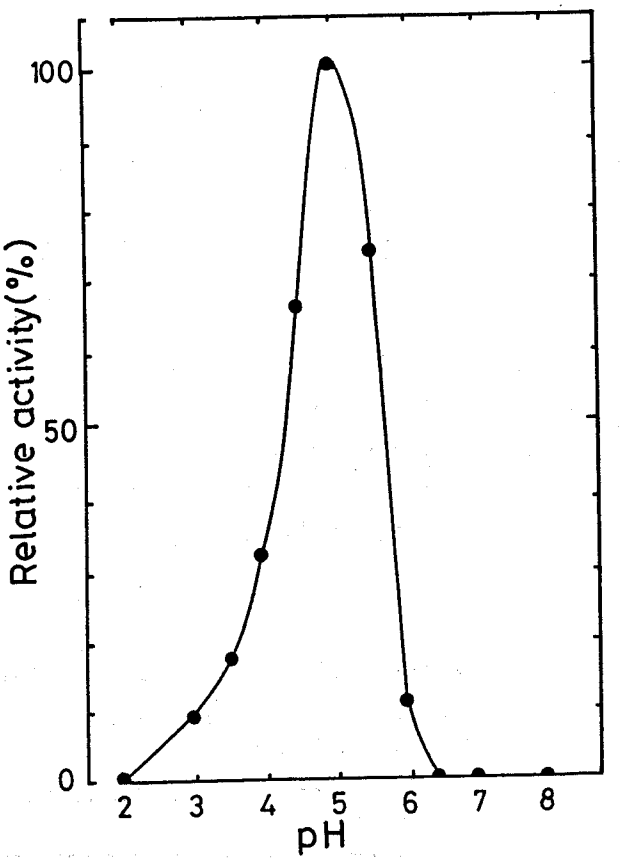

Fig.20. Effect of pH on the hydrolysis of Z-Glu-Phe by carp muscle cathepsin A. The buffer contained 10mM acetic acid and 10mM Tricine was adjusted to various pH values with 1M HCl or 1M NaOH.

Table 7. Substrate specificity of carp muscle cathepsin A.

Substrate	Relative activity(%)	Km(mM)
Z-Glu-Phe	100	3.52
Z-Glu-Tyr	61	4.76
Z-Phe-Tyr	81	N.D*
Z-Gly-Phe	31	N.D*
Z-Gly-Pro	0	N.D*

*Not determined.

Table 8. Carp muscle cathepsin A activity on various protein substrates.

Substrate	Relative activity(%)
Z-Glu-Phe	100
Hemoglobin	0
Myoglobin	0
Bovine serum albumin	0
Ovalbumin	0
Chymotrypsinogen A	0
Cytochrome c	0
Insulin	0
Glucagon	0
Muscular homogenate	0
Myofibrillar protein	0
Sarcoplasmic protein	0

nity for Z-Glu-Phe than Z-Glu-Tyr(Table 7), as already reported[136]. The Km values determined by Lineweaver-Burk plot for Z-Glu-Phe and Z-Glu-Tyr were 3.52mM and 4.76mM, respectively. Kawamura *et al.*[157] reported that pig kidney cathepsin A hydrolyzed Z-Gly-Pro slightly, but carp muscle cathepsin A did not hydrolyze it at all.

Bovine spleen cathepsin A was reported to show certain endopeptidase activity[158,159]. Therefore, the author examined the activity on commercially obtained protein substrates and endogenous proteins (Table 8). As a result, carp muscle cathepsin A did not show any proteolytic activity against such protein substrates.

(5) Effect of the enzyme on autolysis

Effect of the enzyme on carp muscle myofibrils was examined by SDS polyacrylamide gel electrophoresis(Fig.21). The mixture of carp muscle myofibrils and the enzyme was incubated at pH 5.0 or 6.5 at 37°C for 24hr.

At pH 5.0, the degradation of high molecular weight protein supposed to be myosin heavy chain, 67,000 dalton protein, 50,000-55,000 dalton protein supposed to be actin was recognized. But the

degradation was not influenced by Dip-F, the inhibitor of the enzyme. Therefore, the enzyme did not seem to concern with the degradation. Cathepsin D shows the activity to some extent at pH 6.0 as described in SECTION I, so this degradation was probably due to cathepsin D contaminated in muscle homogenate. After death, the muscle pH dropped around 6.5 in carp muscle[62]. At pH 6.5, however, no change was observed than the degradation of myosin heavy chain and the increase of the protein band above 67,000 dalton protein. Conclusively, cathepsin A did not concern with the protein breakdown at pH 6.5 as well as at pH 5.0. Recently, Konagaya[160] reported that several peptidases exist in fish muscle and they act at neutral pH range. At present, it remains to be elucidated to clarify the relation between these peptidases and autolysis.

Makinodan and Ikeda[136] suggested that carp muscle cathepsin A acted on sarcoplasmic protein, but in this study, the purified enzyme did not degrade endogenous proteins(Table 8). The difference of the results was probably due to the fact that autolysis of sarcoplasmic protein which might be caused by cathepsin D was not completely eliminate in the experiments by

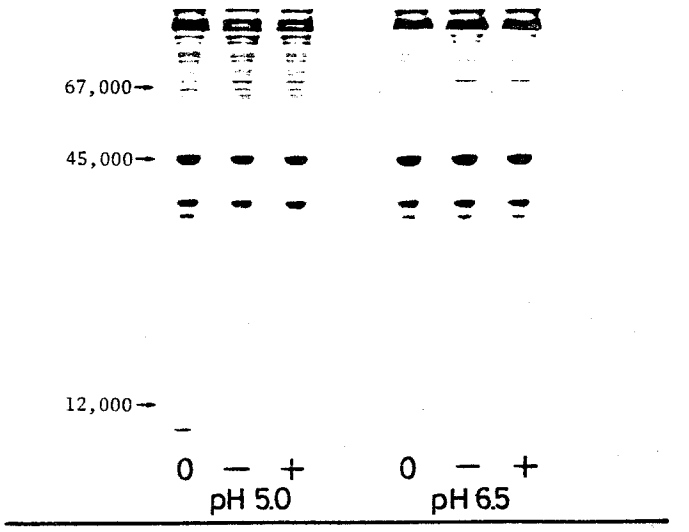

Fig.21. SDS polyacrylamide gel electrophoretic analysis of carp muscle myofibrils treated with carp muscle cathepsin A. A reaction mixture contained 2μg of cathepsin A, 130mM acetate(pH 5.0) or phosphate(pH 6.5) buffer, 1.26mg of myofibrils, 100ppm chloramphenicol and 1mM Dip-F(+) or above mentioned buffer(-) in a total volume of 2.5mℓ was incubated at 37°C for 24hr.

Makinodan and Ikeda[136].

The author examined the subcellular localization of the enzyme in carp muscle, but clear data were not obtained. Judging from the properties described above, however, carp muscle cathepsin A belongs to lysosomal carboxypeptidase A defined by Barret[140].

(6) Combined action of carp muscle cathepsins A and D[161)]

Combined action of cathepsins A and D on hemoglobin at pH 4.5 was originally reported using partially purified chicken muscle enzymes by Iodice *et al.*[55)]. Later, Makinodan and Ikeda[136)] recognized the combined action of carp muscle cathepsins A and D. These reports are important as the data that a combined proteolytic action for the protein degradation was manifested, although it was on the exogenous protein, hemoglobin. However, the enzyme preparation used in the above mentioned study still contained contaminated proteins, so the author re-investigated the combined action using the purified enzymes.

Fig.22 shows the combined action of carp muscle cathepsins A and D on hemoglobin at pH 4.5. Cathepsin A alone did not act on hemoglobin, while cathepsin D hydrolyzed it almost linear with respect to the incubation time. On the other hand, the increase of ninhydrin positive matter observed when both enzymes were added simultaneously was much greater than when cathepsin D alone was added. The results show that peptides hydrolyzed from hemo-

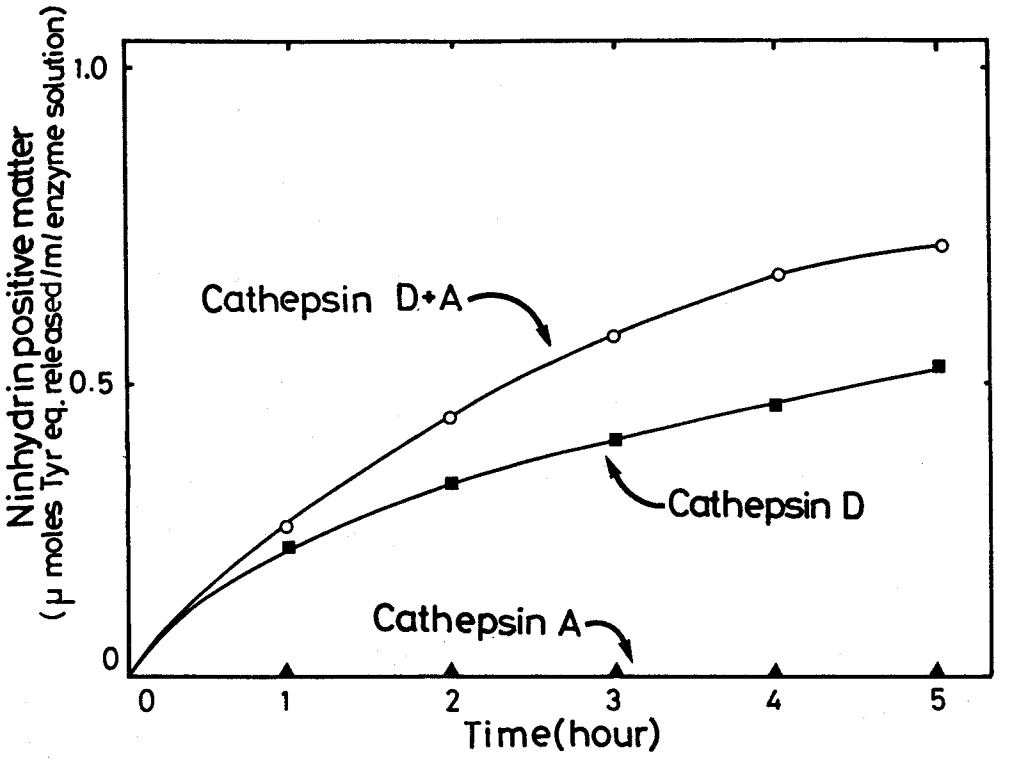

Fig.22. The combined action of carp muscle cathepsins A and D on hemoglobin at pH 4.5. A reaction mixture contained 25mg hemoglobin, cathepsins(A;5μg, D;11μg) and 100mM acetate buffer, pH 4.5.

globin by cathepsin D was further hydrolyzed by cathepsin A. Thus, the combined action of carp muscle cathepsins A and D on hemoglobin was confirmed. Subsequently, the author examined the action of cathepsin A against the condensate of 5% TCA soluble products formed by cathepsin D from hemoglobin. TCA was removed from the filtrate by shaking with ethylether and condensed by a rotary evaporator. But,

any ninhydrin positive matter was not detected. For the combined action, the co-existence of cathepsins A and D in the reaction mixture may be absolutely required, or the substrate for cathepsin A exists in 5% TCA insoluble fraction.

In addition, the author also examined the combined action of these enzymes on carp muscle myofibrils prepared by the method of Tokiwa and Matsumiya[13] and on the sarcoplasmic proteins prepared as the supernatant fraction of the muscle homogenate by the centrifugation at 13,000×g for 30min. Cathepsin D showed the proteolytic activity on them, but cathepsin A did not. On the basis of the present results, it may be concluded that carp muscle cathepsin A is not included in autolysis.

CHAPTER V

PARTIAL PURIFICATION AND PROPERTIES OF Ca^{2+}-DEPENDENT CYSTEINE PROTEINASE(CALPAIN) AND ITS ENDOGENOUS INHIBITOR(CALPASTATIN) FROM CARP MUSCLE

Since a Ca^{2+}-dependent cysteine proteinase(calpain, EC.3.4.22.17) was first reported in rat brain[162], and its endogenous inhibitor(calpastatin) was first reported in rat liver[163] and bovine cardiac muscle[164,165], calpain and calpastatin have been known to distribute widely in various animal tissues[58]. There are two subclasses of calpain(calpains I and II) and both of them show the maximum activity at neutral pH range[58]. Calpain I requires μmolar Ca^{2+} concentration and calpain II requires mmolar Ca^{2+} concentration for their full activities, respectively.

On the other hand, since post-mortem pH in muscle of white meat fish is thought to be around 6.5[59-63] and post-mortem intracellular Ca^{2+} concentration in chicken muscle to be 10^{-4}M[166], calpain possibly takes part in the post-mortem protein degradation. Thus, the author sought calpain and calpastatin and detected them in carp muscle. In this chapter, partial puri-

fication and characterization of them from carp muscle will be described.

MATERIALS AND METHODS

Measurement of the proteinase activities

Calpains I, II, trypsin(Sigma Type I), α-chymotrypsin(Sigma Type II) and papain(Sigma) activities were assayed with casein according to the method of Kunitz[130]. The standard reaction mixture contained an appropriate volume of the proteinase solution, 0.5%(w/v) casein and 50mM Tris-HCl buffer, pH 7.5 for trypsin, papain and calpains or pH 8.1 for α-chymotrypsin in a total volume of 1.6mℓ. The mixture also contained 10mM cysteine and 2mM EDTA in the case of papain assay, 20mM cysteine and 0.1mM Ca^{2+} in the case of calpain I assay or 20mM cysteine and 5mM Ca^{2+} in the case of calpain II assay. After incubation at 30°C for 10min in the case of trypsin, α-chymotrypsin and papain assays, or for 30min in the case of calpains assays, the reaction was stopped by 5%(w/v) TCA and the acid soluble products formed were colorimetrically determined according to the method of Ross and Schatz[167] or

in the case of trypsin and α-chymotrypsin assays, according to the method of Lowry *et al.*[71]. A blank value was obtained from the identical reaction mixture that lacked casein for trypsin, α-chymotrypsin and papain assays or that lacked Ca^{2+} for calpain assays. One unit of each enzyme was defined as the amount that caused an icrease in the absorbance at 750nm of 1.0 for the above mentioned incubation time. Specific assay conditions and other additions are detailed in the legends.

Measurement of the calpastatin activity

For determining of the effect of calpastatin on the proteinase activity, an appropriate volume of the calpastatin and proteinase solution was pre-incubated at 30°C for 5min. The proteinase activity was then measured as described above. The inhibitory activity which reduced 1 unit of the proteinase was defined as 1 unit of calpastatin. In the case of the assays in the chromatography 50μℓ of rat liver calpain II which was kindly gifted from Dr.Tanaka and Dr.Murachi, Department of Clinical Science, Faculty of Medicine, Kyoto University, was used.

Detection of calpains I, II and calpastatin in carp muscle

All procedures were performed according to the method of Murachi *et al.*[168], unless otherwise described. For the purification, 20mM Tris-HCl buffer, pH 7.5, containing 5mM 2-mercaptoethanol, 1mM EGTA (buffer A) was used. Minced carp(*Cyprinus carpio*) muscle was homogenized with 3 volumes of buffer A containing 0.25M sucrose in a Waring blendor for 3min at top speed. After centrifugation at 105,000xg for 60min, the supernatant was condensed to 8.0mℓ by Amicon PM-10 membrane and applied on a column (3x87cm) of Ultrogel AcA 34(from LKB) equilibrated with buffer A. Elution was carried out with the same buffer.

Partial purification of calpain II and calpastatin from carp muscle

Minced carp muscle(247g) was homogenized with 988mℓ of buffer A containing 0.25M sucrose in a Waring blendor for 3min at top speed. The homogenate was centrifuged at 105,000xg for 60min. The obtained supernatant was dialyzed against buffer A overnight to get the crude solution. The crude solution was applied on a column(5x22cm) of DEAE-

cellulose(from Whatman) and eluted with a stepwise manner. The calpastatin fractions eluted by 0.1M NaCl and calpain II fractions eluted by 0.5M NaCl were condensed by Amicon PM-10 membrane and then applied on columns(2.5x97cm for calpain II and 1.5x 100cm for calpastatin) of Ultrogel AcA 34, respectively.

The molecular weight estimation was obtained after calibration of the column of Ultrogel AcA 34 (1.5x100cm) with the known marker proteins(ferritin, catalase, aldolase and bovine serum albumin from Pharmacia Fine Chemicals).

Protein was determined according to the method of Lowry *et al.*[71] using bovine serum albumin as the standard or the absorbance at 280nm.

RESULTS

1. Detection of calpains I, II and calpastatin in carp muscle

Fig. 23 showed the elution profile of the crude solution from Ultrogel AcA 34. Calpastatin was eluted near void volume, followed by calpains I and II.

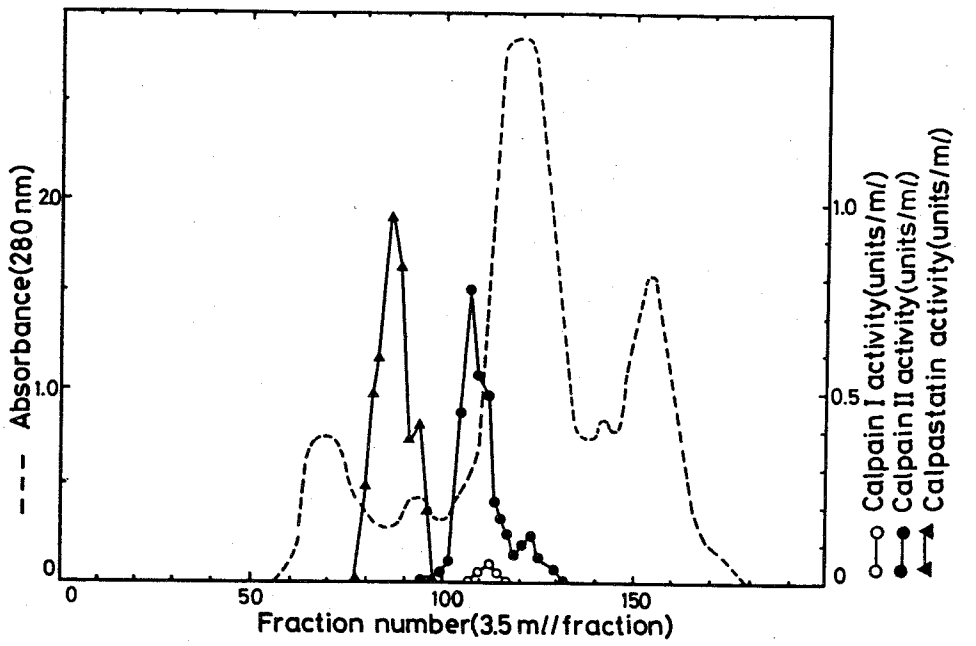

Fig.23. Detection of calpains I, II and calpastatin in the fractions eluted from Ultrogel AcA 34 gel filtration. The condensate of the crude solution (860mg) was applied on a column(3x87cm) of Ultrogel AcA 34 and eluted with buffer A at a flow rate of 13.8mℓ/hr. Then, 3.5mℓ of each fraction was collected.

In comparison with the activity of calpain II, that of calpain I was very weak. The amount of calpain I in carp muscle seems to be much less than that of calpain II, so the author could not isolate calpain I from carp muscle in this study.

2. Partial purification of calpain II and calpastatin from carp muscle

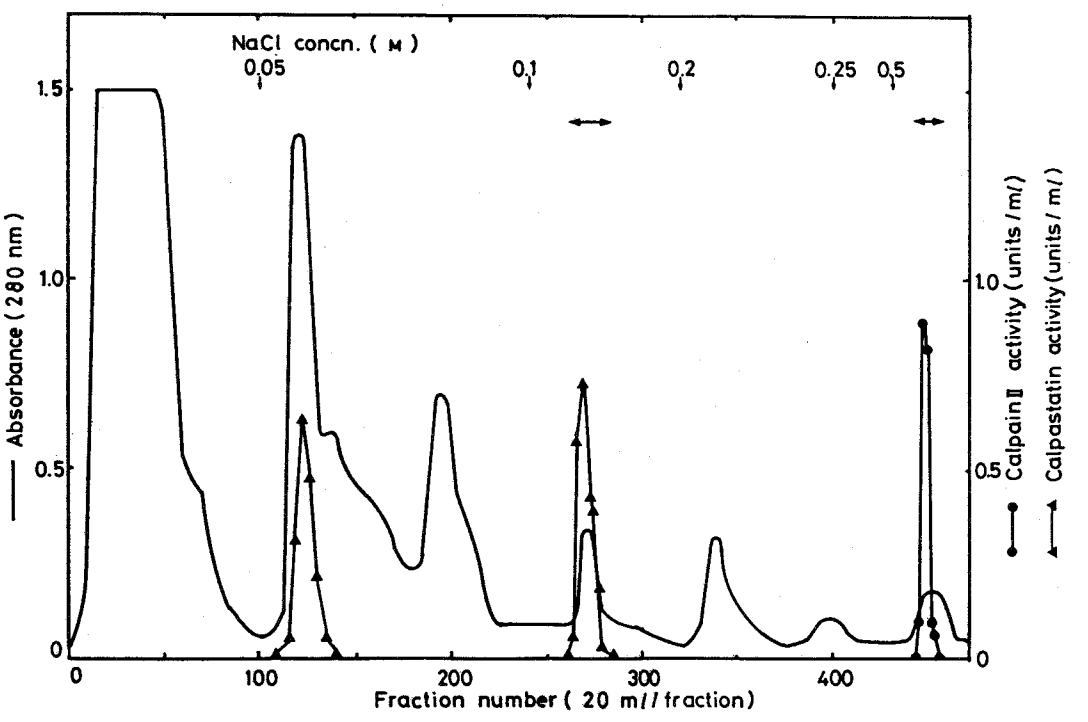

Fig.24. Separation of carp muscle calpain II and calpastatin by DEAE-cellulose chromatography. The crude solution(7,300mg) was applied on a column(5x22cm) of DEAE-cellulose equilibrated with buffer A. A stepwise elution was carried out with buffer A containing 0.05M, 0.1M, 0.25M and 0.5M NaCl. Twenty mℓ of each fraction was collected at a flow rate of 46mℓ/hr.

Fig.24 shows the elution profile of the crude solution from DEAE-cellulose. While calpastatin was eluted in 0.05M and 0.1M NaCl fractions, calpain II was eluted in 0.5M NaCl fractions. Calpain I activity was not detected in any fractions. Calpastatin fractions eluted by 0.1M NaCl and calpain II fractions eluted by 0.5M NaCl were pooled and used for further

purification by Ultrogel AcA 34, respectively.

Figs.25 and 26 show the elution profiles of calpain II and calpastatin from the columns of Ultrogel AcA 34, respectively. Active fractions of calpain II and calpastatin were pooled and used in the experiments described below.

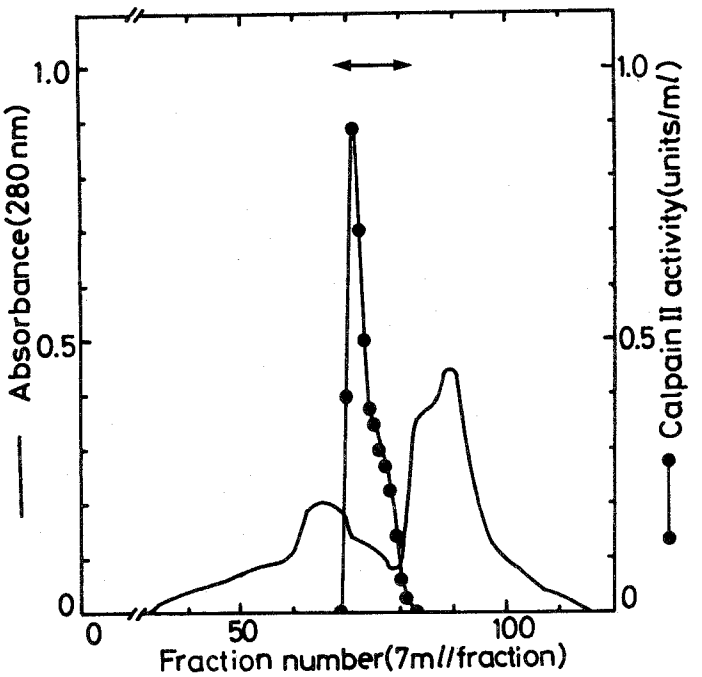

Fig.25. Ultrogel AcA 34 chromatography of 0.5M NaCl fraction of the carp muscle calpain II eluted from DEAE-cellulose. The condensate of 0.5M NaCl fraction (240mg) was applied on a column(2.5x90cm) of Ultrogel AcA 34 equilibrated with buffer A. Seven mℓ of each fraction was collected at a flow rate of 11.8mℓ/hr.

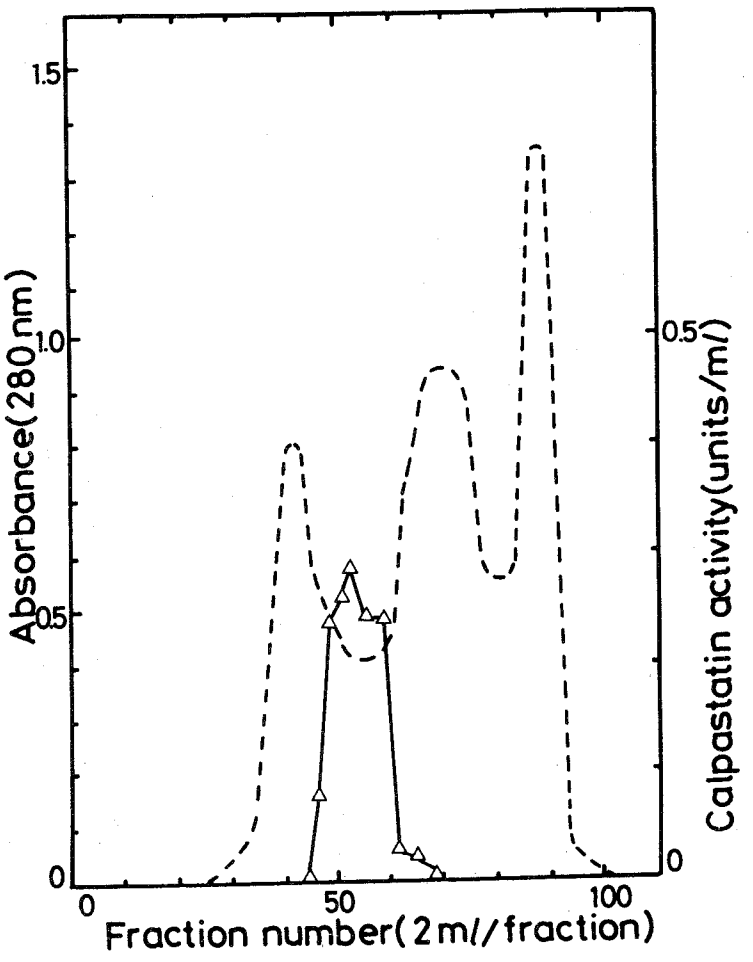

Fig.26. Ultrogel AcA 34 chromatography of 0.1M NaCl fraction of carp muscle calpastatin eluted from DEAE-cellulose. The condensate of 0.1M fraction(350mg) was applied on a column(1.5x100cm) of Ultrogel AcA 34 equilibrated with buffer A. Two mℓ of each fractions was collected at a flow rate of 12.6mℓ/hr.

3. Some properties of carp muscle calpain II and calpastatin

(1) Effect of pH on the calpain II activity

Fig.27 shows the effect of pH on the activity of

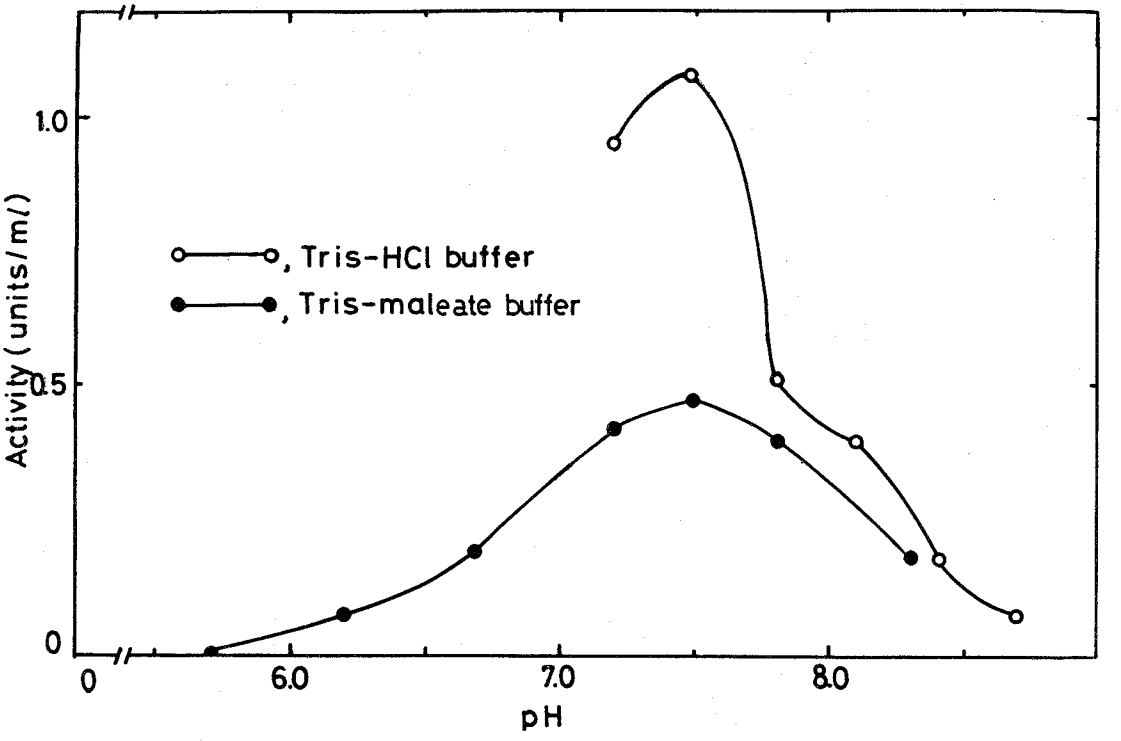

Fig.27. Effect of pH on the activity of carp muscle calpain II. Assay conditions were as follows: 20mM cysteine, 5mM $CaCl_2$, 0.5%(w/v) casein, 0.2mℓ of calpain II preparation and 50mM Tris-HCl(pH 7.2-8.7) or Tris-maleate(pH 5.7-8.3) buffer, in a total volume of 1.6mℓ.

carp muscle calpain II. Calpain II showed the maximum activity at pH 7.5, either with Tris-HCl or with Tris-maleate buffer, but higher activity was obtained with Tris-HCl buffer.

(2) Effect of Ca^{2+} concentration on the calpain II activity

Fig.28 shows the effect of Ca^{2+} concentration on

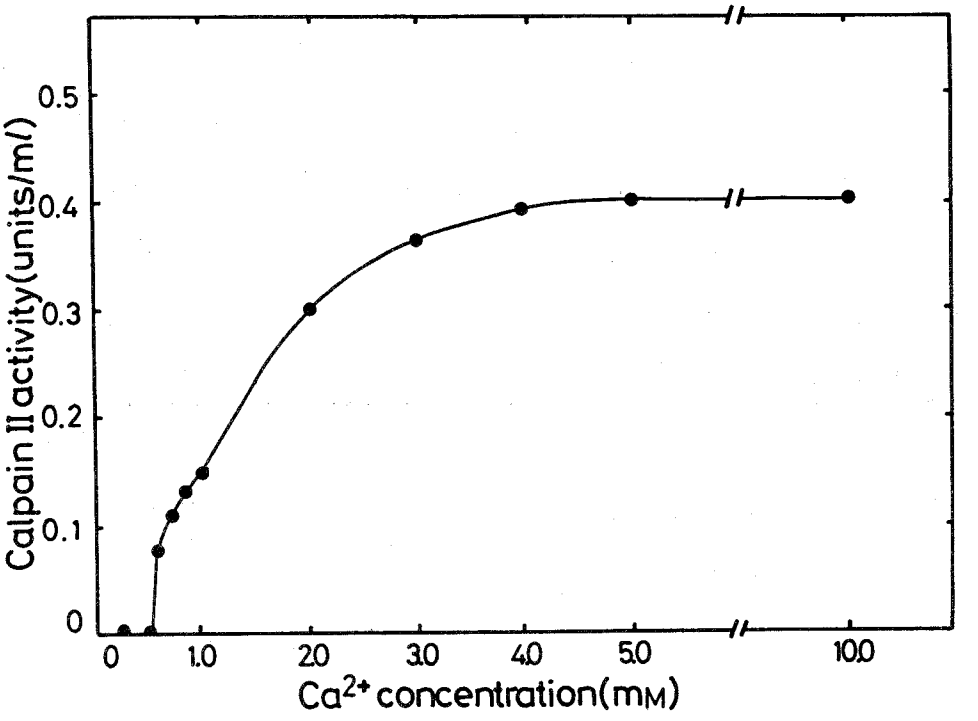

Fig.28. Effect of calcium concentration on the activity of carp muscle calpain II. Assay conditions were as follows: 20mM cysteine, indicated concentration of $CaCl_2$, 0.5%(w/v) casein , 0.2mℓ of calpain II preparation and 50mM Tris-HCl buffer, pH 7.5, in a total volume of 1.6mℓ.

the activity of carp muscle calpain II. Carp muscle calpain II required 4-5mM Ca^{2+} for its full activity and did not show any activity in the absence of Ca^{2+}. This absolute requirement of carp muscle calpain II for Ca^{2+} coincided with the common property of calpain II from various mammalian tissues[58].

(3) Effect of metal ions on the calpain II activity

Table 9 shows the effect of various divalent

metal ions on the activity. Carp muscle calpain II required 4-5mM Ca^{2+} for its full activation(Fig.28). Instead of Ca^{2+}, it required 15mM Sr^{2+} to obtain the same degree of the activity, whereas it showed no activity with Mn^{2+}, Mg^{2+} or Hg^{2+}.

(4) Effect of cysteine concentration on the calpain II activity

As shown in Fig.29, carp muscle calpain II required 20mM cysteine for its full activity in the presence of 5mM Ca^{2+}.

(5) Effect of antibiotics on the calpain II activity

Antipain, leupeptin and E-64 showed the dose dependent inhibition on the calpain II activity, as shown in Fig.30. Leupeptin and E-64 showed more

Table 9. Effect of various divalent metal ions on the activity of carp muscle calpain II.

Metal ions(mM)	Relative activity(%)
Ca^{2+} (5)	100
Sr^{2+} (5)	52
(15)	96
Mn^{2+} (5)	0
Mg^{2+} (5)	0
Hg^{2+} (5)	0

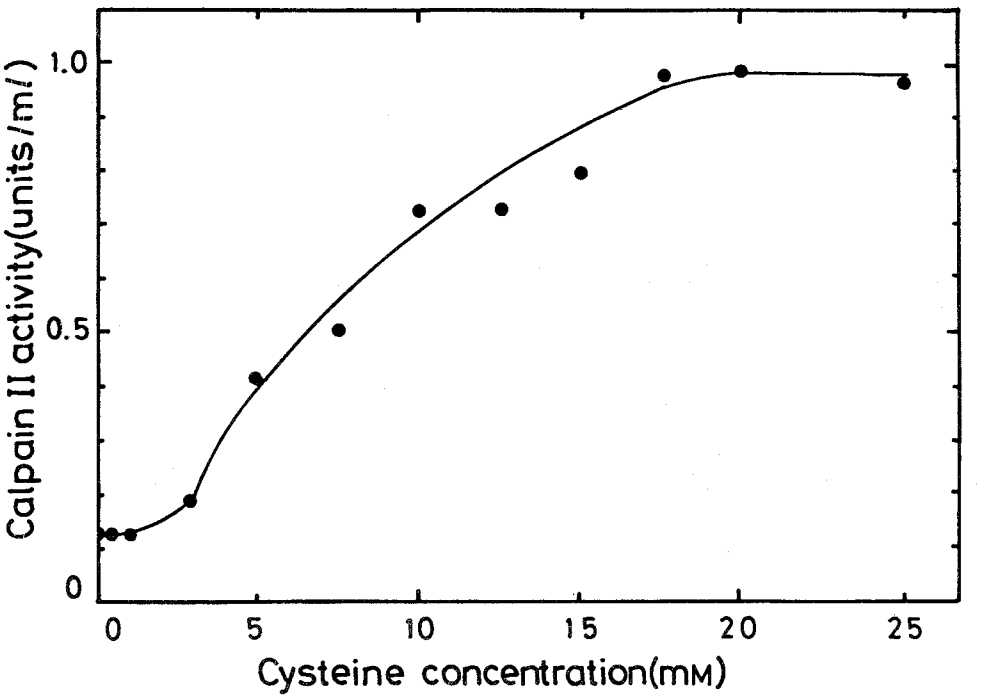

Fig.29. Effect of cysteine concentration on the activity of carp muscle calpain II. Assay conditions were as follows: indicated concentration of cysteine, 5mM $CaCl_2$, 0.5%(w/v) casein , 0.2mℓ of calpain II preparation and 50mM Tris-HCl buffer, pH 7.5, in a total volume of 1.6mℓ.

remarkable inhibition than antipain did.

(6) Autolytic inactivation of calpain II in the presence of Ca^{2+}

As shown in Fig.31, calpain II lost its activity by the pre-incubation at 30°C in the presence of 5mM Ca^{2+}, while it did not lose any in the absence of Ca^{2+}.

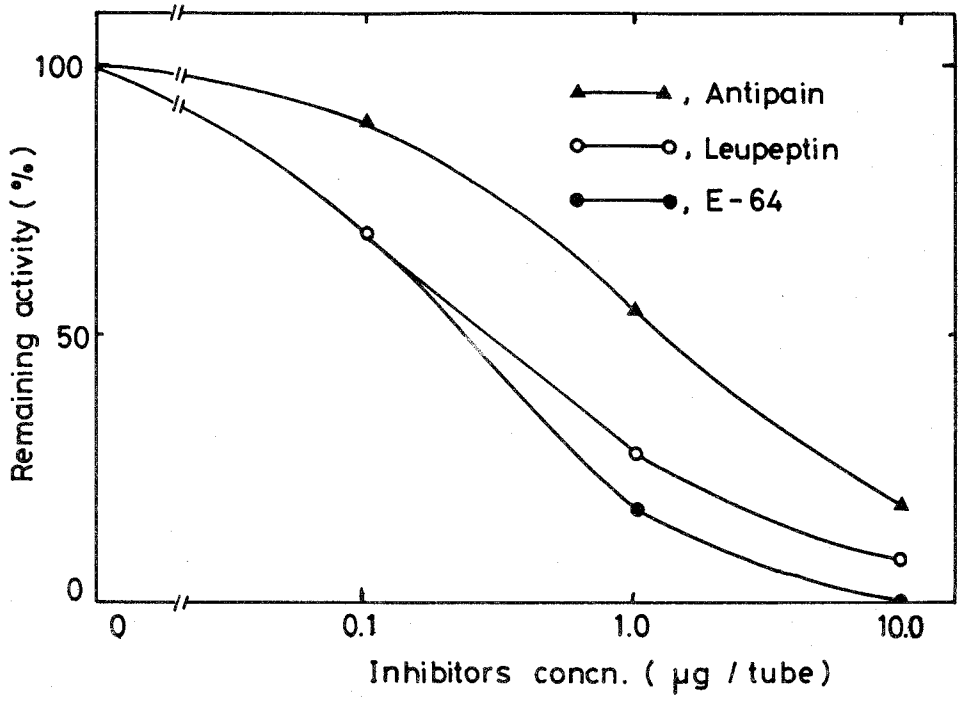

Fig.30. Effect of leupeptin(○——○), antipain(▲——▲) and E-64(●——●) on the activity of carp muscle calpain II. Assay conditions were as follows: indicated concentration of each antibiotic and 0.2mℓ of calpain II preparation were pre-incubated at 30°C for 5min and then the remaining calpain II activity was determined. Antibiotic concentration was expressed as μg/tube of the complete reaction mixture.

(7) Effect of calpastatin on the activities of several proteinases

As shown in Fig.32, carp muscle calpastatin showed the dose dependent inhibition on the caseinolytic activities of calpain II from not only carp muscle but also rat liver, while it showed no inhibitory effect on

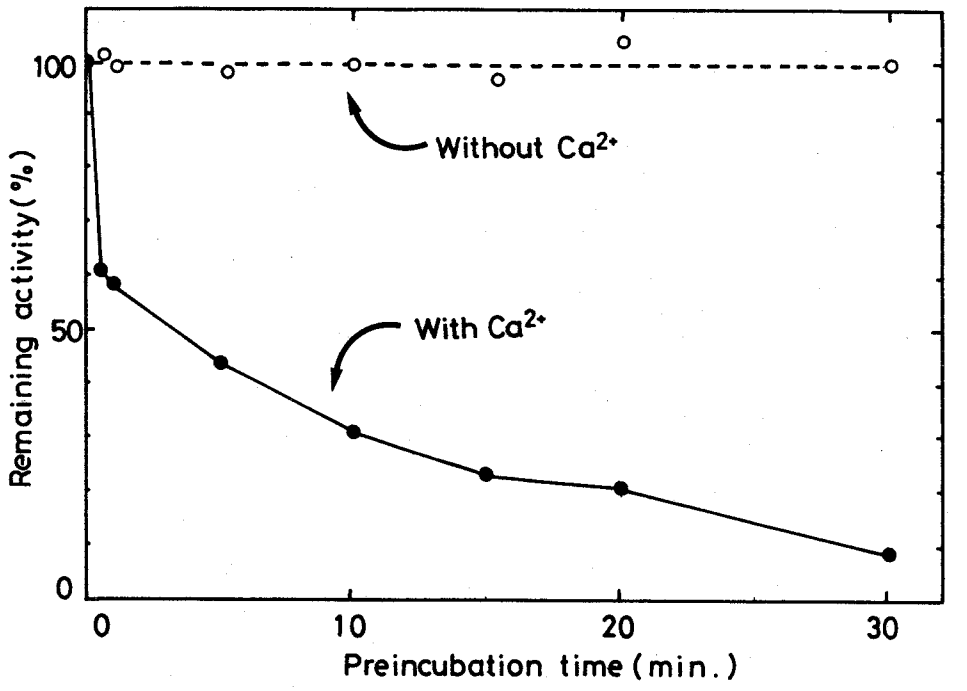

Fig.31. Autolytic inactivation of carp muscle calpain II. In the presence(●——●) or absence(o---o) of 5mM Ca^{2+}, 0.2mℓ of calpain II preparation was pre-incubated at 30°C for indicated time and then the remaining calpain II activity was determined.

those of trypsin, α-chymotrypsin and papain.

(8) Effect of Ca^{2+} concentration on the activity of rat liver calpain II in the presence of carp muscle calpastatin

As shown in Fig.33, the inhibition of carp muscle calpastatin on rat liver calpain II was not reversed when large excess of Ca^{2+} was added to the reaction mixture. Therefore, the nature of inhibition of calpain II by calpastatin was not due to sequestering

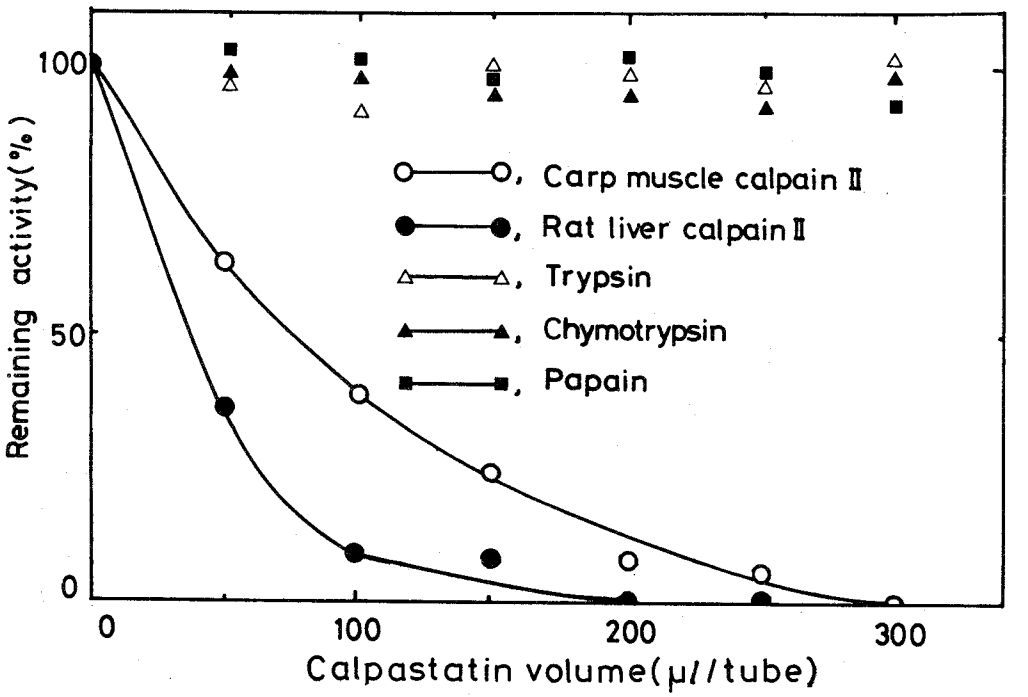

Fig.32. Effect of carp muscle calpastatin on the caseinolytic activities of trypsin(△——△), α-chymotrypsin(▲——▲), papain(■——■), rat liver calpain II (●——●) and carp muscle calpain II(○——○). Assay conditions were as follows: indicated volume of calpastatin preparation and appropriate amount of the proteinase solution(i.e. 2.5μg trypsin, 2.5μg α-chymotrypsin, 5μg papain, 0.05mℓ of rat liver calpain II preparation or 0.2mℓ of carp muscle calpain II preparation) were pre-incubated at 30°C for 5min and then the remaining proteinase activity was determined.

Ca^{2+} in the reaction mixture but due to forming calpain-calpastatin complex in the presence of Ca^{2+}.

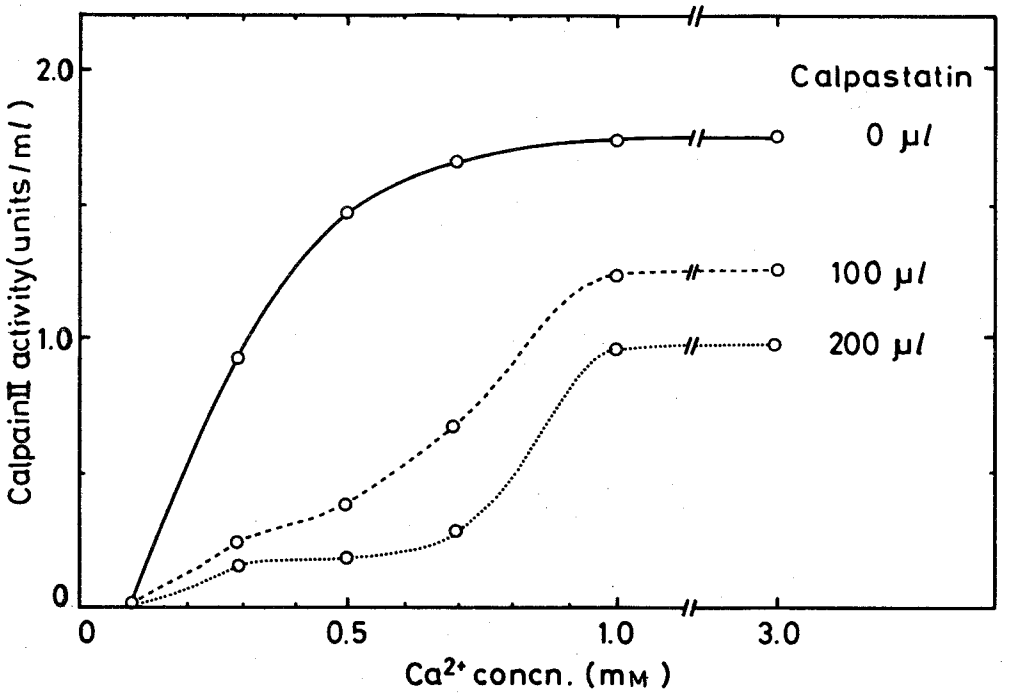

Fig.33. Effect of calcium concentration on the activity of rat liver calpain II in the presence of various amounts of carp muscle calpastatin. Various amounts of carp muscle calpastatin and 0.05mℓ of rat liver calpain II were pre-incubated at 30°C for 5min. Then, the remaining calpain II activity was determined in the presence of various concentration of $CaCl_2$ as described in MATERIALS AND METHODS.

(9) Molecular weight estimation of carp muscle calpain II and calpastatin

As shown in Fig.34, the molecular weights of calpain II and calpastatin were estimated to be 100,000 and 300,000 by gel filtration, respectively.

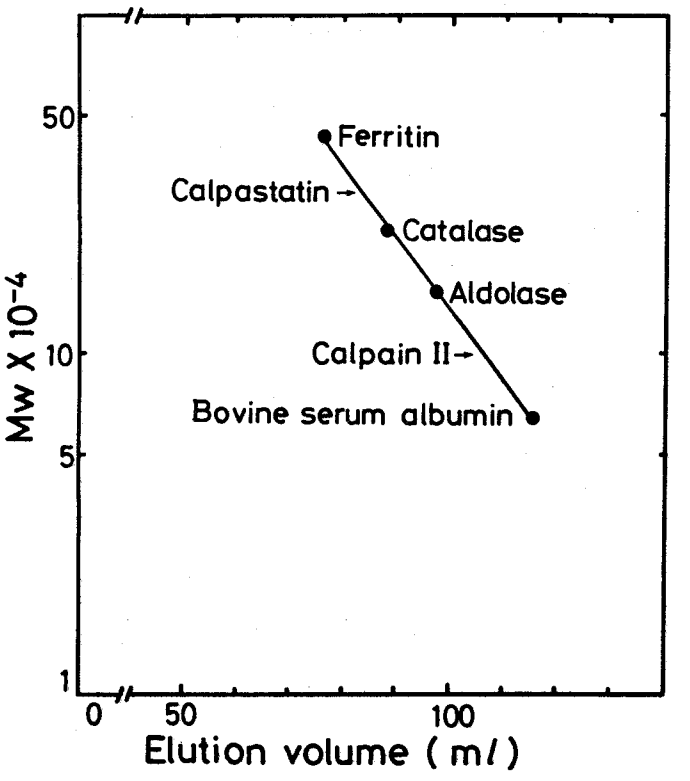

Fig.34. Molecular weight estimation of carp muscle calpain II and calpastatin on a column(1.5x100cm) of Ultrogel AcA 34. Elution conditions were as same as those in Fig.25. The molecular weight markers were as follows: ferritin(440,000), catalase(232,000), aldolase(158,000) and bovine serum albumin(67,000).

DISCUSSION

As described in CHAPTER IV, cathepsins A and D seem not to take part in the post-mortem protein degradation, since both proteases did not show any proteolytic activity on endogenous proteins at pH 6.5. Makinodan *et al.*[15,121] reported a neutral proteinase(tentatively called a sub-endopeptidase) which splits hemoglobin optimally at pH 7.2. Sub-endopeptidase distributes widely in the muscle of various fish as described in CHAPTER III and may take part in the post-mortem protein degradation. Apart from this proteinase, the author found another neutral proteinase, calpain, in carp muscle.

At first, the author tried to detect the calpain activity in the crude enzyme solution, but could not detect any. This is probably due to the excess calpastatin co-existing in the crude solution. Chromatography in the presence of EGTA could separate calpain and calpastatin. When the condensate of the crude solution was applied on a column of Ultrogel AcA 34, not only calpain II and calpastatin but also calpain I were detected(Fig.23). However, by the use of DEAE-cellulose chromatography, any activity of calpain I could not be found. Calpain I might be contained in

the calpastatin fractions eluted from DEAE-cellulose. So calpastatin fractions were further chromatographed by Ultrogel AcA 34. Nevertheless, no calpain I activity was detected in any fraction eluted from Ultrogel AcA 34. The amount of calpain I in carp muscle seems to be much less than that in mammalian muscle, while the amount of calpastatin in carp muscle seems as same as that in mammalian muscle[168]. Since calpain I was not isolated, the author examined the properties of calpain II and calpastatin using partially purified preparations obtained by DEAE-cellulose chromatography and the subsequent gel filtration by Ultrogel AcA 34.

Carp muscle calpain II optimally hydrolyzed casein at pH 7.5 and required 1.5mM Ca^{2+} or 5mM Sr^{2+} for half maximum activity in the presence of 20mM cysteine, while other divalent metal ions had no effect on the activity.

The cysteine dependency and the effect of antibiotics showed that carp muscle calpain II belongs to the cysteine proteinase[140]. These properties of carp muscle calpain II coincides with those from porcine skeletal muscle[169,170], bovine cardiac muscle[165], chicken skeletal muscle[171,172], rabbit skeletal muscle[173-175], bovine skeletal muscle[99], and rat

liver[58].

On the other hand, the molecular weight of carp muscle calpastatin was estimated to be 300,000. This value coincided with those of bovine cardiac muscle[164,165], rat liver[58], rat brain[176], rabbit skeletal muscle[177], chicken skeletal muscle[178] and human erythrocyte[179] (see review by Murachi[180]). Some properties of carp muscle calpastatin will be described in CHAPTER VI. As shown in Fig.10, carp muscle calpastatin inhibited calpains II from carp muscle and rat liver, but did not inhibit trypsin, α-chymotrypsin and papain. Considering the result shown in Fig.33, calpastatin seemed to form a complex with calpain II. Murachi *et al.*[58] showed the cross-reactivity between calpain and calpastatin from different tissues. Data shown in Fig.32 confirm the cross-reactivity between calpain and calpastatin over tissues and species. Waxman[181] showed the ubiquitous distribution of calpain II and calpastatin in bovine tissues. The ubiquity and the cross-reactivity of calpain-calpastatin system suggest that this system might play a physiologically important role *in vivo*.

There have been some reports suggesting that the proteolysis being activated by Ca^{2+} exists in mammalian muscle[77,78,83-86,89,91,95,100,183,184] (see reviews by

Fujimaki and Okitani[50], Okitani[51] and Suzuki[101]). Seki *et al.*[75] showed the effect of Ca^{2+} on the changes of the myofibrillar proteins when incubated at 25°C. Recently, Kameyama and Etlinger[184] and Lewis and Anderson[185] revealed the Ca^{2+}-dependent regulation of the protein degradation in skeletal muscle. However, Hattori and Takahashi[102-104] showed that calpain II had no effect on the meat ageing after death. They[105] purified a myofibrillar component which modified the actin-myosin interaction from Z-disks(see reviews[106,107]).

Carp muscle calpain II could show activity to some extent at the post-mortem muscle pH[59-63]. But, the concentration of the intracellular Ca^{2+} in the post-mortem chicken muscle was reported to be around 10^{-4} M[166]. Carp muscle calpain II could not show any activity in such a range of Ca^{2+} concentration. These facts make it doubtful to include calpain II in autolysis. Recently, Mellgren[186] revealed another Ca^{2+}-dependent cysteine proteinase which has more Ca^{2+} sensitivity. Later, this proteinase was named calpain I by Murachi *et al.*[58]. It is also known to distribute widely in mammalian tissues[168,187]. Dayton *et al.*[189] purified calpain I from porcine skeletal muscle. The author showed the existence of calpain I in carp muscle, but

could not isolate it. Purification of calpain I will be pursued to clarify whether it takes part in autolysis. Recently, Kubota *et al.*[190] and Suzuki *et al.*[191,192] showed the conversion of m-CANP to more Ca^{2+} sensitive form, μ-CANP, by a limited proteolysis. If it is the case for carp muscle calpain II, such conversion of calpain II might concern with autolysis.

On the other hand, Rodeman *et al.*[193] reported that the stimulation of the protein degradation is mediated by prostaglandin E_2 and does not require Ca^{2+}-activated protease. Therefore, in order to clarify the relation between autolysis and muscle protease, the investigation about other proteases, such as cathepsins B, H, L and sub-endopeptidase will be necessary. In CHAPTER VII, the author will show the detection and some properties of cysteine proteinases(tentatively called BANA hydrolases) in carp muscle.

Fujimaki and
Seki *et al*
of the
Rec

CHAPTER VI

TRYPSIN INHIBITOR AND A PAPAIN
SCLE

In the previous chapter, the author demonstrated the existence of calpain and calpastatin in carp muscle. Carp muscle calpastatin inhibited only calpain II among various proteinases examined. During purification of calpastatin, the author found other proteinase inhibitors in carp muscle, namely a trypsin inhibitor and a papain inhibitor.

Laskowski Jr. and Kato[57] reviewed about many proteinase inhibitors in animals and plants. However, there is only limited knowledge on muscle proteinase inhibitor. The author attempted to purify a trypsin inhibitor and a papain inhibitor from carp muscle in the present study. These proteinase inhibitors showed different heat stability. Some other properties and possible physiological functions of these inhibitors will be also described, in comparison with those of calpastatin.

Measurement of the proteinase inhibitor activities

The caseinolytic activities of trypsin(Sigma Type I), α-chymotrypsin(Sigma Type II), papain (Sigma) and calpain II were assayed as described in CHAPTER V. BANA hydrolyzing activity of papain was assayed as follows. A reaction mixture that contained 100mM Tris-maleate buffer, pH 7.5, 0.5mM BANA, 20mM cysteine, 2mM EDTA and 2.5μg of papain in a total volume of 1.0mℓ was incubated at 30°C for 10min. After incubation the activity was colorimetrically determined according to the method of Matsutani *et al.*[194] One unit of BANA hydrolyzing activity of papain was defined as the amount that caused an increase of 1.0 in the absorbance at 540nm for 10min. Inhibitory activities of trypsin inhibitor and papain inhibitor were assayed by the same method for calpastatin as described in CHAPTER V. The inhibitory activities which decreased 1 unit of the caseinolytic activity of trypsin and 1 unit of BANA hydrolyzing activity of papain were defined as 1 unit of trypsin inhibitor and papain inhibitor, respectively. Specific conditions and

other additions are detailed in the legends.

protein was measured according to the method of Lowry *et al.*[71].

Partial purification of a trypsin inhibitor and a papain inhibitor from carp muscle

All purification procedures were performed at 4°C, unless otherwise indicated. For the purification, 20mM Tris-HCl buffer, pH 7.5, containing 5mM 2-mercaptoethanol and 1mM EGTA(buffer A) was used. Minced carp (*Cyprinus carpio*) muscle(382g) was homogenized with 1,538mℓ of buffer A containing 0.25M sucrose in a Waring blendor for 3min at top speed. The homogenate was centrifuged at 105,000xg for 60min. The obtained supernatant was dialyzed against buffer A overnight to get the crude solution. The crude solution was applied on a column(5x25cm) of DEAE-cellulose(from Whatman) equilibrated with buffer A and eluted by NaCl with a stepwise manner. Fractions eluted by 0.15M NaCl were pooled, condensed by Amicon PM-10 membrane to 2.8mℓ and applied on a column(1.5x100cm) of Ultrogel AcA 34(from LKB) equilibrated with buffer A.

RESULTS

1. Detection of a trypsin inhibitor and a papain inhibitor in carp muscle

Fig.35 shows the elution profile of a trypsin inhibitor from DEAE-cellulose. Trypsin inhibitor was eluted in 0.1M and 0.15M NaCl fractions, while papain inhibitor was not detected in any fraction. Calpastatin was eluted in 0.05M and 0.1M NaCl fractions, as described in CHAPTER V.

Fig.36 shows the elution profile of the above mentioned 0.1M NaCl fractions from Ultrogel AcA 34. Trypsin inhibitor was eluted separately in high and low molecular weight fractions. By this purification step, papain inhibitor was detected in the low molecular weight trypsin inhibitor fractions for the first time. Those fractions that contained both inhibitory activities were pooled and used in the further experiments.

2. Some properties of carp muscle trypsin inhibitor and papain inhibitor

Fig.37 shows the effects of trypsin inhibitor and papain inhibitor on several proteinases. The inhibitor solution inhibited serine proteinases,

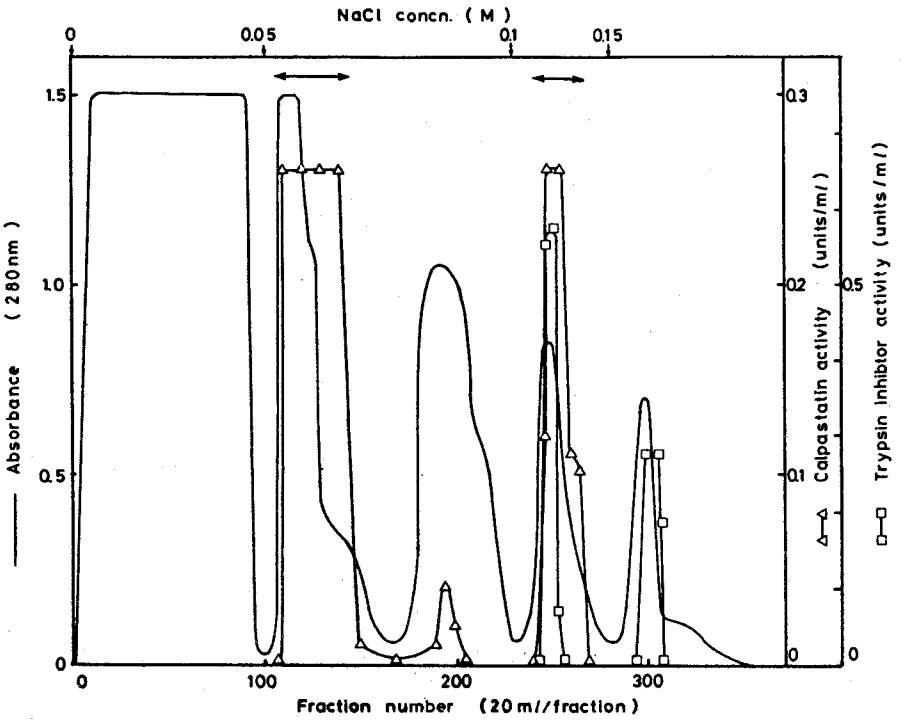

Fig.35. Separation of carp muscle calpastatin and trypsin inhibitor by DEAE-cellulose chromatography. The crude solution(8,400mg) was applied on a column (5x22cm) of DEAE-cellulose equilibrated with buffer A containing 0.05M, 0.1M and 0.15M NaCl. Twenty mℓ of each fraction was collected at a flow rate of 52mℓ/hr.

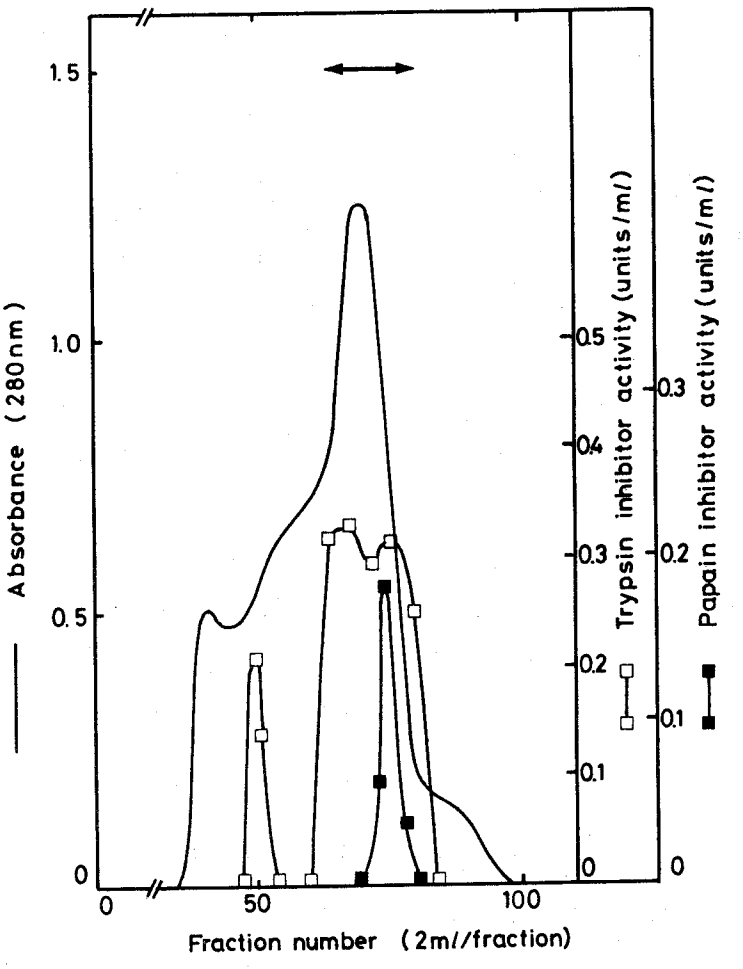

Fig.36. Chromatography of 0.15M NaCl fraction(210mg) on a column(1.5x100cm) of Ultrogel AcA 34. Elution was carried out with buffer A at a flow rate of 14.2mℓ/hr and 2mℓ of each fraction was collected.

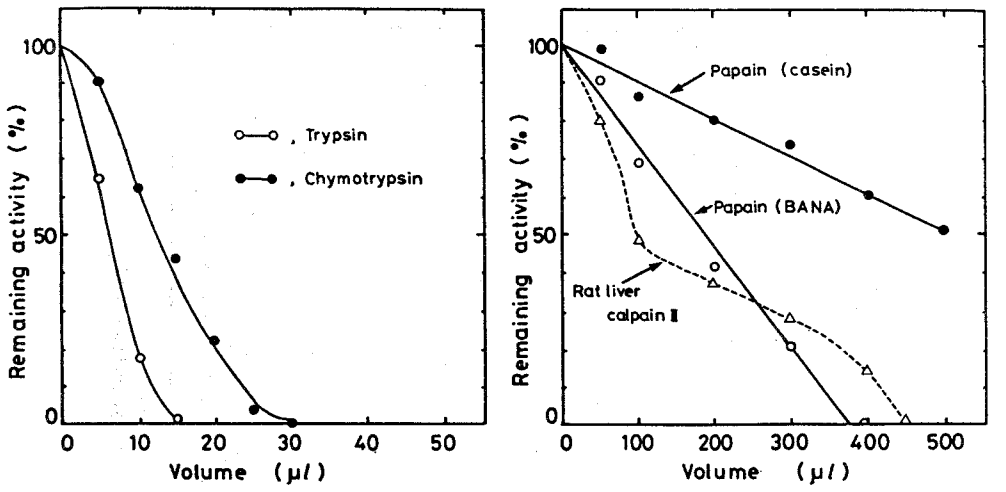

Fig.37. Inhibitory effect of the inhibitor solution eluted from Ultrogel AcA 34 on trypsin and α-chymotrypsin(left) and papain and calpain II(right). Assay conditions were detailed in MATERIALS AND METHODS.

such as trypsin and α-chymotrypsin, and cysteine proteinases, such as papain and rat liver calpain II. While the caseinolytic activities of trypsin and α-chymotrypsin were completely inhibited by less than 30μℓ of the solution, those of papain was not inhibited completely by more than 500μℓ of it. Those findings suggested that the solution contained different types of the proteinase inhibitor. Therefore, the author examined the heat stability of both inhibitory activities of the solution.

Fig.38 shows the heat stability of both inhi-

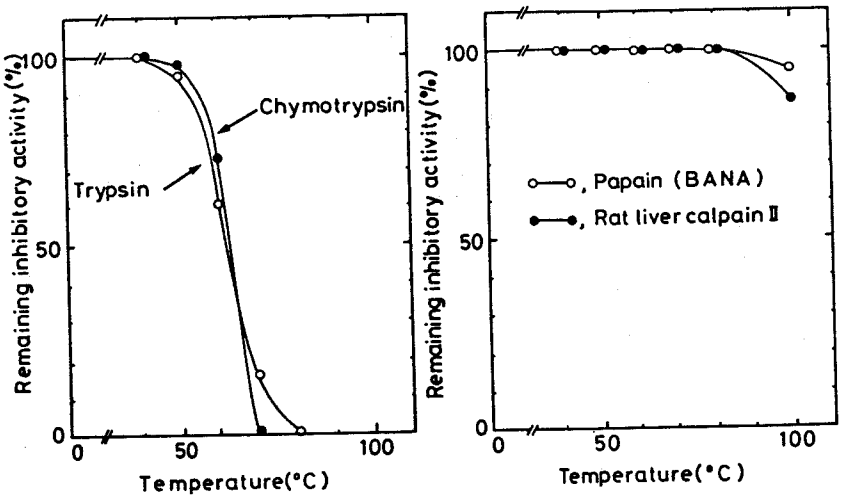

Fig.38. Heat stability of the proteinase inhibitor solution. The proteinase inhibitor solution was pre-incubated at indicated temperature for 5min, then the remaining inhibitory activities on trypsin and α-chymotrypsin (left) and papain and calpain II(right) were determined as described in MATERIALS AND METHODS.

bitory activities. Inhibitory activities on the caseinolytic activities of trypsin and α-chymotrypsin were completely lost by heating at 80°C for 5min, while inhibitory activities on the caseinolytic activity of rat liver calpain II and the amidase activity of papain were not affected at all by heating at 80°C for 5min. Such two types of the heat stability of the solution confirmed that the solution contained two different types of proteinase inhibitor, namely trypsin inhibitor and papain inhibitor.

As described in CHAPTER V, calpastatin that might

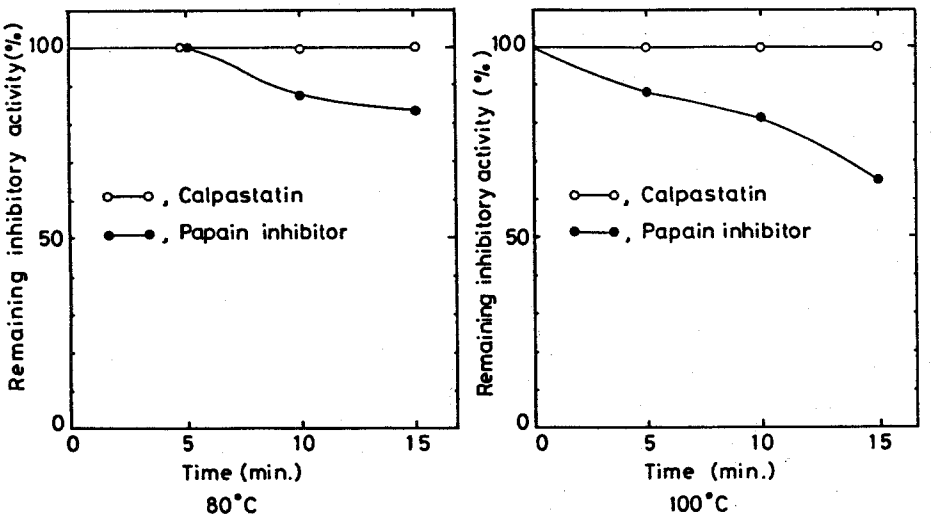

Fig.39. Heat stability of carp muscle calpastatin and papain inhibitor. Each inhibitor solution was pre-incubated at 80°C(left) or 100°C(right) for indicated time and then the remaining inhibitory activity on rat liver calpain II was determined as described in MATERIALS AND METHODS.

be a specific inhibitor for calpain exists in carp muscle. Murachi *et al.*[58] reported the absolute heat stability of rat liver calpastatin. Therefore, the author examined the heat stability of carp muscle calpastatin and papain inhibitor by determining the inhibitory activities on rat liver calpain II. As shown in Fig.39, papain inhibitor was reduced its activity to 65% after heating at 100°C for 15min, whereas calpastatin was not affected at all by the same treatment.

DISCUSSION

In this chapter, the author showed two types of proteinase inhibitors which were differed each other in the inhibition spectra and heat stability.

Reports on proteinase inhibitors in muscle are very few, while those in serum, egg white and plant seeds are well-studied[57]. In 1968, Drummond and Duncan[195] first reported the inhibitory activity which reduced the activation of phosphorylase b kinase by trypsin. Noguchi and Kandatsu[196] found a trypsin inhibitor in rat skeletal muscle and showed its inhibitory effect on the alkaline autolytic activity, which was caused by an alkaline serine proteinase[197]. Noguchi *et al.*[198,199] postulated that this alkaline proteinase might be a target for muscle trypsin inhibitor. Later, this proteinase activity was reported to be due to a proteinase from mast cells[200]. The author sought a serine proteinase as a target enzyme for muscle trypsin inhibitor in carp muscle, but could not detect any. Therefore, the physiological function of muscle trypsin inhibitor still remains to be elucidated. Tschesche[201] purified trypsin-kallikrein inhibitor from cuttle fish, which seems to be distinguished from carp muscle trypsin inhi-

bitor in the molecular weight. Waxman and Krebs[164] and Waxman[165] reported the purification and some properties of bovine cardiac muscle trypsin inhibitor. This trypsin inhibitor seemed to have similar properties in the inhibition spectra, heat stability and molecular weight with carp muscle trypsin inhibitor which was not so heat stable(Fig.38) and of which molecular weight was estimated to be around 50,000 from the calibration of the column shown in Fig.36. Those properties of carp muscle trypsin inhibitor are also similar to those of serum α_1-antitrypsin[202-205]. It is very attractive to suppose that muscle trypsin inhibitor and α_1-antitrypsin is an immunologically identical protein. To ascertain this possibility, it is necessary to obtain the purified preparation of carp muscle trypsin inhibitor.

On the other hand, Lenny *et al.*[205] reported that heat stable cathepsin B and H inhibitors of which molecular weight were 11,000-14,000 distribute in rat and human tissues. However, it is unknown whether those cathepsins B and H inhibitors in the various tissues of rat and human are immunologically identical. Schwartz and Bird[143] found cathepsin B inhibitors in rat skeletal muscle. Recently, Hirado

et al.[206] and Kominami *et al.*[207] purified thiol protease inhibitors from rat liver. In regard to the heat stability, those protease inhibitors above mentioned are similar to carp muscle papain inhibitor. Unlike muscle trypsin inhibitor, carp muscle papain inhibitor may play a protective role against unwanted proteolysis by endogenous cysteine proteinases, since it inhibited intracellular proteinase, calpain II. Thus, the author sought another intracellular target proteinase for papain inhibitor in carp muscle. The results will be described in the following chapter.

CHAPTER VII

OCCURRENCE OF BANA HYDROLASES IN CARP MUSCLE

In the previous chapter, the author showed that carp muscle papain inhibitor inhibited an endogenous cysteine proteinase, calpain II. Papain inhibitor is different from calpastatin in the molecular weight, heat stability and inhibitory effect against papain. In particular, the effect of the muscle papain inhibitor against papain that is an exogenous protein suggests that the inhibitor may have the similar inhibitory effects against cysteine proteinases. Thus, the author attempted to find an endogenous target proteinase other than calpain in carp muscle.

In earlier studies, cathepsin B which deamidates BAA has been the only endogenous cysteine proteinase [137]. At present, cathepsins B, H, and L are known to distribute ubiquitously on mammalian tissues as for cysteine proteinase[208], in addition to a heat stable fish muscle alkaline proteinase[127]. The author attempted to find a proteinase which deamidates BANA in the presence of cysteine and detected two BANA hydrolases in the fractions eluted from a column

of papain-Sepharose 4B. In this chapter, the author call those cysteine proteinase as BANA hydrolases I and II tentatively and show some properties of them.

MATERIALS AND METHODS

Measurement of the proteinase activities

Caseinolytic activities of calpain II and BANA hydrolases were assayed in the presence of 20mM cysteine, 5mM Ca^{2+}(calpain II) or 2mM EDTA(BANA hydrolases), and 0.5%(w/v) casein in a total volume of 1.6mℓ. BANA hydrolyzing activities of papain(Sigma) and BANA hydrolases were assayed in the presence of 20mM cysteine, 2mM EDTA and 0.5mM BANA in a total volume of 1.0mℓ. Caseinolytic and BANA hydrolyzing activity of papain were assayed with 100mM Tris-HCl buffer, pH 7.5. Caseinolytic activities of BANA hydrolases were assayed with 100mM Tris-maleate buffer, pH 6.2 or 8.3. BANA hydrolyzing activities of BANA hydrolases were assayed at pH 6.7. One unit of BANA hydrolyzing activities of papain and BANA hydrolases was defined as the amount that caused an increase in the absorbance at 540nm of 1.0 for 10min or 30min, respectively. Papain inhibitor activity was assayed

according to the same method for calpastatin as described in CHAPTER V. One unit of papain inhibitor was defined as the amount that decreased 1 unit of BANA hydrolyzing activity of papain. Specific assay conditions and other additions are detailed in the legends.

Separation of BANA hydrolases from carp muscle

Calpastatin fractions eluted by 0.05M NaCl from DEAE-cellulose(Fig.24) were pooled, condensed to 2.4mℓ by Amicon PM-10 membrane and applied on a column(1.5x 100cm) of Ultrogel AcA 34(from LKB) equilibrated with 20mM Tris-HCl buffer, pH 7.5, containing 5mM 2-mercaptoethanol and 1mM EGTA(buffer A). Fractions which showed BANA hydrolyzing activity were pooled and applied on a column(2.5x2cm) of papain-Sepharose 4B prepared by the method of Kominami *et al.*[207]. Elution was carried out with buffer A, buffer A containing 1M NaCl, and 1mM HCl.

RESULTS

1. Detection of BANA hydrolases I and II in carp muscle

As shown in Fig.40, BANA hydrolyzing activity and papain inhibitor activity was eluted overlapped from Ultrogel AcA 34. These activities were sepa-

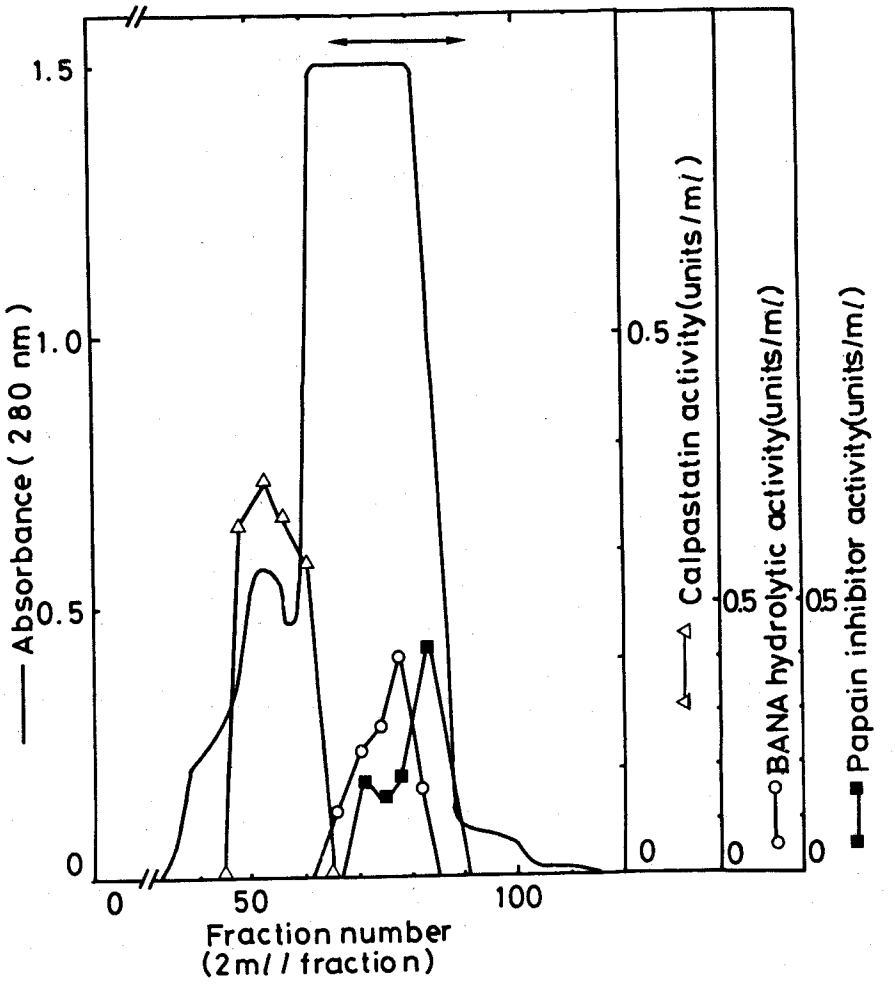

Fig.40. Chromatography of 0.05M NaCl fraction eluted from DEAE-cellulose on a column(1.5x100cm) of Ultrogel AcA 34. Elution was carried out with buffer A at a flow rate of 13.2mℓ/hr and 2mℓ of each fraction was collected.

rated by papain-Sepharose 4B as shown in Fig.41. Papain inhibitor was adsorbed to papain-Sepharose 4B and eluted by 1mM HCl. Two peaks of BANA hydrolyzing activity were observed in the fractions eluted from papain-Sepharose 4B. One BANA hydrolyzing fraction was not adsorbed, while the other was adsorbed and eluted with buffer A containing 1M NaCl. The author tentatively called the former as BANA hydrolase I and the latter as BANA hydrolase II, respectively, and used them for the experiments described below.

2. Some properties of carp muscle BANA hydrolases I and II

Fig.42 shows the effect of pH on BANA hydrolyzing and caseinolytic activities of BANA hydrolases I and II. Both preparations deamidated BANA at pH 6.7 and hydrolyzed casein at pH 8.3 optimally. However, BANA hydrolase I also hydrolyzed casein at pH 6.2. These results suggest that both preparations contained more than one proteinase.

Fig.43 shows the effect of cysteine concentration on BANA hydrolyzing activities of BANA hydrolases I and II in the presence or absence of EDTA. Both preparations showed the absolute cysteine dependency for BANA hydrolyzing activity. Higher activities of both

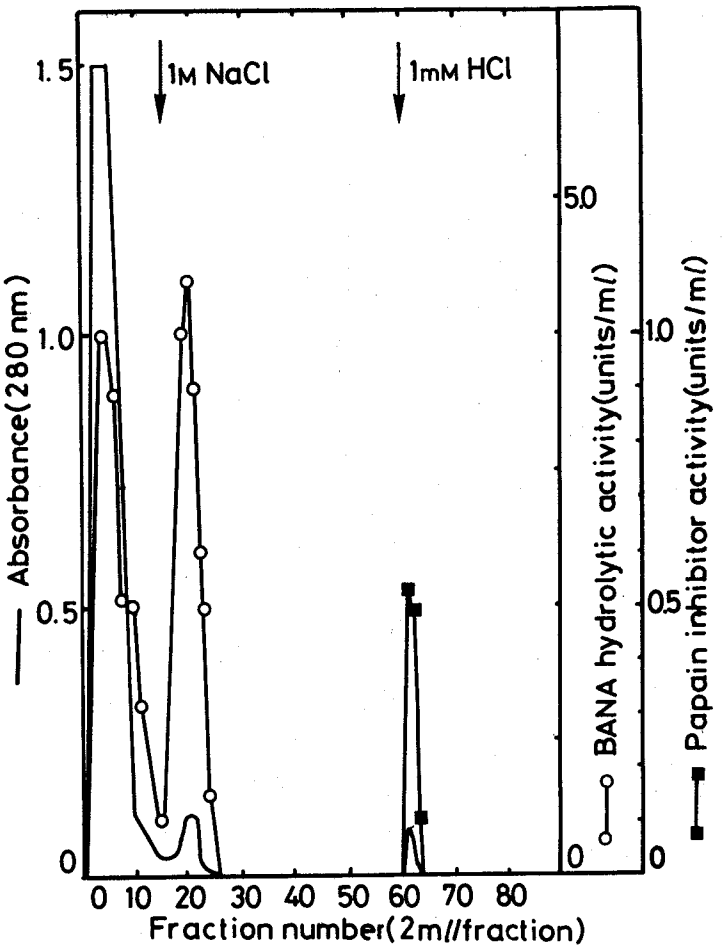

Fig.41. Chromatography of the fraction eluted from Ultrogel AcA 34 on a column(2.5x2cm) of papain-Sepharose 4B. Elution was carried out with buffer A, buffer A containing 1M NaCl, and 1mM HCl at a flow rate of 18mℓ/hr, then 2mℓ of each fraction was collected.

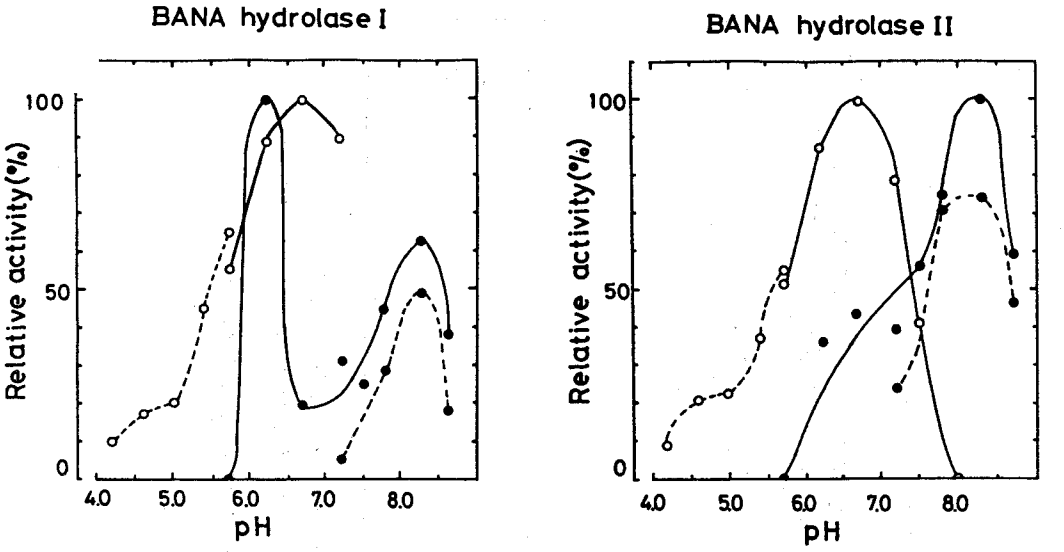

Fig.42. Effect of pH on BANA hydrolyzing and caseinolytic activities of BANA hydrolases I and II. Buffers used were as follows: acetate(O---O) or Tris-maleate (O——O) buffer for the assay of BANA hydrolyzing activity and Tris-maleate(●——●) or Tris-HCl(●---●) buffer for the assay of caseinolytic activity.

preparations were obtained in the presence of EDTA than in the absence of it. These observations suggest that BANA hydrolases I and II contained some kinds of cysteine proteinases[74].

This consideration is also supported by the effect of antibiotics on BANA hydrolyzing activities of both preparations. As shown in Table 10, 0.1μg/mℓ of leupeptin, antipain and E-64 inhibited the activities almost completely. Chymostatin also inhibited the activities to almost the same degree.

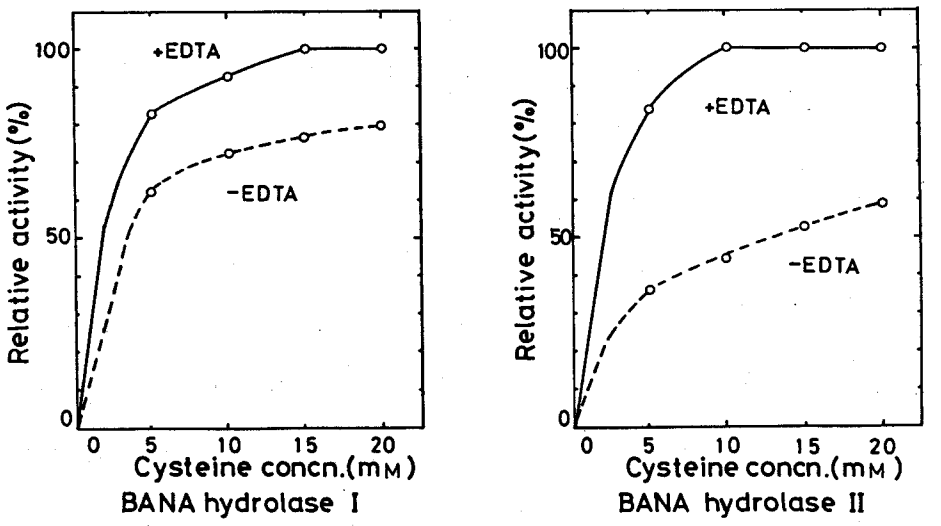

Fig.43. Effect of cysteine concentration on BANA hydrolyzing activity of BANA hydrolases I and II. Assay conditions were as follows: indicated concentration of cysteine, 0.05mℓ of the enzyme solution, 0.5mM BANA and 100mM Tris-maleate buffer, pH 6.7, in the presence (O——O) or absence(O- - -O) of 2mM EDTA in a total volume of 1.0mℓ.

Table10. Effect of antibiotics on BANA hydrolyzing activity of BANA hydrolases I and II.

BANA hydrolase I

Antibiotics	Concn.(μg/ml) 0.01	0.1	1.0	10
	Relative activity (%)			
None	100	100	100	100
Leupeptin	39	0	0	0
Antipain	50	5	0	0
E-64	19	0	0	0
Pepstatin	100	100	100	100
Elastatinal	100	100	77	23
Chymostatin	52	7	0	0
Phospho-ramidon	100	60	65	51

BANA hydrolase II

Antibiotics	Concn.(μg/ml) 0.01	0.1	1.0	10
	Relative activity(%)			
None	100	100	100	100
Leupeptin	38	0	0	0
Antipain	67	7	0	0
E-64	15	0	0	0
Pepstatin	100	100	100	100
Elastatinal	100	100	82	21
Chymostatin	62	12	0	0
Phospho-ramidon	100	90	98	70

Both preparations of BANA hydrolases I and II showed not only BANA hydrolyzing activity but also caseinolytic activity. Then, the author examined some properties of caseinolytic activities of both preparations.

Fig.44 shows caseinolytic activities of both preparations as a function of time. While both preparations hydrolyzed casein at pH 8.3 almost dependent on

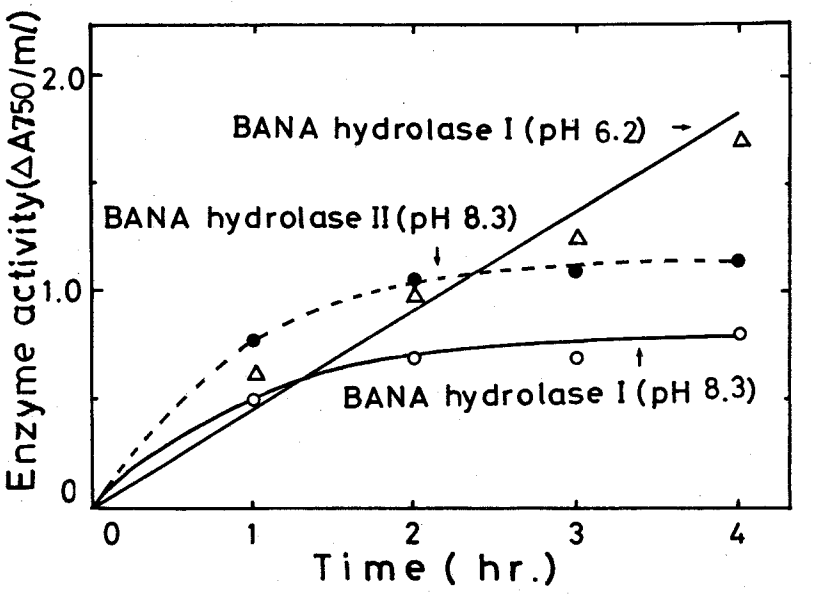

Fig.44. Caseinolytic activities of BANA hydrolases I and II as a function of time. BANA hydrolase I activity was assayed at pH 6.2(△——△) or pH 8.3(○——○) and BANA hydrolase II activity was assayed at pH 8.3(●---●) as described in MATERIALS AND METHODS.

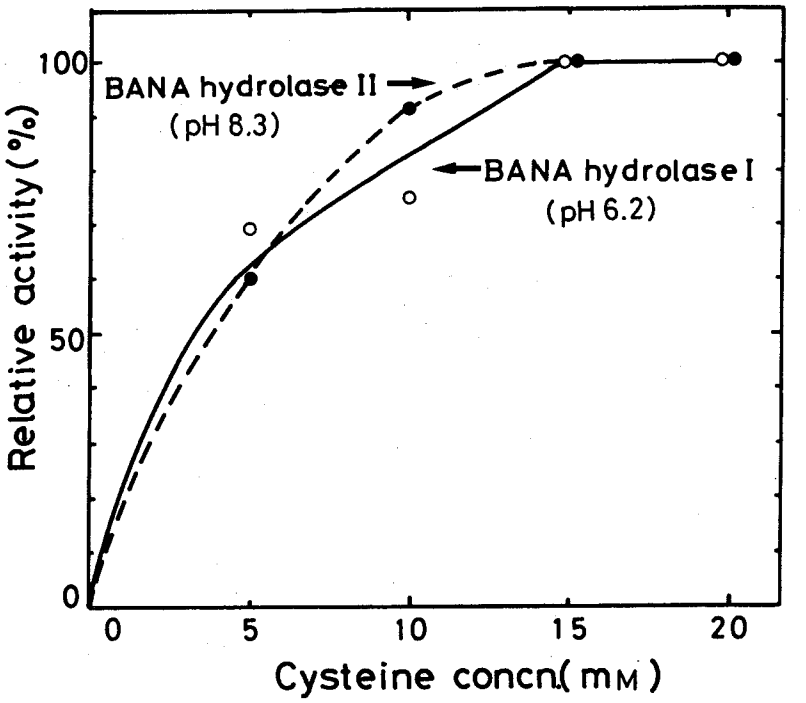

Fig.45. Effect of cysteine concentration on caseinolytic activities of BANA hydrolases I and II. Assay conditions were as follows: indicated concentration of cysteine, 0.2mℓ of the enzyme solution, 0.5%(w/v) casein, 2mM EDTA and 100mM Tris-maleate buffer, pH 6.2 for BANA hydrolase I(O——O) or pH 8.3 for BANA hydrolase II(●---●).

the incubation time, BANA hydrolase I did more markedly at pH 6.2.

As illustrated in Fig.45, caseinolytic activities (at pH 6.2 for BANA hydrolase I and pH 8.3 for BANA hydrolase II) showed the absolute cysteine dependency as well as in the case of BANA hydrolyzing activity.

DISCUSSION

Tallan *et al.*[209] first reported the existence of cathepsin B in bovine spleen in 1952. Later, some investigators have revealed the existence of the enzyme in the crude extract of mammalian muscle[41,48,54,89,92,210]. Recently, it has become clear that the crude extract prepared from various mammalian tissues contain at least three cysteine proteinases which deamidate BAA or BANA[137,208].

Makinodan and Ikeda[14] showed the existence of cathepsin B in carp muscle extract. As described above, it is likely that fish muscle extract contained more than one cysteine proteinase that deamidated BAA or BANA. In the present study, however, the author could not detect any BANA hydrolyzing activity in the crude

solution and in the fractions eluted from DEAE-cellulose. BANA hydrolyzing activity was detected in the fractions eluted from Ultrogel AcA 34 for the first time, accompanied with papain inhibitor(Fig.40). Since both activities eluted in the overlapped fractions, papain inhibitor might surpress BANA hydrolase in the co-existed fractions. BANA hydrolyzing activity could be separated into BANA hydrolases I and II by papain-Sepharose 4B and papain inhibitor was also isolated by the same chromatography. Since BANA hydrolases I and II were eluted in the same fractions from ion-exchange chromatography and gel filtration, both preparations seemed to contain proteinases that have almost the same isoelectric points and molecular weights. From the enzymatic properties of them, both preparations obviously contained some cysteine proteinases. Particularly, BANA hydrolase I seems to contain at least two cysteine proteinases that optimally hydrolyzed casein at pH 6.2 and 8.3, respectively.

Schwart and Bird[143] purified cathepsin B from rat skeletal muscle and reported that this proteinase optimally deamidated BANA at pH 6.0. Hardy and Pennington[211] also revealed that rat skeletal muscle contained several BANA hydrolases of which molecular weight were 25,500-45,000. Molecular weights and pH dependencies

of the activity of rat skeletal muscle BANA hydrolases reported by Schwart and Bird[143] and Hardy and Pennington[211] are similar to those of carp muscle BANA hydrolases. On the other hand, Okitani[212] purified BANA hydrolase H from rabbit skeletal muscle and estimated its molecular weight to be 340,000. The author, however, could not detect such a high molecular weight BANA hydrolase in carp muscle.

Recently, Rodeman *et al.*[193] reported that protein turnover activated by Ca^{2+} was due to lysosomal cysteine proteinases mediated by prostaglandin E_2. After death of animal, sarcoplasmic reticulum loses its ability to adsorb Ca^{2+} [213] and Ca^{2+} concentration in the sarcoplasm increases around 10^{-4}M[166]. Carp muscle BANA hydrolases as well as calpain possibly take part in the post-mortem protein degradation, considering their optimum pHs, although intracellular localization of BANA hydrolases is unknown.

SUMMARY AND CONCLUSIONS

In this study, the author examined fish muscle autolysis as post-mortem protein degradation by muscle proteases. At first, the author recognized the increase of TCA soluble peptides and amino acids and changes of SDS polyacrylamide gel electrophoretic pattern during autolysis. This observation suggested the possible participation of muscle proteases in autolysis(CHAPTER II).

Then, the author studied the distribution of known three proteinases(cathepsin D, sub-endopeptidase and alkaline proteinase) in the muscle of various fish and confirmed their existence in the muscle of all fish examined(CHAPTER III).

Cathepsin D has known to possibly take part in autolysis for a long time. The author purified cathepsin D from carp muscle as an electrophoretically homogeneous preparation by heat treatment, acid treatment, ammonium sulfate fractionation, acetone fractionation, DEAE-Sephadex A-50 chromatography, preparative electrofocusing by Multiphor and Sepharose 6B gel filtration. The purified preparation represented about 2,000-fold purification with a yield of 4%.

The activity against hemoglobin was maximum at pH 2.6-2.8 with 0.6M formate buffer and around pH 3.2 with 0.12M formate buffer. The molecular weight was found to be 41,000 and the iso-electric point to be pH 5.4. From the effect of inhibitors on the activity, carp muscle cathepsin D was classified as an aspartic proteinase. Carp muscle cathepsin D hydrolyzed myofibrils optimally at pH 3-4, but did not above pH 6.0. Therefore, the participation of carp muscle cathepsin D in autolysis is very doubtful(CHAPTER IV, SECTION I).

Cathepsin A has not been purified as an homogeneous preparation as yet from muscle. In this study, the author first purified it from carp muscle as an electrophoretically homogeneous preparation by almost the same purification scheme for cathepsin D. The purified preparation represented about 1,700-fold purification with a yield of 1.5%. The enzyme hydrolyzed Z-Glu-Phe more preferably than Z-Glu-Tyr, with the optimum pH 5.0. The Km values for Z-Glu-Phe and Z-Glu-Tyr were estimated to be 3.52mM and 4,76mM, respectively. From the effect of inhibitors on the activity, carp muscle cathepsin A was classified as a lysosomal carboxypeptidase A. On the other hand, carp muscle cathepsin A did not hydrolyze any protein substrate including some intracellular

proteins. Furthermore, the enzyme had no electrophoretically recognizable effect on autolysis. Therefore, carp muscle cathepsin A does not seem to participate directly in the post-mortem protein degradation of fish muscle(CHAPTER IV, SECTION II).

There is only limited knowledge on neutral proteases in comparison with those on acid proteases. Recently, it was found that calcium-dependent cysteine proteinase(calpain) and its endogenous inhibitor(calpastatin) distribute widely in mammalian tissues. The author, however, could not detect any calpain activity in the crude extract of carp muscle. When the condensate of the crude solution was chromatographed by Ultrogel AcA 34, the author could detect calpains I, II and calpastatin. But, further purification of calpain I was difficult since its activity was very weak. Then, the author obtained partially purified preparations of calpain II and calpastatin from carp muscle and examined their properties. Carp muscle calpain II of which molecular weight was 100,000 hydrolyzed casein optimally at pH 7.5 in the presence of 5mM Ca^{2+} and 20mM cysteine. From the results of cysteine dependency and the effect of inhibitors, carp muscle calpain II was classified as a cysteine

proteinase. In the presence of 5mM Ca^{2+}, calpain II lost its activity completely by heating at 30°C for 30min by autolysis. On the other hand, calpastatin of which molecular weight was 300,000 did not inhibit trypsin, α-chymotrypsin and papain but inhibited calpain II from rat liver as well as carp muscle. The inhibition on calpain II was due to forming calpain-calpastatin complex in the presence of Ca^{2+}(CHAPTER V).

During the purification of calpain II and calpastatin from carp muscle, the author found other proteinase inhibitors, namely trypsin inhibitor and papain inhibitor. By DEAE-cellulose chromatography and following Ultrogel AcA 34 gel filtration, trypsin inhibitor and papain inhibitor were obtained in the overlapped fractions. These two proteinase inhibitors were distinguished in the inhibition spectra and the heat stability. Carp muscle trypsin inhibitor which was not so heat stable inhibited serine proteinases, such as trypsin and α-chymotrypsin. On the other hand, papain inhibitor which was rather heat stable inhibited cysteine proteinases, such as papain and calpain II. The author could not detect any intracellular target enzyme for trypsin inhibitor in carp muscle, so the physiological function of carp muscle

trypsin inhibitor is still unknown. Unlike trypsin inhibitor, carp muscle papain inhibitor may play a protective role against unwanted proteolysis by endogenous cysteine proteinases, since it inhibited intracellular Ca^{2+}-dependent cysteine proteinase, calpain II. Thus, the author sought another intracellular target proteinase for papain inhibitor in carp muscle(CHAPTER VI).

By DEAE-cellulose chromatography and following Ultrogel AcA 34 gel filtration, the author obtained the fraction which showed BANA hydrolyzing activity in the presence of cysteine. This fraction also contained papain inhibitor. BANA hydrolase and papain inhibitor were separated by the affinity chromatography on papain-Sepharose 4B. BANA hydrolase fraction was also separated into BANA hydrolases I and II by this chromatography. Both preparations degraded BANA and casein around neutral pH range. These proteinases required cysteine for the activities and were inhibited by leupeptin, antipain and E-64. Each preparation, however, seemed to contain more than one enzyme (CHAPTER VII).

As above mentioned, there exist some neutral proteinases, such as calpain and BANA hydrolase other

than cathepsin D, sub-endopeptidase and alkaline proteinase in fish muscle. The activities of these neutral proteinases which are regulated by Ca^{2+}, intracellular SH compounds and/or endogenous inhibitors are thought to be possibly involved in the post-mortem protein degradation of fish muscle.

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Professor Shizunori Ikeda, Laboratory of Fishery Chemistry, Department of Fisheries, Faculty of Agriculture, Kyoto University. The author is also grateful to Dr. Yasuo Makinodan, for his continuous guidance and advice throughout the work. Thanks are also due to the members of the Seminar of Fishery Chemistry for their helpful discussion and encouragement.

The author is also indebted to Professor Takashi Murachi, Dr. Kazuyoshi Tanaka and the members of Clinical Science, Faculty of Medicine, Kyoto University, for their fruitful discussion as to CHAPTERS V, VI and VII in this study.

REFERENCES

1) J. Sakai: *Nōgakkaihō*, 137, 28(1914).

2) T. Ohya and K. Shimada: *Suisankōsyūjo Shikenhōkoku*. 19, 1(1923).

3) K. Saitō and M. Samejima: *Bull. Japan. Soc. Sci. Fish.*, 24, 201(1958).

4) T. Hata, T. Asao, and E. Doi: *Syokuryōkagakukenkyūjo Hōkoku, Kyoto Univ.*, 24, 43(1960).

5) Y. Makinodan, M. Yamamoto, and W. Shimidu: *Bull. Japan. Soc. Sci. Fish.*, 29, 776(1963).

6) H. S. Groninger, Jr.: *Arch. Biochem. Biophys.*, 108, 175(1964).

7) G. Siebert, A. Schmidt, and R. von. Malortie: *Hoppe-Seyler's Z. Physiol. Chem.*, 342, 20(1965).

8) J. W. C. Bird, T. Berg, A. Milanesi, and W. T. Stauber: *Comp. Biochem. Physiol.*, 30B, 457(1968).

9) C. Y. Ting, M. W. Montgomery, and A. F. Anglemier: *J. Food. Sci.*, 33, 617(1968).

10) Y. Makinodan and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 35, 749(1969).

11) Y. Makinodan and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 35, 758(1969).

12) H. Manita, T. Koizumi, and J. Nonaka: *Bull. Japan. Soc. Sci. Fish.*, 35, 1027(1969).

13) T. Tokiwa and H. Matsumiya: *Bull. Japan. Soc. Sci. Fish.*, 35, 1027(1969).

14) Y. Makinodan and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 37, 1002(1971).

15) Y. Makinodan, M.Hirotsuka, and S. Ikeda: *J. Food. Sci.*, 44, 1110(1979).

16) Y. Makinodan: Minor components of fish and shellfish- Their biochemistry and food chemistry, ed. by S. Ikeda,p.81(1981),Kōseisya Kōseikaku Co., Tokyo.

17) Y. Makinodan: *New Food Industry*, 24, 53(1982).

18) R. Zender, C. Lataste-Dorolle, R. A. Collet, P. Rowinski, and R. F. Mouton: *Food Research*, 23, 305(1958).

19) M. Fujimaki: *Nogeikagakukaishi*, 32, 689(1958).

20) M. Fujimaki and Y. Nakajima: *Nogeikagakukaishi*, 32, 695(1968).

21) T. Hata and E. Doi: *Nogeikagakukaishi*, 33, 369 (1958).

22) D. DE. Fremery and M. E. Pool: *Food Research*, 24, 73(1959).

23) C. Raudouco-Thomas, C. Lataste-Dorolle, R. Zender, R. Busset, M. M. Meyer and R. F. Mouton: *Food Research*, 24, 453(1959).

24) R. A. Sliwinski, D. M. Doty, and W. A. Landmann: *Agric. Food. Chem.*, 7, 788(1959).

25) R. H. Locker: *J. Sci. Food. Agric.*, 11, 520(1960).

26) R. A. Sliwinski, R. Margolis, K. Pih, W. A. Landmann, and D. M. Doty: *Ann. Rep. Amer. Meat. Institut. Found. Bull.*, 45, 20(1961).

27) R. K. Scopes and R. A. Lawrie: *Nature*, 197, 1202 (1963).

28) J. G. Sharp: *J. Sci. Food. Agric.*, 14, 468(1963).

29) R. K. Scopes: *Biochem. J.*, 91, 201(1964).

30) M. Fujimaki and F. E. Deatherage: *J. Food. Sci.*, 29, 316(1964).

31) J. M. Neelin and D. Rose: *J. Food. Sci.*, 29, 544 (1964).

32) D. E. Goll, D. W. Henderson, and E. A. Kline: *J. Food. Sci.*, 29, 590(1964).

33) A. W. Khan and L. van. DE. Berg: *J. Food. Sci.*, 29, 597(1964).

34) C. E. Bodwell and A. M. Pearson: *J. Food. Sci.*, 29, 601(1964).

35) G. A. Garder and D. J. Stewart: *J. Sci. Food. Agric.*, 17, 491(1966).

36) C. L. Davey and K. V. Gilbert: *J. Food. Sci.*, 31, 135(1966).

37) F. C. Parrish, Jr. and M. E. Bailey: *J. Agr. Food. Chem.*, 14, 232(1966).

38) A. Suzuki, M. Nakazato, and M. Fujimaki: *Agric. Biol. Chem.*, 31, 953(1967).

39) T. Fukazawa and T. Yasui: *Biochem. Biophys. Acta.*, 140, 534(1967).

40) M. H. Stromer, D. E. Goll, and L. E. Roth: *J. Cell. Biol.*, 34, 431(1967).

41) C. J. Randall and H. F. MacRae: *J. Food. Sci.*, 32, 182(1967).

42) F. C. Parrish, Jr. and M. E. Bailey: *J. Agr. Food. Chem.*, 15, 88(1967).

43) M. B. Berman: *J. Food. Sci.*, 32, 568(1967).

44) A. Suzuki and M. Fujimaki: *Agric. Biol. Chem.*, 32, 975(1968).

45) C. B. Martins and J. R. Whitaker: *J. Food. Sci.*, 33, 59(1968).

46) A. Suzuki, A. Okitani, and M. Fujimaki: *Agric. Biol. Chem.*, 33, 579(1969).

47) A. Suzuki, A. Okitani, and M. Fujimaki: *Agric. Biol. Chem.*, 33, 1723(1969).

48) A. J. Lutalo-Bosa and H. F. MacRae: *J. Food. Sci.*, 34, 401(1969).

49) T. Fukazawa, E. J. Briskey, F. Takahashi, and T. Yasui: *J. Food. Sci.*, 34, 606(1969).

50) M. Fujimaki and A. Okitani: *Nippon Shokuhin Kōgyō Gakkaishi*, 22, 34(1975).

51) A. Okitani: *Kagaku to Seibutsu*, 19, 108(1981).

52) J. E. Snoke and H. Neurath: *J. Biol. Chem.*, 187, 127(1950).

53) A. L. Tappel, H. Zalkin, K. A. Caldwell, I. D. Desai and S. Shibko: *Arch. Biochem. Biophys.*, 96, 340(1962).

54) J. M. W. Bouma and M. Gruber: *Biochem. Biophys. Acta.*, 89, 545(1964).

55) A. A. Iodice, V. Leong, and I. M. Weinstock: *Arch. Biochem. Biophys.*, 117, 477(1966).

56) A. A. Iodice: *Arch. Biochem. Biophys.*, 121, 241 (1967).

57) M. Laskowski, Jr. and I. Kato: *Ann. Rev. Biochem.*, 49, 593(1980).

58) T. Murachi, K. Tanaka, M. Hatanaka, and T. Murakami: *Adv. Enz. Reg.*, 19, 407(1981).

59) H. Uchiyama, T. Suzuki, S. Ehira, and E. Noguchi: *Bull. Japan. Soc. Sci. Fish.*, 32, 280(1966).

60) S. V. Manohar: *J. Fish. Res. Board. Can.*, 27, 1977(1970).

61) M. Tamazawa, M. Murase, and K. Shiga: *Bull. Japan. Soc. Sci. Fish.*, 46, 191(1980).

62) Y. Makinodan, M. Hirotsuka, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 46, 1507(1980).

63) S. Ikeda: Minor components of Fish and Shellfish - Their biochemistry and food chemistry, ed. by S. Ikeda, p.66(1981), Kōseisya Kōseikaku Co., Tokyo.

64) T. Ohya, E. Sumi, and K. Shimada: *Suisankōsyūjo Shikenhōkoku*, 21, 149(1925).

65) T. Ohya and E. Sumi: *Suisankōsyūjo Shikenhōkoku*, 22, 20(1926).

66) K. Yoshimura, S. Kubo, and Y. Itō: *Hokkaidō Daigaku Suisankenkyūjo Ihō*, 4, 157(1953).

67) M. Fujii and T. Tomita: *Suisankōsyūjo Kenkyūhōkoku*, 4, 33(1955).

68) W. Shimidu and T. Kiriyama: *Bull. Japan. Soc. Sci. Fish.*, 20, 33(1954).

69) J. M. Shewan and N. R. Jones: *J. Sci. Food. Agric.*, 8, 491(1957).

70) E. W. Yemm and E. C. Cocking: *Analyst*, 80, 209(1955).

71) O. H. Lowry, N. J. Rosebough, A. L. Farr, and R. J. Randall: *J. Biol. Chem.*, 193, 265(1951).

72) K. Weber and M. Osborn: *J. Biol. Chem.*, 224, 4406 (1969).

73) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 49, 481(1983).

74) A. J. Barret: *Ciba Found. Symp.*, 75, 1(1980).

75) N. Seki and T. Watanabe: *Bull. Japan. Soc. Sci. Fish.*, 48, 517(1982).

76) K. A. Caldwell: *J. Agr. Food. Chem.*, 18, 276(1970).

77) A. Okitani and M. Fujimaki: *Agric. Biol. Chem.*, 36, 7(1972).

78) W. A. Busch, M. H. Stromer, D. E. Goll, and A. Suzuki: *J. Cell. Biol.*, 52, 367(1972).

79) A. Okitani, K. Shinohara, M. Sugitani, and M. Fujimaki: *Agric. Biol. Chem.*, 37, 321(1973).

80) M. F. Eino and D. W. Stanley: *J. Food. Sci.*, 38, 45 (1973).

81) M. F. Eino and D. W. Stanley: *J. Food. Sci.*, 38, 51 (1973).

82) J. D. Hay, R. W. Currie, and F. H. Wolfe: *J. Food. Sci.*, 38, 987(1973).

83) A. Okitani, Y. Otsuka, M. Sugitani, and M. Fujimaki: *Agric. Biol. Chem.*, 38, 573(1974).

84) A. Suzuki and D. E. Goll: *Agric. Biol. Chem.*, 38, 2167(1974).

85) M. K. Reddy, J. D. Etlinger, M. Rabinowitz, D. A. Fischman, and R. Zak: *J. Biol. Chem.*, 250, 4278 (1975).

86) W. J. Reville, D. E. Goll, M. H. Stromer, R. M. Robson, and W. R. Dayton: *J. Cell. Biol.*, 70, 1 (1976).

87) N. Arakawa, S. Fujiki, C. Inagaki, and Fujimaki: *Agric. Biol. Chem.*, 40, 1265(1976).

88) D. G. Olson, F. C. Parrish, Jr., W. R. Dayton, and D. E. Goll: *J. Food. Sci.*, 42, 117(1977).

89) W. T. Stauber, A. M. Hedge, and B. A. Schottelius: *Acta. Biol. Med. Germ.*, 36, 1645(1977).

90) A. Okitani, U. Matsukura, Y. Ohtsuka, M. Watanabe, and M. Fujimaki: *Agric. Biol. Chem.*, 41, 1821(1977).

91) C. S. Cheng and F. C. Parrish, Jr.,: *J. Food. Sci.*, 42, 1621(1977).

92) B. Venugopal and M. E. Bailey: *Meat Sci.*, 2, 227 (1978).

93) J. F. Penny and F. Pryce: *Meat Sci.*, 3, 121(1979).

94) F. M. Robbins, J. E. Walker, S. H. Cohen, and S. Chatterjee: *J. Food. Sci.*, 44, 1672(1979).

95) K. Yamamoto, K. Samejima, and T. Yasui: *J. Food. Sci.*, 44, 51(1979).

96) A. Okitani, U. Matsukura, H. Kato, and M. Fujimaki: *J. Biochem.*, 87, 1133(1980).

97) U. Matsukura, A. Okitani, T. Nishimura, and H. Kato: *Biochem. Biophys. Acta.*, 662(1981).

98) A Okitani, T. Matsumoto, and H. Kato: *Biochem. Biophys. Acta.*, 662, 202(1981).

99) C. K. Kang, T. H. Donnelly, R. F. Jodlowski, and E. D. Warner: *J. Food. Chem.*, 46, 702(1981).

100) P. Nagainis and F. H. Wolfe: *J. Food. Chem.*, 47, 1358 (1982).

101) A. Suzuki: *Kagaku to Seibutsu*, 19, 182(1981).

102) A. Hattori and K. Takahashi: *J. Biochem.*, 85, 47(1979).

103) K. Takahashi. F. Nakamura, and A. Inoue: *J. Biochem.*, 89, 321(1981).

104) A. Hattori and K. Takahashi: *J. Biochem.*, 92, 381 (1982).

105) K. Takahashi, F. Nakamura, and M. Okamoto: *J. Biochem.*, 92, 809(1982).

106) K. Takahashi: *New Food Industry*, 23, 59(1981).

107) K. Takahashi: *Kagaku to Seibutsu*, 19, 261(1981).

108) G. Siebert: *Experientia*, 14, 65(1958).

109) G. Siebert and A. Schmidt: The Technology of Fish Utilization, ed. by R. K. Kreuzer, p.47(1965), Fishing News L.T.D., London.

110) Y. Makinodan, and S. Ikeda: *Bull. Jap. Soc. Sci. Fish.*, 35, 672(1969).

111) T. Takahashi and M. Yamazawa : *J. Tokyo. Univ. Fish.*, 55, 99(1969).

112) K. Mush and G. Siebert: *Hoppe-Seyler's Z. Physiol. Chem.*, 312, 878(1971).

113) W. B. Wojtowicz and P. H. Odese: *J. Fish. Res. Board. Canada*, 29, 85(1972).

114) P. K. Reddi, S. M. Constantinides, and H. A. Dymsza: *J. Food. Sci.*, 37, 643(1972).

115) G. M. Geist and D. L. Crawford: *J. Food. Sci.*, 39, 548(1974).

116) S. N. Doke, V. Ninjoor, and G. B. Nadkarni: *Agric. Biol. Chem.*, 44, 1521(1980).

117) R. R. Eitenmiller: *J. Food. Sci.*, 39, 6(1974).

118) J. Sakai and J. J. Matsumoto: *Comp. Biochem. Physiol.*, 68B, 389(1981).

119) J. Sakai, Y. Sakaguchi, and J. J. Matsumoto: *Comp. Biochem. Physiol.*, 70B, 791(1981).

120) Y. Makinodan, T. Akasaka, H. Toyohara, and S. Ikeda: *J. Food. Sci.*, 47, 647(1982).

121) Y. Makinodan and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 42, 665(1976).

122) K. Iwata, K. Kobashi, and J. Hase: *Bull. Japan. Soc. Sci. Fish.*, 39, 1325(1973).

123) K. Iwata, K. Kobashi, and J. Hase: *Bull. Japan. Soc. Sci. Fish.*, 40, 201(1974).

124) K. Iwata, K. Kobashi, and J. Hase: *Bull. Japan. Soc. Sci. Fish.*, 40, 189(1974).

125) K. Iwata, K. Kobashi, and J. Hase: *Bull. Japan. Soc. Sci. Fish.*, 40, 1043(1974).

126) Y. Makinodan and S. Ikeda: *J. Food. Sci.*, 42, 1026 (1977).

127) Y. Makinodan, N. N. Kyaw, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 48, 479(1982).

128) Y. Makinodan, N. N. Kyaw, and S. Ikeda: *Comp. Biochem. Physiol.*, 73B, 785(1982).

129) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 49, 109(1983).

130) M. Kunitz: *J. Gen. Physiol.*, 30, 291(1947).

131) M. L. Anson: *J. Gen. Physiol.*, 22, 79(1938).

132) H. L. A. Tarr: *Ann. Rev. Biochem.*, 27, 223(1958).

133) R. Ueno, T. Morishita, and T. Takahashi: *Bull. Japan. Soc. Sci. Fish.*, 47, 207(1981).

134) A. J. Barret: Proteinases in Mammalian Cells and Tissues, ed. by A. J. Barret, p.209(1977), North-Holland Publishing Co., Amsterdam.

135) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 48, 1501(1982).

136) Y. Makinodan and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 42, 239(1976).

137) A. J. Barret: Proteinases in Mammalian Cells and Tissues, ed. by A. J. Barret, p.181(1977), North-Holland Publishing Co., Amsterdam.

138) G. DE. la Haba, P. S. Cammarata, S. N. Timasheff: *J. Biol. Chem.*, 234, 316(1959).

139) B. J. Davis: *Ann. N. Y. Acad. Sci.*, 121, 424(1964).

140) A. J. Barret: Proteinases in Mammalian Cells and Tissues, ed. by A. J. Barret, p.3(1977), North-Holland Publishing Co., Amsterdam.

141) G. Siebert: Wiss. Zeits. Martin-Luther-Univ. Halle-Wittenberg, Intracellular Protein Catabolism, Proc. Symp., p.80(1973).

142) W. N. Schwart and J. W. C. Bird: *Biochem. J.*, 167, 811(1977).

143) K. Fukushima, G. H. Gnoh, and S. Shinano: *Agric. Biol. Chem.*, 35, 1495(1971).

144) M. Cunningham and J. Tang: *J. Biol. Chem.*, 251, 4528(1976).

145) M. B. Wojtowics and P. H. Odense: *Can, J. Biochem.*, 48, 1050(1970).

146) O. V. Kazakova and V. N. Orekhovich: *Int. J. Peptide. Protein. Res.*, 7, 23(1975).

147) H. Toyohara , Y. Makinodan, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 48, 1145(1982).

148) J. S. Fruton and M. Bergmann: *J. Biol. Chem.*, 130, 19(1939).

149) J. K. McDonald and C. Schwabe: Proteinases in Mammalian Cells and Tissues, ed. by A. J. Barret, p.329(1977). North-Holland Publishing Co., Amsterdam.

150) A. I. Logunov and V. N. Orekhovich: *Biokhimiya*, 37, 715(1972).

151) K. Matsuda and E. Misaka: *J. Biochem.*, 76, 639 (1974).

152) S. L. Taylor and A. L. Tappel: *Biochem. Biophys. Acta.*, 341, 99(1974).

153) Y. Kawamura, T. Matoba, T. Hata, and E. Doi: *J. Biochem.*, 76, 915(1974).

154) Y. Kawamura, T. Matoba, T. Hata, and E. Doi: *J. Biochem.*, 77, 729(1975).

155) K. Iwata, K. Kobashi, and J. Hase: *Bull. Japan. Soc. Sci. Fish.*, 43, 181(1977).

156) K. Iwata, N. Nakai, K. Kobashi, and J. Hase: *Bull. Japan. Soc. Sci. Fish.*, 43, 307(1977).

157) Y. Kawamura, T. Matoba, T. Hata, and E. Doi: *J. Biochem.*, 81, 435(1977).

158) N. Lichtenstein and J. S. Fruton: *Proc. Natl. Acad. Sci., U.S.* 46, 781(1960).

159) A. I. Logunov and V. N. Orekhovich: *Biochem. Biophys. Res. Commun.*, 46, 1161(1972).

160) S. Konagaya: *Bull. Tokai. Reg. Fish. Res. Lab.*, 94, 1(1979).

161) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 49, 1153(1983).

162) G. Guroff: *J. Biol. Chem.*, 239, 149(1964).

163) I. Nishiura, K. Tanaka, S. Yamato, and T. Murachi: *J. Biochem.*, 84, 1657(1978).

164) L. Waxman and E. G. Krebs: *J. Biol. Chem.*, 253, 5888(1978).

165) L. Waxman: Protein turnover and Lysosomal Function, ed. by H. L. Segal and D. J. Doyle, p.363(1978), Academic Press Inc., New York, N. Y.

166) R. Nakamura: *Anal. Biochem.*, 53, 531(1973).

167) E. Ross and G. Schatz: *Anal. Biochem.*, 54, 304 (1973).

168) T. Murachi, M. Hatanaka, Y. Yasumoto, N. Nakayama, and K. Tanaka: *Biochem. Intern.*, 2, 651(1981).

169) W. R. Dayton, D. E. Goll, M. G. Zeece, R. M. Robson, and W. J. Reville: *Biochemistry*, 15, 2150(1976).

170) W. R. Dayton, W. J. Reville, D. E. Goll, and M. H. Stromer: *Biochemistry*, 15, 2159(1976).

171) S. Ishiura, H. Murofushi, K. Suzuki, and K. Imahori: *J. Biochem.*, 85, 225(1978).

172) S. Ishiura, H. Sugita, K. Suzuki, and K. Imahori: *J. Biochem.*, 86, 579(1979).

173) J. L. Azanza, J. Raymond, J. M. Robin, P. Cottin, and A. Ducastaing: *Biochem. J.*, 183, 339(1979).

174) J. L. A. Mellgren, A. Repetti, T. C. Muck, and J. Easly: *J. Biol. Chem.*, 257, 7203(1982).

175) K. Suzuki, S. Ishiura, S. Tsuji, T. Katamoto, H. Sugita, and K. Imahori: *FEBS Lett.*, 104, 355(1979).

176) I. Nishiura and T. Murachi: *Experientia*, 35, 1006(1979).

177) M. Takahashi-Nakamuram.S. Tsuji, K. Suzuki, and K. Imahori: *J. Biochem.*, 90, 1583(1981).

178) S. Ishiura, S. Tsuji, H. Murofushi, and K. Suzuki: *Biochem. Biophys. Acta.*, 701, 216(1982).

179) E. Takano and T. Murachi: *J. Biochem.*, 92, 2021 (1980).

180) T. Murachi: *Protein, Nucleic acid and Enzyme*, 27, 107(1982).

181) L. Waxman: *Fed. Proc.*, 38, 479(1979).

182) P. D. Weiner and A. M. Pearson: *J. Food. Sci.*, 34, 592(1969).

183) M. A. Macbride and F. C. Parrish, Jr.: *J. Food. Sci.*, 42, 1627(1977).

184) T. Kameyama and J. D. Etlinger: *Nature*, 279, 344 (1977).

185) S. E. M. Lewis, P. Anderson, and D. F. Goldspink: *Biochem. J.*, 204, 257(1982).

186) R. L. Mellgren: *FEBS Lett.*, 109, 129(1980).

187) A. Kishimoto, N. Kajiwara, H. Tabuchi, M. Shiota, and Y. Nishizuka: *J. Biochem.*, 90, 889(1981).

188) L. Waxman: *Methods in Enzymology*, 80, 664(1982).

189) W. R. Dayton, J. V. Schollmeyer, R. A. Lepley, and L. R. Cortes: *Biochem. Biophys. Acta.*, 659, 48(1981).

190) S. Kubota, K. Suzuki, and K. Imahori: *Biochem. Biophys. Res. Commun.*, 100, 1189(1981).

191) K. Suzuki, S. Tsuji, S. Ishiura, Y. Kimura, S. Kubota, and K. Imahori: *J. Biochem.*, 90, 1783 (1981).

192) K. Suzuki, S. Tsuji, S. Kubota, Y. Kimura, and K. Imahori: *J. Biochem.*, 90, 275(1981).

193) H. P. Rodeman, L. Waxman, and A. L. Goldberg: *J. Biol. Chem.*, 257, 8716(1982).

194) M. Matsutani, M. Takehisa, R. Fukuba, A. Shimasue, and N. Kikukawa: *J. Med. Technol.*, 11, 300(1967).

195) G. I. Drummond and L. Duncan: *J. Biol. Chem.*, 243, 5532(1968).

196) T. Noguchi and M. Kandatsu: *Agric. Biol. Chem.*, 33, 1226(1969)

197) T. Noguchi and M. Kandatsu: *Agric. Biol. Chem.*, 35, 1092(1971).

198) T. Noguchi, Y. Takano, and M. Kandatsu: *Agric. Biol. Chem.*, 36, 1667(1972).

199) T. Noguchi, E. Miyazawa, and M. Kametaka: *Agric. Biol. Chem.*, 38, 253(1974).

200) R. G. Woodbury, M. Everitt, Y. Sanada, N. Katsunuma, D. Lagunoff, and H. Neurath: *Proc. Natl. Acad. Sci.*, 75, 5311(1978).

201) H. Tschesche: *Methods in Enzymology*, 45, 792(1976).

202) F. C. Moll, S. F. Sunden, and J. R. Brown: *J. Biol. Chem.*, 233, 121(1958).

203) H. L. Bundy and J. W. Mehl: *J. Biol. Chem.*, 234, 1124(1959).

204) R. Pannell, D. Johnson, and J. Travis: *Biochemistry*, 13, 5439(1974).

205) J. F. Lenny, J. R. Tolan, W. J. Sugai, and A. G. Lee: *Eur. J. Biochem.*, 101, 153(1979).

206) M. Hirado. D. Iwata, M. Ninobe, and S. Fujii: *Biochem. Biophys. Acta.*, 669, 21(1981).

207) E. Kominami, N. Wakamatsu, and N. Katsunuma: *Biochem. Biophys. Res. Commun.*, 99, 568(1981).

208) A. J. Barret and H. Kirschke: *Methods in Enzymology*, 80, 535(1982).

209) H. H. Tallan, M. E. Jones, and J. S. Fruton: *J. Biol. Chem.*, 194, 793(1952).

210) K. A. Caldwell and O. K. Grosjean: *J. Agric. Food. Chem.*, 19, 108(1971).

211) M. F. Hardy and R. J. T. Pennington: *Biochem. Biophys. Acta.* 577, 253(1979).

212) A. Okitani, T. Nishimura, Y. Ohtsuka, U. Matsukura, and H. Kato: *Agric. Biol. Chem.*, 44, 1705 (1980).

213) M. L. Greaser, R. G. Cassens, and W. G. Hoekstra: *J. Agr. Food. Chem.*, 15, 1112(1967).

214) A. A. Milanesi and J. W. C. Bird: *Comp. Biochem. Physiol.*, 41B, 573(1972).

215) R. C. Whiting, M. W. Montgomery, and A. F. Anglemier: *J. Food. Sci.*, 40, 854(1975).

216) C. DE. Duve, B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Applemans: *Biochem. J.*, 60, 604(1955).

217) P. Harikumar, V. Ninjoor, S. B. K. Warrier, B. Bhushan, and U. S. Kumta: *J. Food. Sci.*, 39, 1224 (1974).

a) Y. Makinodan, T. Akasaka, H. Toyohara, and S. Ikeda: *J. Food. Sci.*, 47, 647(1982).

b) H. Toyohara, Y. Makinodan, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 48, 1145(1982).

c) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 48, 1501(1982).

d) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 49, 109(1983).

e) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 49, 481(1983).

f) Y. Makinodan, H. Toyohara, and S. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, 49, 1153(1983).

g) H. Toyohara, Y. Makinodan, K. Tanaka, and S. Ikeda: *Agric. Biol. Chem.*, 47, 1151(1983).