STUDIES ON THE MECHANISM OF *MODORI* (THERMAL GEL DEGRADATION)-PHENOMENON OCCURRING IN *KAMABOKO* PROCESSING

MASATO KINOSHITA

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INTRODUCTION

Kamaboko is a traditional and special fish jelly product of Japan, which was described in a literature in the Heian period, the 12th century.[1] The making process of kamaboko is as follows; at first fish meat is ground with 2-3 % table salt to make meat sol, and then the meat sol is heated up to 80 °C or more. The resulting gel is kamaboko. It is very elastic just like rubber and has a unique texture which is never seen in mammalian meat gel.

In the heating process of the fish meat sol, two characteristic phenomena are observed. The one is so-called suwari(gelation)-phenomenon observed in the lower temperature range between 20 and 50 °C. Suwari-phenomenon is considered to occur due to that actomyosin is cross-linked [2-4] by heat to make three dimensional network in which water is trapped. The strong elasticity of kamaboko is attributed to this network structure of actomyosin. The other one is so-called modori(thermal gel degradation)-phenomenon which observed sometimes in the temperature range between 50 and 70 °C. Once this phenomenon occurs, such a elastic kamaboko gel becomes frail just like normal cooked meat and loses its commercial value significantly. Therefore, the modori-phenomenon is a troubled problem for the fish-jelly industry.

Regarding the characteristics of modori-phenomenon many things have been revealed, for example, this phenomenon occurs at 50-70 °C,[5,6] and its occurrence depends on fish species[7], season, fishing ground and physiological condition of the fish.[8,9] As to the mechanism of this phenomenon, roughly four hypotheses have

been built up so far; modori-phenomenon is caused (1) by the thermal denaturation of proteins and the subsequent dehydration from three dimensional protein network,[3,5,10-12] (2) by the non-enzymatic modori-inducing proteins which exist in the sarcoplasmic fraction,[13-14] (3) by the modori-inducing protein which associates with myosin,[15] and 4) by endogenous proteinase(s).[6,16-22] Among these hypotheses, because of the facts that modori-phenomenon is repressed by the addition of some kinds of proteinase inhibitor[20,23] and accompanied by the degradation of myosin heavy chain,[23] it is most likely that certain proteinase(s) concerns this phenomenon. Indeed, a heat-stable alkaline proteinase (HAP) has been thought to be a most possible candidate because of its strong casein degrading activity at 65 °C, an optimum temperature for modori-phenomenon.[6,21,22] However, there exist some contradictions that the amount of HAP in muscle of a certain fish species dose not correlate well with the modori occurring property of the muscle[24] and that the proteolytic activity of HAP is inhibited by 2 % sodium chloride[25,26].

In spite of numbers of studies, an established theory have not been built up about this subject because of the lack of direct evidence. In this thesis, the author attempts to identify the modori-inducing factor and make clear the mechanism of this-phenomenon.

CHAPTER 1. PURIFICATION AND CHARACTERIZATION OF A HEAT-STABLE ALKALINE PROTEINASE

Participation of a heat-stable alkaline proteinase (HAP) in *modori*-phenomenon has long been suspected because the optimum temperature of casein-degrading activity of HAP was well coincident with that of the occurrence of *modori*-phenomenon. However, induction of *modori*-phenomenon by adding HAP has not yet been clearly demonstrated. Therefore, the hypothesis that HAP causes *modori*-phenomenon has not been completely accepted. In this chapter, some characteristics of HAP are investigated.

MATERIALS AND METHODS

Materials

Carp (*Cyprinus carpio*) weighing about 620 g were killed by decapitation. Only dorsal muscle was collected and used for the purification. The sources of other materials used in this work were as follows: DEAE-cellulose (DE-52) from Whatman; hydroxylapatite from Bio-rad; Ultrogel AcA34 from LKB; Hammarsten grade casein from Merck. Suc-Leu-Leu-Val-Tyr-methylcoumarinamide (SLLVY), Boc-Leu-Thr-Arg-methylcoumarinamide (BLTR) and 7-amino-4-methylcoumarin were obtained from Protein Research Foundation, Osaka. Other reagents grade chemicals were purchased from Wako Pure Chemicals, Kyoto.

Measurement of heat-stable alkaline proteinase activity

The hydrolysis of synthetic substrates was measured in the reaction mixture containing 10 µl of proteinase, 22 mM KH_2PO_4-7.2 mM $Na_2B_4O_7$ buffer, pH 8.0, 0.5 mM substrate in a total volume 0.1 ml.[27] Trypsin- and chymotrypsin-

like activities were determined using BLTR and SLLVY as substrates, respectively. After incubation at fixed temperatures (30-65 °C) for 10 min, the increase of fluorescence of 7-amino-4-methylcoumarin (excitation at 380 nm; emission at 460 nm) was measured by a HITACHI 204 Fluorescence Spectrophotometer. Sodium dodecyl sulfate (SDS) was used for the induction of the activities in the experiments of Figs. 4 and 5 and Table 2. One unit of synthetic substrate-degrading activity was defined as the amount that released 1 pmol aminomethylcoumarin for 1 min. Caseinolytic activity was determined in the reaction mixture containing 20-200 µl of proteinase, 22 mM KH_2PO_4-7.2 mM $Na_2B_4O_7$ buffer, pH 8.0, 1 % casein in a total volume 1 ml.[28] The reaction was stopped by the addition of 1 ml of 5 % trichloroacetic acid after the incubation at fixed temperatures (30-65 °C) for 60 min and acid soluble products of filtrate were colorimetrically determined by the method of Lowry et al..[29] One unit of caseinolytic activity was defined as the amount that caused an increase in the absorbance at 750 nm of 1.0 for 60 min. For examining the effect of pH (Fig. 5), 5 mM citric acid, 5 mM K_3PO_4, 5 mM boric acid and 5 mM veronal buffer adjusted to fixed pH by 1 N NaOH was used.

Purification procedures

Fifty grams of minced muscle was homogenized with 200 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.1 M NaCl in a non-bubbling homogenizer (Nihon Seiki Co. Ltd.) for 2 min at top speed. The homogenate was centrifuged at 10,000 x g for 30 min and the obtained supernatant was used as crude extract. Carp muscle HAP was

purified to homogeneity using a combination of DEAE-cellulose, hydroxylapatite and Ultrogel AcA34 according to the method of Makinodan et al..[28)]

Electrophoresis

Polyacrylamide gel electrophoresis under non-denaturing condition was carried out according to the method of Davis[30)] and that with sodium dodecyl sulfate (SDS-PAGE) was carried out according to the method of Laemmli[31)], respectively. Gel was stained with Coomassie Brilliant Blue R-250 and scanned by a Shimadzu Chromato Scanner CS-930 at the absorbance of 550 nm.

RESULTS

Purification

The purification fold of the final preparation against crude extract was 464, 350 and 186 on the basis of caseinolytic, SLLVY-degrading and BLTR-degrading activities, respectively (Table 1). This final preparation was homogeneous judged from polyacrylamide gel electrophoresis under non-denaturing condition (Fig. 1) and its relative molecular mass was estimated 570 kDa by the gel filtration on Ultrogel AcA34 (data not shown). The proteinase gave ten bands with a relative molecular mass of 20-40 kDa on a polyacrylamide gel electrophoretic analysis with SDS (Fig. 2). Such high-molecular-mass and multiple-subunit-structure showed a close similarity to those of multicatalytic proteases (MCPs) purified from various animal cells and tissues.[28,32-43)]

Table 1. Purification of carp muscle HAP.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	5250				
hydrolysis of					
casein		730	0.140	1	100
SLLVY		1370	0.261	1	100
BLTR		1890	0.360	1	100
DEAE-cellulose	52				
hydrolysis of					
casein		210	4.04	29	29
SLLVY		330	6.35	24	24
BLTR		400	7.70	21	21
Hydroxylapatite	6.3				
hydrolysis of					
casein		130	20.6	147	18
SLLVY		198	31.4	120	15
BLTR		136	21.6	60	7
Ultrogel AcA34	2.0				
hydrolysis of					
casein		130	65.0	464	18
SLLVY		183	91.5	350	13
BLTR		134	67.0	186	7

Each activity was measured at 65°C in the absence of SDS.

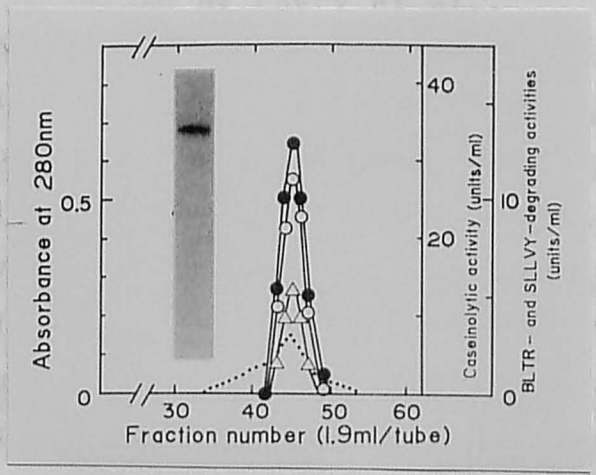

Fig. 1. Elution profile of carp muscle HAP from a column (1.5x100 cm) of Ultrogel AcA 34.
Elution was monitored by the absorbance at 280nm(•••).
Caseinolytic (△), BLTR (○)- and SLLVY (●)-degrading activities were measured at 65°C. Inset; 5 % polyacrylamide gel electrophoresis of the purified proteinase (fraction number 45, 30 μg protein) under non-denaturing condition.

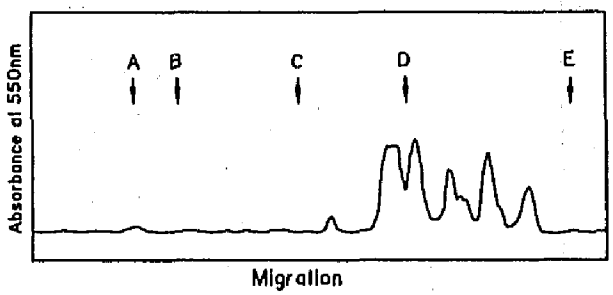

Fig. 2. Densitogram of a 15% SDS-polyacrylamide gel electrophoresis pattern of purified carp muscle HAP. Purified proteinase of 1.5 μg was applied on 15 % polyacrylamide gel with SDS. The destained gel was scanned at 550 nm. Marker proteins: A, rabbit muscle phosphorylase b (M_r 94,000); B, bovine serum albumin (67,000); C, ovalbumin (43,000); D, bovine erythrocyte carbonic anhydrase (30,000); E, soybean trypsin inhibitor (20,000).

Comparison of inducing effect by heating and SDS

Figs. 3 and 4 show the induction of the activities by heating and SDS, respectively. As already reported,[32] the proteinase hardly showed any activity below 50 °C without any induction. The optimum temperature of the activities were observed at 65 °C (Fig. 3). On the other hand, SDS showed the inducing effect only on SLLVY-degrading activity, while it showed little or no induction of caseinolytic and BLTR-degrading activities at any concentration tested (Fig. 4). This result is inconsistent with the fact that azocasein-degrading activity of white croaker muscle proteinase II, proposed to be the same as our proteinase, was stimulated by the addition of 3 mM SDS.[44]

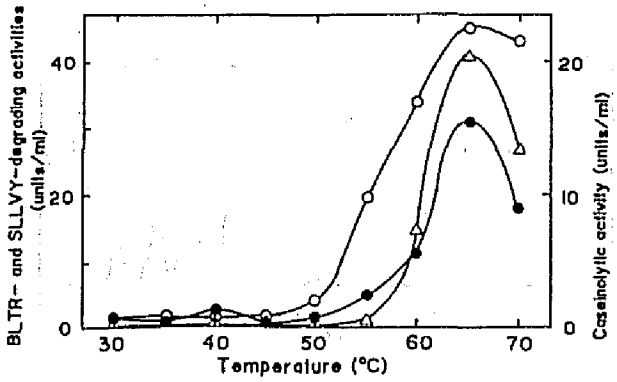

Fig. 3. Inducing effect by heating on caseinolytic (△), BLTR (○)- and SLLVY (●)-degrading activities of carp muscle heat-stable alkaline proteinase. Assay condition is described in the text.

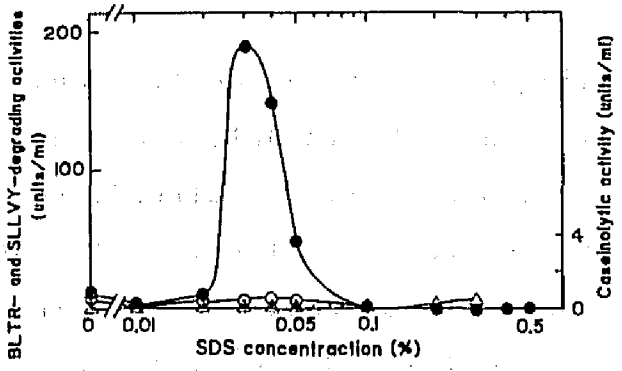

Fig. 4. Effect of SDS concentration on the inducing effect on caseinolytic (△), BLTR (○)- and SLLVY (●)-degrading activities of carp muscle heat-stable alkaline proteinase. Each activity was measured at 37°C.

pH dependence of the activities

Fig. 5 shows pH dependence of the activities. Caseinolytic activity was observed around neutral to slightly alkaline pH range, while BLTR- and SLLVY-degrading activities were detected around relatively wide

pH range from 6 to 10. This result suggests that the proteinase properly induced can reveal its activities under physiological condition. As to SLLVY-degrading activity, SDS-induced form showed higher activity than heat-induced one.

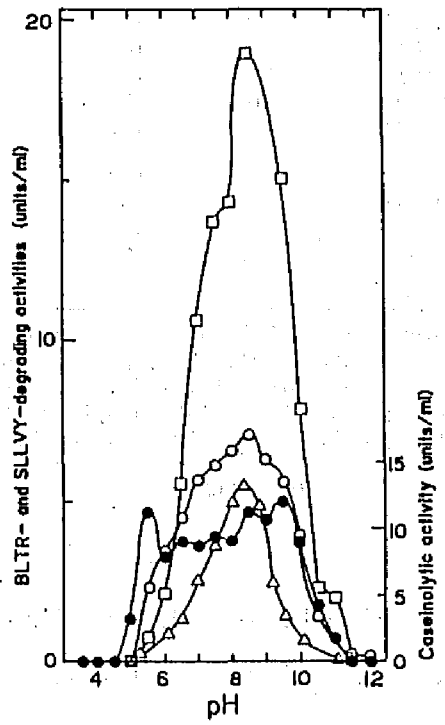

Fig. 5. Effect of pH on carp muscle HAP. Heat-induced caseinolytic (△), BLTR (○)- and SLLVY (●)-degrading activities were measured at 65°C without SDS. SDS-induced SLLVY-degrading (□) activity was measured with 0.03 % SDS at 37°C.

Effect of reagents

The effect of reagents on the activities is summarized in Table 2. Antipain and leupeptin effectively inhibited BLTR-degrading activity, while chymostatin did SDS- and heat-induced forms of SLLVY-degrading activity.

Table 2. Effect of reagents on the activities of carp muscle HAP.

Reagents		Hydrolysis of			
		Casein	BLTR	SLLVY	
		65°C*	65°C*	65°C*	37°C+
No addition		100	100	100	100
Antipain	(100 μg/ml)	59	38	78	78
Leupeptin	(100 μg/ml)	115	16	83	84
Chymostatin	(100 μg/ml)	67	52	21	15
Adriamycin	(100 μg/ml)	73	67	72	5
Pepstatin	(20 μg/ml)	100	N.D.	N.D.	100
EDTA	(5 mM)	60	88	77	103
E-64	(100 μg/ml)	119	72	100	92
2-mercaptoethanol	(10 mM)	85	100	96	41
PMSF	(1 mM)	90	161	148	112
DFP	(1 mM)	106	180	91	96
	(25 mM)	10	320	86	38
MIA	(10 mM)	2	127	104	132
$HgCl_2$	(10 mM)	0	0	0	0
$CaCl_2$	(10 mM)	103	100	111	114
ATP	(10 mM)	24	85	65	98
TLCK	(1 mM)	138	71	87	98
TPCK	(1 mM)	27	74	56	2030
STI	(2 mg/ml)	60	109	86	43

N.D., not determined; E-64, L-trans-epoxysuccinyl-leucylamido-(4-guanidinobutane); PMSF, phenylmethanesulfonyl fluoride; MIA, monoiodoacetic acid; TLCK, N-tosyl-L-lysyl chloromethyl ketone; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; STI, soybean trypsin inhibitor.
*: activity was measured at 65°C. +: activity was measured at 37°C in the presence of 0.03 % SDS.

Adriamycin showed a selective inhibition on SDS-induced form of SLLVY-degrading activity. On the other hand, monoiodoacetic acid (MIA) and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) had markedly inhibitory effects on caseinolytic activity. E-64 and Ca^{2+} showed no inhibitory effect on any activity.

As already reported by Makinodan et al.[45] and Iwata et al.[46], 1 mM DFP did not show significant inhibitory effect on caseinolytic activity. However, DFP at the concentration of 25 mM effectively inhibited caseinolytic activity, whereas it activated BLTR-degrading activity as much as 3.2-fold. Such a equivocal effect was also

observed for TPCK. TPCK inhibited caseinolytic activity as well as DFP, but it activated SDS-induced form of SLLVY-degrading activity as much as 20.3-fold. Interestingly, such activating effect by TPCK was not observed for heat-induced form of SLLVY-degrading activity. The different sensitivity to reagents between SDS-induced and heat-induced forms of SLLVY-degrading activity was also observed for adriamycin, 2-mercaptoethanol, DFP (25 mM) and STI.

Effect of NaCl

The effect of NaCl on the activity of this proteinase was shown in Fig. 6. Only caseinolytic activity was completely inhibited by 0.5 M NaCl, while both SLLVY- and BLTR-degrading activity were activated.

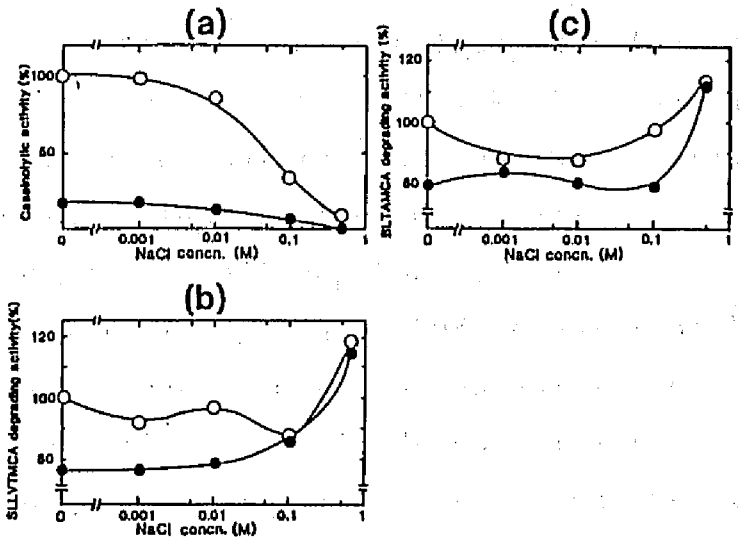

Fig. 6. Effect of NaCl on carp muscle HAP.
The caseinolytic (a), SLLVY degrading (b), and BLTR degrading (c) activities were measured at pH 6.8 at 65°C (●), and at pH 8.0 at 65°C (○).

DISCUSSION

The data presented herein show that heat-stable alkaline proteinase (HAP) purified from carp muscle in the present study should be classified as multicatalytic protease (MCP). However, the degree of endopeptidase activity was apparently distinguishable between carp muscle HAP and other mammalian MCPs. The endopeptidase activity of mammalian MCPs seems not so strong because ^{14}C- or ^{3}H-labeled casein has been usually used as substrates.[34,38,47] On the contrary, that of carp muscle HAP was so prominent as to be easily detectable by the conventional Lowry's method.[29] It should be emphasized that caseinolytic activity of carp muscle HAP was not due to BLTR- and/or SLLVY-degrading activity for the reasons described below. (1) It is improbable that caseinolytic activity was due to SLLVY-degrading activity, because addition of SDS was only effective for SLLVY-degrading activity, but not for caseinolytic activity. (2) Effects of leupeptin, DFP (25 mM), TLCK and TPCK were clearly distinguishable among these activities. Such difference in endopeptidase activity may be attributable to the difference of induction method (heating for carp muscle HAP,[6,32,46] addition of SDS or poly-L-Lys for mammalian MCPs[35,37-39] or difference of animals and tissues. However, the former is improbable from following two reasons. (1) Casein-degrading activity was not detected for heat- and SDS-induced forms of human erythrocyte MCPs[43] and (2) casein-degrading activity of rat skeletal muscle HAP was rather weak compared with that of carp muscle HAP.[48] Therefore, such strong endopeptidase

activity could be characterized peculiar to fish muscle HAP. It is generally accepted that fish can stand starvation much longer than mammals by spending body protein, especially muscular protein for energy source.[49] In particular, degenerated muscle was observed for salmon in spawning stage probably due to the higher proteolytic activities during this stage including MCP[50,51] In these cases, fish muscle HAP is likely to be involved in the urgent muscle protein degradation.

Comparing the inducing effect between addition of SDS and heating, the former induced only chymotrypsin-like activity selectively, while latter was effective for all catalytic activities. As to chymotrypsin-like activity, addition of SDS seems to more closely mimic the *in vivo*-inducing mechanism, because SDS-induced form showed higher activity (Fig. 5) and was more sensitive to reagents (Table 2). It was also speculated from the difference of the degree of latency that each catalytic activity is induced by different mechanism *in vivo* depending on necessity.

Furthermore, it should be remarked that leupeptin, PMSF, MIA, TLCK, DFP and TPCK showed equivocal effects on some catalytic activities (Table 2) as already suggested by Dahlmann *et al.* for rat skeletal muscle MCP.[47] In particular, DFP and TPCK showed striking activating effects on heat-induced trypsin-like activity (3.2-fold) and SDS-induced chymotrypsin-like activity (20.3-fold), respectively. These results together with the unsuccessful dissociation of the proteinase molecule to active subunits (data not show) suggest that each catalytic activity is

depend on different subunit and regulated systematically under unknown mechanism. It should be also emphasized that DFP or TPCK alone could not turn a latent-form of the proteinase into induced-form instead of heating or addition of SDS (data not show).

Thus, it is proposed that the activities of the proteinase seem to be controlled by at least two different steps *in vivo*. Initially, the proteinase is turned into an induced-form from a latent-form by some unknown factor mimicked as the addition of SDS or heating in our *in vitro* assay. Then, the once induced-MCP is further regulated by another mechanism which was observed as the equivocal effect of DFP or TPCK in our experiments in order to precede protein degradation continuously and effectively.

Although the participation HAP in *modori*-phenomenon has been suggested,[14,18,21,22,52-54] it still remained doubtful because of the following reasons. (1) The proteolytic activity of HAP at 65°C was inhibited by the addition of NaCl. (2) HAP also exists in mammalian muscle, which dose not show *modori*-phenomenon at all.[7] In the next chapter, the possibility that HAP is a candidate for *modori*-phenomenon will be examined.

SUMMARY

Heat-stable alkaline proteinase (HAP) was purified from carp muscle by chromatography on DEAE-cellulose, hydroxylapatite, and AcA 34 to homogeneous. This HAP had three distinct catalytic activities, endopeptidase, trypsin-like and chymotrypsin-like activities and all activities were latent. The optimum temperature of the

activities were 65 °C. Because of the inhibition of endopeptidase activity by NaCl, it was doubtful that HAP is a candidate of *modori*-phenomenon.

CHAPTER 2. INVESTIGATION OF A HEAT-STABLE ALKALINE PROTEINASE AS A CANDIDATE FOR A *MODORI*-INDUCING FACTOR

The participation of HAP in *modori*-phenomenon is doubtful because of the inhibition of its endopeptidase activity by NaCl added in *kamaboko* processing. In this chapter, it is elucidated whether HAP is a true candidate for *modori*-phenomenon or not.

MATERIALS AND METHODS

Materials

Threadfin bream (*Nemipterus bathybius*, 250 g in body weight) was purchased in Kyoto wholesale market in a fresh state.

Preparation of the sarcoplasmic fraction and myofibrils

White dorsal muscle was collected and minced by 5 mm mesh meat chopper (Meiko, type no. 10). The minced meat was homogenized with 5 volumes of 50 mM NaCl with nonbubling homogenizer (Nihon Seiki. Co., Ltd.) for 1 min. Adequate volume of 1 N NaOH was added to the homogenate (pH 6.2-6.3) so as to adjust pH to 6.8. Then the homogenate was centrifuged at 10,000 x g for 15 min. The supernatant was used as the sarcoplasmic fraction. The precipitate was washed similarly two more times. The final precipitate was tentatively referred to as thrice-washed myofibrils and used for further experiments. Gel made from the thrice-washed myofibrils did not show *modori*-phenomenon at all (data not shown).

Separation of sarcoplasmic fraction by Ultrogel AcA 34 column chromatography

The sarcoplasmic fraction extracted from 50 g muscle was concentrated to 5 ml by dialysis against polyethylene glycol #6000 and applied on a column of Ultrogel AcA 34 equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl. The elution was performed with the same buffer.

Preparation of fish meat gel

A part of fraction I (fraction numbers 46-49 in Fig. 7), II (fraction numbers 53-60), III (fraction numbers 82-89) and IV (fraction numbers 107-114), eluted from a column of Ultrogel AcA 34 equivalent to the amount extracted from 5 g muscle, was concentrated to 0.5 ml by dialysis polyethylene glycol #6000, added to 5 g of the thrice-washed myofibrils which were free of gel degradation material, ground with 2.5 % NaCl for 3 min and then heated at 65 °C for 120 min in a small glass tube (diameter, 15 mm; height, 15 mm).

Evaluation of fish meat gel

Sensory evaluation of gel was carried out by an experienced panelist with teeth-cutting and folding tests according to the method of Shimizu et al..[55] Mean score for two test pieces was determined with the teeth-cutting test. The teeth-cutting test was done by cutting off the test pieces in 15 mm diameter and in 3 mm thickness by the fore-teeth and scoring toughness according to 10 point scale (10, extremely tough; 8, tough; 6, moderately tough; 4, fragile; 2, very fragile; 0, like mud). The folding test was done by folding the test pieces having in the same diameter and thickness as those for teeth-cutting test neatly in half and evaluating the toughness into four

grades (A, nothing occurred; B, cracked; C, cracked and separated into two pieces; D, easy to break like mud). The folding test was also examined for two test pieces, and the same grade was obtained as to each test in the experiment shown in Fig. 7.

Estimation of the breakdown of myosin heavy chain of fish meat gels

The muscle protein gel was homogenized with 10 volumes of distilled water, and 0.1 ml of the homogenate was heated in boiling water for 5 min with 0.4 ml of 100 mM Tris-HCl buffer, pH 6.8, containing 1 % SDS and 50 mM 2-mercaptoethanol. Breakdown of myosin heavy chain (MHC) was estimated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the 10 % gel.

Estimation of the proteolytic activity

MHC breakdown activity was determined with SDS-PAGE in 10 % gel. The reaction mixture contained 0.15 ml of fraction II (HAP fraction) or III. After dialysis against distilled water, 0.15 ml of the thrice-washed myofibrils were suspended in 6 volume of distilled water and 0.7 ml of 20 mM Tris-HCl buffer, pH 7.0 or 8.0 and heated at 65 °C for 60 min. The reaction was stopped by the addition of 0.1 ml of 10 % SDS containing 50 mM 2-mercaptoethanol and the subsequent heating at 95 °C for 3 min. For the examination of the effect of NaCl was directly added to the reaction mixture to give the final concentration of 2.5 %. HAP activity shown in Fig.7 was determined as caseinolytic activity according to the method of Makinodan and Ikeda.[56]

Immunological methods

Rabbit polyclonal antisera directed against carp muscle HAP were raised using Freund's complete adjuvant.[57] The antisera were applied on a column of DEAE-cellulose equilibrated with 20 mM Tris-HCl, pH 7.5. The unadsorbed fractions were collected as IgG fraction and used for the experiments. Dot blotting was carried out with vitro-cellulose membrane and the membrane was stained in peroxidase anti-peroxidase method[58].

RESULTS AND DISCUSSION

The sarcoplasmic fraction was chromatographed in a column of Ultrogel AcA 34 (Fig. 7). As shown in Fig. 7, strong gel degradation activity was eluted in fraction III,

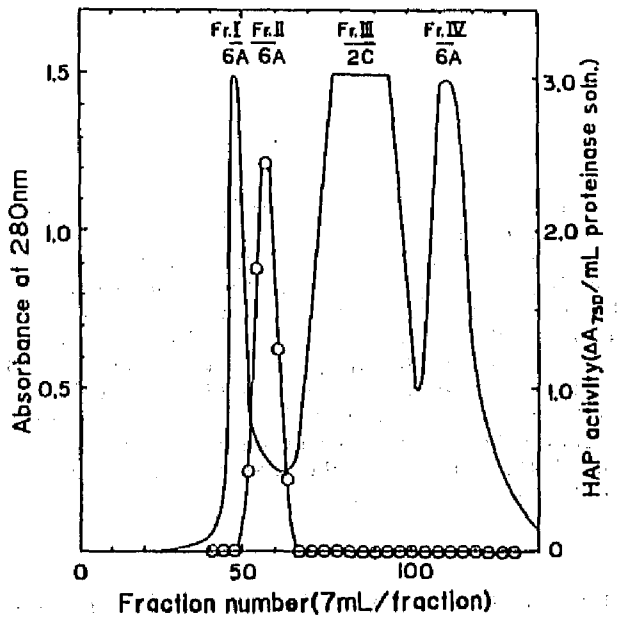

Fig. 7. The elution profile of the sarcoplasmic fraction from a column (2.5x100 cm) of Ultrogel AcA 34.
The number and alphabet are the score of teeth-cutting test and the grade in folding test, respectively. —— , protein concentration monitored at 280nm; ○ , caseinolytic activity of HAP.

which showed typical modori-phenomenon in reducing the toughness score to 2C, while fraction II (HAP fraction) as well as fractions I and IV did not show modori-phenomenon at all. Fraction III was estimated to consist of 40-70 kDa proteins by the calibration of the column. This result suggested that modori-phenomenon was caused by a certain modori-inducing factor, which was clearly distinguished from HAP in molecular weight.

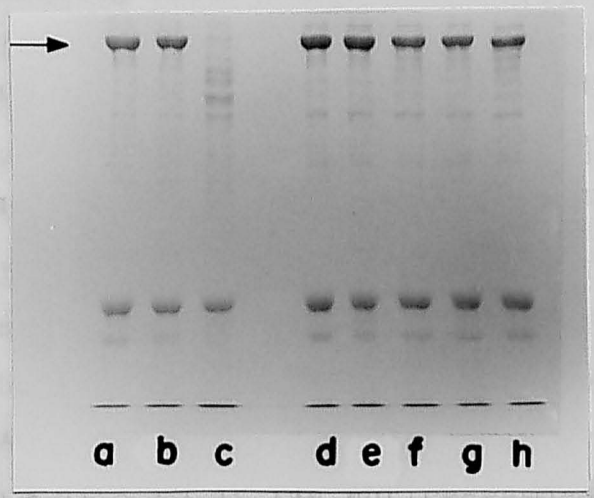

Fig. 8. Effect of NaCl on myosin heavy chain (MHC) degrading activities of fraction II (HAP fraction) and III. Arrow indicates the MHC band. (a), myofibrils and fraction II before heating; (b), myofibrils heated with fraction II at pH 7.0 in the absence of NaCl; (c), myofibrils heated with fraction II at pH 7.0 in the presence of NaCl; (d), myofibrils and fraction II before heating; (e), myofibrils heated with fraction II at pH 7.0 in the absence of NaCl; (f), myofibrils heated with fraction II at pH 7.0 in the presence of NaCl; (g), myofibrils heated with fraction II at pH 8.0 in the absence of NaCl; (h), myofibrils heated with fraction II at pH 8.0 in the presence of NaCl.

The effects of NaCl on MHC breakdown activity by fraction II (HAP fraction) and fraction III are shown in Fig. 8. Fraction II showed strong MHC breakdown activity at pH 7.0 in the presence of NaCl (Fig. 8c), while this activity was hardly detected in the absence of the NaCl

(Fig. 8b). In the contrast, HAP fraction hardly showed any MHC breakdown activity at pH 7.0 and 8.0 (optimum pH for the caseinolytic activity for HAP[56]) in the presence or absence of NaCl (Fig. 8 e-h).

As described in chapter 1, HAP consists of many subunit and different substrate specificity of HAP seems to be attributed to distinct active site. Then, it might be probable that fraction II, which induced modori-phenomenon, consists of some subunits derived from HAP. In order to clarify this probability, immunological investigation was carried out. As shown in Fig. 9, fraction III did not cross with anti HAP antibody, while fraction II (HAP fraction) did. This means that the modori-inducing factor contained in fraction III is immunological different from HAP.

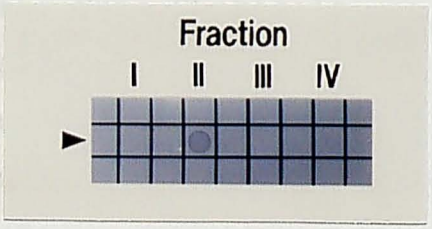

Fig. 9. Dot blotting of sarcoplasmic fractions of threadfin bream against anti-HAP antibody from carp muscle.

These results clearly show that modori-phenomenon is caused not by HAP but by other modori-inducing factor. Then, the factor was tentatively called sarcoplasmic-60 °C-modori-inducing proteinase, Sp-60-MIP, because it existed in sarcoplasmic fraction and active around 60 °C. In the

next chapter, the purification of Sp-60-MIP will be carried out.

SUMMARY

From the results of reconstituted experiment of modori-phenomenon by adding sarcoplasmic fraction to myofibrils which was free of modori-property, it was elucidated that HAP could not induce modori-phenomenon. Additionally, the existence of other modori-inducing factor was recognized. The factor was distinguishable from HAP by its molecular weight and by immunological cross-reactivity. The factor was tentatively designated as sarcoplasmic-60°C modori-inducing proteinase, Sp-60-MIP, because it was likely to exist in sarcoplasm and showed modori-inducing activity around 60°C.

CHAPTER 3. PURIFICATION AND PROPERTIES OF A SARCOPLASMIC-60 °C *MODORI*-INDUCING PROTEINASE

In the former chapter, the existence of true *modori*-inducing factor, designated as sarcoplasmic-60 °C *modori*-inducing proteinase (Sp-60-MIP) was confirmed. Then, in this chapter Sp-60-MIP was purified and its some properties were investigated.

MATERIALS AND METHODS

Materials

Threadfin bream (*Nemipterus bathybius*, 405 g in body weight and 28 cm in body length) was purchased in Kyoto wholesale market in a fresh state. The source of other materials used in this work was as follows: DEAE-cellulose (DE-52) from Whatman, Con A-Sepharose and Arginine-Sepharose from Pharmacia LKB Biotechnology, Shimpack-HAC from Shimadzu. Other reagent grade chemicals were purchased from Wako Pure Chemicals.

Purification procedures

All procedures were carried out at 4 °C, except for Shimpack-HAC column chromatography which was carried out at room temperature.

Step 1. Dorsal white muscle (120 g) was collected and homogenized with 480 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA in a non-bubbling homogenizer (Nihon Seiki Co. Ltd.) for 3 min at top speed. The homogenate was centrifuged at 12,000 x g for 15 min and the supernatant obtained was used as crude extract for the further purification. On the other hand, the precipitate was further washed three more times with 4 volumes of

39 mM borate buffer, pH 7.0, containing 5 mM EDTA and 90 mM KCl to obtain myofibrils free from MHC-degrading activity and used as the substrate.

Step 2. Crude extract was applied on a DEAE-cellulose column (2 x 10 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, and washed thoroughly with the same buffer. Elution was performed with NaCl stepwise in 20 mM Tris-HCl, pH 7.5 (Fig. 10).

Step 3. Active fractions from DEAE-cellulose column (fraction number 57-66 in Fig. 10) were concentrated by dialysis against polyethylene glycol #6000 and then dialyzed against 20 mM Tris-HCl, pH 7.5, including 0.5 M NaCl, 1 mM $CaCl_2$ and $MnCl_2$. The dialysate was applied on a column (1 x 5 cm) of Con A-Sepharose equilibrated with the same buffer. Elution was performed with a 0-0.5 M methyl-α-D-pyranoside gradient in a total volume of 100 ml (Fig. 11).

Step 4. Active fractions from Con A-Sepharose (fraction number 22-37 in Fig. 11) were pooled and concentrated by dialysis against polyethylene glycol #6000 and then dialyzed against 20 mM Tris-HCl, pH 7.5. The dialysate was applied on a column (1 x 3 cm) of Arg-Sepharose equilibrated with the same buffer. Elution was performed with a 0-0.5 M NaCl gradient in a total volume of 60 ml (Fig. 12).

Step 5. Active fractions from Arg-Sepharose (fraction number 25-29 in Fig. 12) were pooled and concentrated by dialysis against polyethylene glycol #6000 and then dialyzed against 1 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl. The dialysate was applied on high

performance liquid chromatography with Shim-pack HAC (Fig. 13). Fraction (retention time around 17 min) was used for the experiment. The homogeneity of purified proteinase was examined in 7.5 % PAGE under non-denaturing condition and in 10 % SDS-PAGE. The standard of molecular mass were rabbit myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), albumin bovine plasma (66 kDa), albumin egg (45 kDa) and carbonic anhydrase (29 kDa). Both gels were stained by silver.

Assay of MHC-degrading activity

The Sp-60-MIP activity was measured as MHC-degrading activity. The standard reaction mixture contained 50 mM phosphate buffer (pH 7.0), 3 % NaCl, 20 mg threadfin bream myofibrils and purified proteinase in a total volume of 1 ml. Reaction was performed at 60 °C for 60 min and stopped by the addition of 0.5 ml of 10 % SDS in Tris-HCl, pH 6.8 and subsequent heating at 100 °C for 3min. The degree of the degradation of MHC was determined by 7.5 or 10 % SDS-PAGE analysis. Gels were stained with Coomassie Brilliant Blue R-250.

Substrate specificity

Substrate specificity was investigated with synthetic fluorogenic peptides as substrates. The reaction mixture was composed of 50 mM phosphate buffer (pH 7.0), 3 % NaCl, 0.5 mM substrate (previously dissolved in dimethylsulfoxide to obtain 10 mM stock solution) and the proteinase in a total volume of 0.1 ml. Reaction was performed at 60 °C for 20 min and stopped by the addition of 2.6 ml of 1 % SDS Tris-HCl (pH 9.0). The increase of fluorescence (excitation at 380 nm; emission at 460 nm) of

AMC was measured by a HITACHI 204 Fluorescence Spectrophotometer.

Effect of heating on the proteinase molecule with or without substrate

The proteinase was incubated at 60°C for 20 min with or without BLTR in the reaction mixture described above. Reaction was stopped by the addition of 0.05 ml of 10 % SDS in Tris-HCl, pH 6.8, and subsequent heating at 100°C for 3 min. SDS-PAGE in 10 % gel was performed and the gel was stained by silver.

RESULTS

Purification

Elution profile from DEAE-cellulose was shown in Fig. 10(A). Strong MHC-degrading activity was eluted around fraction number 59 (Fig. 10 B). Several bands observed below MHC band in fraction number 6 (Fig. 10 B) were not degraded products of MHC but proteins contained in this

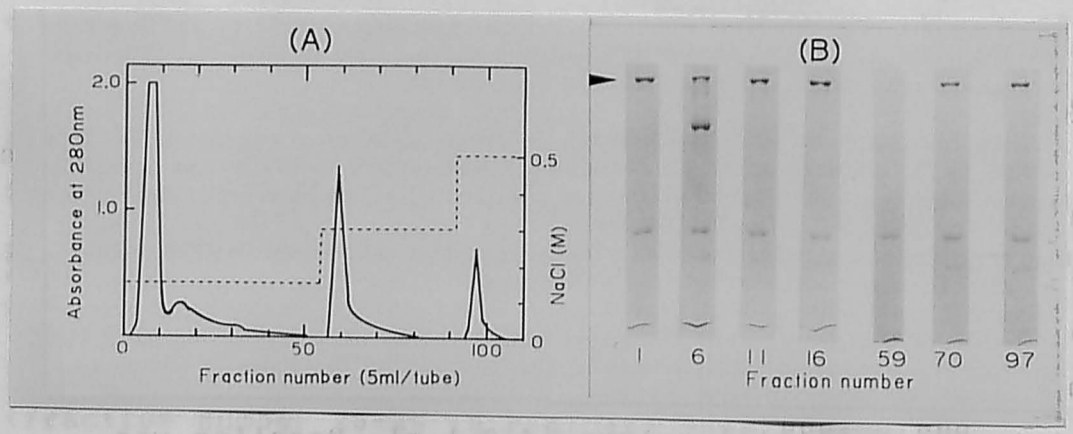

Fig. 10. (A) Elution profile of the Sp-60-MIP from a column (2x10 cm) of DEAE-cellulose. (B) MHC-degrading activity of each fraction determined on SDS-PAGE. Activity was measured at 60°C in the presence of 3 % NaCl. Arrow indicates MHC.

fraction. From Con A-Sepharose column (Fig. 11), MHC-degrading activities were eluted in both the non-adsorbed fractions (fraction number 5 and 9) and the adsorbed fractions (fraction number 24 and 33). The latter showed much stronger MHC-degrading activity despite of lower protein concentration. Therefore, the latter fractions were used for further purification.

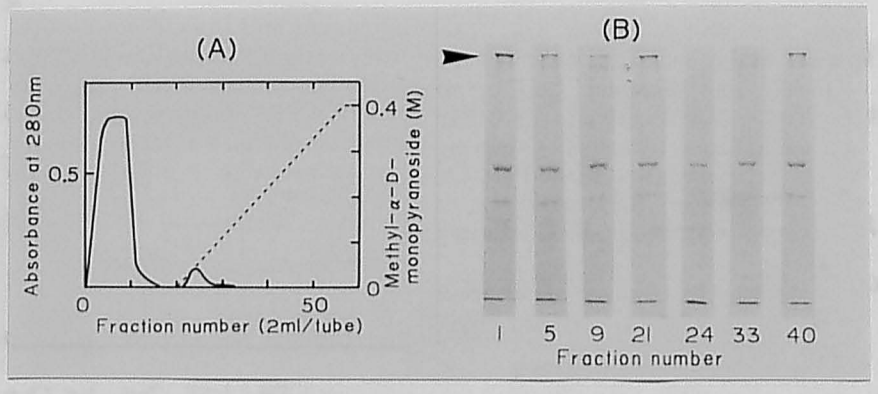

Fig. 11. (A) Elution profile of the Sp-60-MIP from a column (1x5 cm) of Con A-Sepharose. (B) MHC-degrading activity of each fraction determined on SDS-PAGE.
Activity was measured at 60°C in the presence of 3 % NaCl.
Arrow indicates MHC.

As shown in Fig. 12, the latent proteinase was bound to Arg-Sepharose and eluted around fraction number 26. From Shim-pack HAC, Sp-60-MIP was eluted as a sharp single peak and judged as homogeneous by the electrophoretic analysis under non-denaturing condition (Fig. 13). Purified proteinase was migrated as a single band in SDS-PAGE (Fig. 13) corresponding to the molecular weight of 77,000, while the value of 70,000 was obtained based on the elution position from TSK gel G3000SWXL calibrated (data not show). This proteinase was a glycoprotein based on the adsorption to Con A-Sepharose (Fig. 11) and being positive to periodic acid-Schiff staining (data not shown).

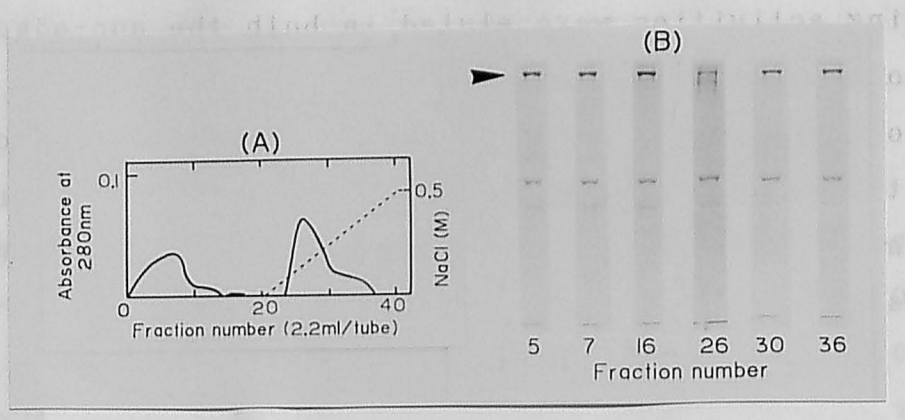

Fig. 12. (A) Elution profile of the Sp-60-MIP from a column (1x3 cm) of Arg-Sepharose. (B) MHC-degrading activity of each fraction determined on SDS-PAGE.
Activity was measured at 60°C in the presence of 3 % NaCl.
Arrow indicates MHC.

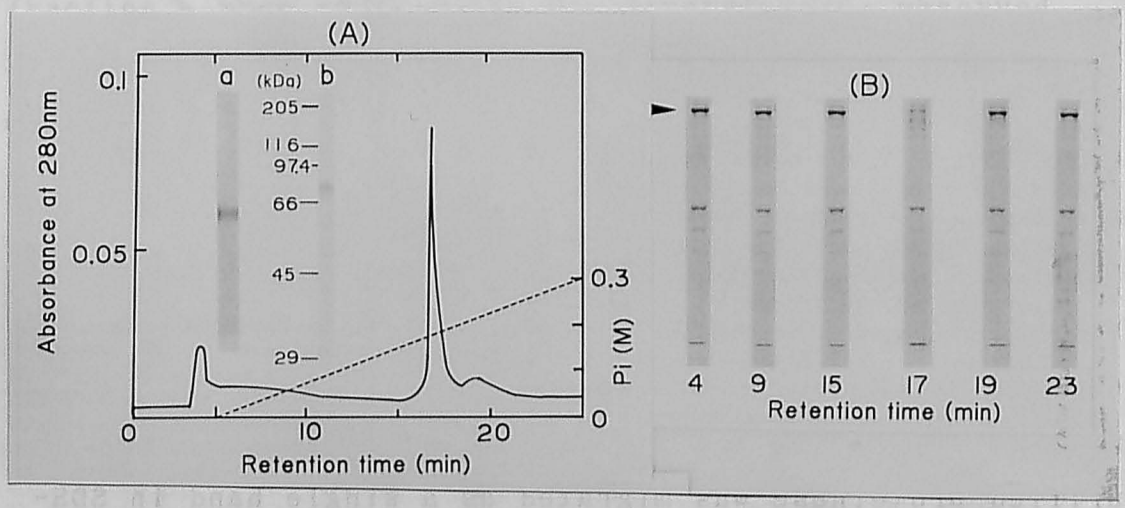

Fig. 13. (A) Elution profile of the Sp-60-MIP from a column (0.4x5 cm) of Shim-pack HAC. Inset: a and b show PAGE analysis without and with SDS of purified proteinase (retention time 17 min), respectively. (B) MHC-degrading activity of each fraction determined on SDS-PAGE.
Activity was measured at 60°C in the presence of 3 % NaCl.
Arrow indicates MHC.

Substrate specificity

Activities on synthetic fluorogenic peptide substrates were shown in Table 3. Sp-60-MIP hydrolyzed Boc-Leu-Thr-Arg-MCA (BLTR) most sufficiently among peptides tested. This proteinase hydrolyzed some peptidyl Arg-MCA derivatives such as Boc-Leu-Gly-Arg-MCA, Z-Phe-Arg-MCA to some degree, while peptidyl Pro-MCA derivatives (substrate of collagenase), peptidyl Phe- or Tyr-derivatives (substrate for chymotrypsin) and peptidyl Ala-derivative (substrate for elastase) were not hydrolyzed at all. These results clearly demonstrated that this proteinase had a trypsin-like substrate specificity. In addition, this proteinase showed little activity on casein in the presence or absence of NaCl despite of its strong MHC-degrading activity.

Table 3. Substrate specificity of the Sp-60-MIP on synthetic fluorogenic substrates.
Activities were measured at 60°C in the presence of 3 % NaCl.

substrates	Activity (pmol AMC/min)
Boc-Leu-Thr-Arg-MCA	4
Boc-Leu-Gly-Arg-MCA	1.2
Z-Phe-Arg-MCA	0.4
Bz-Arg-MCA	0.08
Arg-MCA	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0
Suc-Gly-Pro-MCA	0
Suc-Ala-Ala-Pro-Phe-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	0
Suc-Ala-Pro-Ala-MCA	0

Effect of temperature

Effect of temperature is shown in Fig. 14. At physiological temperature around 30-40 °C, no MHC- and

BLTR-degrading activity was detected, but strong activities towards them were observed at 60 ℃. However, these activities were remarkably reduced above 70 ℃. This result can be explained by assuming that this proteinase existed as a latent-form and turned into active-form by heating.

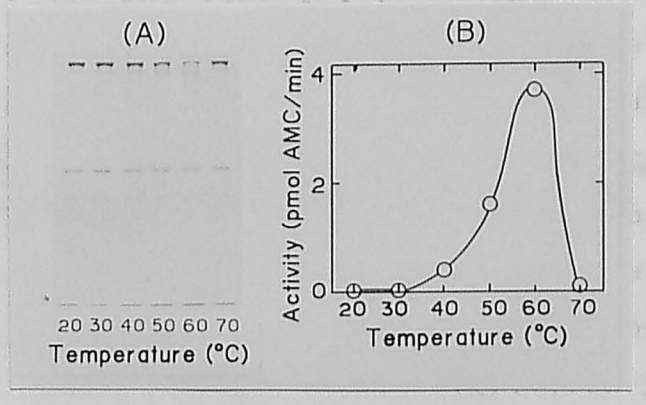

Fig. 14. Effect of temperature on MHC-degrading activity (A) and BLTR-degrading activity (B) of the Sp-60-MIP. Activities were measured in the presence of 3 % NaCl. Arrow indicates MHC.

Effect of pH

Effect of pH on MHC- and BLTR-degrading activities are shown in Fig. 15. Sp-60-MIP showed these activities around pH 6-8. Therefore, this proteinase is considered as a neutral proteinase.

Effect of NaCl

Sp-60-MIP revealed the optimum activities on MHC and BLTR in the presence of 2-4 % NaCl (Fig. 16). Both activities were markedly decreased in the presence of lower concentration of NaCl.

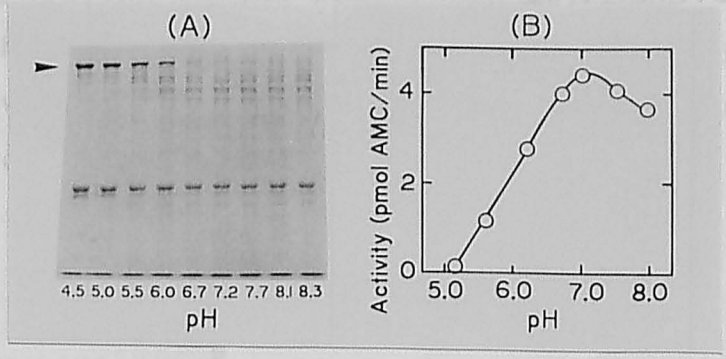

Fig. 15. Effect of pH on MHC-degrading activity (A) and BLTR-degrading activity (B) of the Sp-60-MIP.
Arrow indicates MHC. Activities were measured at 60°C in the presence of 3 % NaCl.

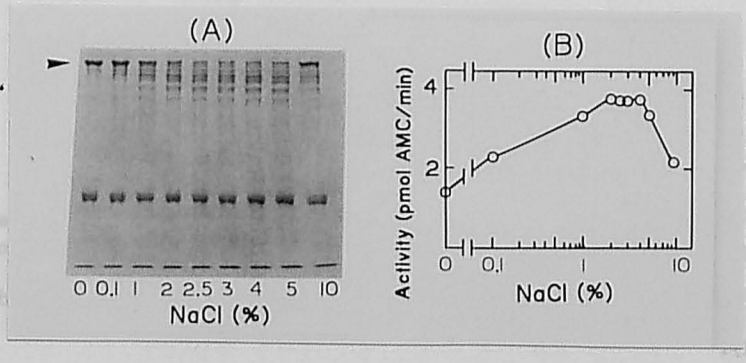

Fig. 16. Effect of NaCl on MHC-degrading activity (A) and BLTR-degrading activity (B) of the Sp-60-MIP.
Arrow indicates MHC. Activities were measured at 60°C.

Effect of reagents

Results are shown in Fig. 17 and Table 4. Soybean trypsin inhibitor and leupeptin effectively inhibited both activities, while E-64 (inhibitor for cysteine proteinase) showed no effect. These facts clearly suggested that this proteinase should be classified as serine proteinase[59]. EDTA inhibited only MHC-degrading activity. This was probably due to the stabilizing effect of EDTA on MHC, because no inhibiting effect was observed for BLTR-

degrading activity.

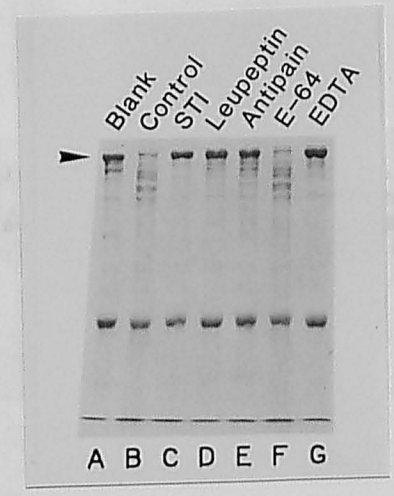

Fig. 17. Effect of inhibitors on MHC-degrading activity of the Sp-60-MIP.
Activities were measured at 60°C in the presence of 3 % NaCl.
A, MHC+purified proteinase at 0 hour incubation; B, MHC+purified proteinase; C, MHC+purified proteinase+soybean trypsin inhibitor (1 mg/ml); D, MHC+purified proteinase+leupeptin (10 μg/ml); E, MHC+purified proteinase+antipain (10 μg/ml); F, MHC+purified proteinase+E-64 (100 μg/ml); G, MHC+purified proteinase+EDTA (10 mM).

Table 4. Effect of inhibitors on BLTR-degrading activity of the Sp-60-MIP.
Activities were measured at 60°C in the presence of 3 % NaCl.

Inhibitors	Final concentration	Relative activity (%)
non		100
Soybean trypsin inhibitor	(1 mg/ml)	15
Leupeptin	(10 μg/ml)	3
Antipain	(10 μg/ml)	16
EDTA	(10 mM)	85
Diisopropyl fluorophosphate	(10 mM)	13
E-64	(100 μg/ml)	101

Effect of heating on the proteinase molecule

As shown in Fig. 18, the mobility of the proteinase molecule was not changed by heating, regardless of the presence of the substrate.

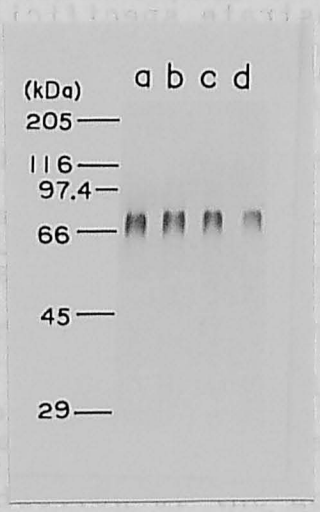

Fig. 18. SDS-PAGE analysis of the heating effect on proteinase molecule.
a, before incubation without BLTR;b, before incubated with BLTR; c, incubated at 60°C for 20 min without BLTR; d, incubated at 60°C for 20 min with BLTR.

DISCUSSION

In chapter 2, it was clarified that the modori-phenomenon accompanied by the degradation of MHC is not caused by HAP, but by Sp-60-MIP. Enzymatic properties of Sp-60-MIP presented herein support the participation of this proteinase to modori phenomenon, specifically occurred around 50-70 °C in the manufacturing process of fish jelly products, from the reasons described below. (1) Sp-60-MIP revealed MHC-degrading activity at unphysiologically higher temperature around 60 °C (Fig. 14), (2) this proteinase degraded MHC at neutral pH range (Fig. 15). (3) this proteinase revealed MHC-degrading

activity optimally only in the presence of 2-4 % NaCl (Fig. 16), around which concentration fish gel products were made.

Sp-60-MIP was classified as a serine proteinase from the inhibition spectra (Fig. 17 and Table 4) and proved to have a trypsin-like substrate specificity (Table 3). The absorption to Arg-Sepharose (Fig. 12) also supports the affinity of this proteinase to basic amino acid residue. A proteinase reported by Busconi et al.[60] and Folco et al.[61] in white croaker (Micropogon opercularis) muscle seemed to be similar to this latent proteinase in respects of enzymatic properties as serine proteinase, induction of the activity by heating and strong MHC-degrading activity, but was distinguishable from this proteinase in the points of inhibition by NaCl, fairly higher molecular weight and alkaline pH dependency around 8.5. The molecular weight of Sp-60-MIP was estimated 77,000 in SDS-PAGE or 70,000 in the gel filtration. This could be ascribed to its glycoprotein nature.

It is noteworthy that MHC- and BLTR-degrading activities of this proteinase were latent and only induced by heating in the presence of NaCl. Because little MHC-degrading activity was observed below 0.1 % NaCl, it was proposed that NaCl may act to solubilize intact myofibrils and increase the digestibility to this proteinase. However, BLTR-degrading activity was also increased in the presence of higher concentration of NaCl, it is also probable that NaCl may act directly to a proteinase molecule. HAP activity was also latent and induced by heating, but reduced in the presence of NaCl.[26] It is

also interesting that SDS was ineffective for the induction of this latent proteinase activity (data not shown), although it effectively induced chymotrypsin-like activity of HAP.[40,64] No change was observed in the mobility on a SDS-PAGE analysis in the presence of absence of the substrate (Fig. 18). From this result, the autolytic activation did not seem to be improbable. Induction of this latent proteinase was probably due to only slight conformational change caused by heating, which was not detected as the change in migration on SDS-PAGE. Because the proteinase which once heated at 60 °C without substrate and then cooled to 4 °C did not show the activity around physiological temperature range and again required heating for the induction of the activity (data not show), the inducing process by heating is supposed to be reversible.

It is generally accepted that neutral proteinase activity is strictly regulated in the cells by the presence of endogenous inhibitor or by the requirement some other factors to avoid unwanted proteolysis. For example, calpain, cytosolic Ca^{2+}-dependent neutral proteinase, is known to co-exist with endogenous inhibitor, calpastatin, and show the absolute requirement of Ca^{2+} for the activity.[63,64] As to Sp-60-MIP, its activity seems to be retained as latent-form under physiological condition and induced according to necessity by some unknown mechanism. The existence of endogenous inhibitor is also probable, because strong trypsin inhibitory activity was detected in fish muscle.[60,61,64,65] On the other hand, biological function

of Sp-60-MIP still remains obscure.

SUMMARY

Sarcoplasmic-60 °C-*modori*-inducing proteinase (Sp-60-MIP) was purified to homogeneity from threadfin bream muscle by a combination of DEAE-cellulose, Con A-Sepharose, Arg-Sepharose and Shim-pack HAC chromatographies. This MIP was a glycoprotein having a monomelic subunit structure whose molecular weight was estimated to be 77,000 on a SDS-PAGE analysis. The activity of Sp-60-MIP existed in a latent form and appeared by heating. Sp-60-MIP hydrolyzed myosin heavy chain as well as Boc-Leu-Thr-Arg-MCA in the presence of 2-4 % NaCl at pH 7.0 and at 60 °C, optimally. Sp-60-MIP was classified as serine proteinase based on the effects of soybean trypsin inhibitor, leupeptin and antipain.

CHAPTER 4. INDUCTION OF MODORI BY A SARCOPLASMIC-60 °C MODORI-INDUCING PROTEINASE

To confirm that Sp-60-MIP actually induces modori, a reconstituted experiment of modori-phenomenon was attempted using purified Sp-60-MIP.

MATERIALS AND METHODS

Materials

Threadfin bream (Nemipterus virgatus, 420 g in weight and 29 cm in body length on the average) was obtained from Whole Sale Market, Kyoto, in a fresh state.

Preparation of Sp-60-MIP

Sp-60-MIP was purified from dorsal white muscle (500g) of threadfin bream using partially modified method described in chapter 3. After purified as described in chapter 3, the proteinase fraction was concentrated by TGC ,Millipore, and then applied on TSK gel G3000SWXL (gel filtration column high performance liquid chromatography). Elution was performed with 2mM Na-phosphate buffer, pH6.8, containing 0.1 M NaCl. Purified MIP was concentrated by TGC ,Millipore, and used for further experiment.

Preparation of myofibrils

Myofibrils (Mf) were prepared according to the method of Perry and Grey[66]. Briefly, minced muscle was homogenized with 4 volumes of 39 mM borate buffer, pH 7.0, containing 5 mM EDTA and 90 mM KCl and then sieved through a net of 2 mm mesh to remove myocommata. The filtrate was centrifuged at 12,000 x g for 15 min. The precipitate was homogenized and centrifuged two more times as described above. The final centrifugation was carried out at

14,000 x g for 15 min to remove excess water and then the final precipitate was collected as the Mf. This Mf prepared from threadfin bream was ascertained to be completely free of modori-inducing activity.[67]

Preparation of fish meat gel

Fish meat gel was prepared as follows: Twenty grams of myofibrils, prepared as described above, was ground with 2.8 % NaCl and 2 ml of the MIP solution (0.5 mg protein/ml) or 2 mM Na-phosphate buffer, pH 6.8, containing 0.1 M NaCl (control gel). Resulting salt-ground sol was packed in a small glass cylinder (15 mm in inside diameter, 15 mm in height, 10 mm in thickness), wrapped with polyvinylidene chloride film and then heated at 60 ℃ for 2 hours.

The final NaCl concentration in gel was 3 %. pH and water content of gel were adjusted to 7.0 and 86 %, respectively.

Evaluation of fish meat gel

Gel strength of fish meat was evaluated by puncture test and sensory tests including teeth-cutting and folding tests. Sensory tests were carried out as described in chapter 2. The puncture test was performed penetrating the column type plunger (diameter, 3 mm) at a speed of 1 mm/sec into test pieces (diameter, 15 mm; thickness, 15 mm) using a Yamaden RE-3305 rheometer.

RESULTS & DISCUSSION

The elution profile of the Sp-60-MIP from TSK gel G3000SWXL was shown in Fig. 19. The proteinase was eluted as a single peak at a retention time around 16.5 min and

judged to be homogeneous on the basis of SDS-PAGE analysis (Fig. 19 inset). In this purification, gel filtration by TSK-gel G3000SWXL was carried out addition to previous purification method[68] to avoid unwanted contamination of other proteins accompanying with mass purification.

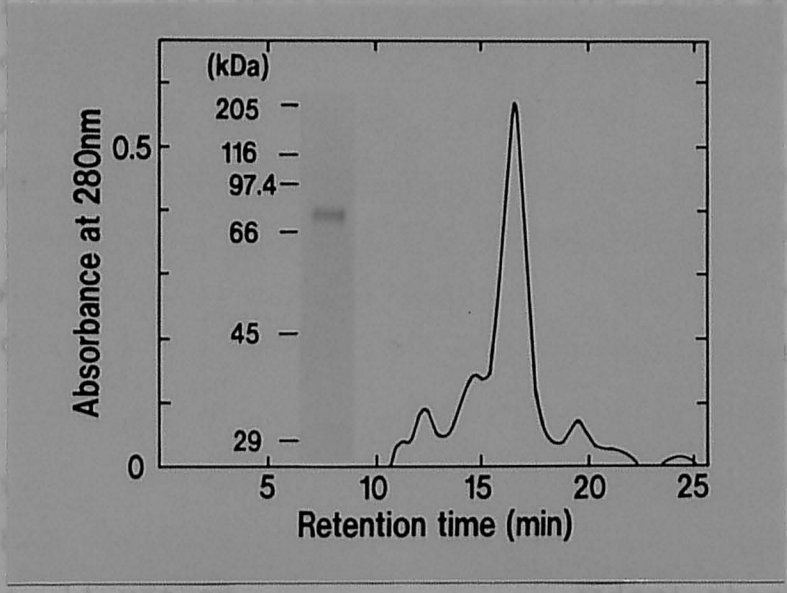

Fig. 19. Elution profile of the Sp-60-MIP from TSK gel G3000SWXL. Inset: 7.5% SDS-PAGE analysis of purified proteinase (retention time 16.5 min)

To examine the effect of this purified proteinase on gel strength, gels were prepared from threadfin bream myofibrils with or without purified Sp-60-MIP. As shown in Table 5, the gel strength of the gel added the Sp-60-MIP significantly reduced to 198 g·cm while that of control gel was 4440 g·cm, and the scores of sensory test of the former gel were obviously lower (1 in teeth-cutting test and C in folding test) than those of the latter gel (7 in teeth-cutting test and A in folding test).

Table 5. Evaluation of fish meat gels prepared by heating at 60°C for 2 hours.

	Fish meat gel with	
	phosphate buffer	Sp-60-MIP
Teeth-cutting test	7	1
Folding test	A	C
Gel strength (g·cm)	4440	198

Additionally, as to the gel added Sp-60-MIP, the density of MHC band fainted and many bands of degraded products could be observed between MHC band and actin band (Fig. 20). Therefore, it is obvious that Sp-60-MIP caused typical modori-phenomenon.

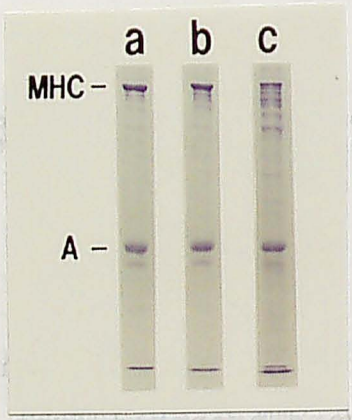

Fig. 20. Estimation of degradation of myosin heavy chain of fish meat gel.
Fish meat gels were prepared by heating at 60°C for 2 hours. SDS-PAGE was performed with 7.5% gel. Details were described in "Materials and Method". MHC, myosin heavy chain; A, actin. a, fish meat gel prepared with 2mM phosphatebuffer, pH 6.8, containing 0.1M NaCl; b, with the Sp-60-MIP without incubation; c, with the Sp-60-MIP.

Some attempts to induce modori-phenomenon artificially by adding endogenous factor had been made.[13,14,22,55] Iwata et al.[13,14] reported the existence of modori-inducing proteins which did not show any proteolytic activity and suggested the possibility of these proteins to induce modori-phenomenon. However, it seems unlikely that these proteins would loose gel structure specifically around 60 °C. Shimizu et al.[55] and Makinodan et al.[22] performed the reconstituted experiments but the added fractions were not homogeneously purified. This is, therfore, the first report that demonstrated the direct induction of modori by adding the homogeneously purified proteinase.

SUMMARY

It was confirmed that purified Sp-60-MIP caused modori-phenomenon by the reconstituted experiment. Therefore, it was substantiated that modori-phenomenon was caused by the protease.

CHAPTER 5. DETECTION OF MYOFIBRIL-ASSOCIATED MODORI-INDUCING PROTEINASE

It has been clarified that Sp-60-MIP purified from threadfin bream[13] causes modori-phenomenon. On the other hand, it seems that the characteristics of modori-phenomenon are different among fish species.[7] There are some papers that myofibrils prepared from oval filefish[69,70] or myosin from nibe croaker,[55,71] which were free of sarcoplasmic component, showed modori-phenomenon. In this chapter, the existence of myofibril-associated MIP will be described.

MATERIALS AND METHODS

Materials

Threadfin bream was purchased from Kyoto Whole Sale Market in a fresh state. Crucian carp (Carassius auraatus cuvieri) was used for the experiments within 24 hours after death.

Preparation of the sarcoplasmic and myofibrillar fractions

The sarcoplasmic fraction (Sp) and the myofibrils (Mf) were prepared from the dorsal white muscle. Mf were prepared as described in chapter 3. The first supernatant resulting in the preparation of Mf was obtained as Sp. The Mf prepared from threadfin bream was used as standard substrates for determining the activity of sarcoplasmic modori-inducing proteinase (MIP) of each species, because threadfin bream Mf was ascertained to be completely free of modori-inducing activity.[67]

Preparation of fish meat gel

Three types of fish meat gel were prepared from each

given fish species; One is the gel of minced meat (designated as minced meat gel). Another one is the gel of the Mf from given fish species (Mf gel) and the other is the gel of threadfin bream Mf added with the Sp from given fish species (Sp-added gel). Mf gel was used to assess the MIP activity associated with Mf, and Sp-added gel was used to assess the extractable MIP activity. Minced meat gels or Mf gels were prepared by grinding the minced meat or the Mf of each species, respectively, with 3 % NaCl, packed in a small glass cylinder (15 mm in inside diameter, 15 mm in height, 10 mm in thickness), wrapped in polyvinylidene chloride film and then heated at 50 °C and at 60 °C for 120 min, respectively. Sp-added gels were prepared as follows; The Sp prepared from 20 g muscle of each species was concentrated to 2 ml by dialysis against polyethylene glycol #6000, added to 20 g of Mf prepared from threadfin bream, and ground with 3 % NaCl. Resulting meat sol was packed, wrapped, and then heated in the same manner as in the Mf gel.

pH of each muscle protein gel was adjusted to 7.0. Water content of each muscle protein gel was not made equal among the fish species.

Estimation of the muscle protein gel

Sensory evaluation of the gel and estimation of breakdown of MHC was carried out as described in chapter 2.

RESULTS AND DISCUSSION

Fig. 21 shows the classification of modori-inducing proteinase (MIP) according to the extractability from muscle. No MHC breakdown was observed before heating in

the meat sols from either threadfin bream or crucian carp (data not shown).

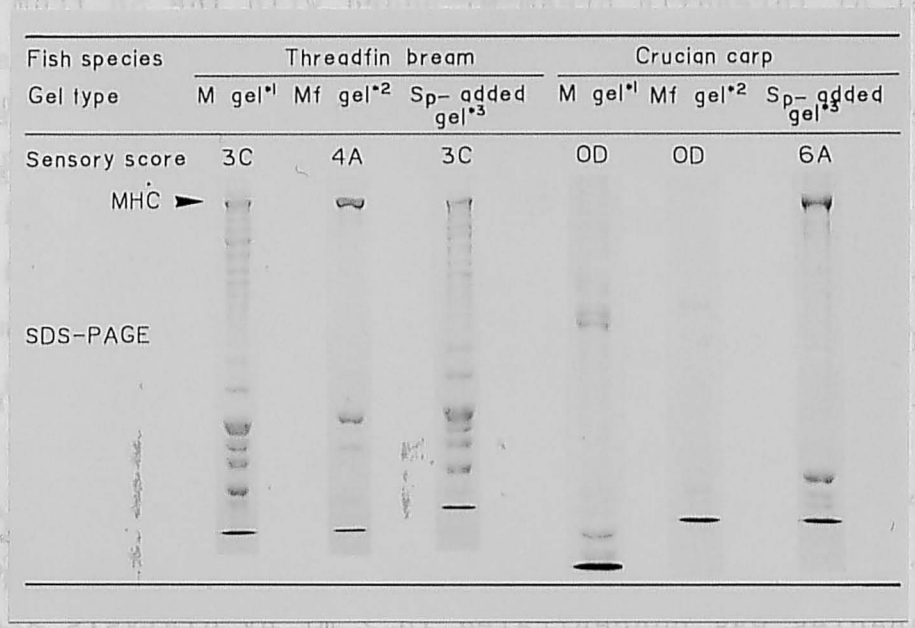

Fig. 21. Classification of modori-inducing proteinases based on extractability from muscle.
Minced meat gels, myofibrillar gels and sarcoplasmic fraction added gels were heated at 60°C for 120 min and was evaluated as described in "Materials and Methods". Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE.
*1 minced meat gel; *2 myofibrillar gel; *3 sarcoplasmic fraction added gel.

In the case of threadfin bream, the Sp-added gel showed the gel degradation (sensory score, 3C) and the breakdown of MHC, while Mf gel did not show the gel degradation (sensory score, 4A) and the breakdown of MHC at all. Judging from these results, it is evident that MIP existed only in sarcoplasmic fraction and was completely separated from myofibrils as to threadfin bream.

On the contrary, in the case of crucian carp, the Mf gel showed the gel degradation (sensory score, OD) and the complete breakdown of MHC while the Sp-added gel showed no

gel degradation (sensory score, 6A) and little MHC breakdown. The gel degradation property (sensory score, 0D) and the MHC breakdown activity in Mf gel were still observed to be associated with Mf even after elaborate washings. Therefore, MIP activity was considered to be tightly associated with Mf.

Tentatively, the former type of MIP, easily extractable one, designated as the sarcoplasmic MIP and the latter type of MIP, which is tightly associated with Mf, as the myofibril-associated MIP.

SUMMARY

The existence of two distinct types of MIP was noticed. They could be distinguished from each other by extractability from myofibrils. The easily extractable MIP was designated as sarcoplasmic MIP and the MIP, which is tightly associated to myofibrils, was designated as myofibril-associated MIP, tentatively.

CHAPTER 6. CLASSIFICATION OF *MODORI*-INDUCING PROTEINASE ACCORDING TO THE OPTIMUM TEMPERATURE AND SENSITIVITY TO n-BUTANOL

On the study of *modori*-phenomenon, the author used the gel heated at 60 °C to observe *modori*-phenomenon and used the gel heated at 50 °C as control gel, which did not show *modori*-phenomenon. However, occasionally typical *modori*-phenomenon was observed in the gel heated at 50 °C.

In this chapter, it will be investigated whether the *modori*-phenomenon observed in the gel heated at 50 °C is caused by the same MIP that induces *modori*-phenomenon at 60 °C. The classification of MIPs was also carried out according to the results in chapter 5 and this chapter.

MATERIALS AND METHODS

Materials

Materials were same as described in chapter 5.

Prepararion of fish meat gel

Minced meat gels were prepared as described in chapter 5. When the effect of *n*-butanol was tested, *n*-butanol (2%) was added to minced meat before grinding.

RESULTS AND DISCUSSION

Toyohara *et al*.[70] demonstrated that the gel degradation of file fish observed at 50 °C and that observed at 60 °C were caused by different proteinases which were distinguishable from each other by the optimum temperature (at 50 °C and 60 °C) and the sensitivity to *n*-butanol, a potent inhibitor for *modori*-phenomenon.[72] To confirm this, minced meat gels from threadfin bream and crucian carp were made at 50 °C and at 60 °C in the absence

or presence of n-butanol (2 %) (Fig. 22). n-Butanol did not show the inhibitory effect on the gel degradation and the breakdown of MHC of both species at 50 °C, while it showed a remarkable inhibitory effect on those of both species at 60 °C.

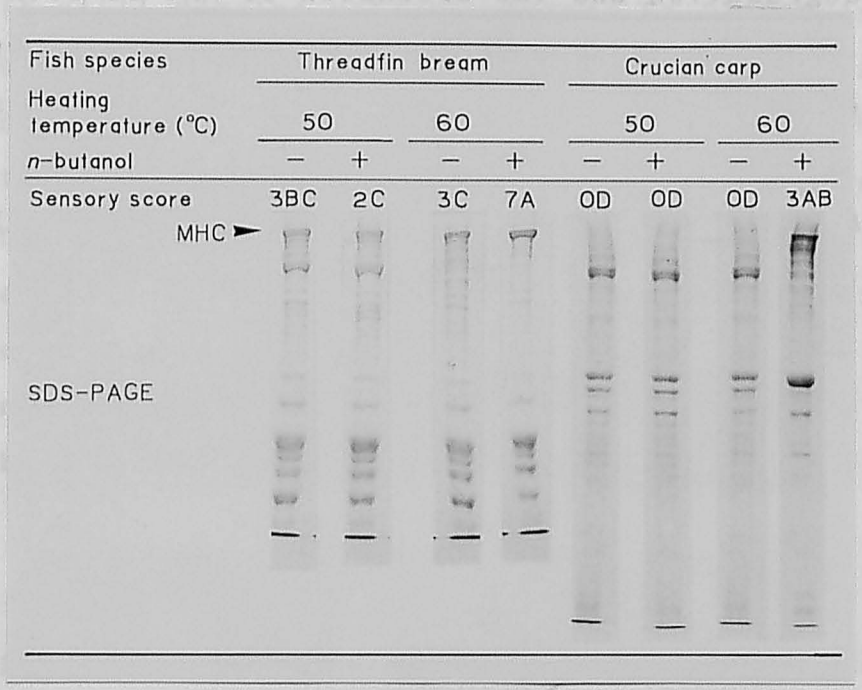

Fig. 22. Classification of modori-inducing proteinases based on optimum temperature and sensitivity to n-butanol. Minced meat gels were heated at 50°C or 60°C for 120 min and was evaluated as described in "Materials and Methods". The concentration of n-butanol is 2% (v/w). Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE.

It seems unlikely that this difference would be ascribed to the difference of the volatility of n-butanol at these temperatures from the following reasons. (1) The boiling point of n-butanol is much higher (117.3 °C) than these temperatures, and (2) even if the effective concentration of n-butanol was lower in the gel heated at 60 °C due to vaporization, reverse result would be

demonstrated. Therefore, it seems probable that the modori-phenomena occurred at 50 °C and 60 °C in these species were caused each other by the distinct MIPs having different sensitivity to n-butanol. Moreover, in the other fish species tested, n-butanol showed inhibitory effect on the gel degradation and the breakdown of MHC of both Mf gels and Sp-added gels heated at 60 °C (date not shown).

Based on the results shown in chapter 5 and this chapter, it seems appropriate to classify MIPs into following four types according to the extractability, the optimum temperature and the sensitivity to n-butanol (Fig. 23).

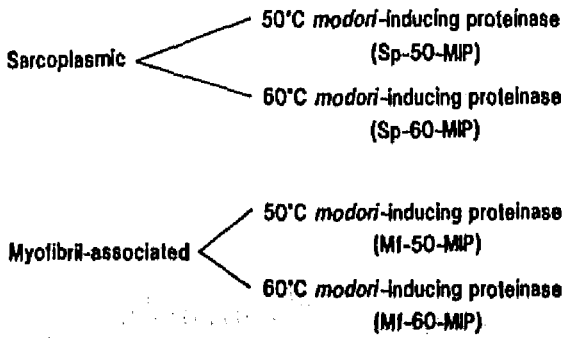

Fig. 23. Four types of *modori*-inducing proteinases.

(1) Sarcoplasmic-50 °C-modori-inducing proteinase (abbreviated as Sp-50-MIP), which is easily extractable, acts around 50 °C and is not sensitive to n-butanol, (2) sarcoplasmic-60 °C-modori-inducing proteinase (abbreviated as Sp-60-MIP), which is also easily extractable, but acts around 60 °C and is sensitive to n-butanol, (3) myofibril-associated-50 °C-modori-inducing proteinase (abbreviated as Mf-50-MIP), which is tightly associated with Mf, acts

around 50 °C and is not sensitive to n-butanol, and (4) myofibril-associated-60 °C-*modori*-inducing proteinase (abbreviated as Mf-60-MIP), which is also tightly associated with Mf, but acts around 60 °C and is sensitive to n-butanol.

It still remains unknown whether Sp-50-MIP and Mf-50-MIP as well as Sp-60-MIP and Mf-60-MIP are intrinsically different or not. In other words, it is obscure whether the difference in the extractability of these MIPs reflects the actual difference in their localization in muscle cells of these MIPs. Mf-MIPs of crucian carp could not be separated from myofibrils at all, even in the presence of several detergents[73]. This suggested the possibility that the Mf-MIPs are intrinsically integrated in Mf for the catabolism of myofibrillar proteins.

SUMMARY

n-Butanol repressed the *modori*-phenomenon occurred at 60 °C while it did not effective on that occurred at 50 °C. Therefore, it was suggested that *modori*-phenomena observed at 50 °C and 60 °C were produced each other by the different *modori*-inducing proteinases (MIPs) which could be distinguished from the other by the effect of n-butanol.

The existence of some other MIPs was demonstrated and thus all MIPs were classified into following four types on the basis of the extractability from muscle, the optimum temperature for the myosin heavy chain breakdown activity and the sensitivity to n-butanol. 1; Sarcoplasmic-50 °C-MIP (Sp-50-MIP), which is easily extractable from muscle tissue, acts optimally at 50 °C, and is not sensitive to

n-butanol, 2; sarcoplasmic-60°C-MIP (Sp-60-MIP) which is also easily extractable, but acts optimally at 60°C, and is sensitive to n-butanol, 3; myofibril-associated-50°C-MIP (Mf-50-MIP) which is tightly associated with myofibrils, acts optimally at 50°C, and is not sensitive to n-butanol, and 4; myofibril-associated-60°C-MIP (Mf-60-MIP) which is also tightly associated with myofibrils, but acts optimally at 60°C, and is sensitive to n-butanol.

CHAPTER 7. DISTRIBUTION OF MODORI-INDUCING PROTEINASES AMONG SEVERAL FISH SPECIES

The attempts were made to clarify the distribution pattern of four types of MIPs among several modori-occurring fish species.

MATERIALS AND METHODS

Materials

Body weight, body length and the date of catch of the fishes used for the experiment are summarized in Table 6. These fishes were used within 24 hours after death except for threadfin bream, which was obtained from the Kyoto Whole Sale Market and used within approximately 48 hours after death.

Methods

Methods used in this chapter is same as described in chapters 5.

RESULTS AND DISCUSSION

As shown in Fig. 24, Mf gels of threadfin bream made at 50 °C and at 60 °C do not show the modori-phenomenon judging from the results of the folding tests A and the SDS-PAGE analysis. However, Sp caused gel degradations when added to threadfin bream Mf and heated at both 50 °C and at 60 °C (sensory score at 50 °C, 3BC; that at 60 °C, 2CD) accompanying the breakdown of MHC, respectively. It was, therefore, suggested that threadfin bream contained Sp-50-MIP and Sp-60-MIP. In addition, the result supported the validity to use threadfin bream Mf as a substrate for the examination of the extracted MIP of each species.

Table 6. Fish used in the experiment.

Fish species	Body length (cm)	Body weight (g)	Date of catch (month/day/year)
Threadfin bream (Nemipterus virgatus)	26.0	410	Oct 20, '88
Mud dab (Limanda yokohamae)	25.5	350	Apr 14, '89
Walleye pollack (Theragra chalcogramma)	58.0	1700	Jul 2, '89
Red sea bream (Pagrus major)	36.2	1200	Jan 24, '89
Rainbow trout (Salmo gairdneni)	27.5	230	May 30, '89
Brown croaker (Argyrosomus miiuy)	31.7	440	Dec 14, '88
Shortfin lizard fish (Saurida elongate)	31.3	310	Sep 13, '89
Nibe croaker (Nibe mitsukurii)	30.5	520	Feb 7, '89
Tilapia (Sarotherodon niloticus)	22.2	400	Jan 11, '90
File fish (Navodon modestus)	16.6	110	Nov 8, '88
Pacific mackerel (Scomber japonicus)	33.0	680	Jan 23, '89
Crucian carp (Carassius auratus cuvieri)	23.5	520	Dec 9, '88

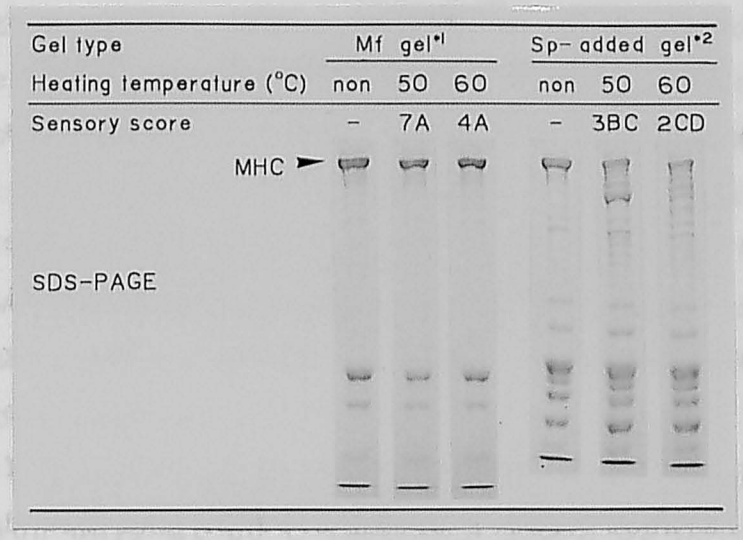

Fig. 24. Classification of modori-inducing proteinases of threadfin bream.
Myofibrillar gels and sarcoplasmic fraction added gels were prepared and evaluated as described in "Materials and Methods". Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE. *1 myofibrillar gel; *2 sarcoplasmic fraction added gel.

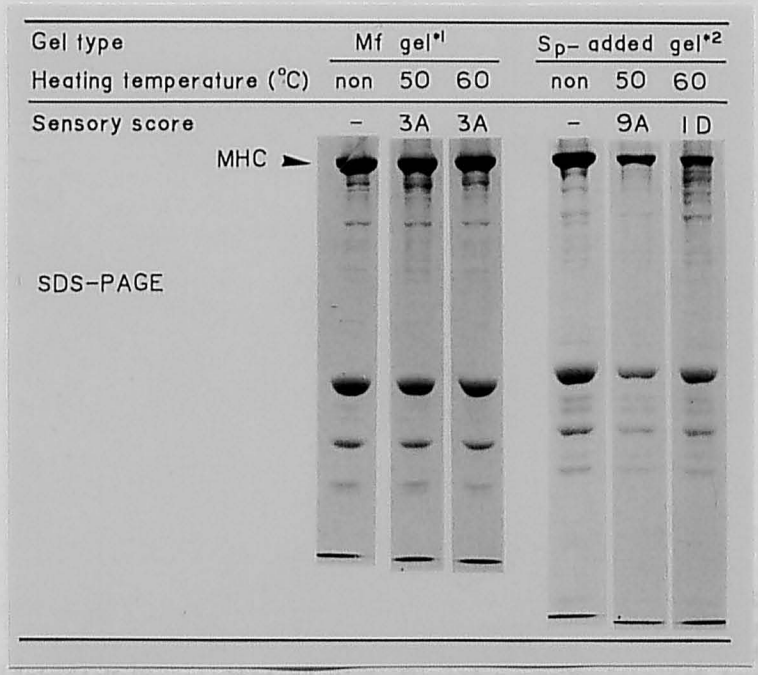

Fig. 25. Classification of modori-inducing proteinases of walleye pollack.
Myofibrillar gels and sarcoplasmic fraction added gels were prepared and evaluated as described in "Materials and Methods". Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE. *1 myofibrillar gel; *2 sarcoplasmic fraction added gel.

As shown in Fig. 25, in the case of walleye pollack typical gel degradation and the breakdown of MHC are observed only in the Sp-added gel made at 60 °C (sensory score was 1D), while Mf gels made at 50 °C and at 60 °C and Sp-added gel made at 50 °C did not show modori-phenomenon. This result suggested that walleye pollack contained only Sp-60-MIP and supported the advantage of leaching process applied for the preparation of surimi of this species in eliminating MIP. The same results were obtained for red sea bream, rainbow trout, brown croaker and mud dab (data not shown).

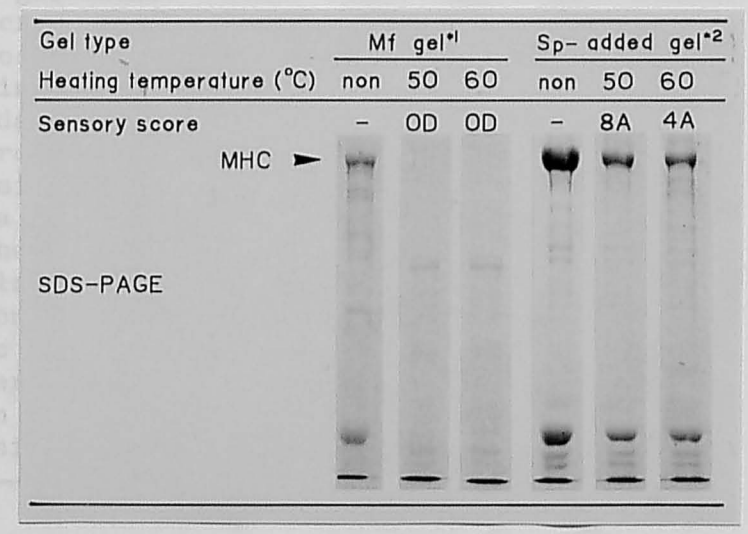

Fig. 26. Classification of modori-inducing proteinases of crucian carp.
Myofibrillar gels and sarcoplasmic fraction added gels were prepared and evaluated as described in "Materials and Methods". Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE. *1 myofibrillar gel; *2 sarcoplasmic fraction added gel.

Mf gels of crucian carp made at 50 °C and at 60 °C show intensive gel degradation as shown in Fig. 26 (sensory score was 0D at 50 °C and at 60 °C) and the breakdown of MHC, but Sp-added gels do not show the gel degradation (sensory score was 8A at 50 °C and 4A at 60 °C). Similar results were obtained for file fish and Pacific mackerel (data not shown). It was, therefore, suggested that these species contained Mf-50-MIP and Mf-60-MIP.

For nibe croaker, both Mf gel and Sp-added gel show degradation only at 60 °C (sensory score of both gels was 1D) as shown in Fig. 27. A similar result was obtained for tilapia. From this result, these species contained Mf-60-MIP and Sp-60-MIP.

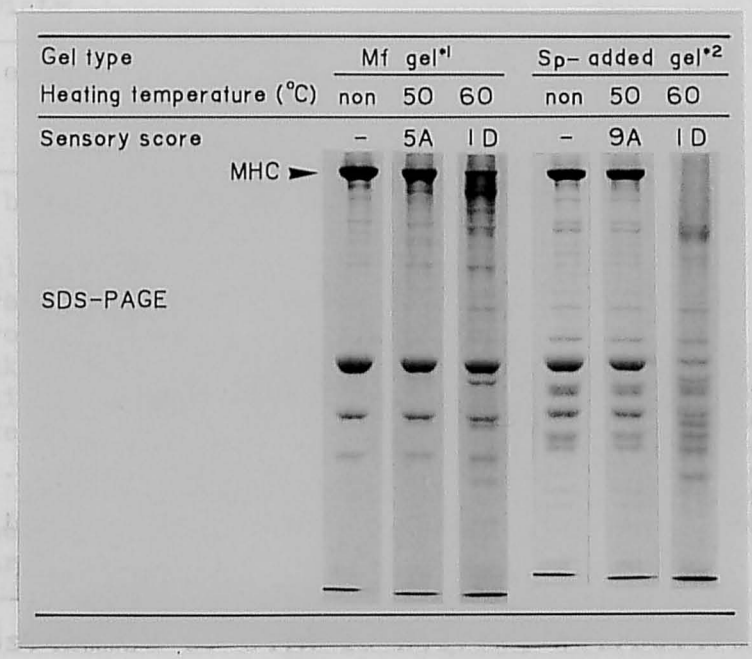

Fig. 27. Classification of modori-inducing proteinases of nibe croaker.
Myofibrillar gels and sarcoplasmic fraction added gels were prepared and evaluated as described in "Materials and Methods". Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE. *1 myofibrillar gel; *2 sarcoplasmic fraction added gel.

For shortfin lizard fish, gel degradations are observed in Sp-added gel heated at 60 °C (sensory score, 1D) as shown in Fig. 28. In the case of Mf gels made at 50 °C and 60 °C, though both of sensory scores were 3A, a partial modori-phenomenon was observed because MHC was degraded to some degree and the scores of teeth-cutting test of them were relatively lower than those of the gels heated for 10 min (data not shown). This result suggested that shortfin lizard fish contained Sp-60-MIP, Mf-50-MIP and Mf-60-MIP.

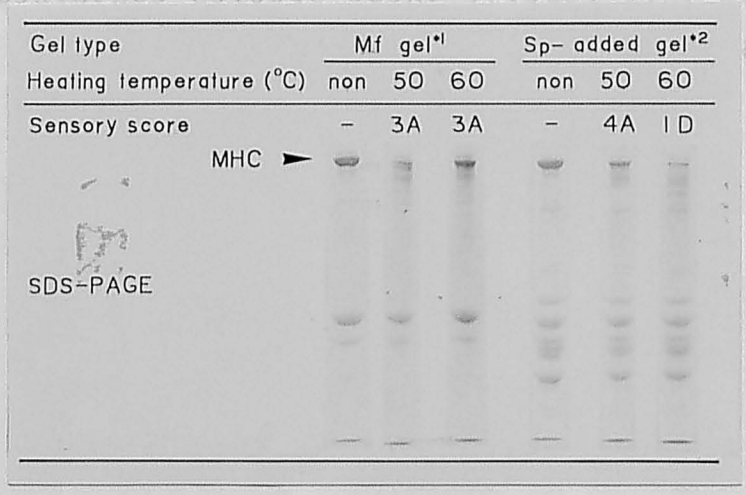

Fig. 28. Classification of modori-inducing proteinases of shortfin lizard fish.
Myofibrillar gels and sarcoplasmic fraction added gels were prepared and evaluated as described in "Materials and Methods". Degradation of myosin heavy chain (MHC) was determined by SDS-PAGE. *1 myofibrillar gel; *2 sarcoplasmic fraction added gel.

The distribution pattern of MIPs is summarized in Table 7. Though only a limited number of species were examined in the present study, the diversity of the distribution pattern of MIPs was clearly demonstrated. This may be partly responsible for the remarkable species-

specificity of the *modori*-phenomenon.[7]

It must be stressed, however, that lack of some type of MIP activity in some fish species does not necessarily mean lack of this type of proteinase in the corresponded fish species. MIP is supposed to exist as a latent form *in vivo* and required some activation for the revelation of its activity.[13] Presumably, the assay conditions for the activation of MIP, such as temperature or ionic strength, are not suitable.

Table 7. Distribution of four types of MIP among fish species.

Fish species	MIP			
	Sp-50-MIP	Sp-60-MIP	Mf-50-MIP	Mf-60-MIP
Threadfin bream	+	+		
Mud dab		+		
Walleye pollack		+		
Red sea bream		+		
Rainbow trout		+		
Brown croaker		+		
Shortfin lizard fish		+	(+)	(+)
Nibe croaker		+		+
Tilapia		+		+
File fish			+	+
Pacific mackerel			+	+
Crucian carp			+	+

(+) showed *modori*-phenomenon partially.

SUMMARY

Twelve fish species were classified into following five groups according to the distribution pattern of four types of MIP. 1; The species having only Sp-60-MIP, such as Alaska pollack, marbled sole, rainbow trout, brown croaker and red sea-bream, 2; the species having both Sp-50-MIP and Sp-60-MIP, such as threadfin-bream, 3; the species retaining both Mf-50-MIP and Mf-60-MIP, such as crucian carp, Pacific mackerel and oval filefish, 4; the species having both Sp-60-MIP and Mf-60-MIP, such as nibe croaker and tilapia and 5; the species possessing Sp-60-MIP, Mf-50-MIP and Mf-60-MIP, such as slender lizard fish.

SUMMARY AND CONCLUSION

(1) It was demonstrated that *modori*-phenomenon is caused by a heat stable proteinase which exist in sarcoplasm of fish muscle. This proteinase was designated as sarcoplasmic-60 °C-*modori*-inducing proteinase (Sp-60-MIP). The mechanism of *modori*-phenomenon was assumed that the degradation of myosin heavy chain by Sp-60-MIP results in destroying three-dimensional network of actomyosin. It was also confirmed that HAP bear no relation to *modori*-phenomenon because *modori*-phenomenon was not induced by the addition of HAP.

(2) Sp-60-MIP purified from threadfin bream was a glycoprotein having a monomelic subunit structure of which molecular weight was estimated to be 77,000 on a SDS-PAGE analysis. This proteinase was classified as a serine protease and its activity seemed to exist in a latent form *in vivo* and was induced by heating. This proteinase degraded myosin heavy chain in the presence of NaCl. This proteinase was distinguished from so-called HAP (heat-stable alkaline proteinase) which had long been supposed to be a *modori*-inducing factor, by its molecular weight and by the non-immunological cross-reactivity with anti heat-stable alkaline proteinase antibody.

(3) It is reasonable to assume that Sp-60-MIP plays a significant role in *kamaboko* processing on commercial base from following reason. Sp-60-MIPs were widely distributed among various fish species, especially among major species for *kamaboko* production (threadfin bream, walleye pollack, nibe croaker, shortfin lizard fish).

(4) The existence of other types of *modori*-inducing

proteinases (MIPs) was demonstrated and then those MIPs were classified into four sub-types as follows on the basis of the extractability from muscle, the optimum temperature for the myosin heavy chain breakdown activity, and the sensitivity to n-butanol. 1; Sarcoplasmic-50°C-*modori*-inducing proteinase (Sp-50-MIP) which is easily extractable, acts optimally at 50°C and is not sensitive to n-butanol, 2; sarcoplasmic-60°C-*modori*-inducing proteinase (Sp-60-MIP) which is also easily extractable, but acts optimally at 60°C, and is sensitive to n-butanol, 3; myofibril-associated-50°C-*modori*-inducing proteinase (Mf-50-MIP) which is tightly associated with myofibrils, acts optimally at 50°C, and is not sensitive to n-butanol, and 4; myofibril-associated-60°C-*modori*-inducing proteinase (Mf-60-MIP) which is also tightly associated with myofibrils, but acts optimally at 60°C, and is sensitive to n-butanol.

(5) Twelve fish species were examined and classified into following five groups according to the distribution pattern of above four types of MIP. 1; The species having only Sp-60-MIP, such as Alaska pollack, marbled sole, rainbow trout, brown croaker and red sea bream, 2; the species having both Sp-50-MIP and Sp-60-MIP, such as threadfin bream, 3; the species having both Mf-50-MIP and Mf-60-MIP, such as crucian carp, Pacific mackerel and oval filefish, 4; the species having both Sp-60-MIP and Mf-60-MIP, such as *nibe* croaker and tilapia and 5; the species having Sp-60-MIP, Mf-50-MIP and Mf-60-MIP, such as shortfin lizard fish.

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