Myofibril-Bound Serine Proteinase (MBP) and its Degradation of Myofibrillar Proteins

M.-J. Cao, K. Hara, K. Osatomi, K. Tachibana, T. Izumi, and T. Ishihara

ABSTRACT

Proteolysis of a myofibril-bound serine proteinase (MBP) from carp *Cyprinus carpio* on myofibrillar proteins and their gel formation ability were investigated. MBP readily decomposed myosin heavy chain as indicated by SDS-PAGE. In the preparation of kamaboko, the gel formation ability was diminished by addition of MBP. The optimum degradation temperatures of MBP to myosin heavy chain in myofibril and kamaboko gel were 55°C and 60°C, respectively. The degradation effects of MBP on actin, α-actinin and tropomyosin were studied by the immunoblotting method. Because of its myofibril-bound and myofibrillar protein degradation characteristics, MBP was regarded as the proteinase most probably involved in the modori effect.

Key Words: antibody, gel formation, immunoblotting, myofibril, serine proteinase

INTRODUCTION

WITH THE FAST DEVELOPMENT OF CULture technology, the harvest of freshwater fish is increasing. Kamaboko preparation has also been utilized in the processing of freshwater fish. Similar to marine fish, the modori effect (thermal gel degradation of fish jelly products) occurred in the production of freshwater fish products (Iwata et al., 1974, 1977; Shimizu et al., 1981). Much research has been conducted to elucidate the cause of the modori effect, and many hypotheses have been proposed to explain it. The heat denaturation of muscle protein (Niwa et al., 1975) and the action of a gel degradation-inducing factor (Iwata et al., 1977; Toyohara et al., 1990a) were considered. The multicatalytic proteinase was also assumed as involved (Makinodan et al., 1985, 1987). It is now regarded as the participation of an endogenous serine proteinase(s) (Kinoshita et al., 1990; Toyohara et al., 1990b; Osatomi et al., 1997). The responsible serine proteinase(s) was supposed to exert its action by degrading the myosin heavy chain (MHC), which was known as the major constituent in myofibril and provided gel forming ability in the preparation of the fish jelly products (Toyohara et al., 1988; Cheng et al., 1979).

In the research of such modori-related serine proteinases, Shimizu et al. (1983, 1986) first reported myosin-associated modori-inducing factors in croaker (*Nibea mitsukurii*) and oval-filefish (*Navodon modestus*). Toyohara et al. (1990b) further described the existence of a proteinase(s), closely associated with myofibril in oval-filefish and presumed it to be a serine proteinase. Therefore, it became evident that both sarcoplasmic serine proteinase (SSP) (Kinoshita et al. 1990) and myofibrillar serine proteinase (MSP) (Toyohara et al., 1990b) were responsible for the modori effect. The MSP is regarded as much more important because extensive washing is generally performed in the production of fish jelly products, and SSP would be mostly eluted out in this process.

In order to elucidate the characteristics of such modori-related serine proteinases in the hydrolysis of myofibrillar proteins, it is necessary to obtain the enzymes in their homogeneous states. As compared to SSP, the purification of myofibril-bound serine proteinases had not been complete. It may be due to the scarcity or the difficulty in solubilizing such enzymes from myofibrils. A myofibril-bound serine proteinase (MBP) from the ordinary muscle of carp has been purified to homogenity (Osatomi et al., 1997). The objective of this study was to further elucidate the actions of MBP on a fish jelly product and myofibrillar proteins.

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MATERIALS & METHODS

Materials

Cultured carp *Cyprinus carpio* (≈1.0 kg) was obtained from a commercial supplier, transported to the laboratory within 1 h, decapitated and eviscerated. Ordinary muscle was collected, stored at −35°C and used for MBP purification. The carp muscle utilized for myofibril preparation and fish jelly manufacturing was collected from fresh fish.

Butyloxycarbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Ultrogel AcA 54 and Arginine-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Casein and gelatin were obtained from Sigma (St. Louis, MO). Electrophoresis calibration kits used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Richmond, CA). All other chemicals were reagent grade.

Enzyme purification, activity assay and preparation of antibodies

The purification procedure and activity assay of MBP were carried out as described (Osatomi et al., 1997). In brief, minced carp ordinary muscle was washed with 25 mM phosphate buffer, pH 7.5 and centrifuged repeatedly four times in order to eliminate fat and sarcoplasmic proteins. MBP was separated from myofibril at pH 4.0 in the presence of 0.5 M KCl. After dialysis against distilled water overnight, the crude enzyme solution was lyophilized and consequently dissolved in a minimum volume of distilled water. Following Ultrogel AcA54 gel filtration, MBP was purified by Arginine-Sepharose 4B affinity chromatography in the presence of 0.5 M KCl. The activity of MBP was determined using Boc-Phe-Ser-Arg-MCA as a substrate at 55°C for 10 min. One unit of enzyme activity was defined as the amount of activity that released 1 nmol of 7-amino-4-methylcoumarin (AMC) per min. Polyclonal antibodies against red sea bream *Pagrus major* actin and carp tropomyosin were raised in rats. Polyclonal antibody against red sea bream α-actinin was raised in a rabbit.

Protein concentration assay and myofibril preparation

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Myofibrils were prepared from carp dorsal lateral muscle according to Tsuchiya et al. (1975) with a slight modification.

Preparation of fish jelly product in the presence or absence of MBP

Minced and three-time washed carp meat (250 g) only was first ground for 10 min in a meat grinder (Ishikawa type 18, Tokyo, Ja-

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pan). NaCl was added to make a final concentration of 3% and the mixture was further ground for 10 min at 4°C. Crude MBP solution (extracted from 1.5 kg of ordinary carp muscle) was evenly added during the additional 10 min grind. The final water content of the meat paste was adjusted to 81%. In the control sample, water was added in place of crude MBP. The ground meat paste (30 g each) was stuffed into stainless steel tubes (dia, 32 mm; ht, 30 mm; thickness, 1 mm). The tubes were wrapped with Saran film, then immersed in water baths of different temperatures (40°C, 50°C, 55°C, 60°C, 70°C, and 80°C) for 1 h. After incubation, the tubes were further heated at 90°C for 20 min to inactivate the enzyme and immediately cooled in ice-water for 30 min. Gel strength was estimated following storage in a cold room of 4°C for 12 h.

Assessment of gel strength

The gel strength was estimated by measuring breaking strength (g) and deformation (cm). Each sample was evaluated with an SD-305 type rheometer (SunScience, Tokyo, Japan) equipped with a spherical plunger (dia, 5 mm) at a table speed of 0.6 mm/s. Mean scores of 6 points in each test gel were obtained. Jelly strength was expressed as the multiplication of breaking strength by deformation (g × cm).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed under reducing conditions according to the method of Laemmli (1970) and stained for protein with Coomassie Brilliant Blue R-250. Immunoblotting was carried out as described (Towbin et al., 1979). Briefly, myofibrillar proteins were subjected to SDS-PAGE and transferred electrophoretically to clear blot membrane-p (ATTO, Tokyo, Japan). Nonspecific sites were blocked with 3% (w/v) gelatin (Sigma) in Tris-HCl buffered saline (TBS=20 mM Tris-HCl, pH 7.5, 0.5M NaCl). The membrane was incubated overnight at 37°C using a 1:150 (v/v) dilution of primary antiserum. Bound antiserum was washed with TTBS (TBS, 0.1% Tween-20). After incubation for 1 h with goat anti-rabbit IgG coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden), the membrane was further washed extensively with TTBS. Immunodetection was carried out following the detection procedure of Amersham and stained with Konica immunostaining HRP-1000 kit (Konica, Tokyo, Japan).

Protein extraction from fish jelly for SDS-PAGE

After the evaluation of jelly strength, a small portion of fish gel was obtained and minced. To a precisely weighed 0.2 g sample was added 4.0 mL sample solution (20 mM Tris-HCl, pH 8.0, containing 5% SDS, 8 M urea and 5% 2-mercaptoethanol). The mixture was homogenized in 30 s period four times at medium speed using a physcotron (NITI-ON, Chiba, Japan). The homogenate was boiled 5 min and mixed with 10 μL of 2% bromophenol blue (BPB) followed by SDS-PAGE analyses.

Hydrolysis of myofibrillar proteins by MBP at various temperatures

Myofibril solution was prepared by dissolving carp myofibril in 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. Reaction mixtures consisting of 150 μL myofibril solution (2 mg protein) and 50 μL of purified MBP (0.1 unit) were incubated at the following temperatures for 1 h: 40°C, 50°C, 55°C, 60°C, 70°C and 80°C. Control test was performed by incubating the myofibril solution at 55°C for 1 h using water in place of MBP. Reactions were terminated by adding 1.0 mL of SDS buffer [0.1M Tris-HCl, pH 6.8, containing 3% SDS, and 50% (v/v) glycerol], the mixtures were then boiled for 5 min in the presence of 5% 2-mercaptoethanol. SDS-PAGEs were all carried out on 7.5–15% gradient gels.

RESULTS & DISCUSSION

Thermal stabilities of MBP at 37°C and 55°C

The thermal stabilities of the purified enzyme at 37°C and 55°C were compared (Fig. 1). MBP was stable at 37°C, the remaining activity of over 89% was retained even when it was preincubated in 50 mM borate buffer (pH 8.0) at that temperature for 3 h. MBP lost 40% of its initial activity in 10 min during preincubation in 50 mM borate buffer (pH 8.0) at its optimum temperature, 55°C. However, in the presence of 2% casein (pH 8.0), the thermal stability increased markedly. MBP retained 56% of its initial activity even preincubated at 55°C for 3 h. This indicated that MBP was a thermostable serine protease when it coexisted with protein(s) at some concentration ranges.

Effect of MBP on fish gel strength

As shown (Fig. 2), although the gel strength of carp meat was lower than that of most marine fish, the slight modori effect around 60°C in the control test was observed. This suggested the existence of a modori-related proteinase. In the presence of crude MBP, the gel strength further decreased as compared with the control, especially around 60°C, relatively corresponding to the optimum temperature of MBP (55°C). When the incubation temperature was >70°C, no difference between gel strengths in the presence or absence of MBP could be detected, which could be ascribed to the heat inactivation of MBP.

In the practical manufacture of kamaboko, modori-related proteinase acted in the presence of other proteins. In our study, instead of purified MBP, lyophilized crude enzyme dissolved in a minimum of water was added to 250 g minced meat for investigation of gel degradation. Considering the activity loss and the incomplete extraction of MBP from myofibril, 1.5 kg ordinary muscle was used for enzyme isolation. The strength of the gel only decreased 13% and 16.7% with the addition of crude MBP even at 55°C and 60°C, respectively (Fig. 2). Two possibilities may be hypothesized. One was that MBP could not act as actively in jelly state as in the solvent state, so its gel degradation exertion was suppressed. The other was that compared with endogeneous myofibril-bound enzyme, extraneously added MBP was in a non-binding state. Its binding ability with myofibrillar proteins was consequently weaker, and eventually affected its hydrolytic ability. SDS-PAGE analysis of the degradation of MHC also revealed that MHC was not so obviously degraded at 55°C and 60°C compared with the control (Fig. 3), confirming the alteration of gel strengths.

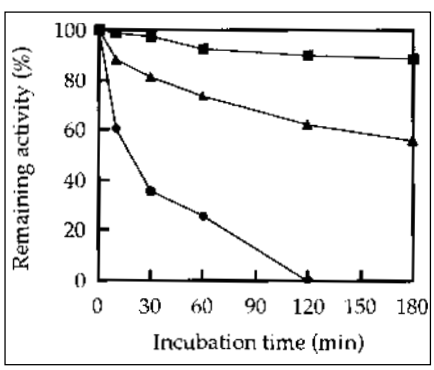

Fig. 1—Thermal stability of MBP at two temperatures. Purified MBP was preincubated at 37°C(■) or 55°C(●) in 50 mM borate buffer (pH 8.0) and at 55°C in the presence of 2% casein (pH 8.0)(▲) for different time intervals, and the remaining activity was determined.

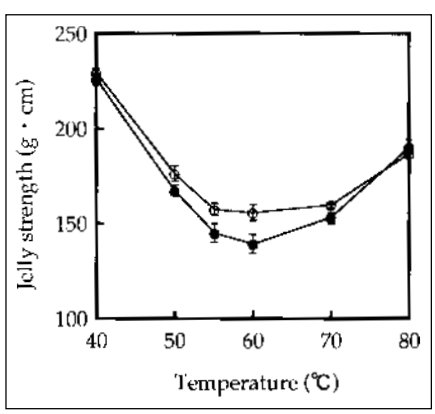

Fig. 2—Changes of gel strength at different temperatures in the presence or absence of crude MBP. With MBP (●); without MBP (○).

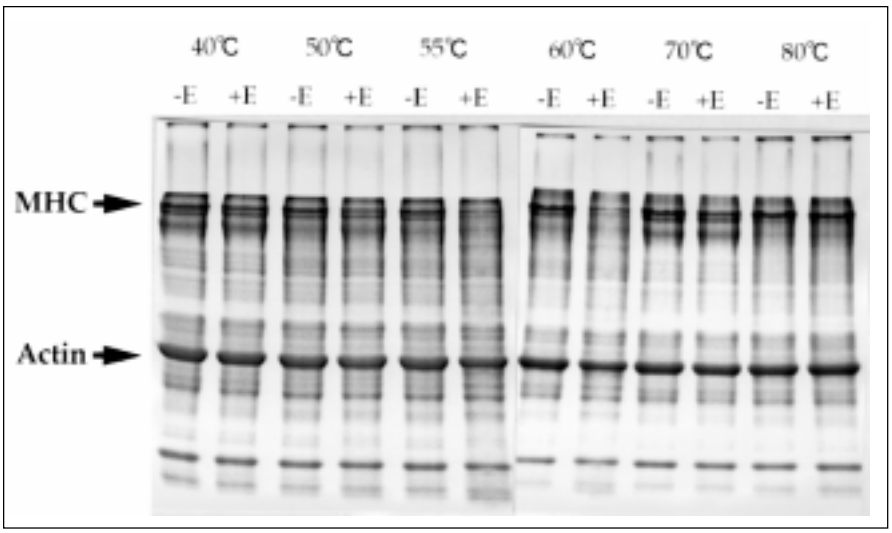

Fig. 3—SDS-PAGE analysis of myofibrillar proteins in fish gels in the presence or absence of MBP at different temperatures. Fish gels were incubated with crude MBP (+E) or without MBP (–E) at different temperatures. Samples (15 μg proteins/lane) were applied to a 7.5-15% gradient gel. MHC, myosin heavy chain.

The modori effect in fresh water fish was different among species, carp was relatively resistant to modori (Shimizu et al., 1981) (Fig. 2). We presumed that it should be ascribed to the scarcity of such modori-related proteinase (MBP) in carp muscle. Actually, only 75 μg of purified MBP was obtained starting from 3 kg of ordinary muscle (Osatomi et al., 1997).

Degradation of myofibrillar proteins by purified MBP at different temperatures

Myosin heavy chain. Myofibril prepared from carp muscle mainly contained myosin heavy chain and actin with tropomyosin, α-actinin, troponin T and myosin light chain as minor constituents. The degradation of myofibril by MBP was investigated by SDS-PAGE (Fig. 4). Myosin heavy chain, the main constituent contributing to gel strength of kamaboko was most rapidly hydrolyzed. Its original band completely disappeared in 1 h at 55°C. This implied that MBP was most probably the serine protease contributing to the modori effect. In the control, myofibril was heated at 55°C for 1 h and a slight degradation of MHC was identified. No degradation, however, was found when the diisopropyl fluorophosphate (DFP)-treated myosin was incubated at the same temperature even for 4 h (Osatomi et al., 1997), suggesting the scarce existence of a myofibril-bound serine proteinase (MBP) in myofibril.

Actin. Actin was regarded as a proteolytically resistant protein compared with MHC and β-tropomyosin/troponin-T (Cheng et al., 1979; An et al., 1994). In the hydrolysis of myofibril by MBP (Fig. 4), actin was also found to be degraded to some degree. After 1 h incubation at 55°C and 60°C, the original actin band became thinner, and several new protein bands appeared below that of actin. As myosin heavy chain was hydrolyzed and more large molecular weight proteins, such as neblin (Labeit et al., 1991) and connectin (Maruyama et al., 1987), were also probably degraded at the same time, it was difficult to ascertain whether these new bands were degraded from actin. To clarify the positions of the degraded actin products, immunoblotting was performed. Actin was partially hydrolyzed into three main protein bands (Fig. 5) with molecular masses of 41 kDa, 38 kDa, and 34 kDa, respectively. Further degradation progressed very slowly (data not shown)

α-actinin. As a minor constituent in myofibril, the degradation of α-actinin whether in autolysis or in the modori effect has been rarely studied. α-Actinin of white croaker was not degraded at all by an endogeneous trypsin-like serine protease (Busconi et al., 1989). However, the immunoblotting result (Fig. 6) in our study showed that the original α-actinin band was degraded to a trace amount after incubation with purified MBP at 55°C for 1 h. This indicated that α-actinin could also be cleaved by MBP at its optimum temperature. The degraded products from α-actinin were not detected.

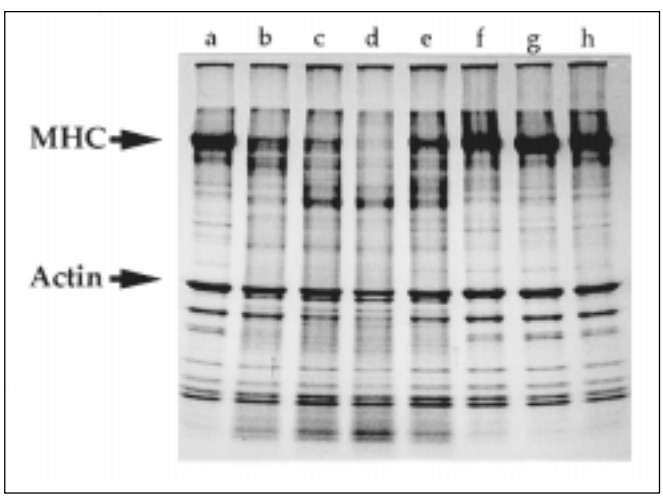

Fig. 4—Degradation of myofibrillar proteins at different temperatures. Myofibril dissolved in 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl was incubated with purified MBP at different temperatures for 1 h. After incubation, SDS buffer was added into the reaction mixtures and boiled for 5 min, samples (17μg proteins/lane) were applied to a 7.5–15% gradient gel. Lane a, 0 h; b, 40°C; c, 50°C; d, 55°C; e, 60°C; f, 70°C; g, 80°C; h, 55°C control (without MBP).

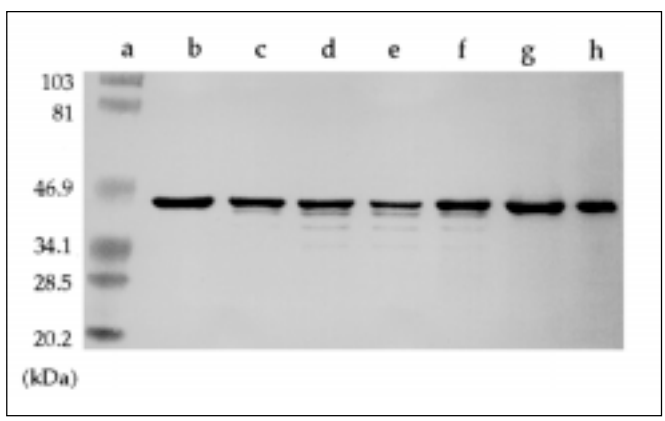

Fig. 5—Immunoblotting of the degradation of actin. Myofibril was first incubated with purified MBP for 1 h as described in Fig. 4, and then applied (lane b to h, 17 μg proteins/lane) to a 7.5–15% gradient gel. After electrophoresis, the gel was transferred to a clear blot membrane-p and immunoblotted with anti-red sea bream actin antibody. Lane a, prestained marker; b, 0 h; c, 40°C; d, 50°C; e, 55°C; f, 60°C; g, 70°C; h, 55°C control (without MBP).

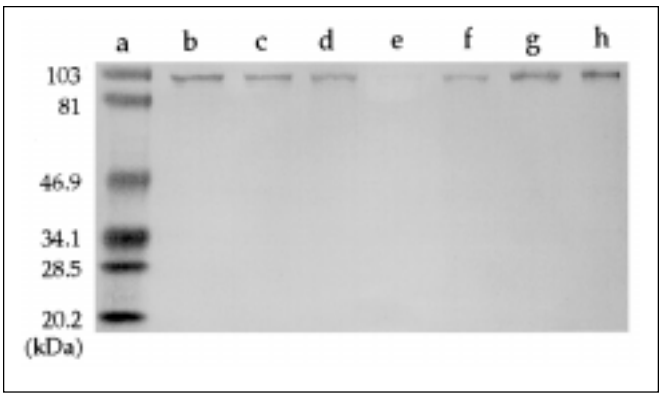

Fig. 6—Immunoblotting of the degradation of α-actinin. Myofibril was first incubated with purified MBP for 1 h as described in Fig. 4, and then applied (lane b to h, 17 μg proteins/lane) to a 7.5–15% gradient gel. After electrophoresis, the gel was transferred to a clear blot membrane-p and immunoblotted with anti-red sea bream α-actinin antibody. Lane a, prestained marker; b, 0 h; c, 40°C; d, 50°C; e, 55°C; f, 60°C; g, 70°C; h, 55°C control (without MBP).

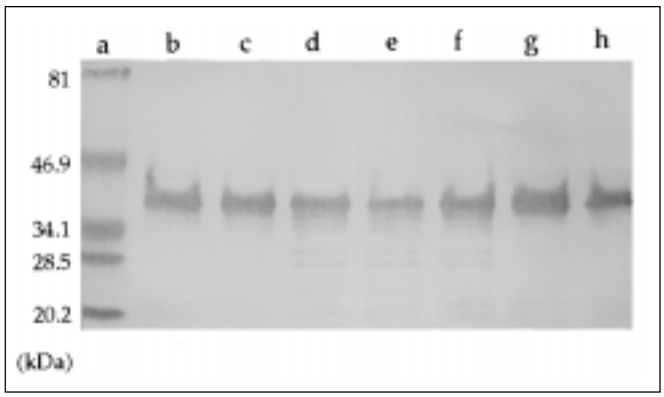

Fig. 7—Immunoblotting of the degradation of tropomyosin. Myofibril was first incubated with purified MBP for 1 h as described in Fig. 4, and then applied (lane b to h, 17 μg proteins/lane) to a 7.5–15% gradient gel. After electrophoresis, the gel was transferred to a clear blot membrane-p and immunoblotted with anti-carp tropomyosin antibody. Lane a, prestained marker; b, 0 h; c, 40°C; d, 50°C; e, 55°C; f, 60°C; g, 70°C; h, 55°C control (without MBP).

Though the antibodies of actin and α-actinin were raised against proteins purified from red sea bream, these two proteins are known to be well-conserved ones in the evolution of gene families (Elzinga et al., 1973; Shah et al., 1982; Beggs et al., 1992; Parr et al., 1992). So antibodies raised against actin and α-actinin of red sea bream could be applied to carp. We found that immunoblotting was an effective way for detection of proteins which could not be clearly identified in SDS-PAGE as was the case with α-actinin.

Tropomyosin. The immunoblotting result of the degradation of tropomyosin by MBP (Fig. 7) showed tropomyosin was also hydrolyzed by MBP at 50–60°C. The tropomyosin band could not be clearly identified when myofibril was treated with the sample solution, instead, SDS solution was used. The reason for that, however, was unclear.

CONCLUSIONS

THE GEL STRENGTH OF FISH JELLY PRODuct of carp muscle further decreased at around 55–60°C with the addition of MBP. The myosin heavy chain (MHC) in myofibril was nearly completely degraded by MBP at 55°C. Actin, α-actinin and tropomyosin were also hydrolyzed by MBP to different degrees. These facts indicated that MBP was the most probable proteinase, which caused the modori effect.

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