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The effect of soybean trypsin inhibitor on the degradation of myofibrillar proteins by an endogenous serine proteinase of crucian carp

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Abstract

The effect of soybean trypsin inhibitor (STI) on the degradation of crucian carp myofibrillar proteins caused by an endogenous myofibril-bound serine proteinase (MBSP) was studied. Soybean trypsin inhibitor was purified to high homogeneity and mixed with myofibrils and its inhibitory effect on myofibrillar protein degradation was investigated. In the absence of STI, proteolysis of myofibrillar proteins including myosin heavy chain, α-actinin, actin and tropomyosin could be identified after incubation at 55 °C for 5–20 min depending on the kind of the protein. In contrast, in the presence of STI, with final concentration of 0.75 μg/ml, proteolysis of these proteins was greatly suppressed even after incubation for 1 h, suggesting STI is an effective inhibitor in preventing myofibrillar protein degradation caused by a serine proteinase that is quite possibly MBSP. Though STI has disadvantages for food digestion, as a native food grade inhibitor, it is safe as a food additive, especially at low concentration. Because in surimi production, the decrease of elasticity is always accompanied with the degradation of myofibrillar proteins, thus, the present result suggested the possibility that STI could be applicable in surimi production in order to enhance the elasticity that is the quality of the final products.

Keywords: Crucian carp; Degradation; Myofibril; Inhibition; Serine proteinase; Soybean trypsin inhibitor

1. Introduction

Recently, a myofibril-bound serine proteinase (MBSP) was identified in the skeletal muscle of crucian carp (*Carasius auratus*) (Cao, Jiang, Zhong, Zhang, & Su, in press). MBSP breaks down myosin heavy chain (MHC) most efficiently at around 55 °C and also degrades other myofibrillar proteins such as α-actinin, actin and tropomyosin to some degree. On the bases of these studies, we proposed that MBSP is most probably the enzyme that is responsible for the so-called modori phenomenon. Crucian carp is a popularly cultured freshwater fish species in different provinces of China and is a potential raw material for surimi production. With the increasing demand for surimi products, more researchers are interested in the utilization of underutilized fish. However, the occurrence of modori phenomenon in the manufacturing process greatly affected the quality of surimi products (Cao et al., 1999; Shimizu, Machida, & Takenami, 1981; Shimizu, Nomura, & Nishioka, 1986). The degradation of MHC by endogenous proteinase(s) is generally regarded as the main

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cause of modori phenomenon (Nakamura, Ogawa, Saito, & Nakai, 1998; Toyohara & Shimizu, 1988). Now, more and more researchers accept the opinion that myofibril-bound serine proteinase(s) is the most important factor responsible for this phenomenon (Cao et al., 1999; Cao, Osatomi, Hara, & Ishihara, 2000; Ramirrez, Garcia-Carreno, Morales, & Sanchez, 2002; Toyohara, Sakata, Yamashita, Kinoshita, & Shimizu, 1990).

In order to make high quality surimi products from freshwater fish, proteinase inhibitors, especially food additive grade inhibitors are calling attention in protecting myofibrillar proteins from proteolysis by endogenous proteinase such as MBSP. Plants contain various kinds of proteinase inhibitors and some of them have been identified as effective in preventing fish myofibrillar protein degradation (Ramirrez et al., 2002). Among serine proteinase inhibitors from plants, soybean trypsin inhibitor (STI) is well characterized. It is relatively thermal stable whether under acidic or basic conditions (Osman, Reid, & Weber, 2002). Though STI is also regarded as anti-nutritional factor, as a soy protein, especially at low concentration, it can be used as food additive.

In the present study, we investigated the inhibitory effect of STI in protecting the proteolysis of crucian carp myofibrillar proteins caused by MBSP with a purpose to use STI as an ingredient in surimi production.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Crucian carp (body weight about 300 g) was obtained alive in early June from a market in Jimei, Xiamen, China. The fish was subdued in iced water and sacrificed instantly. After decapitation and evisceration, the fish was filleted and the fillets were immediately used for myofibril preparation or kept at a −80 °C freezer for further use.

2.1.2. Chemicals

Protein standards for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot were from Bio-Rad (Richmond, CA, USA) and New England BioLabs (Beverly, MA, USA), respectively. Antibodies of rat anti-red sea bream α-actinin and actin were kind gifts from Dr. Tachibana of the Faculty of Fisheries, Nagasaki University, Japan. Antibody of rat anti-carp tropomyosin was prepared in our own laboratory. The second antibody of rabbit anti-rat IgG-HRP was from DAKO (Denmark). *t*-Butyloxycarbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) was a product from Peptide Institute (Osaka, Japan). Porcine trypsin was a product of Sigma. Other reagents were all of analytical grade.

2.2. Preparation of soybean trypsin inhibitor

Soybean was soaked in 20 mM Tris–HCl buffer, pH 8.0 overnight and homogenized using a homogenizer (Kinematica, Switzerland). Soybean trypsin inhibitor was briefly purified by heat treatment of the homogenate at 60 °C for 90 min. After centrifugation at 15,000*g* for 15 min, the supernatant was applied to a DEAE-Sepharose column. Elution was carried out using a gradient of NaCl from 0 to 0.5 M. Inhibitory active fractions against porcine trypsin were collected and concentrated by ultrofiltration using a YM-10 membrane (Millipore, USA). Concentrated fractions were then applied to Sephacryl S-200 column (1.64 × 98 cm) and eluted with 20 mM Tris–HCl, pH 8.0 containing 0.2 M NaCl. Active fractions were obtained and the purity of STI was checked by SDS–PAGE.

2.3. Preparation of myofibrils from skeletal muscle

Crucian carp skeletal muscle (20 g wet weight) stored at −80 °C was minced and homogenized with four volumes of ice-cold 20 mM phosphate buffer (pH 7.5) using a homogenizer. The homogenization process was performed on ice using a polytron of PT-DA 2120 at the speed indicator of 15, and the operation was carried out for 2 times with each time of 30 s and an interval of 1 min. The resulting homogenate was centrifuged at 8000*g*, 4 °C for 10 min in a centrifuge (Avanti J-25, Beckman coulter, USA). The supernatant was discarded while the pellet collected and resuspended in fourfold of ice-cold phosphate buffer. After three repeating cycles of homogenization and centrifugation, the resulting pellet was suspended in 20 mM phosphate buffer and further homogenized. Finally, after centrifugation at 8000*g* for 10 min, the pellet was resuspended in 20 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl and this suspension was regarded as crucian carp myofibrils. Myofibrils were immediately used for experiment or stored at a −80 °C freezer for further use. The protein concentration of myofibrils was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) after proper dilution. Bovine serum albumin was used as standard.

2.4. Extraction of MBSP from myofibril and enzymatic activity assay

Crucian carp myofibrils prepared as above were homogenized with fourfold of 20 mM Tris–HCl buffer, pH 8.0. The homogenate was immediately heated in boiling water while stirring until the temperature reached 55 °C and further incubated at the same temperature in a water bath for 5 min, followed by immediate cooling in ice water to 4 °C. After centrifugation, the supernatant, which was regarded as crude MBSP, was

used for enzymatic characterization. MBSP activity was determined using Boc-Phe-Ser-Arg-MCA as substrate. Briefly, MBSP (50 μl) was added to 850 μl of 50 mM Tris–HCl buffer, pH 8.0. The reaction was initiated by the addition of 100 μl of 10 μM substrate and incubated at 55 °C for 20 min. The reaction was immediately stopped by addition of 1.5 ml stopping agent (methyl alcohol:*n*-butyl alcohol:distilled water = 35:30:35, v/v). MBSP enzymatic activity was detected by measuring the fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm using a fluorescence spectrophotometer (FP-6200, Jasco, Japan). Control tests were performed under same conditions except that MBSP was replaced by Tris–HCl buffer (50 mM, pH 8.0).

2.5. Analysis of thermal stability and inhibitory activity of STI toward MBSP

The thermo stability of STI was examined by incubating the purified STI (0.75 μg/ml, pH 7.5) at different temperatures (60, 70, 80, 90 and 100 °C) for 30 min. After heating, the inhibitor solution was chilled on ice water and the residual inhibitory activity against MBSP was determined. Control tests were investigated without incubating the solution of STI. Inhibitor activity was expressed as a percentage of inhibition (I) of a control assay using the following equation:

$$I(\%) = ((T - T_i)/T) \times 100,$$

where T and T_i are protease activities without and with STI, respectively.

2.6. Inhibitory effect of STI on myofibrillar protein degradation

To investigate the inhibitory effect of STI on the degradation of myosin heavy chain and other myofibrillar proteins caused by MBSP, purified STI was utilized. The experiment was performed using 100 μl myofibril dissolved in 50 mM phosphate buffer, pH 8.0 containing 0.5 M NaCl and STI was mixed with myofibril to the final concentration of 0.75 μg/ml. In the control group, instead of STI, distilled water was added. Followed incubation at room temperature for 30 min to allow complete penetration of STI to myofibril, the mixture was incubated at 55 °C for different time intervals. After incubation, 100 μl of 4× SDS loading buffer was added to each tube and boiled in the presence of 5% of 2-mercaptoethanol for 5 min, followed by SDS–PAGE or Western blotting. The inhibitory effect on MHC degradation was detected by SDS–PAGE, while that on the decomposition of α-actinin, actin and tropomyosin was investigated by Western blotting using specific polyclonal antibodies against these proteins, respectively.

2.7. Protein concentration assay

Protein concentrations of myofibrils and soybean trypsin inhibitor were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.8. SDS–PAGE analysis and Western blotting

SDS–PAGE was performed under reducing conditions using 12% mini-slab gels according to the method of Laemmli (1970) and the gels were stained for protein with Coomassie Brilliant Blue R-250. Western blotting was carried out as described by Towbin, Staehelin, and Gordon (1979). Briefly, myofibrillar proteins on acrylamide gels were electrophoretically transferred onto nitrocellulose membranes in transfer solution. Non-specific protein sites were blocked with 5% non-fat milk in Tris–HCl-buffered saline (TBS = 20 mM Tris–HCl, pH 7.5, containing 0.145 M NaCl). The blotted proteins were incubated with antibodies at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with horseradish peroxidase conjugated secondary antibody, the nitrocellulose membrane was washed extensively with TBST. Immunodetection was carried out using a detection kit from Bio-Rad.

3. Results and discussion

In our previous study (Cao et al., in press), we identified the existence of a myofibril-bound serine proteinase (MBSP) in the skeletal muscle of crucian carp. MBSP with optimum temperature of 55 °C degrades myosin heavy chain and tropomyosin markedly. It also breaks down other myofibrillar proteins such as α-actinin and actin to some degree and MBSP is thus regarded as a proteinase most probably involved in the modori phenomenon. In the present study, we tried to find an inhibitor to MBSP, which is both effective to the enzyme and can be used as food additive. We focused this on soybean trypsin inhibitor (STI), purified the inhibitor and studied its inhibitory effect toward MBSP. As shown in Fig. 1(a), STI with the molecular weight of 21.5 kDa was obtained in high homogeneity by heat treatment, DEAE-Sepharose ion-exchange and gel-filtration. The inhibitory effect of STI against MBSP was revealed in Fig. 1(b). MBSP activity to synthetic substrate Boc-Phe-Ser-Arg-MCA decreased with the increasing addition of STI, suggesting it is a specific inhibitor toward MBSP. Furthermore, STI is a relatively thermal stable protein as more than 55% of its initial inhibitory activity remained even after pretreatment at 100 °C for 30 min (Fig. 2).

The inhibitory effect of STI in preventing the degradation of myofibrillar proteins was further investigated

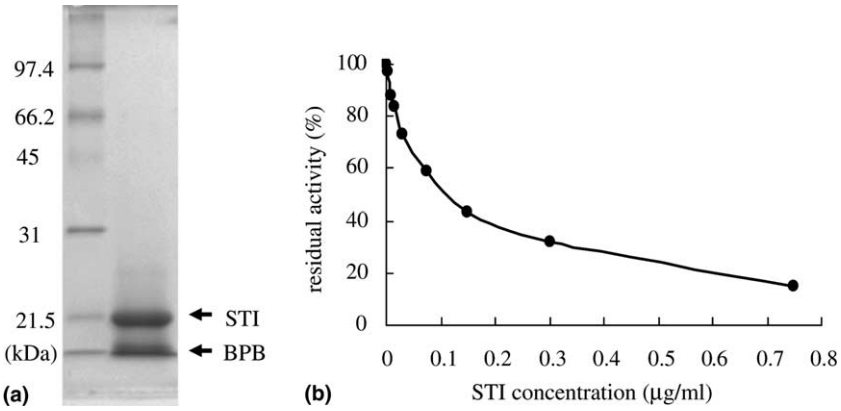

Fig. 1. SDS–PAGE of purified soybean trypsin inhibitor (STI) and inhibition effect of STI on crucian carp MBSP activity. (a) SDS–PAGE. Positions of molecular weight standards are labeled on the left, purified STI and bromphenol blue (BPB) on the right. (b) Crude MBSP was preincubated with different amounts of STI at room temperature for 20 min, the residual enzymatic activity toward Boc-Phe-Ser-Arg-MCA was determined. Assays were performed in duplicate and variation between the duplicate samples was less than 10%.

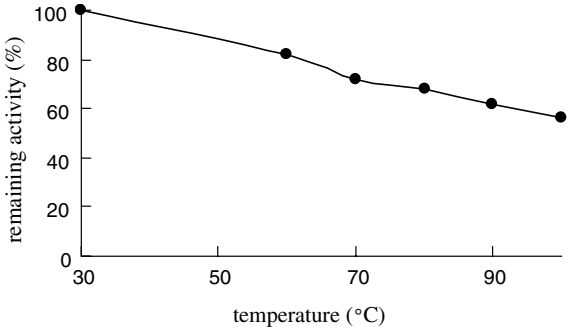

Fig. 2. Thermo stability of purified STI. STI (75 μg/ml) in 20 mM Tris–HCl buffer, pH 8.0 was incubated at different temperatures (30, 60, 70, 80, 90 and 100 °C) for 30 min. The remaining inhibitory activity was determined as described in Section 2. STI without heating was used as control. Assays were performed in duplicate and variation between the duplicate samples was less than 10%.

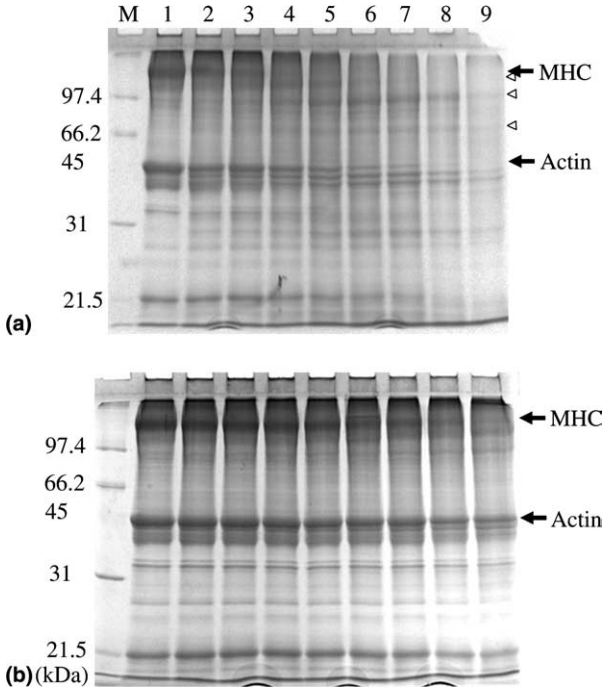

Fig. 3. Degradation of myofibrillar proteins by MBSP in the absence and presence of STI. Myofibrillar proteins in the absence (a) and presence (b) of STI were incubated at 55 °C for different time intervals (lanes 1–9 corresponding to incubation time of 0, 2, 5, 10, 20, 30, 60, 120, 240 min, respectively). After incubation, samples were SDS-treated and applied to SDS–PAGE. Positions of molecular weight standards are labeled on the left. MHC, myosin heavy chain.

as these proteins could be substrates of MBSP during the manufacturing of surimi and most probably substrates in vivo. Myofibrils mainly consisted of myosin heavy chain (MHC, 200 kDa), actin (42 kDa) and other minor proteins such as α-actinin (100 kDa) and tropomyosin (34 kDa). Myofibrils in the absence and presence of STI were incubated at 55 °C for different time intervals and the degradation of myofibrillar proteins was studied. As shown in Fig. 3(a), where no STI was added, the degradation of myofibrillar proteins, especially myosin heavy chain (MHC) could distinctly be identified even after incubation for 5 min (Fig. 3(a), lane 3). Prolonged incubation produced three new bands with sizes of approximately 120, 90 and 70 kDa, which are quite possibly the degraded products of MHC (Fig. 3(a), open arrowheads), although the possibility that these bands are decomposed products of other higher molecular weight proteins such as neblin and connectin could not be excluded. In contrast, no apparent degradation was observed in the presence of STI even after 1 h (Fig. 3(b), lane 7). This result strongly suggested that STI is an effective inhibitor toward the endogenous proteinase.

To further investigate the degradation of other myofibrillar proteins, Western blots using specific polyclonal antibodies against these proteins were carried out. In the absence of STI, α-actinin, actin and tropomyosin all degraded after incubation for 20 min (Figs. 4(a), 5(a) and

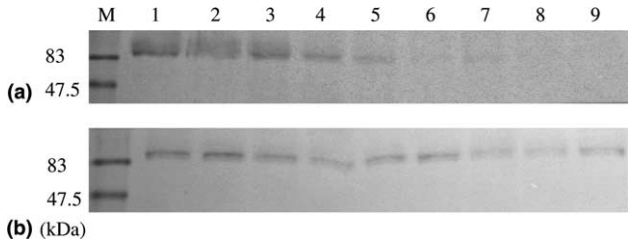

Fig. 4. Western blot detection of the degradation of α-actinin by MBSP in the absence (a) and presence (b) of STI. Myofibrillar proteins in the absence and presence of STI were incubated at 55 °C for different time intervals (lanes 1–9 corresponding to incubation time of 0, 2, 5, 10, 20, 30, 60, 120, 240 min, respectively). After incubation, samples were SDS-treated and applied to SDS–PAGE. Proteins were then electrophoretically transferred from SDS–PAGE to nitrocellulose membranes. Immunological detection was performed using a polyclonal antibody against α-actinin. Positions of prestained molecular weight standards are labeled on the left.

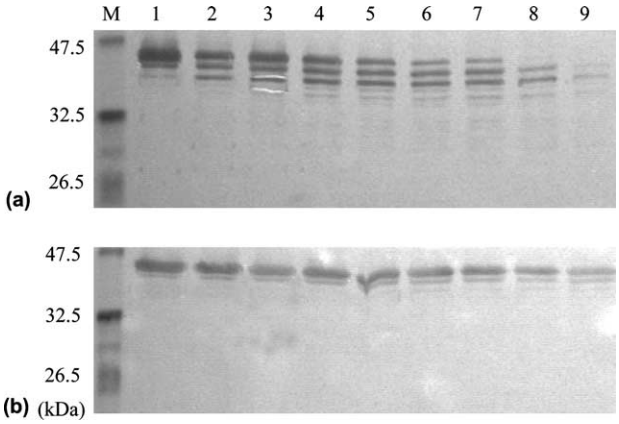

Fig. 5. Western blot detection of the degradation of actin by MBSP in the absence (a) and presence (b) of STI. Myofibrillar proteins were incubated and applied to electrophoresis and electrophoretically transferred from SDS–PAGE to nitrocellulose membranes as described in Fig. 4. Immunological detection was performed using a polyclonal antibody against actin. Positions of prestained molecular weight standards are labeled on the left.

6(a)). Actin was especially susceptible to degradation as its degradation products with molecular masses of 41 and 39 kDa could immediately be identified even in 2 min incubation, indicating actin was cleaved by the endogenous proteinase at specific sites, while its complete disappearance occurred after 4 h (Fig. 5(a)). The degradation of tropomyosin was also remarkable (Fig. 6(a)). At the beginning (2 and 5 min of incubation), no degradation could be detected, whereas at 20 min, nearly complete degradation to small peptides was identified as its degradation products could not be identified. In contrast, with the addition of STI (Figs. 4(b), 5(b) and 6(b)), degradation of all three myofibrillar proteins (α-actinin, actin and tropomyosin) was greatly suppressed even after 4 h of incubation. As group-specific inhibitor has characterized STI as a serine proteinase inhibitor, thus, the endogenous proteinase(s) responsible

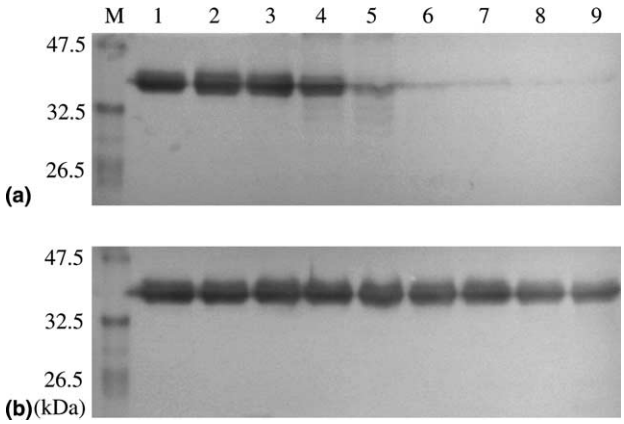

Fig. 6. Western blot detection of the degradation of tropomyosin by MBSP in the absence (a) and presence (b) of STI. Myofibrillar proteins were incubated and applied to electrophoresis and electrophoretically transferred from SDS–PAGE to nitrocellulose membranes as described in Fig. 4. Immunological detection was performed using a polyclonal antibody against tropomyosin. Positions of prestained molecular weight standards are labeled on the left.

for myofibrillar protein (MHC, α-actinin, actin and tropomyosin) degradation should be a serine proteinase. This result is consistent with our previous work that a myofibril-bound serine proteinase (MBSP) is mainly involved in the degradation of myofibrillar proteins (Cao et al., in press).

The present study only investigated the inhibitory effect of STI on myofibrillar proteins in the reaction system of myofibrils. The contribution of STI to the elasticity and quality of surimi is surely worth studying in further work. Though STI is a soy protein and soybean is a good protein source for feeding animals as well as humans, STI is regarded as an anti-nutritional factor (Osman et al., 2002). To improve the nutritional quality of soy foods, STIs are generally inactivated by heat treatment or eliminated by fractionation during food processing (Friedman & Brandon, 2001). It should also be emphasized that all of these adverse effects are seen when STIs are present in relatively high concentrations in the diet. In the present study, the final concentration of STI in myofibrils is 0.75 μg/ml, which is a low concentration compared with other soy foods. Considering the application of STI in surimi production, usually, boiling treatment is necessary in the last procedure, which will surely inactivate the activity of STI to some degree. Thus, the remaining adverse effects of STI in the final products of surimi, if any, should be negligible.

MBSP associates tightly with myofibrils and it could not be dissociated from myofibrils by addition of detergents such as Triton X-100 and other treatments, so we prepared the crude enzyme by heating myofibrils at 55 °C for 5 min. This treatment denatured some contaminating proteins while MBSP was thermal stable and released into the supernatant. Substrate specificity and

inhibitory characteristic analysis revealed that the crude enzyme is a trypsin-type serine proteinase, which is consistent with our previous result (Cao et al., in press). Interestingly, in the present study, we found that the enzymatic activity of MBSP toward a fluorescence substrate of Boc-Phe-Ser-Arg-MCA was more than 10-fold higher compared with the same enzyme we reported previously (Cao et al., in press). The degradation of myofibrillar proteins also confirmed this result as incubation of myofibrils at 55 °C for 5 min caused the apparent degradation of MHC. In our previous study, when the fish was caught later in February, similar degradation only happened after 30 min incubation. Why the same species of fish revealed significant enzymatic activity difference in three months is of much interest to us. This phenomenon is quite possibly that the activity of MBSP is seasonally variable.

The degradation action of MBSP on myofibrillar proteins strongly suggested its involvement in myofibrillar protein turnover in vivo. To further analyze the characteristics of this proteinase enzymatically and its complete protein sequence, we are now trying to isolate this proteinase to high purity and molecular cloning work of this enzyme is also on the way.

Acknowledgements

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