



Cite this: DOI: 10.1039/c9fo01547g

# Glycation by saccharides of different molecular sizes affected the allergenicity of shrimp tropomyosin *via* epitope loss and the generation of advanced glycation end products

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Tropomyosin is the most potent allergen of shrimp that can cause severe food allergy. However, to date, an effective approach to eliminate this allergenicity is still lacking. Glycation is a promising approach that can reduce the allergenicity of shrimp tropomyosin by destroying the epitopes; however, advanced glycation end products (AGEs) are also generated during glycation, which can function as neoallergens to strengthen the allergenicity; therefore, it is hard to tell how the glycation of an allergen with different saccharides affects the allergenicity *via* epitope loss and neoallergen generation. This study was aimed at the elucidation of how the glycation of tropomyosin (TM) with saccharides of different molecular sizes (glucose, maltose, and maltotriose) affected the allergenicity through epitope loss and the generation of neoallergens that belonged to advanced glycation end products (AGEs). Saccharides of higher molecular sizes (maltotriose) could lead to higher glycated TM than saccharides of smaller molecular sizes (glucose and maltose). Compared with TM, the TM glycated by glucose (TM-G) and maltotriose (TM-MTS) had lower allergenicity and contributed to weaker mouse allergy symptoms; on the other hand, the TM glycated by maltose (TM-M) had no significant impact on the allergenicity due to the generation of AGE-related neoallergens, which might offset the glycation-induced epitope loss. The glycation of TM by maltotriose led to lower generation of AGE neoallergens (e.g. CML) than that in the cases of glucose and maltose; therefore, maltotriose could be applied to desensitize TM-induced food allergy through glycation, and this could be a potential immunotherapy for shrimp allergy patients.

Received 15th July 2019,  
Accepted 20th September 2019

DOI: 10.1039/c9fo01547g

rsc.li/food-function

## 1. Introduction

Shrimp is among the most common and potent food allergens that can cause severe food allergy.<sup>1</sup> The main allergen in shrimp is tropomyosin; however, to date, an effective approach to eliminate or weaken its allergenicity is still lacking.<sup>2</sup> Glycation is a nonenzymatic reaction that occurs between a reducing sugar and free amino groups (e.g. between lysine and a free  $\epsilon$ -amino group).<sup>3</sup> As shrimp tropomyosin has high content of lysine, a promising approach to reduce shrimp tropomyosin allergenicity is the destruction of the epitopes by glycation, resulting in epitope loss.<sup>4</sup>

However, glycation can generate advanced glycation end products (AGEs), which may function as neoallergens that affect allergenicity. Previous studies have proved that AGEs,

e.g. N<sup>ε</sup>-(carboxymethyl) lysine (CML), can function as immune epitopes.<sup>5–9</sup> The receptors for AGEs (RAGE) are present on the surface of a variety of immune cells;<sup>3,10,11</sup> when AGEs bind to RAGE, proinflammatory and allergic responses occur.<sup>12,13</sup> Previous studies have proved that CML functions as a potent epitope and aggravates the allergenicity.<sup>3,5–9</sup> Mueller *et al.*<sup>8</sup> found that peanut allergens (Ara h 1 and Ara h 3) modified with CML could be recognized by RAGE, leading to the activation of allergy symptoms.

Furthermore, the influence of glycation on allergenicity is heavily dependent on the sources of saccharides.<sup>4</sup> As the glycation of allergens by different saccharides could either weaken the allergenicity by destructing epitopes or exacerbate the allergenicity by generating neoallergens (e.g. CML), it is hard to tell how the glycation of allergens affects the allergenicity *via* epitope loss and neoallergen generation. Previous data have shown that saccharides of greater molecular sizes can reduce the allergenicity of allergens.<sup>14</sup> However, only few studies have been reported on how the glycation of TM by saccharides of different sizes influences the epitope loss and neoallergen generation. The objective of this study was to

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elucidate how the glycation of shrimp tropomyosin by saccharides of different molecular sizes affected the allergenicity, which might provide a new insight into the desensitization of shrimp-induced allergy.

## 2. Materials and methods

### 2.1 Materials

The shrimp tropomyosin (Exo m 1) was extracted and purified from *Exopalaemon modestus*.<sup>15</sup> Cholera toxin (CT), glucose (G), maltose (M) and maltotriose (MTS) were purchased from Sigma-Aldrich (St Louis, MO, USA). The BCA protein assay kit was procured from Thermo Scientific (Rockford, IL, USA). BALB/c mice were obtained from JOINN Laboratories (Suzhou, Jiangsu, China).

### 2.2 Preparation of glycated TMs (GTMs)

At first, TM was dissolved in a solution (1 mM NaHCO<sub>3</sub> and 5 mM  $\beta$ -mercaptoethanol) containing glucose, maltose or maltotriose with the final TM concentration of 0.2 mg mL<sup>-1</sup>, mixed well and lyophilized. The lyophilized mixtures of TM and saccharides were incubated in a desiccator (60 °C for 48 h) to generate TM-G (TM glycated by glucose), TM-M (TM glycated by maltose) and TM-MTS (TM glycated by maltotriose). The glycated TM mixtures were dialyzed (10 kDa, Union Carbide, USA) for 48 h and lyophilized. The glycation degree of GTMs was determined by investigating the molecular weight and carbohydrate content using SDS-PAGE and the phenol-sulfuric acid method, respectively.<sup>16</sup>

### 2.3 Glycation site of TM

**2.3.1 Digestion of GTMs.** GTMs were first gastric digested using pepsin (Sigma, USA) with the pepsin to TM ratio of 1 : 50 (w/w), incubated at 37 °C for 1 h and terminated by adjusting the pH value to 7.5. Subsequently, trypsin (Sigma, USA) was added with the trypsin to GTM ratio of 1 : 100 (w/w) followed by incubation at 37 °C for 3 h, and digestion was immediately terminated by bringing the pH to 2.0 using 1 M HCl.

**2.3.2 Analysis of the glycation site.** The glycation site analysis was carried out by investigating the glycated peptides in the GI digests using quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) (MALDI SYNAPT MS, Waters Corp., Manchester, UK). The mass detector was coupled to an electrospray ionization source (ESI). A Waters Acquity ultra performance liquid chromatography (UPLC) system equipped with the Acquity BEH C<sub>18</sub> column (2.1 × 50 mm, 1.7  $\mu$ m, Waters Corp., Milford, MA, USA) was applied to separate the peptides in tryptic digests. The measurement was performed in a positive ionization mode in the scan *m/z* range from 100 to 3000. The low collision energy was set at 6 V, and the high collision energy was set at 25 V. The capillary and sampling cone voltages were 3.5 kV and 30 V, respectively. The source and desolvation temperatures were 100 °C and 400 °C, respectively. The results were analyzed using MassLynx V4.1 and MassEnt

(Waters Corp., Manchester, UK) to process the MS spectra and perform peptide sequencing, respectively.

### 2.4 Advanced glycation end products (AGEs)

**2.4.1 AGE assay.** The AGEs were determined using spectrofluorimetry.<sup>17</sup> Fluorescence intensity was determined at maximum emission (~440 nm) using a spectrofluorometer (F-7000, Hitachi Corp., Tokyo, Japan) with excitation at 350 nm and expressed in arbitrary units (AU). The AGE concentration was normalized to the total protein concentration determined by the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and expressed in AU per gram of protein.

**2.4.2 CML determination.** CML is a common marker of AGEs. Basically, CML was determined using the RP-HPLC method.<sup>18</sup> At first, GTMs (2 mg) were reduced overnight at 4 °C in 500  $\mu$ L buffer (0.1 M sodium tetraborate decahydrate and 0.1 M sodium borate, pH 9.0), then washed with ultrapure water and concentrated with centrifugal filter units (Amicon Ultra-4, 10 kDa, Millipore, Ireland). After this, they were hydrolyzed in 1 mL of 6 M HCl under nitrogen (110 °C for 22 h). The hydrolysate (20  $\mu$ L) was diluted with 580  $\mu$ L of 0.1 M sodium borate buffer (pH 9.0). After this, the OPA reagent (50  $\mu$ L) was pipetted to the sample vial and mixed for derivatisation. After reaction for 3 min, the derivatized hydrolysate (10  $\mu$ L) was injected into the Waters Symmetry C<sub>18</sub> column (5  $\mu$ m, 4.6 × 250 mm, USA). The analysis was performed using an Alliance HPLC (Waters e2695, Waters Corp., USA) system equipped with the Waters FLR 2475 detector. The OPA-CML derivatives were detected fluorimetrically with excitation at 340 nm and emission at 455 nm. Quantification was performed by an external CML standard (Cayman Chemical, Ann Arbor, MI, USA).

### 2.5 In vitro allergenicity

The *in vitro* allergenicity of TM and GTMs were determined using a basophil cell line (KU812). The IgE-sensitized basophils were prepared according to Zhang *et al.*<sup>19</sup> The supernatant of the IgE-sensitized basophil culture medium was applied to investigate the histamine release using a histamine ELISA kit (IBL, Hamburg, Germany).

The intracellular ROS was determined using H<sub>2</sub>DCF-DA (2',7'-dichlorodihydrofluorescein diacetate). The IgE-sensitized basophils (1 × 10<sup>5</sup> cells per well) were pre-incubated with H<sub>2</sub>DCF-DA (5  $\mu$ M) at 37 °C for 30 min, then washed to remove excess H<sub>2</sub>DCF-DA, and incubated with TM or GTMs for 30 min. The fluorescence intensity was measured with excitation at 485 nm and emission at 530 nm using a plate reader (SpectraMax M5, Molecular Devices, USA).

### 2.6 Mouse model

**2.6.1 Mouse sensitization and challenge protocol.** The BALB/c mice (female, six-week old) were housed in the animal center of Jiangnan University (Wuxi, China). The experimental protocol for oral immunization and challenge of the animals was approved by the Ethics Committee of Jiangnan University for the Care and Use of Laboratory Animals (JN. no. 20170930b1920120[38]) and performed as previously reported

(Fig. 4A).<sup>20,21</sup> After the mice adapted to the new environment for seven days, they were randomized into five groups (eight mice per group): a PBS negative control group [cholera toxin (CT) in PBS served as the adjuvant], TM positive control group, TM-G group, TM-M group and TM-MTS group. The PBS negative control mice were treated by intragastric (i.g.) gavage of 0.2 mL PBS containing CT (10 µg per mouse). In the immunized group, the mice were sensitized by i.g. gavage of 0.2 mL PBS containing TM or GTMs (0.1 mg per mouse) and CT (10 µg per mouse) once a week (on day 0, 7, 14, 21 and 28). On day 35, after challenging with the i.g. administration of 0.5 mg TM or GTMs, the mice were fasted overnight and sacrificed on day 36. The anaphylactic symptoms were evaluated using an established scoring system.<sup>22</sup> The rectal temperatures were measured for 0–1 h (0, 15 min, 30 min, 45 min and 60 min) after the challenge using a digital thermometer (Prosper, Shanghai, China).

**2.6.2 Serum analysis.** The serum histamine level was determined using a histamine ELISA kit (IBL, Hamburg, Germany). The serum TM-specific IgE, IgG1 and IgG2a levels were determined using ELISA.<sup>23</sup> Briefly, a 96-well plate was coated with a 100 µL TM/GTM solution and incubated overnight at 4 °C. Each well was washed with 300 µL TBST buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20, and pH 7.5) and blocked with a blocking solution (5% defatted milk in TBST) at 37 °C for 2 h. After washing, 100 µL diluted sera (1 : 10 dilution) were added to each well, and the wells were incubated overnight at 4 °C. After the wells were washed, 100 µL of HRP-conjugated goat anti-mouse IgE secondary antibody (1 : 1000 dilution, KPL Inc., Gaithersburg, MD, USA), HRP-conjugated goat anti-mouse IgG1 heavy chain secondary antibody (1 : 5000, Abcam), or HRP-conjugated goat polyclonal secondary antibody to mouse IgG2a-heavy chain (1 : 5000, Abcam) was added to the wells followed by incubation at 37 °C for 2 h. Then, the plates were washed with TBST, and the TMB substrate solution (100 µL per well, Beyotime Biotech, Shanghai, China) was added to it. The plates were incubated at 37 °C for 20 min. The color reactions were stopped with 2 M H<sub>2</sub>SO<sub>4</sub> (50 µL per well). The absorbance (OD<sub>450 nm</sub>) was read using a plate reader (SpectraMax M5, Molecular Devices, USA).

## 2.7 Statistical analysis

The data obtained from three triplicate determinations are presented as means ± standard deviation (SD). Statistical analysis was performed using the SPSS software (SPSS Inc., Version 19, IL, USA).

## 3. Results

### 3.1 Glycation degree of GTMs

Molecular weight alterations could be used to reflect the glycation degree.<sup>3</sup> Compared with TM (Fig. 1A), after glycation, the GTMs had higher molecular weight, and the molecular weight of GTMs increased with an increase in the molecular weight of saccharides (TM-MTS > TM-M > TM-G > TM). Nakamura *et al.*<sup>24</sup> and Zhang *et al.*<sup>14</sup> had also observed similar molecular weight change tendency for α-lactalbumin glycated by glucose, maltose and maltotriose. Moreover, the alteration of carbohydrate content is an important marker that reflects the glycation degree.<sup>19</sup> As shown by the data (Fig. 1B), the carbohydrate content of TM was 3.016%, which was consistent with the previously reported data.<sup>16</sup> Compared with that of TM, the carbohydrate content of GTMs increased (TM-MTS > TM-M > TM-G > TM), which had similar variation tendency as the molecular weight (Fig. 1A). Maltotriose led to higher glycation degree on TM than glucose and maltose. This may be because the molecular weight of maltotriose is almost 3 times that of glucose and 1.5 times that of maltose, and the amount of glycated lysines in TM-G, TM-M and TM-MTS exhibits insignificant changes (Tables 1–3, 28 glycated lysines for TM-G, 25 glycated lysines for TM-M and 23 glycated lysines for TM-MTS); therefore, the saccharides of greater molecular weight contribute to greater molecular change and higher glycation degree on TM. Using SDS-PAGE, Zhang *et al.*<sup>14</sup> found that α-lactalbumin glycated by saccharides with higher molecular weight had greater molecular weight than those glycated by saccharides with smaller molecular weight.

### 3.2 AGE analysis

The AGEs (*e.g.* CML) can reportedly function as potent epitopes and promote the risk of food allergy.<sup>5</sup> As shown in

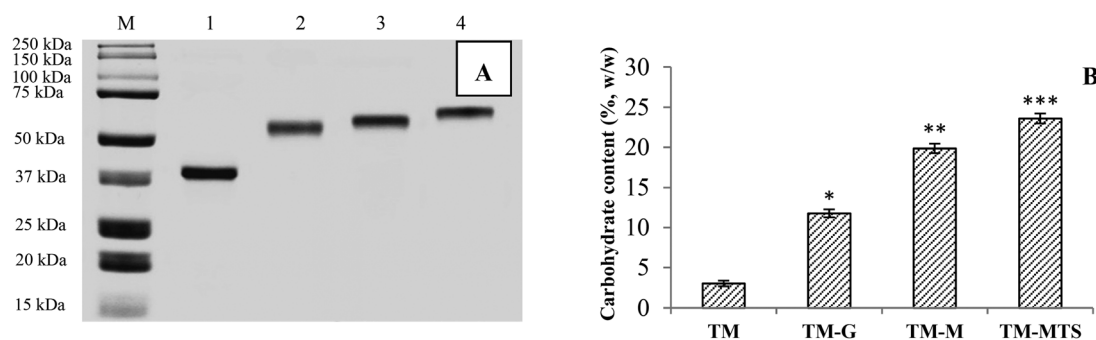


**Fig. 1** SDS-PAGE (A) and carbohydrate content (B) of TM and GTMs. Note: A: M, marker; lane 1, TM; lane 2, TM-G (TM glycated by glucose); lane 3, TM-M (TM glycated by maltose); and lane 4, TM-MTS (TM glycated by maltotriose). \*Shown significant differences when compared with TM, \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

**Table 1** The glycated peptides with one glucose in the digests of TM glycated by glucose

| Start-end | <i>M<sub>w</sub></i> (obs) | <i>M<sub>w</sub></i> (calc) | Sequence          | Glycation site | Epitope                                |
|-----------|----------------------------|-----------------------------|-------------------|----------------|----------------------------------------|
| 1-6       | 867.4993                   | 867.5410                    | MDAIKK            | K5             | —                                      |
| 1-7       | 995.6348                   | 995.6360                    | MDAIKKK           | K6             | —                                      |
| 7-12      | 898.559                    | 898.5291                    | KMQAMK            | K7             | —                                      |
| 7-12      | 898.579                    | 898.5291                    | KMQAMK            | K12            | —                                      |
| 15-26     | 1528.7107                  | 1528.7537                   | KDNAMDRALTCE      | K15            | —                                      |
| 27-32     | 879.4807                   | 879.4973                    | QQNKEA            | K30            | —                                      |
| 37-47     | 1503.8034                  | 1503.7729                   | EKSEEEVHNQ        | K38            | —                                      |
| 47-54     | 1222.7015                  | 1222.7016                   | QKRMQQL           | K48            | VHNLQKRMQQLENDLDS                      |
| 62-69     | 991.6152                   | 991.6225                    | EALLKANA          | K66            | —                                      |
| 72-82     | 1395.7921                  | 1395.7405                   | EKDKALSNAE        | K74            | —                                      |
| 75-91     | 1976.0983                  | 1976.0961                   | DKALSNAEGEVAALNRR | K76            | —                                      |
| 107-115   | 1110.6357                  | 1110.6444                   | NTATTKLAE         | K112           | —                                      |
| 123-130   | 1180.7722                  | 1180.7274                   | SERMRKVL          | K128           | —                                      |
| 144-150   | 1035.6489                  | 1035.6124                   | LENQLKE           | K149           | ENRSLDEERMDALENLKEARFLAEADRKYDE        |
| 155-162   | 1143.5717                  | 1143.6084                   | AEADRKY           | K161           | ENRSLDEERMDALENLKEARFLAEADRKYDE        |
| 163-169   | 992.6484                   | 992.6177                    | DEVARKL           | K168           | —                                      |
| 183-198   | 1994.1443                  | 1994.0730                   | AETGESKIVELEEELR  | K189           | ESKIVELEEELRVVG                        |
| 199-205   | 905.5635                   | 905.5857                    | VVGNNLK           | K205           | —                                      |
| 213-219   | 1036.6099                  | 1036.5825                   | KANQREE           | K213           | —                                      |
| 219-223   | 801.4728                   | 801.4431                    | EAYKE             | K222           | —                                      |
| 224-228   | 764.5353                   | 764.5319                    | QIKTL             | K226           | —                                      |
| 229-234   | 836.5614                   | 836.5642                    | TNKLKA            | K231           | —                                      |
| 229-237   | 1107.6162                  | 1107.6811                   | TNKLKAAEA         | K233           | —                                      |
| 241-250   | 1367.8309                  | 1367.8085                   | FAERSVQKLQ        | K248           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 249-255   | 1049.6318                  | 1049.6393                   | LQKEVDR           | K251           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 260-265   | 893.5924                   | 893.5381                    | LVNEKE            | K264           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 261-267   | 1071.5837                  | 1071.6124                   | VNEKEY            | K266           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 266-273   | 1145.6492                  | 1145.6492                   | KYKSITDE          | K268           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |

**Table 2** The glycated peptides with one maltose in the digests of TM glycated by maltose

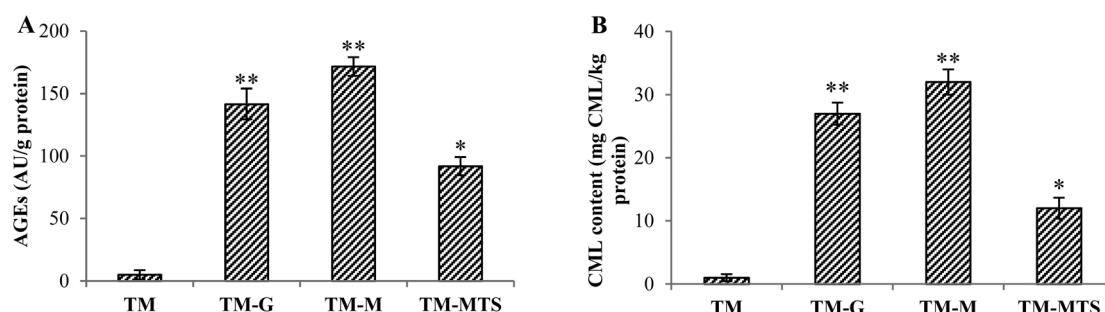
| Start-end | <i>M<sub>w</sub></i> (obs) | <i>M<sub>w</sub></i> (calc) | Sequence        | Glycation site | Epitope                                |
|-----------|----------------------------|-----------------------------|-----------------|----------------|----------------------------------------|
| 1-9       | 1434.8627                  | 1434.8951                   | MDAIKKKMQ       | K5             | —                                      |
| 11-18     | 1290.7117                  | 1290.7866                   | MKLEKDNA        | K12            | —                                      |
| 14-27     | 1966.0029                  | 1966.0148                   | EKDNAMDRALTCEQ  | K15            | —                                      |
| 23-31     | 1434.8627                  | 1434.8037                   | LTCEQQNKE       | K30            | —                                      |
| 37-42     | 1092.6177                  | 1092.6199                   | EKSEEE          | K38            | —                                      |
| 43-51     | 1495.9333                  | 1495.9306                   | VHNLQKRMQ       | K48            | VHNLQKRMQQLENDLDS                      |
| 58-67     | 1415.8701                  | 1415.8884                   | DSVQEALLKA      | K66            | —                                      |
| 72-78     | 1174.8024                  | 1174.7457                   | EKDKAL          | K74            | —                                      |
| 74-78     | 916.6656                   | 916.6606                    | KDKAL           | K76            | —                                      |
| 110-116   | 1075.7983                  | 1075.7137                   | TTKLAE          | K112           | —                                      |
| 125-131   | 1273.8457                  | 1273.8553                   | RMRKVLE         | K128           | —                                      |
| 144-151   | 1286.8351                  | 1286.8094                   | LENQLKEA        | K149           | ENRSLDEERMDALENLKEARFLAEADRKYDE        |
| 157-166   | 1537.8601                  | 1537.8636                   | EADRYKYDEVA     | K161           | ENRSLDEERMDALENLKEARFLAEADRKYDE        |
| 184-193   | 1446.8936                  | 1446.8830                   | ETGESKIVEL      | K189           | ESKIVELEEELRVVG                        |
| 197-211   | 1999.2329                  | 1999.2320                   | LRVVGNNLKSLEVSE | K205           | —                                      |
| 209-216   | 1246.7498                  | 1246.7417                   | VSEKANQ         | K213           | —                                      |
| 217-224   | 1394.856                   | 1394.8054                   | REEAYKEQ        | K222           | —                                      |
| 224-228   | 944.6376                   | 944.6919                    | QIKTL           | K226           | —                                      |
| 229-234   | 1016.7357                  | 1016.7242                   | TNKLKA          | K231           | —                                      |
| 233-239   | 1058.7378                  | 1058.7096                   | KAAEARA         | K233           | —                                      |
| 244-250   | 1200.8281                  | 1200.8203                   | RSVQKLQ         | K248           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 251-257   | 1230.8337                  | 1230.7832                   | KEVDRL          | K251           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 260-265   | 1073.7202                  | 1073.6981                   | LVNEKE          | K264           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 264-267   | 909.5382                   | 909.6184                    | KEY             | K266           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |
| 266-273   | 1325.9032                  | 1325.9155                   | KYKSITDE        | K268           | ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE |

Fig. 2A, compared with the case of TM, after glycation, AGEs were generated in GTMs; moreover, the AGE amount increased as follows: TM-M > TM-G > TM-MTS > TM, and TM-M had highest AGE content. A previous study has stated that the production rate and extent of AGEs

depend upon different types of reducing sugars.<sup>3</sup> The lowest AGE content in TM-MTS, as shown in Fig. 2A, may be because MTS has relatively greater molecular sizes, which has lower accessibility to interact with TM and thus generate AGEs.

**Table 3** The glycosylated peptides with one maltotriose in the digests of TM glycosylated by maltotriose

| Start-end | $M_w$ (obs) | $M_w$ (calc) | Sequence       | Glycation site | Epitope                                 |
|-----------|-------------|--------------|----------------|----------------|-----------------------------------------|
| 14–18     | 1062.7229   | 1062.6871    | EKDNA          | K15            | —                                       |
| 24–36     | 1992.1863   | 1992.1008    | TCEQQNKEANNRA  | K30            | —                                       |
| 37–41     | 1107.6653   | 1107.6973    | EKSEE          | K38            | —                                       |
| 47–51     | 1176.7487   | 1176.7962    | QKRMQ          | K48            | VHNLQKRMQQLNDLDS                        |
| 58–71     | 1995.2637   | 1995.2314    | DSVQEALLKANAH  | K66            | —                                       |
| 72–82     | 1720.1037   | 1720.0204    | EEKDKALSNAE    | K74            | —                                       |
| 73–77     | 1076.7413   | 1076.7391    | EKDKA          | K76            | —                                       |
| 105–116   | 1775.1866   | 1775.1466    | RLNTATTKLAEA   | K112           | —                                       |
| 120–131   | 1949.081    | 1949.1565    | ADESERMRKVLE   | K128           | —                                       |
| 146–150   | 1117.7584   | 1117.7656    | NQLKE          | K149           | ENRSLSDERMDALENQLKEARFLAEADRKYDE        |
| 158–162   | 1138.7288   | 1138.7660    | ADRYK          | K161           | ENRSLSDERMDALENQLKEARFLAEADRKYDE        |
| 163–169   | 1316.9004   | 1316.8977    | DEVARKL        | K168           | —                                       |
| 188–193   | 1174.8275   | 1174.8486    | SKIVEL         | K189           | ESKIVELEELRVVG                          |
| 197–207   | 1699.0948   | 1699.1669    | LRVVGNNLKSL    | K205           | —                                       |
| 212–216   | 1075.7015   | 1075.7187    | EKANQ          | K213           | —                                       |
| 215–223   | 1652.9867   | 1652.9683    | NQREEAYKE      | K222           | —                                       |
| 224–228   | 1088.7836   | 1088.8119    | QIKTL          | K226           | —                                       |
| 229–236   | 1360.9019   | 1360.9239    | TNKLKAAE       | K231           | —                                       |
| 233–240   | 1331.9894   | 1331.8722    | KAAEARAE       | K233           | —                                       |
| 248–259   | 1988.2868   | 1988.2103    | KLQKEVDRLDE    | K248           | ERSVQKLQKEVDRLDELDVNEKEKYKSITDELDTQTFSE |
| 250–256   | 1373.9442   | 1373.9192    | QKEVDRL        | K251           | ERSVQKLQKEVDRLDELDVNEKEKYKSITDELDTQTFSE |
| 261–267   | 1395.9297   | 1395.8923    | VNEKEKY        | K264           | ERSVQKLQKEVDRLDELDVNEKEKYKSITDELDTQTFSE |
| 266–273   | 1469.891    | 1469.9291    | KYKSITDE       | K266           | ERSVQKLQKEVDRLDELDVNEKEKYKSITDELDTQTFSE |
| 268–280   | 1999.1506   | 1999.1311    | KSITDELDTQTFSE | K268           | ERSVQKLQKEVDRLDELDVNEKEKYKSITDELDTQTFSE |

**Fig. 2** AGE content (A) and CML content (B) analysis of TM and GTMs. Note: TM-G: TM glycosylated by glucose; TM-M: TM glycosylated by maltose; TM-MTS: TM glycosylated by maltotriose. \*Showed significant differences when compared with TM, \* $p < 0.05$ ; \*\* $p < 0.01$ .

With respect to CML, compared with TM, glycosylated TM had significantly higher CML contents (TM-M > TM-G > TM-MTS > TM, Fig. 2B), which shared a similar trend to the AGE amount (Fig. 2A), thus suggesting that the saccharide of greater molecular size (maltotriose) contributed towards less generation of AGEs than the saccharides of smaller molecular size (glucose and maltose). In addition, glucose and maltose lead to higher CML generation than maltotriose in GTMs; this may be because glucose and maltose have smaller molecular sizes and therefore more accessibility to react with TM and generate more CML.

### 3.3 Glycation-induced epitope loss

As shown in Tables 1–3, the lysines of TM-G (28 out of 28 lysines), TM-M (25 out of 28 lysines, except for K6, K7 and K168) and TM-MTS (23 out of 28 lysines, except for K5, K6, K7, K12 and K30) have been glycosylated (glycosylated lysine amount: TM-G > TM-M > TM-MTS). Smaller saccharides (glucose) resulted in more glycosylated lysines than larger saccharides (maltotriose); this

might be because smaller saccharides had less steric hindrance and higher accessibility to glycosylate TM, whereas longer saccharide chains on TM-MTS provided more steric hindrance to the glycosylation of TM, leading to a lower glycosylation degree.<sup>19</sup>

The IgE-epitope regions of TM that contain potential glycosylation sites (lysine, K) are as follows: VHNLQKRMQQLNDLDS (43–59), ENRSLSDERMDALENQLKEARFLAEADRKYDE (131–164), ESKIVELEELRVVG (187–201) and ERSVQKLQKEVDRLDELDVNEKEKYKSITDELDTQTFSE (243–280).<sup>25–30</sup> As shown in Tables 1–3, all the potential lysine glycosylation sites (K48, K149, K161, K189, K248, K251, K264, K266 and K268) that were located in the epitopes had been glycosylated; this suggested that these epitopes with lysines could be affected by glycosylation-induced lysine loss and/or the masking effects of sugar residues.

### 3.4 *In vitro* allergenicity

Compared to TM-sensitized basophils, TM-G and TM-MTS had lower production of histamine (Fig. 3A, TM > TM-M > TM-G >

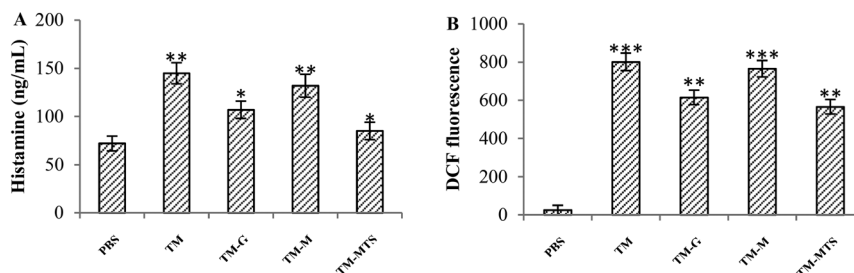


Fig. 3 The histamine content (A) and ROS content (B) of basophil sensitized by TM and GTMs. Note: TM-G: TM glycated by glucose; TM-M: TM glycated by maltose; TM-MTS: TM glycated by maltotriose. \*Showed significant differences when compared with PBS, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

TM-MTS), whereas the allergenicity of TM-M showed no significant alterations. Moreover, the TM glycated by the saccharides with greater sizes led to lower ROS generation (Fig. 3B, TM > TM-M > TM-G > TM-MTS); this might be because the lower AGE production (Fig. 2A and B) could lead to weaker RAGE activation. Moreover, TM-G and TM-MTS had lower AGE (CML) content; the greater epitope loss (Tables 1–3) and lower generation of AGE neoallergens (Fig. 2) might lead to weaker activation of RAGE and allergic reactions. In the case of TM-M, its insignificant allergenicity might be because there were higher AGEs that could activate the RAGE on the basophil to induce more severe allergy symptoms on the basophil.

### 3.5. Mouse allergy symptoms and serum antibody analysis

As shown in Fig. 4B and C, compared with the case of the PBS negative group, the mouse allergy symptoms in the TM and GTM groups exacerbated upon subsequent challenges. As shown in Fig. 4D, compared with the case of the PBS group, the rectal temperature in the TM and GTM groups decreased. As shown in Fig. 4E–H, compared with the case of the PBS group, after TM and GTM sensitization and challenge, the serum histamine and Th2-dependent antibodies (IgE and IgG1) were significantly increased, whereas the Th1-dependent antibody (IgG2a) was slightly increased. Compared with the mice of the TM group, the mice of the TM-G and TM-MTS groups had weaker anaphylactic symptoms and lesser decrease in the rectal temperature; this indicated that the decrease in the number of accessible epitopes on GTM resulted in weaker allergic responses and lower secretion of TM-specific antibodies.

## 4. Discussion

Tropomyosin is the most potent allergen of shrimp that can cause severe food allergy.<sup>1</sup> However, to date, an effective approach to eliminate or weaken its allergenicity is still lacking. Previous studies have reported that glycation is a promising approach that can reduce shrimp tropomyosin allergenicity by destroying the epitopes.<sup>3</sup> On the other hand, other studies have stated that glycation can generate advanced glycation end products (AGEs), which can activate the receptors for AGEs (RAGE) that are present on the surface of immune cells (e.g. basophil); therefore, AGEs can function as neoallergens

that affect allergenicity.<sup>3,10,11</sup> As the glycation of allergens by different sources of saccharides can either weaken the allergenicity by destroying epitopes or exacerbate the allergenicity by generating neoallergens (e.g., CML), it is hard to tell how the glycation of an allergen with different saccharides affects the allergenicity *via* epitope loss and neoallergen generation. In this study, how the glycation of shrimp tropomyosin by saccharides of different molecular sizes affected the allergenicity *via* epitope loss and neoallergen generation was investigated.

Compared to the case of TM, after glycation, the lysines of TM-G (28 out of 28 lysines), TM-M (25 out of 28 lysines, except for K6, K7 and K168) and TM-MTS (23 out of 28 lysines, except for K5, K6, K7, K12 and K30) were glycated (glycated lysine amount: TM-G > TM-M > TM-MTS), and smaller saccharides (glucose) resulted in more glycated lysines than larger saccharides (maltotriose); this might be because smaller saccharides had lesser steric hindrance and higher accessibility to the glycation of TM, whereas longer saccharide chains provided more steric hindrance to the glycation of TM, leading to fewer glycated lysines.<sup>19</sup> An epitope is the part of an allergen that is recognized by the immune system.<sup>31</sup> As shown in Tables 1–3, all the potential lysine glycation sites (K48, K149, K161, K189, K248, K251, K264, K266 and K268) that were located in the epitopes had been glycated in GTMs; this suggested that these epitopes could be affected by glycation-induced lysine loss and/or the masking effects of sugar residues, which could result in weaker allergenicity.

With regard to the AGEs, compared with the case of TM, after glycation, AGEs were generated in the GTMs, and the AGE amount increased as follows: TM-M > TM-G > TM-MTS > TM. The higher CML generation in TM-G and TM-M may be because glucose and maltose have smaller molecular sizes and therefore more accessibility to react with TM and generate more CML; on the other hand, the lower AGE content in TM-MTS may be because MTS has relatively greater molecular sizes, which has lower accessibility to interact with TM and generate AGEs.

Basophil plays an important role in activating allergic reactions, which can secrete histamine that is responsible for many clinical symptoms.<sup>32,33</sup> Compared to TM-sensitized basophils, TM-G and TM-MTS inhibited the release and production of histamine (Fig. 3A, TM > TM-M > TM-G > TM-MTS), whereas the allergenicity of TM-M showed no significant alterations. As

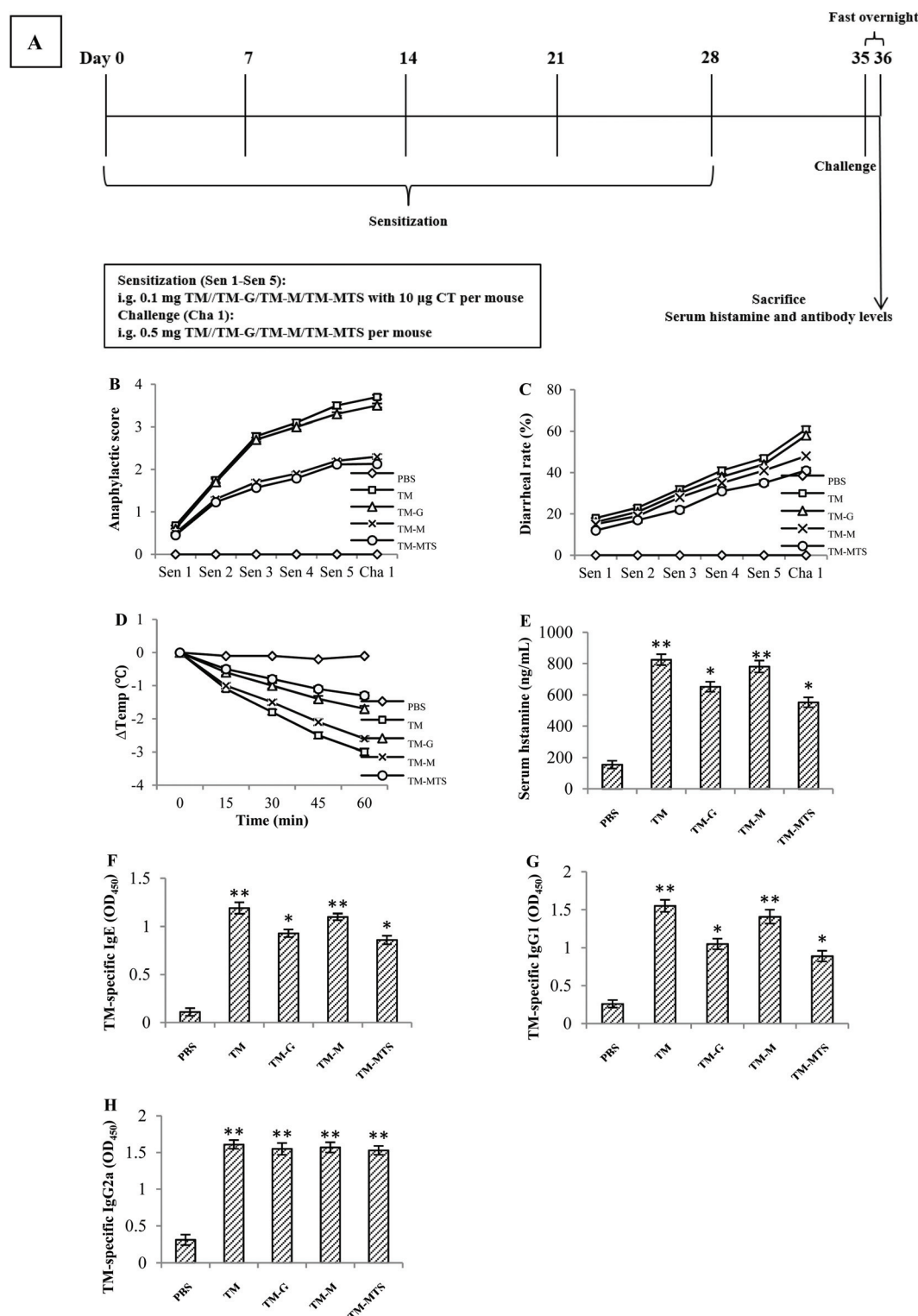


**Fig. 4** Immunizing protocol (A), anaphylactic score (B), diarrhea rate (C), rectal temperature changes (D), serum histamine (E), serum TM-specific IgE (F), serum TM-specific IgG1 (G), and serum TM-specific IgG2a (H) of BALB/c mice immunized by TM and GTMs. Note: TM-G: TM glycosylated by glucose; TM-M: TM glycosylated by maltose; TM-MTS: TM glycosylated by maltotriose. \*Showed significant differences when compared with PBS, \* $p < 0.05$ ; \*\* $p < 0.01$ .

lysine was present in TM epitopes,<sup>25</sup> the reduced allergenicity of TM-MTS might be because the epitopes with lysines had been glycosylated (Tables 1–3), and the longer saccharide residues

of maltotriose on TM-MTS had stronger masking effects on the epitopes, therefore diminishing their affinity to bind IgE and leading to weaker allergenicity.

Moreover, there are receptors for AGEs (RAGE) on the basophil;<sup>13</sup> the interaction of AGEs with their receptor (RAGE) on the basophil surface can induce ROS generation and activate the inflammatory cascades that implicate the allergic diseases.<sup>12,33,34</sup> The TM glycated by the saccharide of greater sizes led to lower ROS generation (Fig. 3B, TM > TM-M > TM-G > TM-MTS), which might be because the lower AGE production (Fig. 2A and B) would lead to weaker RAGE activation. Moreover, TM-MTS had lower AGE (CML) content; greater epitope loss (Tables 1–3) and lower generation of AGE neoallergens (Fig. 2) might contribute to lower activation of RAGE and weaker allergenicity. As for TM-M, its insignificant allergenicity might be because there were higher AGEs that could activate RAGE on the basophil to induce more severe allergic symptoms.

Therefore, the weaker allergic response of TM-MTS-sensitized basophil might be because of three main reasons: (1) during glycation, TM epitopes with glycation sites (*e.g.* lysine) were destroyed or masked by saccharide residues on GTM; (2) there were fewer neoallergens (*e.g.* CML) generated in TM-MTS, resulting in weaker RAGE activation; and (3) the epitope loss was heavier than neoallergen generation. The glycation-destroyed epitopes and less generation of neoallergens may result in the weaker allergenicity of TM-MTS.

Mouse allergy symptoms could reflect the allergenicity of allergens.<sup>16</sup> Compared with the case of the TM-sensitized group, the mouse of the TM-MTS group had the weakest allergy symptoms. This might be because of the following reasons: (1) MTS had longer saccharide residues; the longer saccharide residue on TM-MTS would occupy more surface space of TM to block or mask the epitopes; (2) Longer saccharide residues on TM-MTS functioned as a resistance to prevent the binding between TM epitopes and IgE on the immune cells (*e.g.* basophil); and (3) TM-MTS had less generation of neoallergens (*e.g.* CML) than TM-G and TM-M. On the other hand, the mouse of the TM-G and TM-M groups had relatively stronger allergy symptoms, which might be because there was more production of AGE neoallergens in TM-G and TM-M than in TM-MTS.

After sensitization and challenge by the allergens, the mouse immune cells could secrete mediator (histamine) and TM-specific antibodies (IgE, IgG1 and IgG2a). IgE and IgG1 are Th2-dependent antibodies, and IgG2a belongs to a Th1-dependent antibody.<sup>35</sup> As shown in Fig. 4E–H, compared with the case of the PBS group, after TM and GTM sensitization and challenge, the serum histamine and Th2-dependent antibodies (IgE and IgG1) were significantly increased and the Th1-dependent antibody (IgG2a) was slightly increased. Compared with the case of the TM group, the mice in the TM-G and TM-MTS groups had weaker anaphylactic symptoms, indicating that the decreased accessible epitopes on GTM resulted in weaker allergy responses and lower secretion of the TM-specific antibodies. The abovementioned results might be because the saccharides of smaller molecular size (glucose) had lesser steric hindrance and higher accessibility to react with TM; therefore, they were more likely to disrupt the epitopes. The saccharides of larger molecular size (maltotriose) provided more steric hindrance and less accessibility to disrupt the epitopes,

while the longer saccharide residues on GTM could not only mask the epitopes but also function as a resistance to prevent the binding between the epitopes and IgE on the immune cells, therefore leading to weaker allergenicity. The lower allergenicity of TM-MTS might be because: (1) MTS had longer saccharide residues than other saccharides; the longer saccharide residues on TM-MTS could highly mask the epitopes and desensitize the allergy reactions; (2) MTS contributed to fewer AGEs than glucose and maltose during glycation. The insignificant allergenicity alteration of TM-M might be because the generation of AGE (*e.g.* CML) neoallergens offset the epitope loss.

In summary, the allergenicity of GTMs might be affected by three main aspects: (1) glycation induced epitope loss, (2) saccharide residues induced masking effects on the epitopes, (3) generation of AGE-related neoallergens during glycation. Firstly, the saccharides could destruct the epitopes with the glycation sites (*e.g.*, lysine). After the saccharide residues were added to the TM molecules, the saccharide residues would function both as a resistance to prevent the binding of IgE to TM and to mask the epitopes by the residues' molecular motions (*e.g.*, bending and stretching vibrations), and the longer saccharide residues, the stronger resistance, and masking effects of the saccharide residues prevent the binding between the allergen and the IgE on the immune cells. Moreover, there were neoallergens (*e.g.*, CML) generated during the glycation of TM, which also influenced the allergenicity. Therefore, the allergenicity alterations of tropomyosin allergens affected by glycation may result from the changed epitope balance induced by epitope loss and neoallergen generation. Compared to TM, the weaker allergenicity of TM-G and TM-MTS may be because during the glycation of TM by glucose or MTS, the saccharides heavily destructed and masked the TM epitopes; the loss of TM epitopes was more than the neoallergen generation. The insignificant allergenicity alteration of TM-M might be because the AGE (*e.g.*, CML) neoallergen generation offset the epitope loss.

## 5. Conclusions

Compared with TM, the TM glycated by glucose and maltotriose produced the GTMs (TM-G and TM-MTS) with lower allergenicity and weaker mouse allergy symptoms. On the other hand, the TM glycated by maltose (TM-M) had no significant impact on the allergenicity because of the greater generation of AGE-related neoallergens, which may offset the glycation-induced epitope loss. Therefore, maltose was not suitable to desensitize the TM-induced allergy. The glycation of TM by maltotriose significantly reduced the allergenicity and alleviated the allergic responses, which could be a potential immunotherapy for shrimp allergic individuals.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

This study was supported by the National First-Class Discipline Program of Food Science and Technology (No. JUFSTR20180201), China Scholarship Council (CSC) Program (No. 201806790023), China Association for Science and Technology Program (No. 2017CASTQNJL028) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (No. KYCX17\_1403).

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