Induction of Modori-Phenomenon (Thermal Gel Degradation) by A Latent Serine Proteinase

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An attempt was made to substantiate a modori (thermal gel degradation) inducing effect of a latent serine proteinase. The proteinase was purified homogeneously from the sarcoplasmic fraction of threadfin bream muscle using a combination of DEAE-cellulose, Con A-Sepharose, Arg-Sepharose, Shim-pack HAC, and TSK-gel G3000SWxl column chromatographies. Its molecular weight was estimated as 77,000 by SDS-PAGE analysis and 70,000 by gel filtration using TSK-gel G3000SWxl. To examine the occurrence of modori-phenomenon, sols prepared from threadfin bream myofibrils lacking in modori-causing property were heated at 60°C in the absence or presence of the proteinase. Only in the presence of the proteinase, marked modori was observed by the puncture test and sensory tests for estimating the physical property of resulting gels, and breakdown of myosin heavy chain was also observed by SDS-PAGE analysis. This evidence clearly suggests that the proteinase is responsible for the modori-phenomenon occurring at 60°C.

Modori-phenomenon (thermal gel degradation of fish jelly products) is known to accompany the breakdown of myosin heavy chain (MHC).1,2) The authors have suggested that a group of serine proteinases, collectively designated modori-inducing proteinases (MIPs), would be involved in modori-phenomenon because of their strong MHC-degrading activities in the presence of NaCl at 50–60°C.2–6) MIPs could be classified into subclasses according to the extractability from muscle tissue (sarcoplasmic and myofibril-associated types) and the optimum temperature range (50°C and 60°C types).7) Among them, a sarcoplasmic-60°C modori-inducing proteinase (Sp-60-MIP) was homogeneously purified from threadfin bream muscle.6) Recently, Yanagihara et al. also reported the possible participation of a similar sarcoplasmic serine proteinase active at 55°C in modori-phenomenon of white croaker.8) However, no direct evidence to substantiate the induction of modori-phenomenon by the purified proteinase has so far been available because of the difficulty to obtain enough amount of the purified proteinase to perform a reconstituted experiment.

In the present study, we purified large amount of Sp-60-MIP from threadfin bream muscle and tried to clarify whether the proteinase can induce modori-phenomenon or not.

Materials and Methods

Materials
Threadfin bream Nemipterus virgatus (420 g in weight and 29 cm in body length on the average) have been obtained from whole sale market, Kyoto, in a fresh state.

Measurement of Sp-60-MIP Activity
Sp-60-MIP activity was measured as MHC-degrading activity. The reaction mixture contained 50mM phosphate buffer (pH 7.0), 3% NaCl, 20 mg threadfin bream myofibrils, and proteinase solution 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 3 min. The breakdown of MHC was determined by the SDS-PAGE analysis using 10% gel by the method of Laemmli.9)

Purification of SP-60-MIP
Sp-60-MIP was purified from dorsal white muscle (500 g) using partially modified method

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of Kinoshita et al.6)
Step 1: Minced muscle was homogenized with 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl and 1 mM EDTA and centrifuged at 14,000×g for 20 min to obtain a crude extract.
Step 2: Crude extract was applied on a DEAE-cellulose (Whatman) column (5 cm in diameter, 7 cm in height) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 50 mM NaCl and washed thoroughly with the same buffer. Elution was performed with NaCl stepwise in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA.
Step 3: Active fractions from DEAE-cellulose column (fractions eluted by 0.15-0.3 M NaCl concentration) were concentrated by dialysis against polyethyleneglycol #6000 and dialyzed against 20 mM Tris-HCl, pH 7.5, including 0.5 M NaCl, 1 mM CaCl₂, and MnCl₂. The dialysate was applied on a column (1.5 × 7 cm) of Con A-sepharose (Pharmacia LKB Biotechnology) 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.
Step 4: Active fractions from Con A-sepharose (fractions eluted around 50 mM methyl-α-D-pyranoside concentration) were pooled and concentrated by dialysis against polyethyleneglycol #6000 and then dialyzed against 20 mM Tris-HCl, pH 7.5. The dialysate was applied on a column (1 × 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 100 ml.
Step 5: Active fractions from Arg-sepharose (fractions eluted around 0.2 M NaCl concentration) were pooled and concentrated by dialysis against polyethyleneglycol #6000 and then dialyzed against 2 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl. The dialysate was applied on high performance liquid chromatography with Shim-pack HAC (Shimadzu). Elution was performed with a 2-500 mM phosphate gradient.
Step 6: Active fractions (fractions eluted around 150 mM phosphate concentration) were collected and concentrated using ultrafiltration membrane UFPI TGC (Millipore). The concentrate was applied on TSK-gel G3000SWxl (Toso) equilibrated with 2 mM sodium-phosphate buffer, pH 6.8, containing 0.1 M NaCl. Sp-60-MIP activity was eluted at a retention time around 16.5 min (Fig. 1) and used for the experiment.

![Fig. 1. Elution profile of the sarcoplasmic-60°C modori-inducing proteinase (Sp-60-MIP) from TSK-gel G3000SWxl. Inset: 7.5% SDS-PAGE analysis of purified proteinase (retention time from 16 to 17 min).](image)

Elution was performed with 2 mM potassium phosphate buffer, pH 6.8, containing 0.1 M NaCl. Sp-60-MIP was eluted at a retention time from 16 to 17 min.

**Preparation of Myofibrils**
Myofibrils were prepared from threadfin bream muscle according to the method of Perry and Grey.10) Gels prepared from these myofibrils did not show modori-phenomenon at all (data not shown), suggesting that myofibrils thus prepared were completely free of MIP as described previously.6)

**Preparation of Myofibril Gel**
Myofibril gels were prepared as follows: twenty grams of myofibrils, prepared as described above, was ground with 2.8% NaCl, which gave 3% in the final gel together with NaCl in purified MIP solution, and 2 ml of MIP (0.5 mg protein/ml). For a control gel, 2 mM sodium-phosphate buffer, pH 6.8, containing 0.1 M NaCl (eluting buffer from TSK gel G3000SWxl) was used instead of MIP. Resulting salt-ground sol, 86% in water content, 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°C for 2 h.

**Evaluation of Gel Strength**
Gel strength was evaluated by the puncture and sensory (teeth-cutting and folding) tests as described previously.10)
Estimation of MHC Breakdown

Breakdown of MHC of myofibril gel was analyzed by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) according to the method of Laemmli.9) Results and Discussion

Different from the previous purification method, gel filtration by TSK-gel G3000SWxL was added as a final step to remove small amounts of contaminated proteins (Fig. 1). The proteinase was eluted as a single peak at a retention time of around 16.5 min and judged to be homogeneous on the basis of SDS-PAGE analysis (Fig. 1 inset). As same as the previous report,6) the molecular weight of this MIP was estimated as 77,000 by SDS-PAGE analysis and 70,000 by TSK gel G3000SWxL. Therefore, Sp-60-MIP was obviously distinguishable from other fish muscle serine proteinases in molecular weight; one from white croaker Argyrosomus argentatus has a molecular weight of 680,0008) and the other from white croaker Micropogon opercularis has a molecular weight of 269,000.11)

To examine the modori-inducing activity of produced with or without Sp-60-MIP. As shown in Table 1, gel strength of the MIP-added gel remarkably decreased to 24 g·cm, which was about 1/20 of that of the control gel. Sensory scores of the MIP-added gel were also markedly lower (“1” in the teeth-cutting test and “C” in the folding test) than those of the control gel (“7” in the teeth-cutting test and “A” in the folding test). Additionally, in the MIP-added gel the density of MHC band was faint and many faster migrated bands probable of degraded products could be observed between MHC and actin bands in the SDS-PAGE analysis (Fig. 2). These findings clearly suggest that Sp-60-MIP induces modori-phenomenon.

Some attempts to induce modori-phenomenon by adding an endogenous factor have so far been carried out. Makinodan et al.13) examined the modori-inducing effect of a partially purified heat-stable alkaline proteinase (HAP) and recognized the modori-inducing effect judging from their result that the gel strength of HAP-added gel was barely reduced to a half of that of the control gel. In our experiment, however, no modori-phenomenon was induced by the addition of partially purified HAP from threadfin bream.9) Therefore, further studies are required to clarify whether HAP is a modori-inducing factor. On the other hand, Iwata et al. reported the existence of modori-inducing proteins free of proteolytic activity from carp muscle.13,14) However, the modori-evaluating method employed in their study seemed not to be suitable because they assessed degree of modori not by the gel strength but by the volume of expressive water from gel. In
addition, modori-inducing activities of the proteins were not clearly demonstrated even by their evaluating method.

This is the first paper to give direct evidence to substantiate that Sp-60-MIP can induce modori-phenomenon. Though the protein concentration of Sp-60-MIP in the threadfin bream muscle has not been examined, it is likely that Sp-60-MIP is a candidate for modori-phenomenon in practical fish jelly production. Additionally, Sp-60-MIP is known to widely distribute among various fish species, such as threadfin bream, walleye pollack, and shortfin lizard fish,\textsuperscript{7} which are especially important for fish jelly production. It is, therefore desirable to suppress this MIP activity to improve quality of fish jelly products. We are now surveying naturally occurring inhibitors of this MIP.

References