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Original article

Effect of spawning on endogenous proteinases in abdominal muscle of threadfin bream *Nemipterus virgatus*

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Introduction

Threadfin bream (*Nemipterus virgatus*) is one of the most important commercial fisheries species in Japan and Southeast Asia, and it is a potential as a raw material for *surimi* products. However, the quality and commercial value of *surimi* products is always reduced by *modori* phenomenon [1]. So-called *modori* phenomenon is that the myofibrillar proteins could be degraded and result in gel softening when *surimi* is passing the temperature zone of 40-60°C to form a gel. Many researches have demonstrated that the degradation of myofibrillar proteins is caused by the endogenous proteinases, especially serine- or cysteine-type proteinases in fish. Sarcoplasmic serine proteinase and myofibril-bound serine proteinase (MBSP) are typical serine proteinases in fish muscle to degrade the myosin heavy chain (MHC) at 50-60°C [2-4]. The myofibril degrading cysteine proteinase include cathepsins B and L [5-7]. Furthermore, the characteristic of myosin gel-forming ability is influenced by seasons [8], especially the spawning period [9]. In this study, we investigated the effects of spawning on endogenous proteinases in the muscle of threadfin bream.

Materials and methods

Captured threadfin bream with body weight of 117.5±8.6 g was obtained from fish market in Nagasaki, Japan. There are three sampling seasons: July (spawning period), November 2016 and March 2017. The autolytic activity was determined by Lowry method. One unit of the autolytic activity was defined as the activity producing 1 μmol of free amino acids per minute. Belly muscle was homogenized with 3-folds of 0.5% KCl and then centrifuged. The supernatant was the sarcoplasmic fraction. The precipitate was washed with 50 mM Tris-HCl containing 5 mM $CaCl_2$ until the sarcoplasmic fraction was completely removed, it was the myofibrillar fraction. The activity of sarcoplasmic and myofibrillar proteinases were measured using fluorogenic synthetic substrates. One unit of enzyme activity was defined as the amount of the activity releasing 1 nmol of 7-amino-4-methylcoumarin (AMC) per minute. The gelatinolytic activities of sarcoplasmic fraction were analyzed by gelatin zymography.

Results and discussion

A preliminary experiment showed that the proteinase activity of threadfin bream belly muscle was much higher than dorsal muscle (data not shown). Therefore, the belly muscle was chosen to conduct the following experiments.

In order to investigate the main working proteinases in different seasons, various inhibitors were used in the autolytic activity assay, such as serine proteinase inhibitor Pefabloc SC, aspartic proteinase inhibitor Pepstatin A and cysteine proteinase inhibitor E-64. The results (Fig. 1) showed that a higher autolytic activity was found in threadfin bream belly muscle in July (spawning period). Similar phenomenon was reported in some fish species, rainbow trout [10] and chum salmon [11] possessed a higher proteinase activity during spawning period. Rabbit fish showed a lower gel-forming property in spawning period [9], and that may be caused by high proteinase activity. A higher proteinase activity in spawning period could promote the degradation of proteins to satisfy the demand of

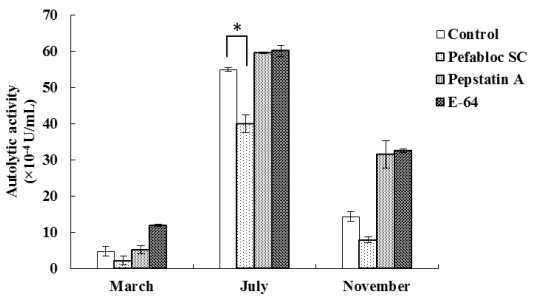

Fig. 1. Effects of inhibitors on autolytic activity of threadfin bream belly muscle in different seasons. The autolytic activity was assayed at 50°C, pH 7.0 for 1 h incubation.

energy and material for reproductive behavior, while it also had bad effects on fish muscle processing, especially *surimi* products.

For the inhibitory effects, though threadfin bream represented different autolytic activities upon seasons, it also exhibited a same inhibitory pattern. The autolytic activity was inhibited by Pefabloc SC, while Pepstatin A and E-64 represented little effect. Therefore, serine proteinase plays an important role in threadfin bream muscle during spawning period.

To further characterize the endogenous proteinase, the sarcoplasmic fraction was extracted and the activity toward Boc-Val-Pro-Arg-MCA was measured in different seasons. The result showed that a highest proteinase activity appeared in July which is in good agreement with that the highest autolytic activity in spawning period.

Through inhibitory effects, substrate specificity and gelatinolytic activity, the endogenous proteinase was further characterized. According to the gelatin zymography (Fig. 2), a clear band with a molecular weight approximately 50 kDa exhibiting high gelatinolytic activity. And the active band was inhibited by Pefabloc SC and EDTA which indicated the proteinase is a serine proteinase.

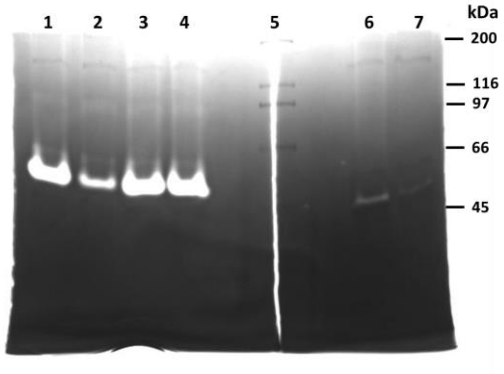

Fig. 2. The inhibitory effects on sarcoplasmic proteinase from threadfin bream belly muscle using gelatin zymography. Lane 1, control; Lane 2, Pefabloc SC (5 mM); Lane 3, Pepstatin A (0.01 mM); Lane 4, E-64 (0.01 mM); Lane 5, protein standard; Lane 6, EDTA (5 mM); Lane 7, mix inhibitors.

Moreover, the proteinase activity toward Boc-Val-Pro-Arg-MCA was extremely inhibited by Pefabloc SC and EDTA. Though the proteinase was not purified, the data of inhibitory effects certified that the main working endogenous proteinase was a serine proteinase. A result of substrate specificity showed that the proteinase selectively cleaved at the C terminal of arginine residues, while little hydrolyzing activity upon substrates of chymotrypsin, Suc-Leu-Leu-Val-Tyr-MCA and Suc-Ala-Ala-Pro-Phe-MCA. Obviously, the main working proteinase in sarcoplasmic fraction is a trypsin-type serine proteinase.

In the present study, we also confirmed that the activity of myofibril-bound serine proteinase was detected in the threadfin bream myofibrillar fraction and exhibited a highest activity in spawning period. The result of inhibition revealed that it was extremely inhibited by Pefabloc SC and EDTA. It is evidence supporting that the proteinase in myofibrillar fraction is a MBSP (Table 1).

Table 1. Effects of inhibitors on Boc-Val-Pro-Arg-MCA hydrolyzing activity of the myofibrillar fraction. The activity was assayed at 50°C, pH 8.0 for 1 h incubation

Inhibitors	Final concentration (mM)	Relative activity (%)
Control		100
Pefabloc SC	5	17
Pepstatin A	0.01	90
E-64	0.01	95
EDTA	5	53

MBSP is a kind of serine proteinase that tightly bound to myofibrils. Because of participating the *modori* phenomenon and have the characteristics of heat-stability, strong proteinase activity and difficulty in removing, MBSP was put into a lot of attention. Interestingly, the MBSP activity only detected in the belly muscle of threadfin bream (data not shown). In any case, it is essential to investigate the properties of the MBSP in solving the *modori* phenomenon in fish *surimi* processing.

Conclusions

A preliminary research on the effects of endogenous proteinases from threadfin bream muscle in different seasons was conducted. There are two endogenous serine proteinases exhibited especially high activities during spawning period. And the high activity was considered to have potential for promoting *modori* phenomenon. The proteinase of sarcoplasmic fraction was confirmed to be the main working proteinase at 50°C and it was characterized to be a trypsin-type serine proteinase. The proteinase in myofibrillar fraction was detected as a MBSP.

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