

**Determining the effect of malondialdehyde on the IgE-binding capacity
of shrimp tropomyosin upon *in vitro* digestion**

Running title: malondialdehyde modulate the IgE-binding Capacity of
tropomyosin *in vitro* digestion

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ABSTRACT

BACKGROUND: Stability in simulated gastric fluids is considered an important
parameter for the estimation of food allergenicity. Moreover, proteins in food are highly

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susceptible to lipid oxidation during processing and preservation. In this study, the change in the IgE-binding capacity of malondialdehyde (MDA)-treated shrimp tropomyosin (TM) following *in vitro* digestion was investigated by SDS-PAGE and Western blot.

RESULTS: Shrimp TM treated with different concentrations of MDA was slightly degraded and became increasingly resistant to pepsin digestion over time. While untreated TM was rapidly degraded, MDA-treated TM showed some resistance and was degraded by trypsin only after increasing the digestion time. Results of immunoblotting studies on IgE using sera from patients allergic to shrimp indicated that the IgE-binding capacity of TM and MDA (50 mM)-treated TM decreased slightly after pepsin digestion and significantly decreased after trypsin digestion.

CONCLUSION: The study indicated that the resistance of TM to degradation increased after oxidation. The treatment with proteases, especially trypsin, is quite effective in decreasing the IgG/IgE-binding capacity of shrimp TM.

Keywords: Shrimp; Simulated gastric fluid; Simulated intestinal fluid; Digestibility; Tropomyosin; Western blot

INTRODUCTION

Shrimp is one of the most popular seafood worldwide, known for its high nutritional value and deliciousness. It provides essential amino acids required to meet nutritional

needs and has a high content of unsaturated fatty acids that is beneficial for health.¹

Tropomyosin (TM) is one of the most allergenic proteins found in the shrimp. Due to such high allergenic protein, TM is known to affect the health of many children and adults worldwide.^{2,3}

To trigger allergic reactions, the integrity of the epitopes on the allergen must remain intact in the gastrointestinal tract. *In vitro* assessments of digestion have been carried out only with isolated allergens so far.⁴ However, allergic individuals are seldom exposed to pure allergens. Indeed, the stability of a protein during digestion could be altered by the presence of other components in the food matrix.

It has been shown that food proteins are an important target for reactive oxygen species and other peroxides.^{5,6,7} The effect of natural oxidation on the functional properties of food proteins, such as water holding ability, gelation, and digestibility, processing has attracted a significant amount of attention. Sante-Lhoutellier⁸ reported a 46%-decrease in the rate of proteolysis of oxidized myofibrillar proteins compared to the control. Furthermore, oxidation enhances the hydrophobicity by exposing hydrophobic amino acids that are buried in the interior of the native protein.⁹ It generates a charge obstacle for the mobility of proteases (pepsin and trypsin) over the protein substrate in the reaction mixture. Additional protective effects of oxidation against the *in vitro* digestion of proteins by pepsin and trypsin during food preservation have been also reported.^{10,11}

Due to exist unsaturated lipids, the shrimp is susceptible to oxidation under non-enzymatic (i.e., oxygen) and enzymatic (i.e., lipoxygenase) conditions.^{12,13} Lipid peroxidation could generate free radicals such as reactive aldehydes and hydroperoxides

that interact with proteins and cause oxidative damage. The association of oxidized shrimp proteins with lipids reduces protein solubility, gel-forming ability, and other functional properties.^{14,15} These changes may be partly attributed to the reaction of a major lipid peroxidative product, malondialdehyde (MDA), with the proteins. However, due to the complexity of lipid peroxidation and the large number of by-products, it is easy to identify the end product. In order to better understand the influence of lipid peroxidation, it is essential to know the mechanism of interaction between the major by-products of lipid peroxidation and the proteins and to understand the impact of these interactions on the functional properties of proteins.¹⁶ So far, there have been no reports on the digestibility of oxidized shrimp TM, its effects on the IgE-binding capacity of these digests, or its association with allergenicity. The main aim of our present study to study the influence of the interactions between shrimp TM and MDA on the digestion and the potential allergenicity of TM. These results will be useful for evaluating and minimizing the risk of allergies associated with processed foods containing shrimp ingredients.

EXPERIMENTAL

Materials and reagents

Shrimp (*Metapenaeus ensis*) were purchased alive from the local fish market in The protein standards for SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) were obtained from Solarbio Co. (Beijing, China). Rabbit polyclonal antibodies against shrimp TM were prepared in Beijing Protein Innovation (Beijing, China). Horseradish peroxidase-labeled goat anti-rabbit IgG and goat anti-human IgE

were also used in the study (from Zhongshan Jinqiao Co., Beijing, China). The 1,1,3,3-tetraethoxypropane (MDA precursor), trypsin, and porcine pepsin were procured from Sigma (St. Louis, MO). All the reagents used in the experiments were of analytical grade.

Human sera

The Affiliated Hospital of Medical College of Qingdao University (Qingdao, China) provided sera from patients allergic to shrimp, which were kept at -80°C . All the patients had well-documented clinical records of shrimp allergy. The levels of IgE specific to the allergen were greater than 0.5 kU/L, as established by an allergy screen (MEDIWISS Analytic GmbH, Moers, Germany). Signed written consent for the use of the sera was obtained from all the patients. The serum collection protocol used was approved by the medical ethics committee (File No, QD-PF-20160816551).

Purification of shrimp TM

Shrimp TM purification was performed according to a previously reported protocol with some modifications.¹⁷ Briefly, shrimp muscle was blended and extracted with 2 of a solution containing 20 mM KCl (pH 7.5), 1 M KHCO_3 , and 0.1 M dithiothreitol. resultant homogenate was centrifuged five times at 4000 xg for approximately 10 min each. The extract was then discarded, and the shrimp homogenate was extracted in 1 L 95% ethanol for 10 minutes and extracted in 1 L of diethyl ether for 10 minutes in triplicate, respectively. Later, the extract was discarded and the shrimp homogenate was dried for 2 hours in a ventilation cabinet and extracted by incubating overnight with 5

volumes of solution containing 1M KCl and 0.01 M NaN₃ (pH 7.5), at 4 °C. The protein was then salted out with precisely 30% (NH₄)₂SO₄. The precipitate was collected and again dissolved in 30% (NH₄)₂SO₄ to remove the residues of the earlier centrifugation. Finally, the solution was heated in a boiling water bath for 10 min in order to remove impurities, and was immediately cooled on ice, followed by a 20-min centrifugation at 8000 xg. The supernatant containing the purified TM was collected and stored at -80 °C prior to use.

MDA treatment of shrimp TM

MDA was obtained by the hydrolysis of 1,1,3,3-tetraethoxypropane in 0.1 M HCl (10 ml) for 40 min at 40 °C (pH 7.4).¹⁸ The MDA concentration was determined by estimating the UV absorbance at 267 nm. It was adjusted to 200 mM via the addition of muriatic acid solution. Based on previous research work on protein modification with MDA, the concentration of MDA arranged from micromolar per litre to millimolar per litre.^[18,19] In this work, MDA-treated TM was prepared by incubating TM with various concentrations (0.0, 0.01, 0.1, 1.0, 10, and 50 mM) of MDA in the dark at 25 °C for 24 h. The final TM concentration in the mixture was 1 mg/ml. Subsequently, the mixture was dialyzed against a phosphate buffer solution at 4 °C for 48 h to remove excess MDA. Following dialysis, the modified TM was freeze-dried and stored at -80 °C prior

Simulated gastric fluid (SGF) digestion stability assay

The digestibility of MDA-treated TM was determined according to the protocol described by Huang et al.²⁰ with minor modifications. The SGF was prepared as described in the United States Pharmacopoeia,²¹ and was composed of porcine pepsin

(275 U/mg) in 25 mM NaCl with pH 1.2. The volume of the reaction solution was made to 1 mL. For the digestion stability assay, a centrifuge tube (2 mL) containing SGF was preheated at 37 °C prior to the addition of the test protein. For the final digestion, a ratio of 0.38 U of pepsin/ μ g protein was selected for all tests (1:100 pepsin to protein, w:w) at 8 different time points (0, 1, 2, 5, 10, 15, 30, and 60 min). The digestion was carried out at 37 °C under continuous shaking and was stopped by adding 30 μ l of 200 mM Na₂CO₃. The TM sample for the 0-min time point was added to SGF and immediately neutralized with Na₂CO₃. For the control, a TM sample was mixed with SGF without pepsin and was incubated at 37 °C for 1 h. All the samples were boiled for 7 min and then analyzed by both SDS–PAGE and Western blot. All the experiments were performed in triplicate.

Simulated intestinal fluid (SIF) digestion stability assay

The SIF for intestinal digestion was also prepared according to the United States Pharmacopoeia²¹ and was composed of trypsin in 0.05 M KH₂PO₄ with pH 7.5. For the digestion stability assay, a centrifuge tube (2 mL) containing SIF was preheated at 37 °C prior to the addition of the test protein. For the final digestion, a ratio of 0.42 U of trypsin/ μ g protein was selected for all tests (1:100 trypsin to protein, w:w) at 8 different time points (0, 1, 15, 30, 60, 120, 180, and 240 min). The digestion process was carried out at 37 °C under continuous shaking and was terminated by heating at 95 °C for 5 min. The TM sample for the 0-min time point was added to the SIF and immediately heated at 95 °C. For the control, a TM sample was mixed with SIF without trypsin. All

the samples were then analyzed by SDS–PAGE and western blot. The experiment was performed in triplicate.

SDS–PAGE and western blot analyses

The protein samples were analyzed by SDS-PAGE, as described by Laemmli.²² All the samples (~6 µg of protein) were mixed with a loading buffer, heat-denatured, and loaded into an SDS-PAGE gel composed of a 5% stacking gel and a 15% running gel. A precision protein standard (14.4-116.0 kDa) was loaded in the first well of the gel as a molecular weight marker. The samples were run through the stacking gel at 80 V and through the resolving gel at 120 V and then visualized with the Coomassie Brilliant Blue R-250 dye in a Tanon-4200SF imaging system (Tanon Science and Technology Co., Shanghai, China).

The IgE/IgG binding potential of the digested TM samples was evaluated using western blot according to the protocol described by Chen et al²³ with some modifications. Briefly, protein samples separated by SDS–PAGE were electrophoretically transferred onto a polyvinylidene fluoride membrane. After determining the transfer efficiency by Ponceau S staining, the membrane was incubated at 37 °C for 2 h in a blocking buffer (5% non-fat dry milk in PBST) to block non-specific binding sites. The membrane was subsequently incubated with rabbit serum (1:5000 v/v dilution in blocking buffer) (Beijing Protein Innovation, Beijing, China) or human serum (1:5 v/v dilution in blocking buffer) for 90 min, and then with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:5000 v/v dilution in blocking buffer) or with HRP-labeled goat anti-human IgE (1:200 v/v dilution in blocking buffer) for 120 min, respectively. The

signal obtained with enhanced chemiluminescence was visualized with a Tanon-4200SF imaging system. The intensity of the bands represents the specific IgG- or IgE-binding capacity of the TM sample.

RESULTS

Digestion of MDA-treated TM by SGF (pepsin)

We previously reported the SDS-PAGE analysis of TM treated with MDA.¹⁹ In this study, we performed SDS-PAGE analysis of SGF-digested, MDA-treated shrimp TM (Fig. 1). TM treated with different concentrations of MDA was only slightly degraded and was resistant to pepsin digestion. Although new cross-linked protein bands with molecular masses of approximately 72-110 kDa were observed when the MDA concentration used was more than 10 mM, the same proteolytic fragments (approximately 22 kDa) were present and the samples showed resistance to digestion even at higher digestion periods (Fig. 1 A-F). Moreover, the degradation was not complete at the end of the reaction, and a large part of the TM samples remained intact.

Digestion of MDA-treated TM by SIF (trypsin)

The stability of TM in SIF was different from that in SGF. When treated with trypsin, the digestion patterns varied based on the concentration of MDA. More number of degraded fragments appeared during the process (Fig. 2). Several proteolytic fragments (~34, 22, 20, and 17 kDa) were detected after a short time of digestion. With increasing digestion time, the number of fragments with higher molecular masses (34 kDa) decreased, while the number of digestion-resistant fragments (~22, 20 kDa) gradually increased (Fig. 2 A-F). When MDA concentration was increased to more than 10 mM

(Fig. 2 E - F), the density of the original TM band (36 kDa) reduced with increasing MDA concentration, and the proteolytic fragments began appearing after digestion for 60 min (Fig. 2 E and F). In addition, a very faint original TM band was still detectable after 4 h of digestion (Fig. 2).

Western blot analysis

The information about proteolytic fragments from MDA-treated TM and pure TM that obtained from SDS-PAGE was limited. Therefore, we used western blot analysis to analyze the fragments for their ability to bind rabbit polyclonal antibodies (IgG) or human serum antibodies (IgE) derived from patients allergic to shrimp, at different time points during simulated gastrointestinal digestion. As shown in Fig. 3 A and B, the intensity of the band corresponding to TM-IgG binding decreased slightly with increasing SGF digestion time. In contrast, during SIF digestion, both MDA (50 mM)-treated TM and pure TM showed degradation in their IgG binding; the immune activity gradually weakened as enzymatic degradation progressed (Fig. 4 A and B). Indeed, we observed that TM aggregates formed after treatment with 50 mM MDA were resistant to trypsin digestion.

The effect of protease (pepsin and trypsin) digestion of MDA-treated TM and pure TM on the IgE-binding capacity was evaluated by IgE immunoblotting (Fig. 3 C and D; Fig. 4 C and D). Smaller TM fragments (23 and 20 kDa) generated after both SGF and SIF digestion showed no IgE reactivity compared to the control and the TM treated with 50 mM MDA. The ability of TM to bind to IgE decreased with increasing digestion time.

DISCUSSION

A significant amount of research has been conducted in recent years in an effort to eliminate allergens from seafood, particularly crustaceans.²⁴ TM is a major allergen in crustaceans, and has been extensively studied.²⁵ However, less attention has been given to the simulated digestion of TM. Therefore, in the present study, we investigated the digestibility of MDA-treated and pure TM by SIF and SGF. Based on the time factor that ingested food normally remains in the human digestive system, the digestion time for SGF and SIF was assigned to 1 and 4 h, respectively. The results indicated that the digestive patterns of TM samples by pepsin and trypsin were very different (Fig. 1 and 2). This difference resulted from the difference in specificity of cleavage of the two proteases. Pepsin cleaves at Tyr or Phe residues and peptide bonds,²⁶ while trypsin shows a higher specificity for cleaving at Lys and Arg residues (hydrophilic amino acids) at the P1 position.²⁷ Pepsin in SGF is known to hydrolyze aromatic amino acids. Aromatic amino acids on the protein surface are also susceptible to oxidation. Both pure and MDA-treated TM samples were slightly degraded and showed similar degrees of resistance to digestion by SGF. A possible reason for this is that the available digestion sites were not modified or masked by cross-linked protein aggregation. Sante et al.²⁸ observed a similar phenomenon during the digestion of muscle fiber proteins after hydroxyl radical oxidation. In this study, they speculated that aggregation might have happened at locations different from the digestion sites on the protein, thereby conferring no resistance to the gastric enzyme.

Trypsin can specifically cleave proteins at Lys and Arg residues. Song²³ showed that treatment with MDA reduced the lysine content of TM, indicating that lysine is

modified during the oxidation process. When peroxide concentration increased above a threshold value, the digestion sites were destroyed. It was observed that the degradation of TM by SIF was clearly delayed. Hence, such modifications might be responsible for the resistance of TM to trypsin digestion. Another possibility is that TM polymers expose fewer lysine molecules on the protein surface and thus decrease the rate of hydrolysis.

The primary structure of shrimp TM contains several cleavage sites for pepsin and trypsin.²⁹ The results of this study indicated that trypsin cleaved TM in a random manner. It is possible that TM was attacked more readily by trypsin than by pepsin due to its tertiary structure. It is worth mentioning that the results of TM degradation obtained from SDS-PAGE (Fig. 1 and 2) were consistent with those obtained from western blot, which is a more sensitive and specific technique (Fig. 3 and 4). In the present study, we were able to detect shrimp TM, both untreated and MDA-treated, and its proteolytic fragments using a polyclonal antibody (IgG) as well as pooled human serum antibodies (IgE).

Treatment of TM with 50 mM MDA appeared to cause protein aggregation. The band intensity for the untreated TM sample slightly decreased with increasing digestion time compared to those for the control and MDA-treated TM samples, suggesting that oxidation by MDA significantly altered the immunogenic epitopes on the surface of TM. The immunogenic activity of TM found to be slightly decreased during digestion with SGF. The digestion sites in the protein were either modified or hidden in the

protein interior, while some epitopes were modified or amino acids that do not comprise pepsin digestion sites were exposed on the protein surface.

TM was resistant to digestion by trypsin after treatment with 50 mM MDA and its IgG-binding capacity was reduced as well. Trypsin probably damaged the lysine molecules that were exposed on the protein surface following digestion. The smaller (~23 and 20 kDa) TM proteolytic fragments generated by SGF and SIF digestion showed no binding to IgE, compared to the control and MDA-treated TM. One reason for this might be that the binding epitopes for IgE in these fragments were obliterated after the digestion. Since the IgE-binding capacity of the various digested samples decreased after treatment with proteases, we proposed that protease treatment is suitable for the elimination of IgE-binding sites on TM.

However, it is important to note that the IgE-binding capacity of TM might not be sufficient to explain its allergenicity. Its allergenicity might depend on its participation in divalent interactions that cause cross-linking of IgE antibodies on basophil or mast cell surfaces. However, in the case of partially digested proteins, some of the fragments contained only one IgE-binding site, which was capable of binding IgE, but not of releasing mediators from basophils or mast cells. Therefore, additional tests such as skin prick tests and histamine release from basophils should be carried out to further assess the alteration in the allergenicity of TM following protease treatment.

CONCLUSIONS

Stability in simulated gastric fluids is an important parameter for the estimation of food allergenicity. The disappearance of epitopes after gastrointestinal digestion might better explain the lower IgE-binding capacity observed. It can be concluded that the resistance of TM to degradation increased after oxidation. The treatment with proteases, especially trypsin, is quite effective in decreasing the IgG/IgE-binding capacity of shrimp TM.

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The authors declare no conflicts of interest.

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Figure 1

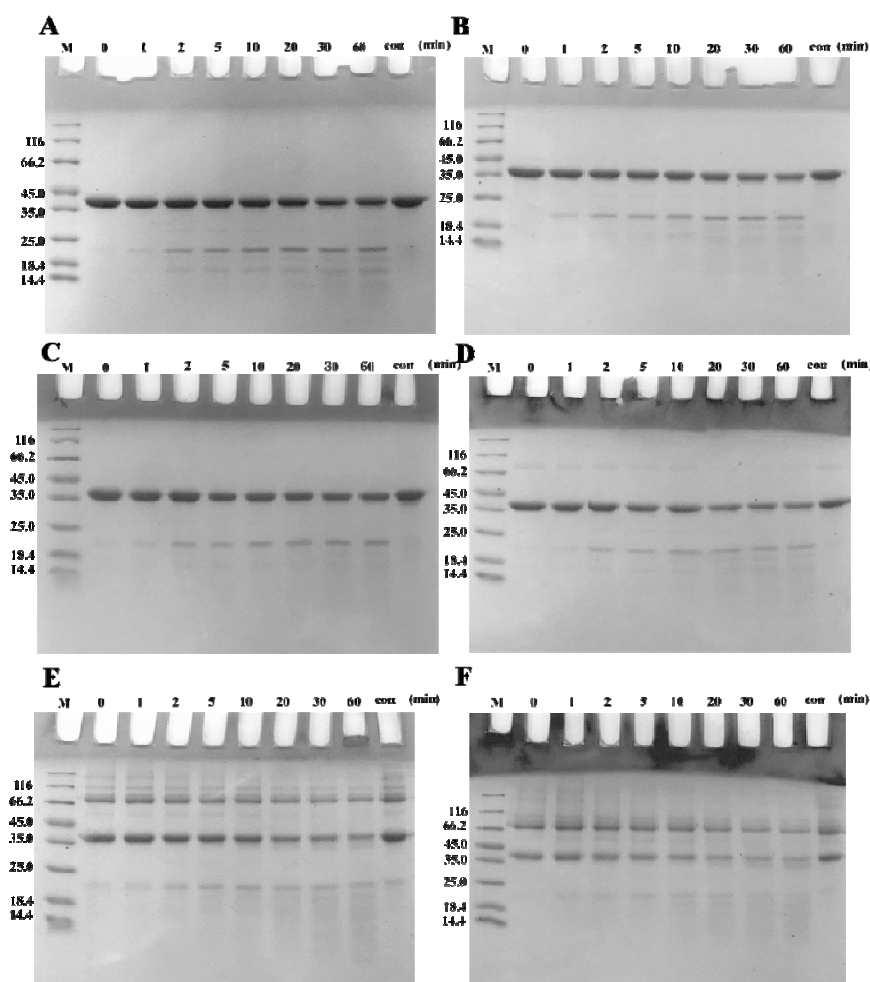


Fig. 1. Effect of simulated intestinal fluid (pepsin) digestion on the shrimp tropomyosin (TM) treated with malondialdehyde (MDA) at different concentrations. The shrimp TM samples were untreated (A); treated with 0.01 mM MDA (B); treated with 0.1 mM MDA (C); treated with 1.0 mM MDA (D); treated with 10 mM MDA (E); and treated with 50 mM MDA (F). Enzymatic digestion was performed, followed by SDS-PAGE.

After electrophoresis, proteins were visualized by staining with Coomassie Brilliant

Blue dye. In the controls (con), proteinase was replaced with 25 mM PBS (pH 7.5).

Molecular masses of the protein markers (M) are shown on the left edges.

Figure 2

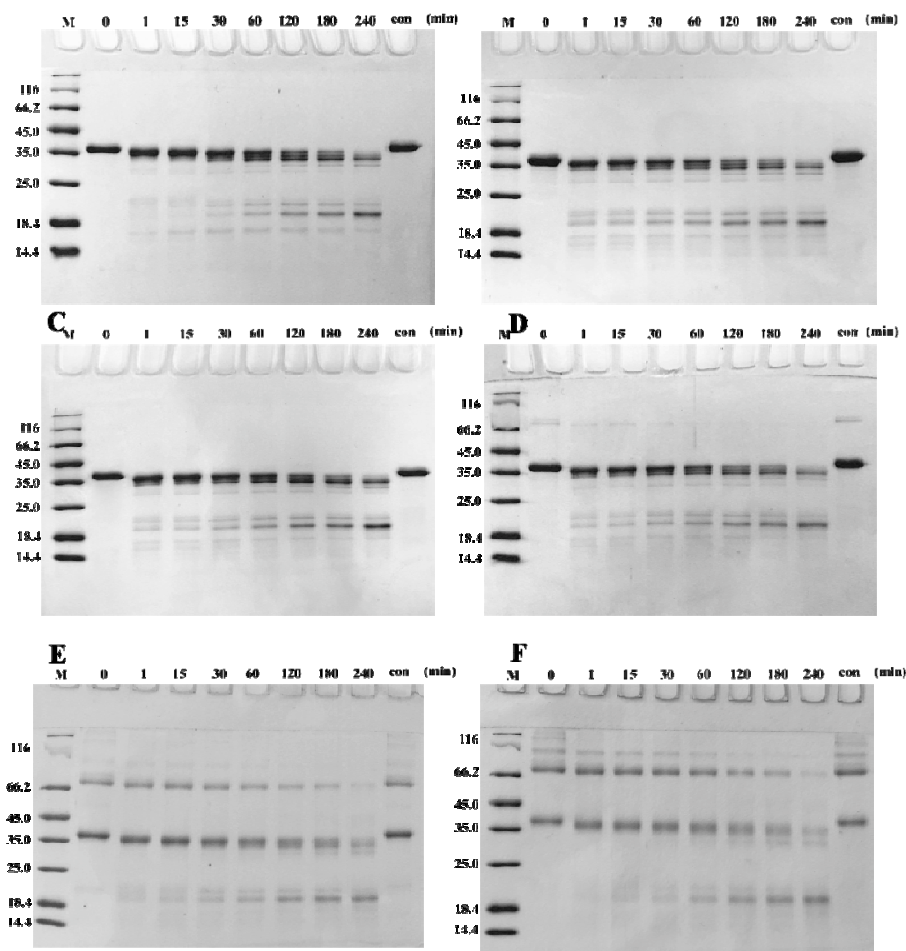


Fig. 2. Effect of simulated intestinal fluid (trypsin) digestion on the shrimp tropomyosin (TM) treated with malondialdehyde (MDA) at different concentrations. The shrimp TM were untreated (A); treated with 0.01 mM MDA (B); treated with 0.1 mM MDA (C); treated with 1.0 mM MDA (D); and treated with 10 mM MDA (E). Enzymatic digestion was performed, followed by SDS-PAGE. After electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue dye. In the controls (con),

proteinase was replaced with 25 mM PBS (pH 7.5). Molecular masses of the protein markers (M) are shown on the left edges.

Figure 3

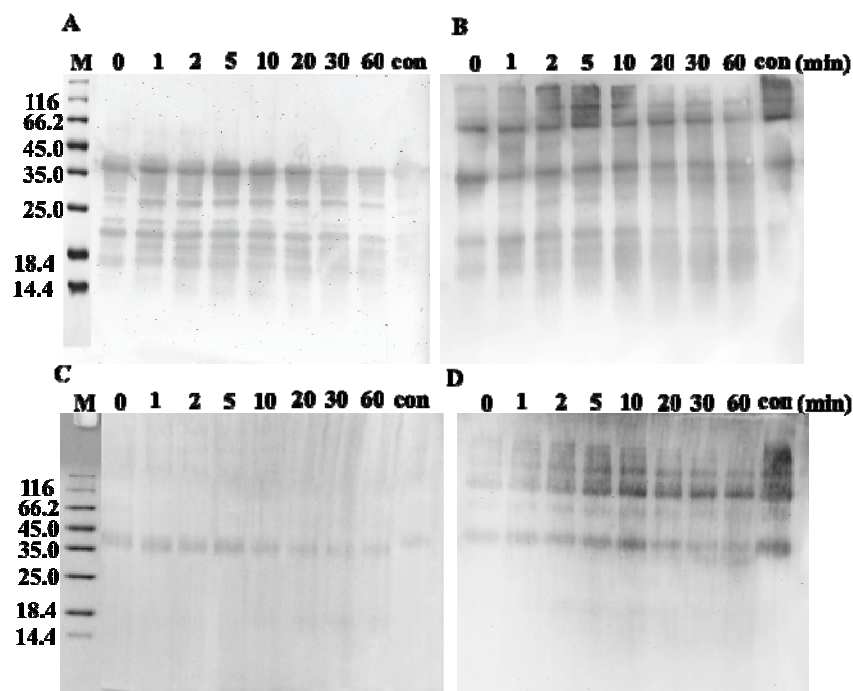


Fig. 3. Western blot analyses of the degradation of the purified shrimp tropomyosin (TM) and 50 mM malondialdehyde (MDA) treated TM in simulated gastric fluid (pepsin). Samples were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The purified shrimp TM (A) and 50 mM MDA-treated TM (B) were detected using polyclonal antisera against shrimp TM. The purified shrimp TM (C) and 50 mM MDA-treated TM (D) were detected using pooled sera from patients allergic to shrimp. Positions of molecular mass standards (M) are labeled on the left edges.

Figure 4

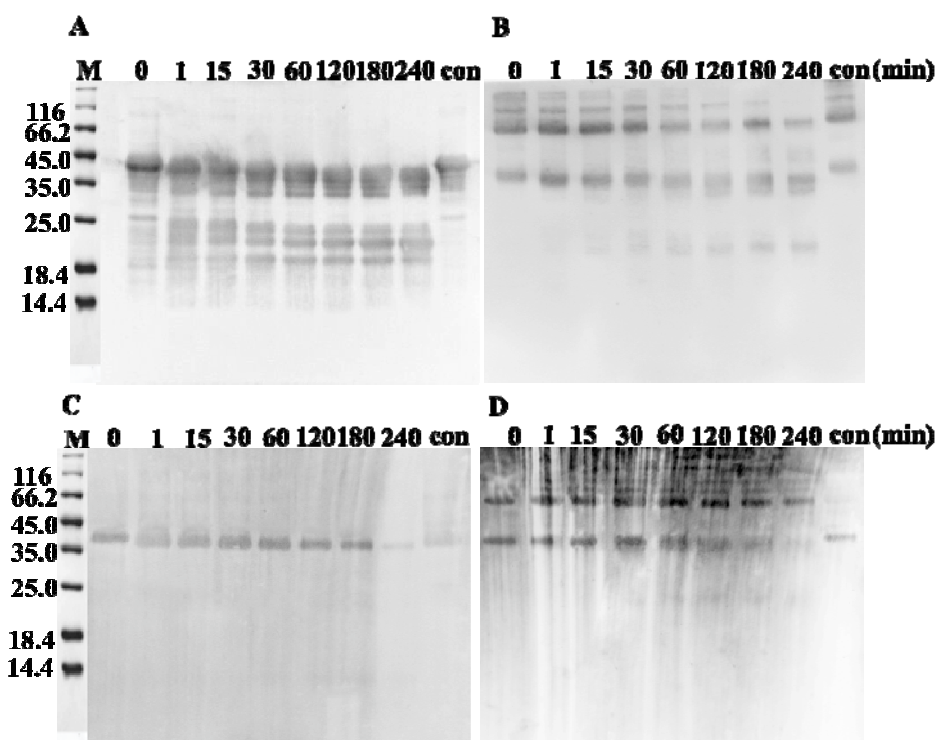


Fig. 4. Western blot analyses of the degradation of the purified shrimp tropomyosin (TM) and 50 mM malondialdehyde (MDA) treated TM in simulated intestinal fluid (trypsin). Samples were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The purified shrimp TM (A) and 50 mM MDA-treated TM (B) were detected using polyclonal antisera against shrimp TM. The purified shrimp TM (C) and 50 mM MDA-treated TM (D) were detected using pooled sera from patients allergic to shrimp. Positions of molecular mass standards (M) are labeled on the left edges.