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ARTICLE

Mung Bean Trypsin Inhibitor Is Effective in Suppressing the Degradation of Myofibrillar Proteins in the Skeletal Muscle of Blue Scad (*Decapterus maruadsi*)

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Mung bean trypsin inhibitor (MBTI) of the Bowman−Birk family was purified to homogeneity with a molecular mass of approximately 9 kDa on tricine−sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and 8887.25 Da as determined by matrix-assisted laser desorption/ionization−quadrupole ion trap−time-of-flight mass spectrometry (MALDI−QIT−TOF MS). Using blue scad myofibrillar proteins as targets, it was found that, in the absence of MBTI, proteolysis of myofibrillar proteins, especially myosin heavy chain (MHC), could be identified after incubation at 55 °C for 2 h, while in the presence of MBTI, with a final concentration of 25 ng/mL, proteolysis of these proteins was greatly suppressed even after incubation for 3 h. Although cysteine proteinase inhibitor E-64 was also effective in preventing protein degradation, inhibitors for metallo- and asparatic proteinases did not reveal obvious inhibitory effects. Our present results strongly suggested that the naturally occurring legume bean seed protein MBTI can be used as an effective additive in preventing marine fish blue scad surimi gel softening, which is quite possibly caused by myofibril-bound serine proteinase (MBSP).

KEYWORDS: Blue scad; degradation; myofibril; mung bean trypsin inhibitor; serine proteinase

INTRODUCTION

Blue scad (*Decapterus maruadsi*) belongs to the family of mackerel that is a kind of popularly harvested marine fish in all tropical and temperate seas, and its production in China reached about 600 000 tons in 2008 (*1*). Among fishery processing products, surimi is much-appreciated in many Asian countries and is available in different shapes and textures. Because of the decrease of marine fish resources and increasing demand of surimi products, low-valued dark-fleshed fish, such as blue scad, become a potential raw material for use in surimi production. However, attention should be paid that the modori phenomenon (thermal gel degradation of fish jelly products at the temperature around 55 °C) occurs in the manufacturing process for most fish species, including marine fish from the family of mackerel (*2*, *3*). The occurrence of modori always accompanies the breakdown of myosin heavy chain (MHC) because it is essential for fish gel formation, and the degradation of MHC finally reduces the elasticity and the commercial value of fish jelly products (*4*). Many studies have indicated that the degradation of MHC in fish muscle is caused by endogenous proteinases, such as cathepsins B and L (*5*, *6*) or serine proteinases (*7*, *8*). Recently, myofibril-bound serine proteinases (MBSPs) are attracting much attention because of their myofibril-binding characteristics and effective degradation of MHC and other myofibrillar proteins, such as titin, nebulin, α-actinin, actin, and tropomyosin, at a neutral or slightly alkaline pH range around 55 °C to different degrees (*4*, *8*−*10*). MBSPs have been purified from not only freshwater fish common carp (*7*) and crucian carp (*11*) but also marine lizard fish (*8*). Its endogenous myofibril-bound serine proteinase inhibitor (MBSPI), which was actually glucose-6-phosphate isomerase, has also been elucidated (*12*, *13*). On the basis of previous research results, we proposed that MBSP is most likely the enzyme that is responsible for the modori phenomenon in fish, although the possibility of the involvement of cathepsin (cathepsins B and L) should not be excluded.

To make high-quality surimi products, proteinase inhibitors, especially food-additive-grade inhibitors, are demanding in protecting myofibrillar proteins from proteolysis by endogenous proteinase. Such inhibitors should be safe, effective, thermally stable, and of a reasonable cost. Legume seeds are rich sources for various kinds of proteinase inhibitors (*14*). Among serine proteinase inhibitors from legume seed, soybean trypsin inhibitor (STI) has been well-characterized, and the effect of Kunitz-type STI on crucian carp muscle protein degradation has been described in our previous work (*15*).

On the other hand, Bowman−Birk proteinase inhibitor (BBI), which consists of two homology regions on the same polypeptide chain, each with a reactive, inhibitory site, is of much interest to us because it is potentially more effective toward serine proteinases, while little information is available for its application in seafood processing. In the present study, mung bean trypsin inhibitor

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(MBTI) was purified to homogeneity and its inhibitory effect on protecting the proteolysis of blue scad myofibrillar proteins caused by MBSP was investigated with a purpose to use MBTI as an ingredient in surimi production.

MATERIALS AND METHODS

Materials. Fresh blue scad (*D. maruadsi*) (body weight, about 100 g; and length, 20 cm) was obtained in winter from a market in Jimei, Xiamen, China. After decapitation and evisceration, the fish was filleted, washed extensively with ice-cold water, and immediately used for experiments. Dried mung bean (*Phaseolus aureus* Roxb.) was purchased from a market in Jimei, Xiamen, China.

Lima bean trypsin inhibitor (LBTI) was from Worthington Biochemical Corporation (Lakewood, NJ). L-3-Carboxy-*trans*-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64) was a product of Amresco (Solon, OH). Pefabloc SC and pepstatin were purchased from Roche (Mannheim, Germany). Ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and protein standards for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were from Bio-Rad (Richmond, CA). Porcine trypsin was a product of Sigma (St. Louis, MO). Prestained protein marker for western blot was from New England BioLabs (Beverly, MA). Polyclonal antibody of rat anti-red sea bream α-actinin was a kind gift from Dr. Tachibana of the Faculty of Fisheries, Nagasaki University, Japan. Polyclonal antibodies of rat anti-carp actin and tropomyosin were prepared in our own laboratory. The secondary antibody of horseradish-peroxidase-labeled rabbit anti-rat IgG was from DAKO (Glostrup, Denmark). *t*-Butyloxycarbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and other synthetic fluorogenic peptide substrates (MCA substrates) were from Peptide Institute (Osaka, Japan). The enhanced chemiluminescent substrate for western blot was from Pierce (Rockford, IL). All other reagents were of analytical grade.

Preparation of Blue Scad Myofibrils and Protein Content Assay. Blue scad skeletal muscle was minced and homogenized with four volumes of ice-cold 50 mM phosphate buffer (pH 7.5) using a homogenizer (Kinematica, Lucerne, Switzerland). The homogenization process was performed on ice using a Polytron, PT-DA 2120 at the speed indicator of 15, and the operation was carried out 3 times with each time of 30 s and an interval of 1 min. The resulting homogenate was centrifuged at 5000*g* and 4 °C for 15 min. The supernatant was discarded, while the pellet was collected and resuspended in a 4-fold volume of ice-cold phosphate buffer. After 3 repeated cycles of homogenization and centrifugation, the resulting pellet was suspended in 50 mM phosphate buffer and further homogenized. Finally, after centrifugation at 8000*g* for 15 min, the pellet was resuspended in 50 mM phosphate buffer containing 0.5 M NaCl, and this suspension was regarded as myofibrils and immediately used for experiments. The protein concentration was determined by the method of Lowry et al. (*16*) after appropriate dilution of the myofibril with 50 mM phosphate buffer (pH 7.5) containing 0.5 M NaCl. Bovine serum albumin was used as the standard.

Purification of MBTI. Dried mung bean (*Vigna radiate*) was powdered in a grinder and soaked in 4-fold 0.1 M H_2SO_4 for 24 h and homogenized using a homogenizer. The mixture was then centrifuged at 12000*g* for 15 min. The resulting supernatant was neutralized with 1 M NaOH followed by heat treatment at 60 °C for 90 min. After centrifugation at 12000*g* for 10 min, the supernatant was ultrafiltrated using a Pellicon XL membrane (30 kDa) (Millipore, Billerica, MA), and the filtrate was collected, dialyzed against 20 mM Tris-HCl at pH 8.0, applied to a Q-Sepharose ion-exchange column, and washed with the dialysis buffer. Elution was carried out using a gradient of NaCl from 0 to 0.5 M. Trypsin inhibitory active fractions were obtained, and the purity of MBTI was checked by tricine−SDS−PAGE.

SDS−PAGE, Tricine−SDS−PAGE, and Western Blot. SDS−PAGE for myofibrillar protein analysis was performed under reducing conditions using 10 or 12% mini-slab gels according to the method of Laemmli (*17*). The gels were stained for protein with Coomassie Brilliant Blue (CBB) R-250. Tricine−SDS−PAGE for the MBTI purity assay was based on glycine-Tris and tricine-Tris buffer systems according to the method of Schägger (*18*), followed by silver staining. Western blot was carried out as described by Towbin et al. (*19*). Briefly, myofibrillar proteins on acrylamide gels were electrophoretically transferred onto a nitrocellulose membrane in transfer solution. Non-specific protein sites were blocked with 5% nonfat milk in Tris-HCl-buffered saline (TBS = 20 mM Tris-HCl at pH 7.5 containing 0.145 M NaCl). The blotted proteins were incubated with corresponding antibody at room temperature for 2 h and washed with TBS and 0.05% Tween-20 (TBST). After incubation for 1 h with horseradish-peroxidase-conjugated secondary antibody, the nitrocellulose membrane was washed extensively with TBST. Immunodetection was carried out using enhanced chemiluminescence (ECL) (Pierce).

Matrix-Assisted Laser Desorption/Ionization Quadrupole Ion Trap Time-of-Flight Mass Spectrometry (MALDI−QIT−TOF MS) Analysis. MS analysis of the purified MBTI was performed using MALDI−QIT−TOF MS, AXIMA Resonance (Shimadzu/Kratos, Kyoto, Japan). The instrument was operated in positive-ion mode using 2,5-dihydroxybenzoic acid (2,5-DHB) as the matrix.

Inhibition of Myofibrillar Protein Degradation by Different Types of Inhibitors. Fish muscle contains different types of proteinases with varying activity at different pH values. The pH of most fish surimi is at neutral or slightly alkaline pH because, during conventional processing, minced fish muscle should be rinsed with ice-cold water or low-concentration alkaline solution several times to remove sarcoplasmic proteinases and undesirable odors. To know which types of the endogenous proteinases that are mainly responsible for myofibrillar protein degradation under neutral or slightly alkaline pH conditions, it is necessary to investigate the inhibition effect of different kinds of proteinase inhibitors on the degradation of myofibrillar proteins. The experiment was carried out in 1.5 mL eppendorf tubes using 100 μL of myofibril (pH 7.5) mixed with different types of proteinase inhibitors (namely, serine, cysteine, asparatic, and metallo-) to their corresponding final concentrations, respectively. After preincubation at room temperature for 20 min to ensure the interaction between inhibitor and target proteinase, the mixture was then incubated at 55 °C for 2 h. After incubation, 25 μL of 4× SDS buffer was added to each tube and boiled for 5 min. Samples were then applied to SDS−PAGE to analyze the inhibitory effect of these inhibitors on MHC degradation.

Extraction of MBSP and Enzymatic Activity Assay. Extraction of MBSP from myofibril was performed according to lizard fish MBSP purification (*8*). Briefly, blue scad myofibrils prepared as described above were homogenized with 4-fold 20 mM Tris-HCl buffer at pH 8.0. The ice-cold homogenate was immediately heated in boiling water, while stirring until the central temperature reached 55 °C and moved to a water bath for further incubation at the same temperature for 5 min, followed by immediate cooling in ice water to 4 °C. After centrifugation, the supernatant was applied to a Q-Sepharose ion-exchange column (2.5 × 14 cm) and eluted with a linear gradient of NaCl from 0 to 0.5 M. Active fractions that were regarded as crude MBSP were pooled and used for enzymatic characterization. MBSP activity was determined using Boc-Phe-Ser-Arg-MCA as the substrate. Briefly, crude MBSP (50 μL) was added to 850 μL of 50 mM Tris-HCl buffer at pH 8.0. The reaction was initiated by the addition of 100 μL of 10 μM substrate and incubated at 55 °C for 30 min. The reaction was immediately terminated by the addition of 1.5 mL stopping agent (35:30:35 methyl alcohol/*n*-butyl alcohol/distilled water, v/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). The enzymatic activity assay was performed in duplicate, and variation between duplicate samples was always less than 10%. One unit of MBSP was to release 1 nmol of AMC/min. On the other hand, for the MBTI inhibitory activity assay, porcine trypsin was used as a target enzyme. One unit of porcine trypsin was defined as the release of 1 μmol of AMC by cleaving substrate Boc-Phe-Ser-Arg-MCA/min at 37 °C.

Analysis of pH and Thermal Stability of MBTI. For the pH stability assay, purified MBTI was preincubated in different buffers with pH ranging from 2.0 to 11.0 at room temperature for 30 min. The remaining inhibitory activity against crude MBSP was determined. The thermal stability of MBTI was examined by incubating purified MBTI at different temperatures (40, 50, 60, 70, 80, 90, and 100 °C) in sealed vials for 0.5, 1, and 2 h, respectively. After heating, the inhibitor solution was immediately chilled in ice water and the residual inhibitory activity against crude MBSP was determined. Control tests were investigated without incubating the

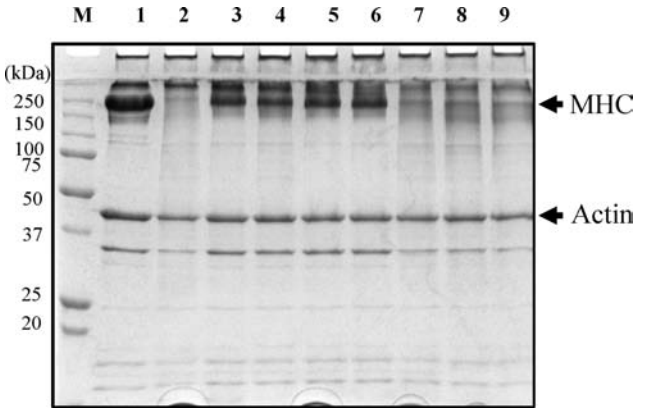

Figure 1. SDS−PAGE analysis of the inhibitory effect of proteinase inhibitors on the degradation of myofibrillar proteins. Blue scad myofibrils were preincubated at room temperature for 20 min with different kinds of inhibitors to correspond with the final concentration followed by incubation at 55 °C for 2 h. After incubation, samples were applied to a 10% gel. Lanes: M, molecular marker; 1, non-incubated control; 2, control (55 °C for 2 h); 3, MBTI (25 ng/mL); 4, LBTI (25 ng/mL); 5, Pefabloc SC (5 mM); 6, E-64 (0.1 mg/mL); 7, pepstatin (10 μg/mL); 8, EDTA (5 mM); and 9, EGTA (5 mM). Arrowheads indicate the positions of MHC and actin.

solution of MBTI. The inhibitory 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 proteinase activities without and with MBTI, respectively. A total of 1 unit of inhibitory activity was defined as the inhibition of 1 unit of porcine trypsin activity.

Inhibitory Effect of MBTI on Myofibrillar Protein Degradation. To investigate the inhibitory effect of MBTI on the degradation of myofibrillar proteins, purified MBTI was used. The experiment was performed using myofibril dissolved in 50 mM phosphate buffer at pH 7.5 containing 0.5 M NaCl in the presence of MBTI with a final concentration of 25 ng/mL. In the control test, phosphate buffer was used to replace MBTI. After preincubation at room temperature for 20 min to allow for well-mixing of MBTI with myofibrils, the mixture was incubated at 55 °C for different time intervals (0, 15, 30, 45, 60, 120, 180, and 240 min). After incubation, SDS buffer was added to samples and boiled in the presence of 5% 2-mercaptoethanol for 5 min, followed by SDS−PAGE or western blot. The inhibitory effect on MHC degradation was detected by SDS−PAGE, while that on the degradation of α-actinin, actin, and tropomyosin was investigated by western blot using specific polyclonal antibodies against these proteins.

Statistical Analysis. Results of enzymatic or inhibitory activity were obtained from three independent experiments performed in triplicate. The data were expressed as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Different kinds of proteinases exist in fish muscle. Purifications of cathepsin B and a novel cysteine proteinase in the skeletal muscle of mackerel (*Scomber australasicus*) have been described (*21*, *22*), and their proteolysis effect on actomyosin was investigated (*5*, *6*). An application of recombinant glycosylated cystatin in preventing roe-herring surimi gel weakening was studied (*3*). However, cathepsins B, D, and L are lysosomal proteinases and are optimally effective at pH values in the range of 3.0−6.5. For surimi production using dark muscle fish, such as mackerel, as raw material, a low concentration of alkaline (0.5% $NaHCO_3$ at pH 7.5) washing is always performed. Thus, we regarded that, except for cathepsins, the action of neutral or alkaline proteinases in the surimi of mackerel, including blue scad, should also be observed.

To know characteristics of the endogenous proteinase responsible for the degradation of blue scad myofibrillar proteins, different kinds of specific proteinase inhibitors were added to myofibrils to investigate the suppression effect on the hydrolytic activity. As shown in **Figure 1**, for the control sample, to which no proteinase inhibitor was added and incubated at 55 °C for 2 h, MHC was markedly degraded to lower molecular-weight products. The addition of serine proteinase inhibitors, such as MBTI, LBTI, and Pefabloc SC, suppressed the reduction of MHC completely, while pepstatin, an inhibitor for asparatic proteinases, EDTA and EGTA, inhibitors for metalloproteinases, did not show any inhibitory effect. Because the protein degradation investigation was performed using fish myofibrils, which had been rinsed with phosphate buffer (pH 7.5) and centrifuged several times, the remaining sarcoplasmic proteins, if any, are trivial. Furthermore, the pH value of the myofibril was 7.5, which nearly excluded the possible involvement of cathepsin L, which had an optimum pH of 5.5, although it also has myofibril binding characteristics and reveals an optimum temperature at 55 °C (*5*). Thus, it is reasonable to propose that the endogenous proteinase responsible for myofibrillar protein decomposition is quite possibly a MBSP.

It is noteworthy that E-64, a specific inhibitor for cysteine proteinases, revealed considerable inhibition toward MHC degradation (**Figure 1**). Generally, E-64 was regarded as an irreversible, potent, and highly selective cysteine proteinase inhibitor. However, Sreedharan et al. reported that E-64 was also one of the most effective low-molecular-weight inhibitors of trypsin-catalyzed hydrolysis (*23*). The inhibitory mechanism of E-64 on many cysteine proteinases is its *trans*-epoxysuccinyl group (active moiety), irreversibly binding to an active thiol group of the enzyme and forming a thioether linkage. MBSP is a trypsin-type serine proteinase; our previous study on common carp MBSP also revealed that its activity was partially inhibited by E-64 (*7*). We propose that the inhibitory effect of E-64 on MBSP was quite possible by the presence of critical thiol groups near its active site. In fact, the existence of two cysteine residues Cys192 and Cys202 around the active site Ser196 in crucian carp MBSP was recognized (*11*). The same results were also found in the MBSP from common carp (*Cyprinus carpio*), where two cysteine residues Cys193 and Cys203 around the active site Ser197 was identified (*24*). It is of interest to notice that both crucian and common carp MBSP mature proteins consist of 222 amino acid residues and share high identities (80.6%) with each other. Although none of the full-length amino acid sequence of MBSP from any marine fish has been determined, on the basis of their similar enzymatic characteristics, it is rational to propose that MBSP from blue scad may have a similar primary structure as MBSPs from crucian and common carp.

To further characterize the endogenous proteinase, the proteinase was extracted from myofibril by a heating treatment, because this process denatured most myofibrillar proteins, and the enzyme was partially released. The proteinase was partially purified by passing through a Q-Sepharose column. Because of the low content and the myofibril-binding characteristics, purification of this proteinase to a high degree could not be reached at the present stage. Similar to MBSPs from common carp (*7*), lizard fish (*8*), and crucian carp (*11*), substrate specificity analysis showed that the enzyme selectively cleaved at the C terminal of arginine and lysine residues, while no cleavage activity was identified toward the substrate of Suc-Leu-Leu-Val-Tyr-MCA, a substrate of chymotrypsin (**Table 1**). Thus, it is reasonable to conclude that the endogenous proteinase is a trypsin-like MBSP.

Increasing attention has been paid to proteinase inhibitors from various sources during the past few decades. Endogenous proteinase inhibitors are important tools for regulating the proteolytic

Table 1. Substrate Specificity of Crude MBSP toward Synthetic MCA Substrates[a]

substrates	relative activity (%)
Boc-Phe-Ser-Arg-MCA	100.0
Boc-Glu-Arg-Arg-MCA	412.7 ± 3.9
Boc-Gln-Arg-Arg-MCA	241.2 ± 1.8
Boc-Leu-Lys-Arg-MCA	167.5 ± 1.4
Boc-Leu-Arg-Arg-MCA	115.6 ± 2.8
Boc-Val-Arg-Arg-MCA	50.4 ± 1.4
Boc-Val-Leu-Lys-MCA	24.6 ± 1.6
Boc-Glu-Lys-Lys-MCA	9.5 ± 0.7
Suc-Leu-Leu-Val-Tyr-MCA	0

[a] Enzymatic activity was determined at pH 8.0 and 55 °C. Each value was the result of three independent assays.

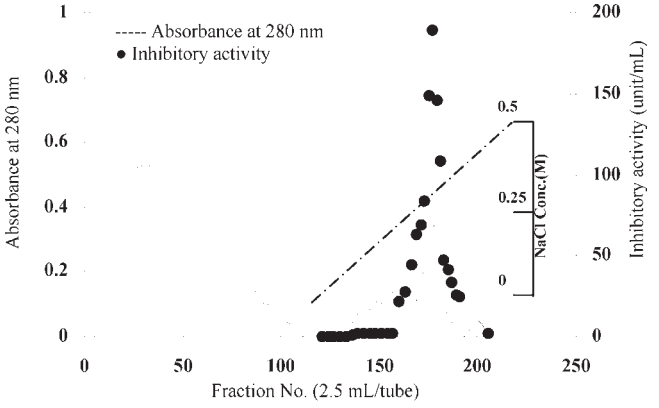

Figure 2. Purification of MBTI by Q-Sepharose chromatography. Absorbance at 280 nm (- · -) and MBTI inhibitory activity (●).

activity of their target proteinases by suppressing these in emergency cases or for signaling receptor interactions or clearance (*20*). Our previous studies have indicated that endogenous inhibitors to MBSP are actually glucose-6-phosphate isomerases with molecular masses of approximately 55 kDa in monomer form (*12*) and 110 kDa in dimer form (*13*). However, the application of glucose-6-phosphate isomerase in fish surimi production is still inappropriate because isolation of this protein from the sarcoplasmic fraction is cost-consuming and loses activity quickly at the temperature above 55 °C. Therefore, in the present study, we tried to find an inhibitor to MBSP that should be thermally stable and effective to the target enzyme and can be used as a food additive. We focused this on naturally occurring substances, especially in plants. MBTI was purified to homogeneity by heating treatment, membrane filtration, and Q-Sepharose chromatography (**Figure 2**). The purity of MBTI was checked by tricine−SDS−PAGE, and the molecular mass was about 9 kDa as estimated by tricine−SDS−PAGE (**Figure 3A**) and 8887.25 Da by MALDI−QIT−TOF MS (**Figure 3B**). MBTI is a well-characterized serine proteinase inhibitor, and the molecular mass result in the present study agreed well with MBTI (8883.0 Da) as reported by Qi et al. (*25*). The inhibitory effect of MBTI against porcine trypsin and MBSP was revealed in **Figure 4**. Both porcine trypsin and MBSP activity against synthetic substrate Boc-Phe-Ser-Arg-MCA sharply decreased with the increasing addition of MBTI, and their inhibitory patterns were similar. As a result, from 100 g (dry weight) of mung bean, approximately 12 mg of highly purified MBTI was obtained. Considering the fact that, in surimi production, as a food additive, the purity of MBTI does not need to be homogeneous. The yield of MBTI can be increased significantly. The pH stability revealed that MBTI is quite stable in the pH range of 2−11 (**Figure 5A**). More recently, a 15 kDa Bowman−Birk-type trypsin inhibitor (VFTI-E1) was purified from

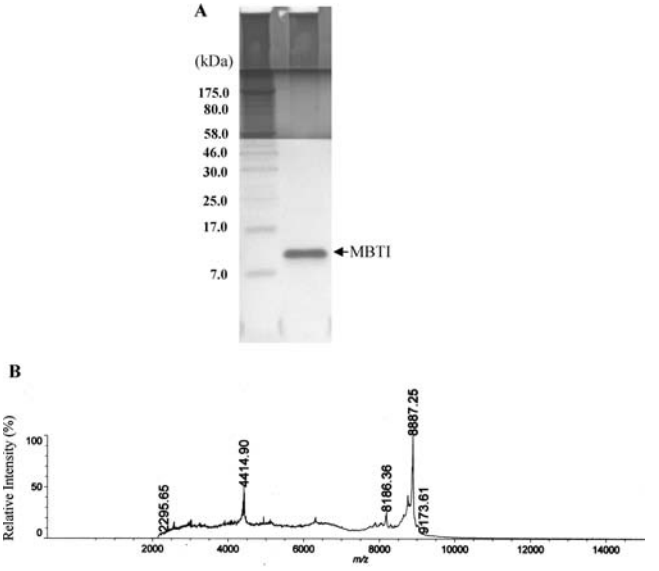

Figure 3. (A) Tricine−SDS−PAGE and (B) MALDI−TOF MS profile of purified MBTI. (A) Positions of molecular-weight standards are labeled on the left. The gel was silver-stained.

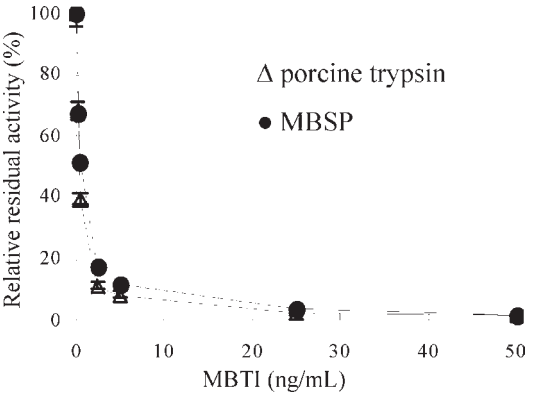

Figure 4. Inhibitory effect of MBTI on porcine trypsin and crude MBSP. Porcine trypsin (0.01 units) and crude MBSP in 20 mM Tris-HCl at pH 8.0 were preincubated with different concentrations of MBTI at room temperature for 30 min. The residual enzymatic activity toward Boc-Phe-Ser-Arg-MCA was determined. Samples without MBTI were used as the control. Each point is the result of three independent assays.

fava beans (*Vicia faba* cv. Egypt 1), which remained approximately 70% of its initial activity at pH 2 and 12, while optimal at pH 8.0 (*26*). MBTI is also a highly thermal-stable protein because more than 85 and 55% of its initial inhibitory activity remained after heat treatment at 100 °C for 1 and 2 h, respectively (**Figure 5B**). In comparison to STI (*15*) and VFTI-E1 from fava beans (*26*), which remained about 55% of its initial inhibitory activity after pretreatment at 100 °C for 30 min, MBTI is much more thermally stable.

The study on protein degradation revealed that the reduction of myofibrillar proteins, especially MHC, by the endogenous proteinase is time-dependent and MBTI is effective in suppressing such degradation. As shown in **Figure 6A**, the MHC band decreased proportionally with the incubation time at 55 °C and its original band nearly completely disappeared after 4 h, suggesting that the endogenous proteinase MBSP is also a thermal-stable enzyme. However, in the presence of MBTI with a final concentration of 25 ng/mL, such degradation was nearly completely suppressed (**Figure 6B**).

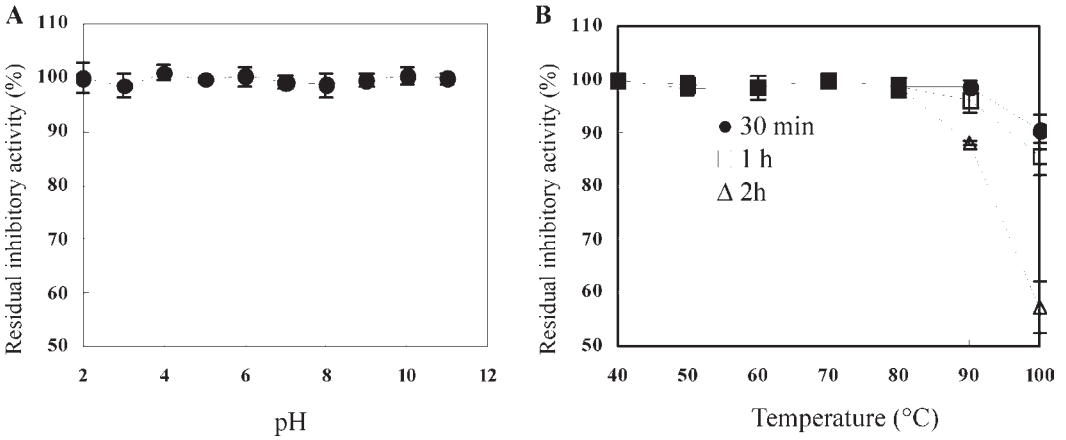

Figure 5. (A) pH and (B) thermal stability of purified MBTI. (A) Purified MBTI was preincubated in different buffers with pH ranging from 2.0 to 11.0 at room temperature for 20 min. The remaining inhibitory activity against crude MBSP was determined in 20 mM Tris-HCl buffer (pH 8.0). (B) Purified MBTI (25 ng/mL) in 20 mM Tris-HCl buffer at pH 8.0 was preincubated at different temperatures (ranging from 40 to 100 °C) for 30 min (●), 1 h (□), and 2 h (△). The remaining inhibitory activity against crude MBSP was determined. MBTI without heating was used as the control. Each point is the result of three independent assays.

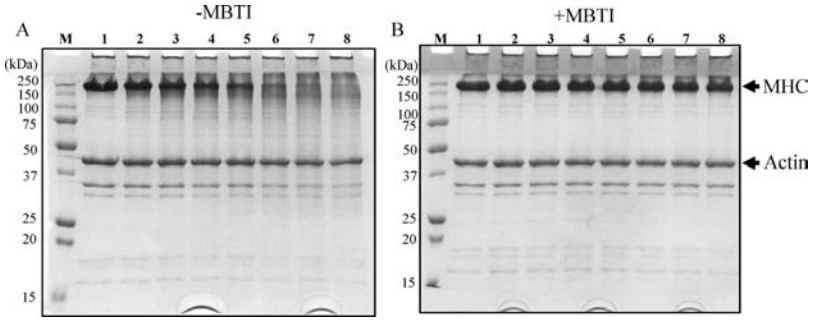

Figure 6. Degradation of myofibrillar proteins in the absence and presence of MBTI. Myofibrillar proteins were incubated at 55 °C in the (A) absence and (B) presence of MBTI (25 ng/mL) for different time intervals. After incubation, samples were applied to SDS−PAGE. MHC, myosin heavy chain; M, protein marker. Lanes 1−8 correspond to incubation times of 0, 15, 30, 45, 60, 120, 180, and 240 min, respectively.

Myofibrils predominantly consist of MHC (200 kDa), actin (42 kDa), and other minor proteins, such as α-actinin (100 kDa) and tropomyosin (34 kDa). Degradation of myofibrillar proteins not only happens on MHC, the breakdown of other larger molecular-mass proteins, such as titin (2000−3500 kDa) and nebulin (650−850 kDa), by MBSP was also observed in our laboratory (*9*). Because the degradation products of these higher molecular-mass proteins may interfere with the interpretation of the breakdown of lower molecular-mass myofibrillar proteins, it is necessary to establish a specific method to investigate the degradation of α-actinin, actin, and tropomyosin. In the present study, western blot using specific antibodies against these proteins was used. In comparison to the dramatic degradation of MHC, after 2 h of incubation, α-actinin and actin revealed negligible reduction, only showed slight decomposition, and even prolonged the incubation to 4 h, while that of tropomyosin was also obvious (**Figure 7**). This cleavage pattern was quite similar to that caused by MBSP in marine lizard fish (*7*) and different from that in freshwater fish, such as common carp (*4*) and crucian carp (*10*), suggesting that MBSPs from marine fish may have more similar enzymatic characteristics. The present results indicated that rod-shaped proteins MHC and tropomyosin are more easily attacked by MBSP than other myofibrillar proteins and MBTI is effective in suppressing such degradation. Because the temperature range of 55−60 °C is a range regarded as modori-causing temperature and MHC is the major component in myofibril and provides gel-forming ability in the preparation of fish jelly products (*20*, *21*), this result strongly suggested that the endogenous proteinase MBSP is the enzyme responsible for modori.

The degradation of myofibrillar proteins during surimi production has been investigated extensively, and many methods have been proposed to prevent its occurrence. Because plant-source BBIs, such as STI and MBTI, have a highly stable structure of disulfide bridges, they can be resistant to cooking temperatures and even survive in the digestive system of animals (*27*). More recently, new functions, such as anti-inflammatory and chemopreventive properties, of STI and MBTI within the gastrointestinal tract have also been reported (*28*). The study revealed that MBTI, even in its truncated active core form composing only 16 residues, is an effective inhibitor toward trypsin. Thus, MBTI and its mutants may be used as potential pharmaceutical agents for the prevention of oncogenesis (*25*).

Because of their high thermal stability and inhibitory activity toward digestive proteinases trypsin and chymotrypsin, STI and MBTI were also regarded as anti-nutritional factors (*29*). At a low concentration, however, they can be used as food additives, especially in surimi, to achieve the purpose of preventing myofibrillar protein degradation caused by serine proteinases. In our previous study, STI was added to a final concentration of 750 ng/mL to suppress myofibrillar protein degradation in crucian carp (*15*), while in the present study, 25 ng/mL is enough to obtain the same result. Although MBSP activity in crucian carp is relatively higher than that in marine fish blue scad, MBTI is still more effective than STI in suppressing the activity of MBSP, especially after heat

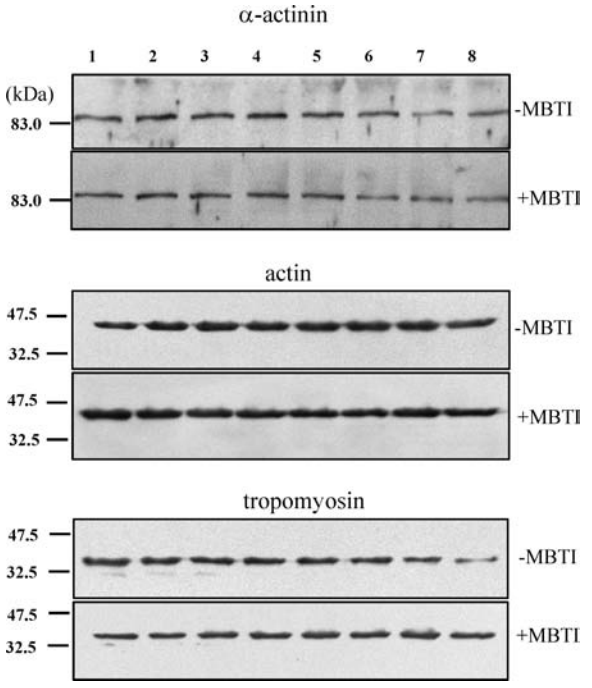

Figure 7. Western blot detection of the time-course degradation of α-actinin, actin, and tropomyosin in the absence and presence of MBTI. Myofibrillar proteins was incubated at 55 °C in the absence and presence of MBTI (25 ng/mL) for different times. After incubation, samples were SDS-treated and applied to SDS−PAGE. Proteins were then electrophoretically transferred to nitrocellulose membranes. Immunological detection was performed using polyclonal antibodies against α-actinin, actin, and tropomyosin. Lanes 1−8 correspond to incubation times of 0, 15, 30, 45, 60, 120, 180, and 240 min, respectively. Positions of prestained molecular-weight standards are labeled on the left.

treatment. Previously, Ramirrez et al. reported that the reduction of the MHC band in surimi prepared from Atlantic croaker and Mexican flounder could be effectively inhibited by the addition of serine proteinase inhibitors from legume seeds (*14*). All of these results strongly suggested that plant-source proteinase inhibitors can be used as additives to prevent protein degradation caused by endogenous MBSPs. The application of MBTI in blue scad surimi production is also carrying out in our collaboration laboratory, and the results will be reported elsewhere.

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