魚類およびウシ血清中に存在する戻り誘発プロテアーゼ阻害物質

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Detection of Inhibitors for *Modori*-Inducing Proteinase in Fish and Calf Serums

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The serums of rainbow trout, Amago salmon, crucian carp, and calf showed inhibitory effects on *modori*-inducing proteinase (MIP) purified from threadfin-bream muscle, while the inhibitory activities per m*l* serum were diverse among the different individuals of the same species. In addition, the inhibitory activity seemed to be also changeable according to the physiological condition in the same species. Taking into consideration the practical use and the high content of the inhibitory activity, calf serum was used for the further study of the MIP inhibitor(s). When calf serum was chromatographed on a column of Ultrogel AcA 34, three distinct MIP inhibitors were detected. The molecular weights of these inhibitors were estimated to be more than 500,000, 58,000, and less than 15,000, respectively. The former two inhibitors were supposed to be α_2-macroglobulin and α_1-proteinase inhibitor, respectively, while the 15,000-dalton inhibitor seemed to be specific for MIP as far as examined. These inhibitors lost their inhibitory activities by heating at 100°C, but fairly stable around *modori*-occurring temperature, at 50–70°C.

Recent studies in our laboratory suggest that the *modori*-phonomenon (thermal fish gel degradation occurring around 60°C) could be caused by a group of newly found heat-activated serine proteinases, tentatively designated as *modori*-inducing proteinases (MIPs).[1-4] Previously, we demonstrated that the bleeding of rainbow trout might promote the *modori*-phenomenon and the addition of the serum suppressed it effectively.[5] These findings suggested that serum contains some MIP inhibitor(s). In the present study, we examined the distribution of MIP inhibitor(s) in serums of some fish species and calf and attempted to partially purify and characterize MIP inhibitor(s) in calf serum.

Materials and Methods

Preparation of Serums

Rainbow trout *Oncorhynchus mykiss* (205–480 g in body weight and 24–33 cm in body length), Amago salmon *Oncorhynchus macrostomus* (790–1130 g in body weight and 38–41 cm in body length), and crucian carp *Carassius auratus* (377–436 g in body weight and 26–27 cm in body length) were obtained alive and killed by striking in the head. Blood was collected from the caudal artery by a syringe as reported previously.[5] The amounts of blood collected were 9.2–11.5 m*l* from rainbow trout, 7.3–8.4 m*l* from Amago salmon, and 3.9–4.8 m*l* from crucian carp, respectively. After coagulating by standing for 5 h at 4°C, each blood was centrifuged at 3,500×g for 10 min. The obtained supernatant was used as the serum. Newborn calf serum was obtained from Nacalai tesque.

Preparation of Threadfin-bream MIP

MIP was partially purified from the sarcoplasmic fraction of threadfin-bream *Nemipterus bathybius* muscle by the method as described previously.[4] Briefly, dorsal white muscle was collected and homogenized with 4 parts of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and the homogenate was centrifuged at 10,000×g for 30 min. The obtained supernatant was chromatographed on DEAE-cellulose, Ultrogel AcA 34, and Con-A Sepharose columns. MIP activity was monitored as Boc-Leu-Thr-Arg-MCA degrading activity as described below. Protein concentration was determined by the absorbance at 280 nm or by the method of Bradford.[6]

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Assays of MIP and Trypsin

Boc-Leu-Thr-Arg-MCA (Peptide Institute, Osaka) degrading activity of MIP was determined as described previously.[4] The reaction mixture containing 50 mM Na-phosphate buffer, pH 7.0, 3% NaCl, 0.5 mM Boc-Leu-Thr-Arg-MCA (previously dissolved in dimethyluslfoxide to obtain 10 mM stock solution), and threadfin-bream MIP in a total volume of 0.1 m*l*, was incubated at 60°C for 20 min and the reaction was stopped by the addition of 2.6 m*l* of 1% SDS and 0.5 M Tris-HCl, pH 7.0. The increase in fluorescence intensity (excitation at 380 nm; emission at 460 nm) of 7-amino-methylcoumarin was measured with Hitachi 204 fluorescence spectrophotometer. One unit of MIP activity was defined as the activity which caused an increase in the fluorescence intensity of 1.0 under this assay condition. One unit of MIP inhibitory activity of serum was defined as the inhibitory activity which reduced one unit of MIP activity.

The myosin heavy chain degrading activity of MIP was determined as follows. The reaction mixture containing 50 mM Na-phosphate buffer, pH 7.0, 3% NaCl, 20 mg threadfin-bream myofibrils prepared as described previously,[2] threadfin-bream MIP and 50 μ*l* of each inhibitor fraction in a total volume of 1 m*l* was incubated at 60°C for 60 min and the reaction was stopped by the addition of 0.1 m*l* of 10% SDS in 100 mM Tris-HCl, pH 6.8, containing 50 mM 2-mercaptoethanol and the subsequent heating at 100°C for 3 min. As a 0-time sample or a control, the eluting buffer of the gel filtration (50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 50 mM NaCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA) was added instead of the inhibitor fraction. Thirty microgram protein equivalent to bovine serum albumin was applied on the analysis by SDS-polyacrylamide gel electrophoresis in 10% gel according to the method of Laemmli.[7] Staining was made by Coomassie Brilliant blue R-250. No polymerized high molecular weight product was recognized in or on the top of the stacking gel.

Trypsin activity was assayed as the caseinolytic activity at 30°C for 10 min according to the method of Lowry *et al.*[8] and one unit of trypsin activity was defined as the activity which increased the absorbance at 750 nm of 1.0 under this assay condition.

Assay of MIP and Trypsin Inhibitory Activity

MIP inhibitory activity of serum or each fraction eluted from a column of Ultrogel AcA 34 was determined as follows. An aliquot of sample solution was added to the reaction mixture for Boc-Leu-Thr-Arg-MCA degrading activity of MIP and remaining MIP activity was determined as described above. One unit of MIP inhibitory activity shown in Fig. 2 was defined as the inhibitory activity which reduced one unit of MIP activity.

Trypsin inhibitory activity shown in Fig. 2 was determined as follows. An aliquot of each fraction was added to the reaction mixture for the caseinolytic activity of trypsin and remaining trypsin activity was determined as described above. One unit of trypsin inhibitory activity was defined as the inhibitory activity which reduced one unit of trypsin activity.

Results and Discussion

Inhibitory Effect of the Serums on MIP Activity

Figure 1 shows a typical dose dependent inhibitory effect of rainbow trout serum on Boc-Leu-Thr-Arg-MCA degrading activity of threadfin-bream MIP. This result clearly indicated the existence of some MIP-inhibitor(s) in the serum. On the other hand, we found that the degree of the dose dependent effect was diverse, depending upon the individual serum samples. To examine this in more detail, MIP inhibitory activities were compared among the serums obtained from the different individuals of rainbow trout, Amago salmon,

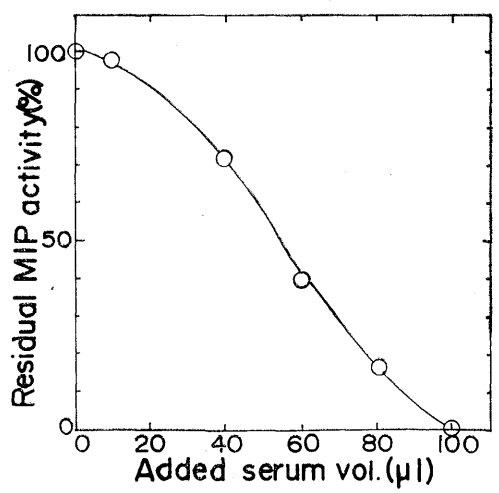

Fig. 1. Dose dependent inhibitory effect of rainbow trout serum on the Boc-Leu-Thr-Arg-MCA degrading activity of threadfin-bream *modori*-inducing proteinase (MIP). MIP activity was assayed at 60°C as described in the text.

Table 1. Comparison of the inhibitory activities on *modori*-inducing proteinase (MIP) in serums

Species	Calf	Amago salmon (May)	Amago salmon (July)	Rainbow trout (July/duploid)	Rainbow trout (July/triploid)	Crucian carp (July)
Number of samples	3	5	5	5	7	5
MIP inhibitory activity (units/m*l*)*[a]	51.1±3.4	41.7±8.2*[b]	24.1±8.7*[b]	22.5±8.9*[c]	18.5±8.0*[c]	19.7±14.3

*[a] Each MIP inhibitory acitivity was expressed as units/m*l* serum.
*[b] Significantly different each other ($p<0.01$).
*[c] Significantly different each other ($p<0.05$).

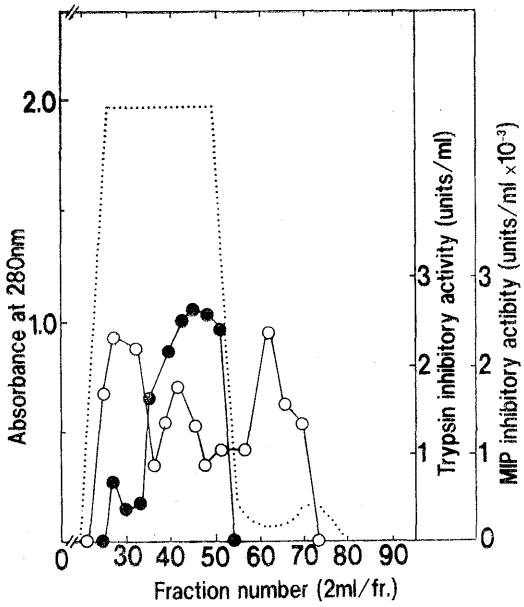

Fig. 2. Elution profile of the inhibitory activities for *modori*-inducing proteinase (MIP, ○—○) and trypsin (●—●) of calf serum from a colum of Ultrogel AcA 34 (2.5×100 cm). Newborn calf serum (25 m*l*) was concentrated with an Amicon PM-10 membrane to 5 m*l* and then applied on the column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA. MIP and trypsin inhibitory activities were determined as described in the text. Protein concentration (------) was determined by the absorbance at 280 nm.

and crucian carp. Commercially obtained newborn calf serum was also examined as a reference. As might be expected, a fairly large variance of the inhibitory activity per m*l* serum was demonstrated even in the serums obtained from the same species (Table 1). It should be noted that the inhibitory activities are different between Amago salmons of May and July at the 1% level of significance and the inhibitory activities of rainbow trout of diploid and triploid are also different at the 5% level of significance. The biological meaning of these differences, however, still remains poorly understood. It is also noteworthy that the calf serum showed the highest inhibitory activity and no noticeable variance was recognized among the lots of calf serums tested.

Similar to rainbow trout serum,[5] the addition of calf serum effectively suppressed the *modori*-phenomenon of walleye pollack (data not shown). Moreover, calf serum seemed to be more suitable as an additive than fish serum, because a larger amount of serum could easily be obtained with high inhibitory activities. Calf serum, accordingly, was used for the further study.

Gel Filtration of Calf Serum

To know whether MIP inhibitory activity in the serum could be ascribed to one or more inhibitors, gel filtration on Ultrogel AcA 34 (Pharmacia LKB Biotechnology) was performed.

Figure 2 shows the elution profile of MIP inhibitors from the column. Three distinct MIP inhibitory activities were detected and tentatively designated as MIP inhibitors I, II, and III according to the eluting order from the column. The molecular weights of MIP inhibitors I, II, and III were roughly estimated to be more than 500,000, 58,000, and less than 15,000, respectively, according to the calibration of the column with known marker proteins. Existence of similar three distinct MIP inhibitors was recognized in rainbow trout serum (data not shown).

Trypsin inhibitory activity was also measured, because MIP is known as a trypsin like serine proteinase.[4] Interestingly, both MIP inhibitors I and II showed trypsin inhibitory activities, while MIP inhibitor III did not. Both MIP inhibitors I and II also showed the inhibitory effect on the caseinolytic activities of chymotrypsin and papain, while MIP inhibitor III did not show any inhibitory effect on these proteases (data not shown).

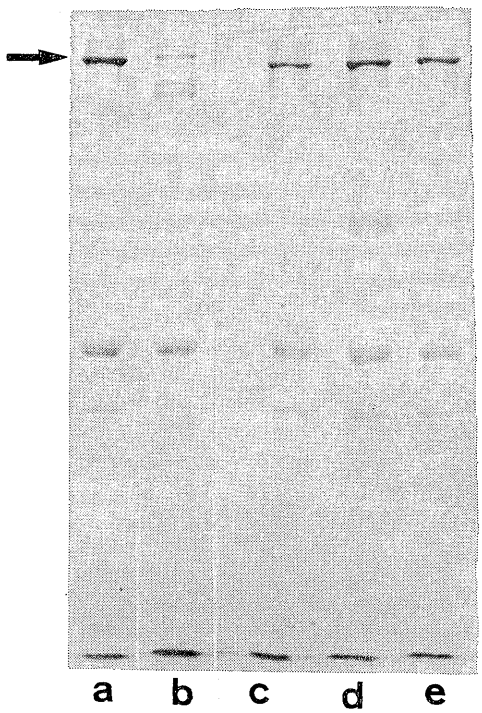

Fig. 3. Effect of calf serum *modori*-inducing proteinase (MIP) inhibitors on myosin heavy chain degrading activity of threadfin bream MIP, a, 0-time; b, control (without inhibitor); c, with MIP inhibitor I (fraction number 35); d, with MIP inhibitor II (fraction number 42); e, with MIP inhibitor III (fraction number 61). The arrow indicates myosin heavy chain.

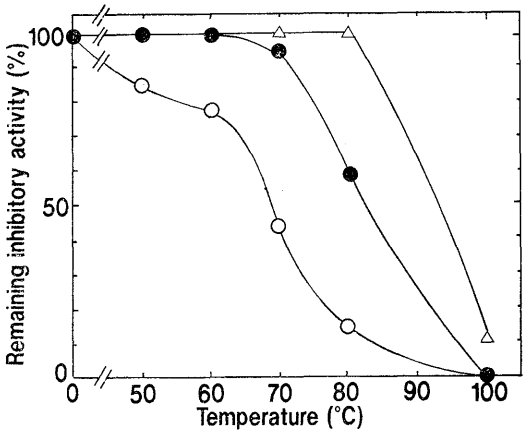

Fig. 4. Heat-stability of the *modori*-inducingproteinase (MIP) inhibitors eluted from the column of Ultrogel AcA 34. Aliquots of each fraction was heated at various temperatures for 10 min and then the remaining inhibitory activity on Boc-Leu-Thr-Arg-MCA degrading activity of threadfin-bream MIP was determined. ○—○, MIP inhibitor I (fraction number 35); ●—●, MIP inhibitor II (fraction number 42); △—△, MIP inhibitor III (fraction number 61).

Therefore, it is probable that MIP inhibitor III would be specific for MIP. On the other hand, based on the molecular weight of more than 500,000 and relatively wide inhibition spectra, MIP inhibitor I is assumed to be α_2-macroglobulin.[10] MIP inhibitor II is supposed to be α_1-proteinase inhibitor based on the molecular weight and the inhibitory effects on trypsin and chymotrypsin,[9] whereas the inhibitory effect of MIP inhibitor II on papain was probably due to the contaminated kininogen.[10]

Properties of MIP Inhibitors

It seemed difficult to examine the *modori*-suppressing effect of each MIP inhibitor by making *kamaboko* gel actually because of the low content of each inhibitor recovered from the column. Therefore, the inhibitory effect on the myosin heavy chain degrading activity of threadfin-bream MIP was investigated instead of producing *kamaboko*, because the *modori*-phenomenon is possibly caused by the proteolytic degradation of myosin heavy chain by MIP.[1-4] Fraction numbers 35, 42, and 61 were used as the samples of MIP inhibitors I, II, and III, respectively. As shown in Fig. 3, all these inhibitors inhibited the degradation of myosin heavy chain by MIP. Therefore, it seems probable that these MIP inhibitors could potentially suppress the *modori*-phenomenon.

The heat-stability of the serum MIP inhibitors was investigated by determining the remaining inhibitory activity on Boc-Leu-Thr-Arg-MCA degrading activity of threadfin-bream MIP after heating at various temperatures (Fig. 4). Although MIP inhibitor III was the most heat-stable, all inhibitors lost their inhibitory activities after heating at 100°C for 10 min. On the other hand, these MIP inhibitors are relatively heat stable around *modori*-occurring temperature, at 50–70°C. Therefore, it seems probable that the *modori*-suppressing effect of serum could be ascribed to the synergistic effect of these inhibitors.

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