JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Changes in Allergenicity and Digestibility of Squid Tropomyosin during the Maillard Reaction with Ribose

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The effect of the Maillard reaction on the allergenicity of squid tropomyosin (TM) was investigated. When TM was reacted with ribose (TM−ribose), its human-specific IgE-binding ability decreased markedly and α-chymotryptic digestibility of TM was also altered at the early stage of the Maillard reaction. On the other hand, the modification of the lysine residues in TM using 2,4,6-trinitrobenzenesulfonic acid had no effect on the allergenicity and α-chymotryptic digestibility of TM. Therefore, the structural change in TM induced by the Maillard reaction would cause the reduction of the allergenicity, rather than the block of lysine residues. Although peptic digestion diminished the specific IgE-binding ability of TM, the reduction of the allergenicity by the Maillard reaction remained after peptic digestion. These results suggest that hypersensitive reaction of TM−ribose in the human body might be lower than that of native TM.

KEYWORDS: Squid; tropomyosin; seafood allergy; allergenicity; Maillard reaction; digestibility

INTRODUCTION

The Maillard reaction is one of the most important reactions in food processing because of its significant effect on food quality such as flavor, color, texture, and nutritional value. Recently, the effect of the Maillard reaction on allergenicity of allergenic food proteins has been investigated from the viewpoint of food safety. For instance, the peanut allergens, Ara h 1 and Ara h 2, increased their allergenicity with roasting and the Maillard reaction with glucose (*1*), and the allergenicity of a major cherry allergen (Pru av 1) decreased as a result of the Maillard reaction with glucose and ribose (*2*). However, less research has been performed on allergenic changes in seafood with the Maillard reaction.

Tropomyosin (TM) has been reported to be the major allergen in marine invertebrates such as shrimp, squid, and scallop (*3*−*9*). Since TM of invertebrates is typically a lysine-rich protein (lysine is 9.2% of the total amino acid in squid TM; GenBank AB218915), it reacts easily with reducing sugars through the Maillard reaction during food processing such as grilling, steaming, and roasting. Particularly, the brown color of dried seafoods is caused by the Maillard reaction (*10*, *11*). In previous work, we found that the allergenicity of scallop TM was enhanced with the progress of the Maillard reaction with glucose and ribose (*12*).

Squid is commonly eaten in dried form in Japan and Korea. Browning reaction in squid mantle meat is often observed during the heating or drying process. It was reported that ATP-related compounds were quickly degraded by enzymes and ribose was accumulated in squid muscle after death (*13*−*15*). Omura et al. reported that the ribose content in squid muscle (>1.0 μmol/g of dry matter) increased more than 8 times during storage and heating processes (*16*) and the browning reaction progressed with a loss of ribose (*10*) in squid mantle muscle. These findings indicate that the Maillard reaction in squid mantle muscle would be closely related to ribose and the reaction affects food quality of dried squid products (*10*, *17*). In this study, the change in allergenicity of squid TM during the Maillard reaction with ribose was then examined using immunochemical procedures to compare the results obtained from scallop TM (*12*).

MATERIALS AND METHODS

Materials. Squid (*Todarodes pacificus*) was purchased from a local fish market. A calibration kit of glycosylated human serum and peroxidase-conjugated rabbit antihuman IgE antibody were purchased from Japan Roche Diagnostic Inc. (Tokyo, Japan) and Daco Cytomation (Glostrup, Denmark), respectively. All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Kanto Chemical Co., Inc. (Tokyo, Japan).

Preparation of Squid TM. Squid TM was prepared by the method of scallop TM (*12*) with slight modifications. In brief, TM was extracted from the mantle muscles (100 g) with 0.4 M LiCl at pH 5.4 and partially purified by ammonium sulfate fractionation at 45−65% saturation, ultracentrifugation, and isoelectric precipitation at pH 4.60. Crude TM

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10.1021/jf061070d CCC: $33.50
Published on Web 11/16/2006

Table 1. Clinical History of Patients' Sera Including Specific IgE Antibody versus Squid TM

serum	age (years)	sex	hypersensitive reaction[a]	CAP-RAST class[b]	dilution rate of serum: competitive ELISA	dilution rate of serum: dot blotting	intake experience of squid[c]
1	4	female	Ur and BA	6	1:100	1:100	Y
2	6	male	BA, AR, OAS, and VOM	3	1:200	1:25	Y
3	2	male	Ur, BA, and AD	6	1:150	1:100	Y
4	5	male	Ur, BA, and Ana	3	1:100	1:25	Y
5	4	male	Ur	6	1:150		Y
6	2	female	Ur	3	1:100	1:50	Y
7	7	female	AD, OAS, and AR	2	1:200	1:25	N
8	3	female	Ana, AD, Ur, and BA	3	1:50	1:25	N
9	8	male	BA and AD	N.D.	1:100		N
10	1	male	AD and AB	5	1:150	1:100	N
11	3	female	Ur, BA, AD, and AR	6	1:300	1:300	N
12	1	female	OAS	3	1:100	1:100	N
13	2	male	AD	6		1:200	N
14	4	female	AD and Ur	3	1:100	1:25	N

[a] Ur, urticaria; BA, bronchial asthma; AD, atopic dermatitis; AR, allergy rhinitis; OAS, oral allergy syndrome; Ana, anaphylaxis; VOM, vomitus. [b] CAP-RAST is a diagnostic test to detect specific IgE antibody against allergen. Measured value is categorized as class 0-6. [c] Patient of "N" has not consumed squid and was not originally sensitized to its TM.

was further purified by anion-exchange chromatography. The protein concentration was determined using the protein assay rapid kit (Wako Pure Chemical Industries, Ltd.) using bovine serum albumin as a standard.

Human Serum. The sera of 14 subjects with the specific IgE antibody to squid TM were used in this study. Squid allergy was diagnosed on the basis of the detection of the TM-specific IgE antibody using the CAP-RAST (Pharmacia & Upjohn, Tokyo, Japan) and a positive response to the oral challenge test and the prick test. The clinical history of the patients is summarized in **Table 1**. Control sera were obtained from two fish-allergic patients without squid TM-specific IgE antibody and two normal subjects who had shown no adverse reactions to any foodstuff. These sera were frozen at −25 °C until use.

Maillard Reaction between TM and Ribose. TM dissolved in 1 mM $NaHCO_3$ and 5 mM 2-ME was mixed with ribose at a final concentration of 0.6 M and the protein concentration was adjusted to 0.2 mg/mL. Each 0.7 mL of the mixture was placed into microtubes and frozen at −25 °C, immediately lyophilized (FDU-506; EYELA, Tokyo, Japan), and stored at −60 °C until use.

The lyophilized mixtures were incubated at 60 °C and relative humidity (RH) of 35% for 180 min in a temperature- and humidity-controlled cabinet (PR-1G; Tabai Espec Corp., Tokyo, Japan) to progress the Maillard reaction between TM and ribose. The TM−ribose was then subjected to the analysis described below.

TNBS Modification to Lysine Residues in TM. Various concentrations of TNBS (0.03−1.0 mM) were added to TM (the final concentration was 0.4 mg/mL) dissolved in 0.2 M $NaHCO_3$ and then incubated at 30 °C for 180 min to react with the lysine residues with TNBS. Before the reaction, TM was pre-incubated at 30 °C for 10 min. TM reacted with TNBS (TM−TNBS) was cooled in ice after 180 min and immediately dialyzed against PBS at 4 °C to remove the unreacted TNBS.

Determination of Available Lysine and Ketoamine Contents. Available lysine and ketoamine assays were carried out to evaluate the progress of the Maillard reaction. The available lysine content was determined by a spectrophotometric analysis using *o*-phtalaldehyde and *N*-acetyl-L-cysteine (*18*). Before the analysis, TM−ribose was dissolved in a 50 mM phosphate buffer (pH 9.5) containing 2% SDS at room temperature.

The ketoamine was assayed by the method of Johnson et al. (*19*) using glycosylated human serum as a standard for the determination of the ketoamine content. Before the analysis of ketoamine, the protein solution was dialyzed against 0.1 M NaCl containing 20 mM Tris-HCl (pH 7.5) overnight at 4 °C to remove unreacted ribose.

Dot Blotting. Dot blotting was performed by the method described previously (*12*) with a slight modification. The subjects' sera were diluted 1/25, 1/50, 1/100, 1/200, or 1/300 (details were listed in **Table 1**). The ECL Western blotting detection reagent (Amersham, Buckinghamshire, U.K.) or the Immobilon Western Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA) was used to detect the enzyme reaction. The higher intensity of spot indicates an increase in the amount of specific IgE bound to TM.

Competitive ELISA. TM−ribose, TM−TNBS, and TM digested by pepsin (digested-TM) were subjected to competitive ELISA to investigate the changes in the specific IgE-binding ability of TM (*12*). Briefly, a flat-bottomed polystyrene microtiter plate (an ELISA plate: IWAKI, Tokyo, Japan) was coated with native TM dissolved in PBS at 2.5 μg/mL overnight at 4 °C. The residual blocking site in the plate was coated with 150 μL of 1% casein in PBS for 3 h at 37 °C. In another microtiter plate (an assay plate; IWAKI, Tokyo, Japan), 125 μL of each of the subjects' sera (diluted 1/25, 1/50, 1/75, 1/100, or 1/150 with 2% casein in PBS; details are listed in **Table 1**) was mixed with equal volumes of TM−ribose, TM−TNBS, or digested-TM as an inhibitor (0.002−200 μg/mL in PBS). After incubation at 37 °C for 2 h, each 70 μL of the solution was placed into the TM-coated ELISA plate described above. After incubation of the ELISA plate at 37 °C for 2 h, the enzyme−substrate reaction was performed using 0.04% *o*-phenylendiamine dihydrochloride and 0.05% H_2O_2 in a 50 mM phosphate−citrate buffer (pH 5.0) or Super Signal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The detection of the enzyme reaction was carried out using the Microplate reader MTP-300 (absorbance at 492 nm; Corona Electric, Ibaraki, Japan) or Luminometer-JNRII AB-2300 (Atto Corp., Tokyo, Japan). The loss of the specific IgE-binding ability of the patients' sera resulting from the treatment with the inhibitors was represented by calculating the inhibition rate using the following formula:

$$\text{inhibition rate (\%)} = (X - Y) \div (X - Z) \times 100$$

where X was the absorbance of each patient's serum without the inhibitors and Y and Z were the absorbance of the patients' and control sera treated with various concentrations of the inhibitors, respectively.

In this study, the inhibitory concentration obtaining a 50% inhibition rate (defined as IC_{50}) was calculated from the inhibition curve in the competitive ELISA as an indicator of the allergenicity of TM.

Circular Dichroism Analysis. To determine the circular dichroism (CD) spectra of unreacted (native) TM and TM−ribose (0.4 mg/mL in 0.1 M NaCl), each TM of 500 μL was placed into a 1 mm path length cuvette and maintained at 20 °C. The change in CD spectrum from 200 to 250 nm was then measured using a JASCO spectropolarimeter equipped with a temperature control system (J-725, Japan Spectroscopic Co., Ltd., Tokyo, Japan). The α-helix content was calculated from the data of the spectrum at 222 nm and the protein concentration (*20*).

Digestion by α-Chymotrypsin. Peptide mapping by α-chymotryptic digestion is a good manner to obtain information about structural change

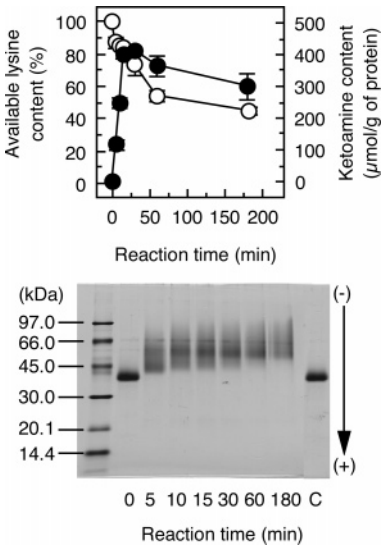

Figure 1. Changes in available lysine (○) and ketoamine (●) contents in TM during reaction with ribose and SDS-PAGE pattern of TM reacted with ribose. "C" shows TM incubated without ribose at 60 °C for 180 min.

of proteins. A series of protein fragments by α-chymotryptic digestion alters with structural change of protein (*21*−*23*). Native TM, TM−TNBS, and TM−ribose (each protein concentration was 0.4 mg/mL) were dissolved in 0.1 M NaCl and 20 mM Tris-HCl (pH 7.5), preincubated at 20 °C for 10 min, and digested with 1/50 weight of α-chymotrypsin for 2−90 min. The reaction was then terminated by adding 0.1 mM PMSF. The digested TM was mixed with a half-volume of 8 M urea, 2% SDS, 2% 2-ME, and 20 mM Tris-HCl (pH 8.0), heated for 2 min in boiling water, and subjected to SDS-PAGE analysis (*24*). The change in relative intensity of TM was monitored by image analysis using NIH Image software (*25*), and the digestibility of TM was expressed using the relative intensity of undigested TM as 100%.

Digestion by Pepsin. Native TM and TM−ribose (protein concentration was 0.4 mg/mL) were dissolved in 0.1 M NaCl (pH 2.0), preincubated at 37 °C for 10 min, and digested with 1/100 weight of pepsin for 5−180 min. The reaction was terminated by adjusting the pH to 7.5 using 0.1 M NaOH. The digested TM was subjected to SDS-PAGE and image analysis using NIH image as well as the α-chymotryptic digestion. The digested TM was also subjected to competitive ELISA to determine the change in allergenicity of TM by the peptic digestion.

Data Analysis. The results of each measurement are the average of three determinations, and error bars correspond to the standard deviations. Statistical analysis was performed using a paired t-test (*26*) at the 1% significance level.

RESULTS AND DISCUSSION

Progress of Maillard Reaction between TM and Ribose. Figure 1 shows the changes in the available lysine and ketoamine contents and SDS-PAGE patterns of TM during the reaction with ribose. When lyophilized TM mixed with ribose was incubated at 60 °C and RH 35%, the available lysine (0.100 g/g of protein) decreased markedly (54.8% of the lysine residues were diminished at 180 min of the reaction time) and ketoamine was produced with the elapse of the reaction time (maximum value: 410 μmol/g of protein for 30 min). In addition, the production of ketoamine with the loss of available lysine occurred at the early periods of the reaction. No lysine loss was observed in TM incubated without ribose (data not shown). Therefore, the results of **Figure 1** clearly indicate that TM was modified with ribose through the Maillard reaction.

In SDS-PAGE analysis, the single band of TM decreased markedly and a broadband having lower mobility simultaneously appeared with the progress of the Maillard reaction. These changes in the SDS-PAGE pattern were often observed in proteins reacted with reducing sugars (*2*, *27*, *28*), and heat treatment without ribose has no effect on the mobility of TM. Additionally, no degradation of TM was observed during the Maillard reaction.

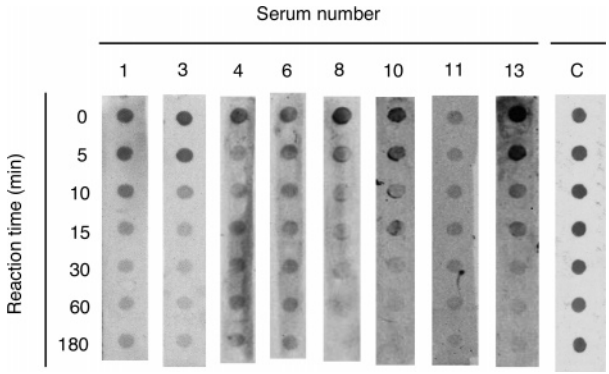

Figure 2. Change in IgE-binding ability of TM during reaction with ribose. Dot blotting of TM–ribose was performed using 8 patients' sera listed in **Table 1**. TM incubated without ribose at 60 °C for 180 min was also examined using patient's sera no. 1 (C), as a control.

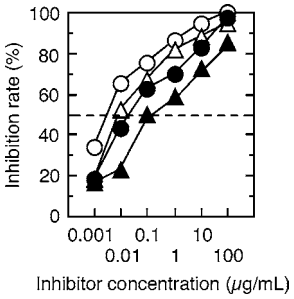

Figure 3. Competitive ELISA of TM–ribose. TMs reacted with ribose for 0 min (○), 10 min (△), 30 min (●), and 180 min (▲) were mixed with patient's serum no. 1.

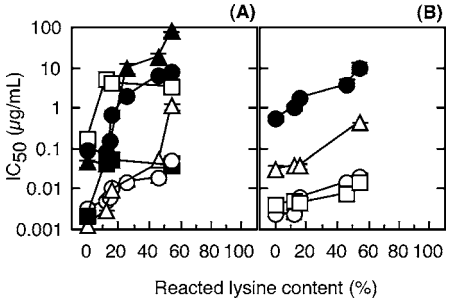

Figure 4. Effect of reaction with ribose on allergenicity of TM: (**A**) Patients' sera 1 (○), 2 (△), 7 (□), 11 (●), 12 (▲), and 14 (■); (**B**) patients' sera 4 (○), 8 (△), 9 (□), and 10 (●). Coated sample and inhibitor were native TM and TM–ribose, respectively.

Effect of Maillard Reaction on Allergenicity of TM. Changes in the allergenicity of TM with the progress of the Maillard reaction were assessed using dot blotting (**Figure 2**) and competitive ELISA (**Figures 3** and **4**). As shown in **Figure 2**, native TM showed a clear reaction with 8 patients' sera. However, the spot intensity of TM weakened with the progress of the Maillard reaction with ribose, and it almost disappeared at 180 min. The spot intensity of TM remained unchanged when TM was incubated under the same condition in the absence of ribose. Therefore, the results of **Figures 1** and **2** indicated that the specific IgE-binding ability of TM was suppressed with the progress of the Maillard reaction.

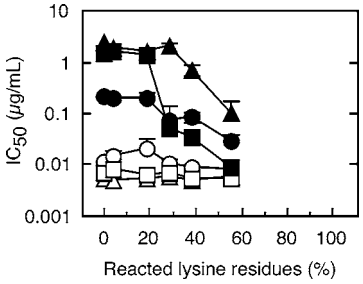

Figure 5. Effect of TNBS modification on allergenicity of TM. Patients' sera 7 (○), 10 (△), 14 (□), 2 (●), 3 (▲), and 5 (■) were used. Coated sample and inhibitors were native TM and TM–TNBS, respectively.

Competitive ELISA using TM−ribose as an inhibitor was performed to estimate the change in the allergenicity of TM by the Maillard reaction. No change was observed in the allergenicity of TM incubated at 60 °C without ribose in the competitive ELISA using all 10 patients' sera (data not shown). In contrast, the reaction between patients' sera and coated native TM was suppressed with an increase in the concentration of all inhibitors. However, the inhibition effect of the TM−ribose was gradually diminished with the progress of the Maillard reaction as shown in **Figure 3**. **Figures 4A** and **4B** show the relation between IC_{50} and the reacted lysine content of TM used as inhibitors. It was confirmed that IC_{50} increased (i.e., the allergenicity of TM was reduced) with the progress of the Maillard reaction in all sera.

Effect of Modification to Lysine on Allergenicity of TM. TM−TNBSs, with various modification rates to lysine (maximum: 58%), were subjected to competitive ELISA using 6 patients' sera, and IC_{50} was plotted against the rate of the lysine residues reacted with TNBS (**Figure 5**). No enhancement of IC_{50} was observed in all 6 patients' sera, regardless of the modification rate of the lysine residues. Therefore, it was apparent that the reduction of the allergenicity in TM−ribose (shown in **Figure 4**) occurred independently of the lysine loss by the Maillard reaction. According to the results of **Figure 5**, the used patients' sera were divided into two groups from the relationship between IC_{50} and the modification rate to lysine residues. In other words, IC_{50} shows no change in spite of the progress of the reaction with TNBS in the patients who had consumed squid (serum nos. 2, 3, and 5), and IC_{50} drastically decreased by reaction of 30−40% of lysine residues with TNBS in the patients who had not consumed it (serum nos. 7, 10, and 14). This result suggests that the epitope site recognized by IgE antibodies differed depending on whether or not the patients had consumed squid, although all sera contained the specific IgE antibodies recognizing squid TM. Therefore, it may be necessary to take into account the intake experience of patients when we identify the epitope sites of squid TM.

Structural Change of TM by Reaction with Ribose. TM of invertebrates consists of two subunits including α-helix of >90% (*29*). The change in the α-helix content of TM during the reaction with ribose was then measured to discuss the mechanism of the reduction of the allergenicity of squid TM by the Maillard reaction. However, the α-helix content of TM was almost unchanged during the reaction with ribose as shown in **Figure 6**, and it was considered that the change in the allergenicity of TM was caused by other factors.

The limited proteolysis using α-chymotrypsin is often used to confirm the structural change of proteins (*21*−*23*). TM−TNBS, and TM−ribose, in which approximately 25% of the lysine residues reacted with ribose, were then digested by

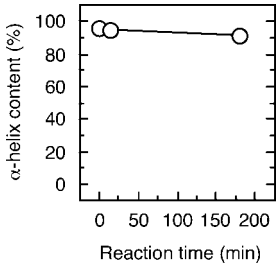

Figure 6. Change in α-helix content of TM during the reaction with ribose.

α-chymotrypsin. **Figure 7** shows the SDS-PAGE patterns of the three kinds of digested TMs. Native TM and TM−TNBS were easily digested by α-chymotrypsin. The reduction rate of TM−TNBS was almost consistent with that of native TM, and each TM of >70% was diminished within 90 min. Several digested fragments were observed in the SDS-PAGEs, in which one major band at approximately 22 kDa and several minor bands around 17 kDa were observed. These results indicate that the digestibility of TM by α-chymotrypsin was not altered by the modification of lysine residues. On the other hand, the digestibility of TM−ribose was significantly lower than that of native TM, and approximately half of the TM−ribose remained at 90 min. Additionally, the digested fragments pattern was apparently different from that of native TM. These results suggest that the structural change of TM by the Maillard reaction with ribose is related to the reduction of the IgE-binding ability.

Effect of Peptic Digestion on Allergenicity of TM. TM−ribose was subjected to peptic digestion to discuss hypersensitive reaction induced by TM in the human body. **Figure 8** shows the SDS-PAGE patterns of native TM and TM−ribose digested by pepsin. Both TMs were digested at approximately 20% at 5 min; however, there was a discrepancy in their reduction rates from 10 to 180 min. In other words, the native TM band diminished completely at 180 min, whereas only 91% around the 45 kDa band was digested at 180 min in TM−ribose. The result in **Figure 8** shows that the digestibility of TM by pepsin also decreased as a result of the progress of the Maillard reaction with ribose.

Competitive ELISA using the two kinds of TMs digested for 180 min as an inhibitor was performed to compare the allergenicity between pre- and post-digestion. As shown in **Figure 9A**, the inhibition rates of both TMs at the same inhibitor concentration diminished by the peptic digestion. However, the lower allergenicity of TM−ribose remained after the peptic digestion. As shown in **Figure 9B**, IC_{50} of the digested TM−ribose was 10 times higher than that of native TM. These results suggest that the digested TM−ribose maintained its lower allergenicity, whereas a part of the epitope sites was cleaved by the peptic digestion. We reproduced the same results by using 3 patients' sera (nos. 1, 3, and 5).

It is known that the trigger of food allergy is the intestinal absorption of digested peptides with epitope sites, and allergenic proteins have a lower degree of digestibility than do ordinary proteins (*30*). Although the digestibility of TM was impaired by the Maillard reaction (**Figures 7** and **8**), the lowered allergenicity remained after peptic digestion (**Figure 9**). Since the pepsin concentration used in this study (1%) was higher than that in the living body (∼0.08%) (*31*), the low allergenicity of TM digested by pepsin seems to be maintained through the digestion process and absorption process. However, there is not enough information concerning the absorbability of the Maillard-reacted TM in vivo. We need to design animal experiments to determine the productivity of specific antibodies against TM−ribose.

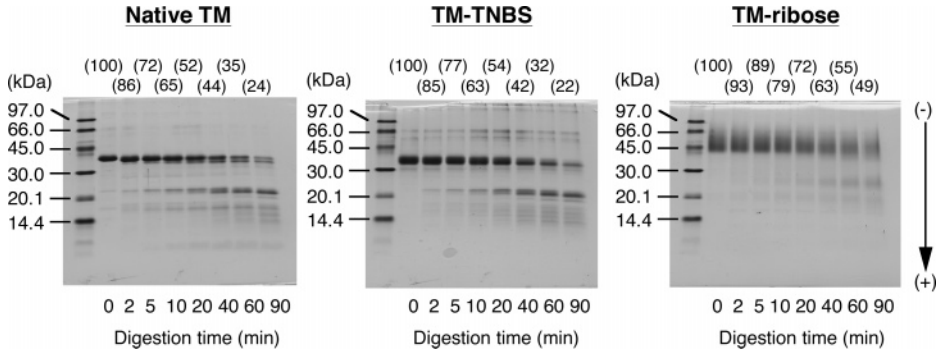

Figure 7. Comparison of SDS-PAGE patterns of α-chymotryptic digestion among native TM, TM–TNBS, and TM–ribose. Numbers in parentheses are the percentage expressed by undigested TM band as 100.

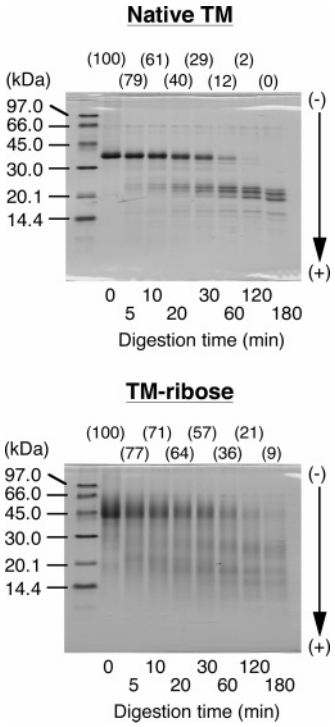

Figure 8. Comparison of patterns of peptic digestion between native TM and TM–ribose. Numbers in parentheses are the percentages expressed by undigested TM band as 100.

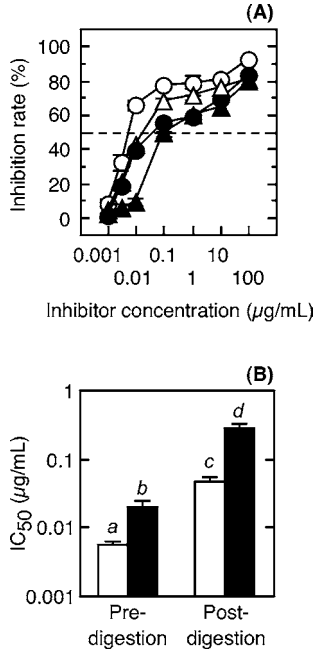

Figure 9. Effect of peptic digestion on allergenicity of native TM and TM–ribose: (**A**) native TM (○), native TM digested for 180 min (●), TM–ribose (△), and TM–ribose digested for 180 min (▲). Coated sample was native TM, and inhibitors were TMs digested by pepsin; (**B**) native TM (□) and TM–ribose (■). This figure shows the result of the experiment with patient serum no. 1 (listed in **Table 1**). The different italic letters show significantly different at $p < 0.01$.

We reported previously that the structural change by the Maillard reaction with glucose and ribose enhanced the IgE-binding ability of scallop TM (*12*). The IgE-binding ability of squid TM was, however, reduced by the Maillard reaction with ribose, as presented in this study. TM consists of two α-helical subunits that form coiled-coil and the structure was highly conserved in various animals (*32*−*34*). On the other hand, there were variations in the amino acid sequence of TMs and only 69.7% of amino acid sequence homology between squid and scallop TMs (GenBank; AB004636/AB218915). Therefore, there may be different epitope sites between both TMs. In addition, different structural changes may be induced by the Maillard reaction with ribose because 21% of lysine residues exists in different position between them. It is necessary to identify epitope sites in TMs and to investigate the conformational change in TMs induced by the Maillard reaction to explain the mechanism of the change in the allergenicity of TM.

In conclusion, the Maillard reaction with ribose reduced the allergenicity of squid TM, which was completely different from that of scallop TM (*12*). The findings in this work may contribute to the development of low-allergenic-processed seafood. However, we need to investigate the allergenicity of the Maillard-reacted TM in various kinds of invertebrates and to understand the relation between the allergenic change and the protein structure, in view of food safety.

ABBREVIATIONS USED

CAP-RAST, capsulated hydrophilic carrier polymer-radio-allergosorbent test; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; ME, mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; TM, tropomyosin; TNBS, 2,4,6-trinitrobenzenesulfonic acid sodium salt dihydrate.

ACKNOWLEDGMENT

We thank Professor Kunihiko Konno (Graduate School of Fisheries Sciences, Hokkaido University) for support involving

the determination of the α-helix content using the JASCO spectropolarimeter.

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Received for review April 17, 2006. Revised manuscript received September 21, 2006. Accepted October 10, 2006. This study was supported by Health and Labor Sciences Research Grants of the Research on Food Safety from the Ministry of Health, Labor, and Welfare of Japan. This study was also supported by a Japan-Korea Basic Scientific Cooperation Program from Japan Society for the Promotion of Science.

JF061070D