



Conformation, allergenicity and human cell allergy sensitization of tropomyosin from *Exopalaemon modestus*: Effects of deglycosylation and Maillard reaction

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ABSTRACT

Tropomyosin (TM) from *Exopalaemon modestus* was glycoprotein, the effects of deglycosylation (enzyme and chemical reagents) and Maillard reaction on the conformation, allergenicity and human cell sensitization of TM were investigated. TM had both N-glycan and O-glycan. After deglycosylation and glycation, the α -helix of TM reduced, while other secondary structures promoted. Deglycosylation enhanced TM allergenicity and digestibility, contributed to digests with lower allergenicity, glucose glycation weakened the allergenicity and digestibility of TM, led to digests with weaker allergenicity. Gastric digestion partly reduced allergenicity of digests, intestinal digestion highly reduced digests' allergenicity, gastrointestinal digestion most efficiently weakened the allergenicity of digests. After deglycosylation, the deglycosylated TM exerted stronger activation of basophil (KU812), while glucose glycated TM weakened KU812 activation. Deglycosylation exerted inhibition on colon cell (Caco-2) proliferation and increase of IL-8 release, while glucose glycated TM suppressed Caco-2 proliferation and IL-8 release.

1. Introduction

Shrimp tropomyosin (TM) is an allergen could trigger severe food allergy anaphylaxis worldwide. *Exopalaemon modestus* (EM) is a famous delicacy with high nutrition in China. As its increased consumption, the appearance of EM-induced allergic reactions also got promoted.

Many food allergens were glycoprotein, glycans often performed physiological roles in anti-proteolytic degradation, and carbohydrate epitopes were highly stable therefore retained immune reactivity even after strong denaturing conditions (Petersen & Mundt, 2001). The main allergen of EM is tropomyosin, a glycoprotein. However, up to now, there were no available data about the glycans of TM from EM and their IgE reactivity in triggering allergy reactions. Therefore, the glycans of TM were investigated using enzymatic and chemical deglycosylation to elucidate their role in triggering allergy.

Maillard reaction (glycation) often occurred between proteins and saccharides, which could modify protein functional properties, such as allergenicity. Gruber, Vieths, Wangorsch, Nerkamp, and Hofman (2004) reported that the allergenicity of allergen in cherry got reduced after glycation by glucose. Taheri-Kafrani et al. (2009) found that

glycation of β -Lg by reducing sugar decreased the allergenicity. As TM was lysine rich, Maillard reaction might be a potent approach to modify TM and weaken the allergenicity.

Generally, food allergens are resistant to digestion in digestive system, which made the allergic peptides in digests could pass through the digestive passage, led to the retention of epitopes to trigger allergy responses (Astwood, Leach, & Fuchs, 1996). After digestion, there were still allergic peptides of TM in digests, which could trigger allergy responses. However, the allergenicity of simulated gastrointestinal digests of the deglycosylated and glycated TM were not well known.

Basophil is a pivotal cell during allergy activation, once activated they could release allergy mediators such as histamine (Gilfillan & Tkaczyk, 2006). KU812 was used in this work to evaluate the allergy sensitization of basophil by the deglycosylated and glucose glycated TM. In addition, Caco-2 cells could represent the function of intestine epithelial cells (Teodorowicz, Fiedorowicz, Kostyra, Wichers, & Kostyra, 2013) and be used to analyze the immunological response of food allergens to intestine cells, and reflected in proliferation of colon cell and release of cytokine, such as Interleukin (IL)-8, which influenced the activation and growth of basophils (Eckmann et al., 1993; Rossi &

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Zlotnik, 2000). Therefore, the IL-8 release from Caco-2 cells activated by the deglycosylated and glycosylated TM may provide new insight into the food allergy immunomodulation.

In this work, the allergenicity of TM treated with deglycosylation and glucose glycation were investigated. The results would help characterize the effects of deglycosylation and glucose glycation on the molecular characteristics of TM including conformation stability, allergenicity, human basophil activation and interaction with human colon epithelium cells (Caco-2).

2. Materials and methods

2.1. Materials

Exopalaemon modestus was obtained from a local fishery market, Wuxi, China. 4-nitrophenyl N-acetyl- β -D-glucosaminide (PNAG, CAS: 3459-18-5), 8-anilino-1-naphthalenesulphonic acid (ANS, CAS: 82-76-8), β -Mercaptoethanol (CAS: 60-24-2), D-glucose (CAS: 50-99-7), pepsin (CAS: 9001-75-6), Triton X-100 (CAS: 9002-93-1) and trypsin (CAS: 9002-07-7) were from Sigma-Aldrich (MO, USA). PNGase F kit, O-glycosidase & α 2-3,6,8 Neuraminidase Bundle were obtained from New England Biolabs Inc. (MA, USA). Anisole (CAS: 100-66-3), *n*-hexane (CAS: 110-54-3) and trifluoromethanesulfonic acid (TFMS, CAS: 1493-13-6) were obtained from Aladdin Industrial Corp. (Shanghai, China). BeyoECL Plus and TMB chromogen solution were obtained from Beyotime Biotech (Shanghai, China), horseradish peroxidase (HRP)-conjugated goat anti-human IgE was from KPL Inc. (MD, USA). Histamine ELISA kit was obtained from IBL International (Hamburg, Germany), human IL-8 ELISA kit was purchased from R&D Systems (MN, USA), BCA protein assay kit and Glycoprotein Staining kit were got from Thermo Inc. (IL, USA).

Human basophil cell line (KU812) and human colon epithelial cell line (Caco-2) were obtained from the Cell Resource Center, Institute of Life Science, Chinese Academy of Sciences (Shanghai, China). The KU812 were grown in IMDM culture medium, Caco-2 were cultured in DMEM culture medium, both with supplement of FBS (10%), penicillin (100 U/mL) and streptomycin (100 μ g/mL) at 37 °C in a CO₂ cell incubator (Forma 371, Thermo Fisher Scientific, MA, USA).

2.2. Human sera from shrimp allergy patients

Human sera from volunteer patients with shrimp-induced food allergy were taken from Wuxi People's Hospital (Wuxi, China) and listed in Table 1.

2.3. Purification of TM

The purification of TM was according to a previous method (Zhang, Zhang, Chen, & Zhou, 2018). TM was lyophilized and kept at –20 °C. The purity and protein concentration was determined using polyacrylamide gel and BCA protein assay kit, respectively.

Table 1
Information of shrimp allergy patients' sera.

No.	Gender	Age (year)	Clinical symptoms	IgE (kU _A /L)
1	Male	5	AS	22.4
2	Female	2	AS	23.2
3	Female	55	HI, EC	6.8
4	Male	4	AS, HI	65.5
5	Female	40	HI, CW	11.4
6	Female	20	HI, NW	19.3
7	Male	40	EC, RH	28.2
8	Male	2	AS, NW	35.9

Note: AS, asthma; CW, chest wheezing; EC, eczema; HI, hives; NW, nose wheezing; RH, rhinitis.

2.4. Detection of glycoprotein

The detection of glycoprotein saccharide moieties in polyacrylamide gels followed the instructions of Glycoprotein Staining kit. After electrophoresis, gel was fixed for 30 min using 50% methanol. Gel was washed by 3% acetic acid for 10 min for twice. Then transfer to oxidizing solution for 15 min. After that, gel was washed by 3% acetic acid for 5 min for three times. Transfer to Glycoprotein Staining Reagent for 15 min, then transfer to the Reducing Solution for 5 min. The gel was washed and stored in 3% acetic acid. The horseradish peroxidase (HRP) and soybean trypsin inhibitor (STI) served as the positive control and negative control, respectively.

2.5. Deglycosylation and glucose glycation of TM

2.5.1. N-deglycosylation

Combining 200 μ g TM, 10 μ L glycoprotein denaturing buffer (10 $\times$) and ultrapure water to 100 μ L, heated in boiling water for 10 min. After cooled to room temperature (25 °C), then adding 20 μ L of glycoBuffer 2 (10 $\times$), 20 μ L 10% NP-40, ultrapure water and 10 μ L PNGase F to make a 200 μ L total reaction volume, incubating at 37 °C for 18 h. Then the deglycosylated TM sample was purified using centrifugal filter units (Amicon Ultra-4, 10 kDa, Meck Millipore Ltd., Tullagreen, Ireland), lyophilized and stored at –20 °C.

2.5.2. O-deglycosylation

Total of 200 μ g TM was mixed with 10 μ L glycoprotein denaturing buffer (10 $\times$) and 90 μ L ultrapure water to reach 100 μ L in microtubes and heated in boiling water for 10 min. After cooled to room temperature (25 °C), then adding 20 μ L glycoBuffer 2 (10 $\times$), 20 μ L 10% NP-40, 20 μ L α 2-3,6,8 Neuraminidase, ultrapure water and 20 μ L O-Glycosidase to 200 μ L, mixed well and incubated at 37 °C for 18 h. Then the deglycosylated TM sample was purified using centrifugal filter units, lyophilized and stored at –20 °C.

2.5.3. N- & O-deglycosylation

Adding 200 μ g TM, 10 μ L glycoprotein denaturing buffer (10 $\times$) and ultrapure water to 100 μ L, heated in boiling water for 10 min. After cooled to room temperature (25 °C), 20 μ L of glycoBuffer 2 (10 $\times$), 20 μ L 10% NP-40, 10 μ L PNGase F, 20 μ L α 2-3,6,8 Neuraminidase, ultrapure water and 20 μ L O-Glycosidase were added to reach 200 μ L, incubated at 37 °C for 18 h. Then TM sample was purified using centrifugal filter units, lyophilized and stored at –20 °C.

2.5.4. KOH treatment (β -elimination)

Native TM (1 mg/mL) in 50 mM KOH was incubated at 25 °C for 12 h, then TM was purified using centrifugal filter units, lyophilized and stored at –20 °C.

2.5.5. TFMS deglycosylation

Deglycosylation with TFMS could remove carbohydrates from glycoproteins with intact protein backbone left. Firstly, mix TFMS and anisole in ratio of 2:1 (v/v), the reaction mixture (1 mL) was used to deglycosylate TM (5 mg). Deglycosylation was under nitrogen for 20 min. Protein was precipitated at –20 °C for 1 h in a 50-fold diethyl ether with 10% *n*-hexane (v/v) for twice. Then protein was dialyzed in ultrapure water for 48 h, lyophilized and stored at –20 °C.

2.5.6. TM glycation by glucose

TM was dissolved in a buffer with 1 mM NaHCO₃, 5 mM β -Mercaptoethanol and 0.6 M glucose, with protein concentration adjusted to 0.2 mg/mL, lyophilized and stored at –20 °C. The glycation of lyophilized TM and glucose was with incubated in a desiccator with saturated Mg (NO₃)₂ solution (*a_w* at 0.51) at 60 °C for 48 h. The glycosylated TM was dialyzed in ultrapure water for 48 h, then lyophilized and stored at –20 °C prior to analysis.

2.6. Conformational changes

2.6.1. Amino acid analysis and circular dichroism (CD)

The amino acid content was determined according to Zhang et al. (2018). The amino acid contents were expressed as the weight percentage (%) in total amino acids. Circular dichroism (CD) spectra were applied to analyze the secondary structures of deglycosylated TM and glycosylated TM (0.1 mg/mL) from 190 to 250 nm using a MOS-450 spectrometer (Bio-Logic, France). DichroWeb was used to analyze secondary structures (<http://dichroweb.cryst.bbk.ac.uk>).

2.6.2. TM surface hydrophobicity and particle characteristics

The ANS was used as a fluorescence probe to determine the surface hydrophobicity (S_0 -ANS) of deglycosylated and glycosylated TM samples. The particle characteristics of deglycosylated TM and glycosylated TM samples were determined by a NanoBrook Omni Particle Analyzer (Brookhaven Corp., NY, USA).

2.7. In vitro simulated gastric and intestinal digestion

The in vitro simulated digestion was according to previous reports (Astwood et al., 1996; Zhang et al., 2018). The simulated gastric digestion was performed with incubation at 37 °C and with supplement 0.2 M Na_2CO_3 to stop digestion. The intestinal digestion performed at 37 °C and was terminated by heated in boiling water for 10 min. As to gastrointestinal digestion, after simulated gastric digestion, adjusted the pH of gastric digests to 7.5, to further simulated intestinal digestion. The digests' allergenicity of deglycosylated TM and glycosylated TM were analyzed using ELISA.

2.8. Allergenicity determination of the deglycosylated and glycosylated TM

Western blot: TM samples were separated using polyacrylamide gel, then the TM proteins were transferred to a membrane and incubated in a blocking solution (5% defatted milk), incubated in a diluted sera (1:10 dilution) and incubated with HRP conjugated goat anti-human IgE (1:5000, KPL Inc., USA). The immobilized protein-antibody complex was determined using ECL luminous liquid and recorded by a gel imager (ChemiDoc XRS⁺, Bio-Rad, USA).

ELISA: the 96-well plates were coated with deglycosylated and glycosylated TM samples (10 µg/mL, 100 µL/well), then blocked by 5% milk, incubated using diluted human sera (50 µL/well, 1:10, v/v) and incubated in HRP conjugated goat anti-human IgE (100 µL/well, 1:5000, KPL Inc., USA). Then color was developed with TMB and terminated the reaction by 2 M H_2SO_4 (50 µL/well), the absorbance was determined by a plate reader.

2.9. KU812 cell culture

2.9.1. KU812 sensitization

KU812 (1×10^5 cells/well) were seeded on a 96-well plate in IMDM cultured at 37 °C for 24 h. Then cells were washed with PBS and sensitized by human sera diluted in IMDM without FBS (100 µL/well). After incubation for 24 h, KU812 were centrifuged and washed with PBS for three times. Allergic activation was induced by incubating with deglycosylated TM or glycosylated TM (37 °C, 60 min).

2.9.2. Histamine release

The KU812 cells were centrifuged and took the supernatant. Histamine content was determined by measuring the histamine in supernatant using the histamine ELISA kit (IBL International, Germany).

2.9.3. Degranulation test

The KU812 degranulation was determined by measuring the β -hexosaminidase in supernatant. Cell supernatant was added to 96-well plate (30 µL/well) and PNAG (50 µL/well, 1 mM) was added with

incubation at 37 °C for 1 h. Then 0.2 M glycine solution (100 µL/well, pH 10.7) was added and measured the absorbance at 450 nm. The results were presented as the percentage of β -hexosaminidase measured in supernatant samples compared to the total levels in cell lysates. For measurement of total β -hexosaminidase, the KU812 were lysed using 0.1% Triton-X 100 prior to removing the supernatant. The β -hexosaminidase was determined by testing the absorbance of the hydrolyzed substrate using a plate reader (SpectraMax M5, Molecular Devices, USA).

2.10. Caco-2 cell culture

2.10.1. Proliferation test

Caco-2 (1×10^5 cells/well) were seeded on 96-well plate in DMEM culture medium, cultured at 37 °C for 24 h. KU812 were then washed with PBS for three times. Then incubated in the presence of deglycosylated TM and glycosylated TM (25 µg/mL) for 24 h. After that, CCK-8 (10 µL/well) were added and incubated for 1 h, the absorbance at 450 nm was determined using a plate reader (SpectraMax M5, USA).

2.10.2. Determination of IL-8 release

Caco-2 was washed with PBS for three times. To determine IL-8 secretion, cells were incubated with deglycosylated TM and glycosylated TM (25 µg/mL) for 24 h. After incubation, the content of IL-8 in culture medium was determined using the ELISA kit of human IL-8 (R&D systems, MN, USA).

2.11. Statistical analysis

The statistical analysis was carried out by SPSS (SPSS Inc., IL, USA). Duncan's multiple range test was applied to determine significant differences between means.

3. Results and discussion

3.1. Glycoprotein detection

There are two types of glycoprotein glycans: O-glycans (at the hydroxyl groups of serine/threonine residues) and N-glycans (generally at asparagine residues with sequence of Asn-X-Ser/Thr). Deglycosylation was to remove the N-glycans and O-glycans of glycoprotein.

Fig. 1A showed the molecular weight of enzymatically and chemically deglycosylated TM, and there were no protein fragments of TM appeared after deglycosylation by enzyme and chemical reagents, which suggested the high stability of TM. Compared with the native TM (lane 1), the de-N-glycosylated TM (TM-DNG, lane 2) by PNGase F had a slight loss of molecular weight, suggested TM had small molecular weight of N-glycans. The molecular weight of the de-O-glycosylated TM (TM-DOG, lane 3) by O-Glycosidases and β -eliminated TM (TM- β E, lane 4) also had a weak reduction, indicating TM had O-glycans and the two methods had the same O-glycan deglycosylation effects on TM. The molecular weight reduction of de-N&O-glycosylated TM (TM-DNOG, lane 5) by PNGase F and O-glycosidases, and TFMS-deglycosylated TM (TM-TFMS, lane 6) further confirmed the existence of both N-glycans and O-glycans of TM, and enzymatic deglycosylation had the same effect with TFMS to completely remove TM glycans. The molecular weight of glucose glycosylated TM (TM-G, lane 7) had a significant increase, suggested TM could be highly glycation modified by glucose.

Fig. 1B showed the glycoprotein detection result. Compared with the positive control (HRP, lane 8) and negative control (STI, lane 9), native TM (lane 1), TM-DNG (lane 2), TM-DOG (lane 3), TM- β E (lane 4) and TM-G (lane 7) had visible bands, while TM-DNOG (lane 5) and TM-TFMS (lane 6) had no visible bands, which suggested de-N-glycosylated TM (lane 2) had O-glycans, the de-O-glycosylated TM (lane 3 and lane 4) had N-glycans, the de-N&O-glycosylated TM (lane 5) and TFMS treated TM (lane 6) had no glycans, and the existence of sugar moieties

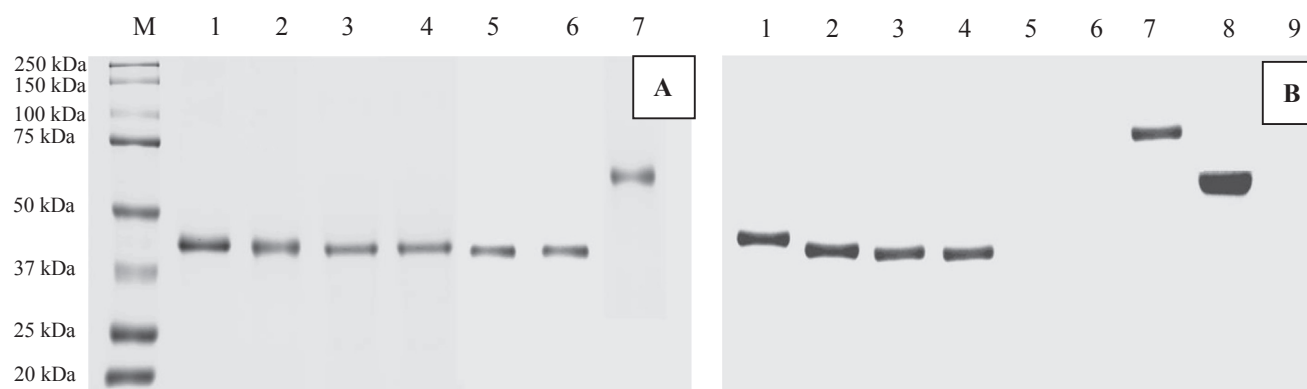


Fig. 1. SDS-PAGE (A) and glycoprotein detection (B) of the deglycosylated and glycosylated TM. (Note: A: M, marker; lane 1, TM; lane 2, de-N-glycosylated TM; lane 3, de-O-deglycosylated TM; lane 4, β -eliminated TM; lane 5, de-N&O-deglycosylated TM; lane 6, TFMS deglycosylated TM; lane 7, Glucose glycosylated TM; B: lane 1, TM; lane 2, de-N-glycosylated TM; lane 3, de-O-deglycosylated TM; lane 4, β -eliminated TM; lane 5, de-N&O-deglycosylated TM; lane 6, TFMS deglycosylated TM; lane 7, glucose glycosylated TM; lane 8, positive control: horseradish peroxidase (HRP); lane 9, negative control: soybean trypsin inhibitor (STI).)

in TM-G. Hoffman, Day and Miller (1981) also found the tropomyosin from shrimp is glycoprotein.

3.2. Amino acid analysis and conformational stability of TM

The alteration of amino acid contents could reflect conformation changes. Table 2 showed the amino acid content in deglycosylated TM and glycosylated TM. Compared with the native TM, most amino acid contents of deglycosylated TM had no significant changes ($p > 0.05$), while the asparagine (Asn) of TM-DNG, TM-DNOG and TM-TFMS slightly increased, the Serine (Ser) content of TM-DOG, TM- β E, TM-DNOG and TM-TFMS also increased. This might be resulted from that deglycosylation removed N-glycans and O-glycans with more Asn and Ser left. As to glucose glycosylated TM (TM-G), the contents of most amino acids decreased, while lysine was most affected by glycation, decreased by 55.22%, which was due to the reaction between the ϵ -amino group of lysine and the carbonyl groups of glucoses, induced the high promotion of molecular weight (Fig. 1A, lane 7). Li, Jiang, You, Luo and Feng (2014) also found the sharp lysine reduction of parvalbumin by Maillard reaction.

As Fig. 2A shown, the native TM consisted of α -helix (78.7%), β -turn (5.1%) and random coils (14.2%), consistent with previous data

(Jin et al., 2015; Zhang et al., 2018). Compared with the native TM, the α -helix content of deglycosylated TM decreased significantly from 78.7% (native TM) to 71.3% (TM-DNG), 66.9% (TM-DOG), 66.7% (TM- β E), 60.7% (TM-DNOG) and 59.5% (TM-TFMS), while β -sheets increased markedly from 0% (native TM) to 4.7% (TM-DNG), 6.1% (TM-DOG), 6.3% (TM- β E), 8.9% (TM-DNOG) and 9.5% (TM-TFMS), β -turns increased from 5.1% (native TM) to 6.8% (TM-DNG), 8.3% (TM-DOG), 8.8% (TM- β E), 10.5% (TM-DNOG) and 10.7% (TM-TFMS), and random coils increased from 14.2% (native TM) to 17.2% (TM-DNG), 18.7% (TM-DOG), 18.2% (TM- β E), 19.9% (TM-DNOG) and 20.3% (TM-TFMS), respectively. These results suggested that the deglycosylation of TM dissociated the protein, induced the transfer of α -helix to other structures (most of random coils). As to Maillard reaction (TM-G), the α -helix content reduced from 78.7% (native TM) to 18.5% (TM-G), while β -sheet, β -turn and random coils rose from 0 to 20%, 5.1% to 21.7% and 14.2 to 39.8%, respectively. Similar results were reported by Yang, Li, Li, and Wang (2013), who found that polysaccharides glycosylated allergen (Fag t 3) led to more random coils.

In Fig. 2B, the S_0 -ANS of deglycosylated TM and glycosylated TM had different increment. Specifically, the S_0 -ANS increased from 193.59 (native TM) to 205.27 (TM-DNG), 201.35 (TM-DOG), 203.61 (TM- β E), 215.52 (TM-TFMS) and TM-G (287.96), respectively. These results

Table 2
Amino acid analysis of deglycosylated TM and glucose glycosylated TM.

Amino acid	Amino acid content (% w/w)						
	TM	TM-DNG	TM-DOG	TM- β E	TM-DNOG	TM-TFMS	TM-G
Ala	7.39 $\pm$ 0.01 ^a	7.40 $\pm$ 0.02 ^a	7.40 $\pm$ 0.02 ^a	7.39 $\pm$ 0.01 ^a	7.39 $\pm$ 0.02 ^a	7.40 $\pm$ 0.01 ^a	8.02 $\pm$ 0.03 ^b
Cys	0.31 $\pm$ 0.03 ^a	0.31 $\pm$ 0.02 ^a	0.30 $\pm$ 0.02 ^a	0.31 $\pm$ 0.01 ^a	0.30 $\pm$ 0.02 ^a	0.31 $\pm$ 0.03 ^a	0.19 $\pm$ 0.02 ^b
Asp	5.97 $\pm$ 0.01 ^a	6.01 $\pm$ 0.03 ^a	5.98 $\pm$ 0.02 ^a	5.98 $\pm$ 0.03 ^a	5.98 $\pm$ 0.02 ^a	5.97 $\pm$ 0.03 ^a	6.16 $\pm$ 0.02 ^b
Glu	21.71 $\pm$ 0.02 ^a	21.68 $\pm$ 0.01 ^a	21.68 $\pm$ 0.02 ^a	21.72 $\pm$ 0.04 ^a	21.69 $\pm$ 0.03 ^a	21.69 $\pm$ 0.02 ^a	19.95 $\pm$ 0.02 ^b
Phe	1.82 $\pm$ 0.03 ^a	1.81 $\pm$ 0.02 ^a	1.81 $\pm$ 0.03 ^a	1.82 $\pm$ 0.01 ^a	1.81 $\pm$ 0.04 ^a	1.83 $\pm$ 0.02 ^a	1.92 $\pm$ 0.03 ^b
Gly	0.72 $\pm$ 0.04 ^a	0.71 $\pm$ 0.03 ^a	0.71 $\pm$ 0.02 ^a	0.72 $\pm$ 0.03 ^a	0.72 $\pm$ 0.04 ^a	0.70 $\pm$ 0.03 ^a	0.61 $\pm$ 0.01 ^b
His	0.42 $\pm$ 0.01 ^a	0.42 $\pm$ 0.02 ^a	0.42 $\pm$ 0.03 ^a	0.43 $\pm$ 0.03 ^a	0.42 $\pm$ 0.01 ^a	0.41 $\pm$ 0.04 ^a	0.65 $\pm$ 0.03 ^b
Ile	1.74 $\pm$ 0.01 ^a	1.75 $\pm$ 0.03 ^a	1.74 $\pm$ 0.02 ^a	1.75 $\pm$ 0.04 ^a	1.74 $\pm$ 0.06 ^a	1.72 $\pm$ 0.04 ^a	1.87 $\pm$ 0.01 ^b
Lys	10.97 $\pm$ 0.04 ^a	10.98 $\pm$ 0.05 ^a	10.97 $\pm$ 0.04 ^a	10.95 $\pm$ 0.06 ^a	10.95 $\pm$ 0.05 ^a	10.92 $\pm$ 0.03 ^a	4.89 $\pm$ 0.02 ^b
Leu	11.44 $\pm$ 0.03 ^a	11.43 $\pm$ 0.04 ^a	11.43 $\pm$ 0.02 ^a	11.45 $\pm$ 0.03 ^a	11.44 $\pm$ 0.03 ^a	11.43 $\pm$ 0.05 ^a	11.15 $\pm$ 0.02 ^b
Met	3.21 $\pm$ 0.02 ^a	3.20 $\pm$ 0.04 ^a	3.19 $\pm$ 0.01 ^a	3.21 $\pm$ 0.03 ^a	3.20 $\pm$ 0.05 ^a	3.19 $\pm$ 0.02 ^a	1.54 $\pm$ 0.03 ^b
Asn	5.57 $\pm$ 0.02 ^a	5.91 $\pm$ 0.01 ^b	5.58 $\pm$ 0.03 ^a	5.57 $\pm$ 0.02 ^a	5.92 $\pm$ 0.03 ^b	5.91 $\pm$ 0.01 ^b	5.02 $\pm$ 0.02 ^b
Gln	5.87 $\pm$ 0.04 ^a	5.87 $\pm$ 0.01 ^a	5.85 $\pm$ 0.03 ^a	5.87 $\pm$ 0.01 ^a	5.86 $\pm$ 0.04 ^a	5.87 $\pm$ 0.02 ^a	5.56 $\pm$ 0.01 ^b
Arg	10.03 $\pm$ 0.06 ^a	10.05 $\pm$ 0.04 ^a	10.03 $\pm$ 0.02 ^a	10.02 $\pm$ 0.03 ^a	10.03 $\pm$ 0.05 ^a	10.04 $\pm$ 0.04 ^a	9.31 $\pm$ 0.02 ^b
Ser	4.26 $\pm$ 0.05 ^a	4.25 $\pm$ 0.02 ^a	4.52 $\pm$ 0.03 ^b	4.51 $\pm$ 0.04 ^b	4.50 $\pm$ 0.02 ^b	4.51 $\pm$ 0.05 ^b	4.86 $\pm$ 0.03 ^b
Thr	2.79 $\pm$ 0.01 ^a	2.78 $\pm$ 0.03 ^a	2.78 $\pm$ 0.02 ^a	2.78 $\pm$ 0.04 ^a	2.78 $\pm$ 0.02 ^a	2.79 $\pm$ 0.05 ^a	2.95 $\pm$ 0.06 ^b
Val	3.95 $\pm$ 0.03 ^a	3.94 $\pm$ 0.05 ^a	3.94 $\pm$ 0.02 ^a	3.95 $\pm$ 0.02 ^a	3.93 $\pm$ 0.04 ^a	3.93 $\pm$ 0.04 ^a	4.17 $\pm$ 0.04 ^b
Tyr	2.01 $\pm$ 0.04 ^a	1.99 $\pm$ 0.03 ^a	1.99 $\pm$ 0.05 ^a	2.00 $\pm$ 0.06 ^a	2.01 $\pm$ 0.04 ^a	2.01 $\pm$ 0.03 ^a	1.26 $\pm$ 0.02 ^b

Note: Different superscripted letter in the same line means significant differences ($p < 0.05$). TM: tropomyosin, TM-DNG: de-N-glycosylated TM, TM-DOG: de-O-deglycosylated TM, TM- β E: β -eliminated TM, TM-DNOG: de-N&O-deglycosylated TM; TM-TFMS: TFMS deglycosylated TM; TM-G: Glucose glycosylated TM.

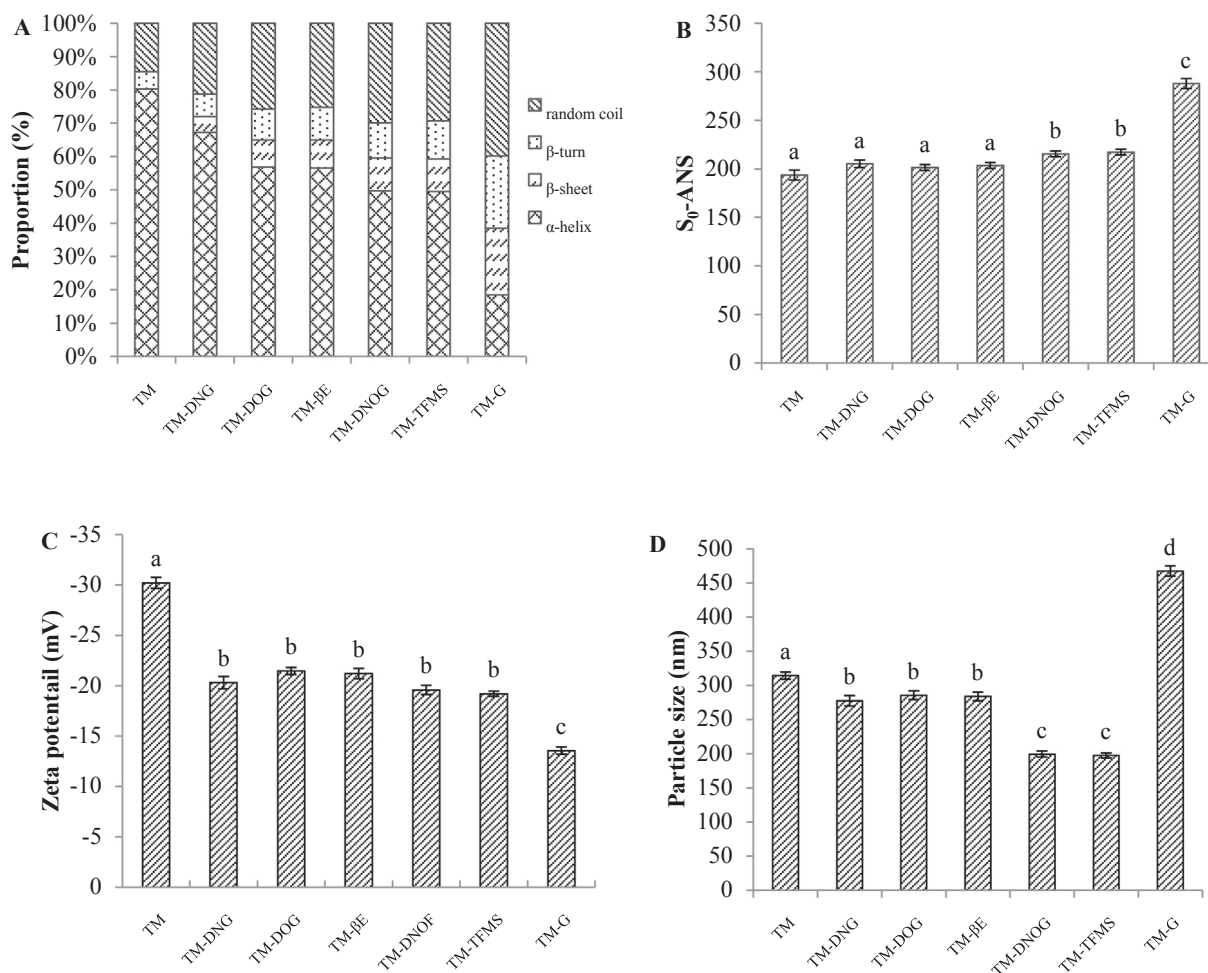


Fig. 2. Effects of deglycosylation and glucose glycation of TM on secondary structures (A), surface hydrophobicity (B), particle size (C) and zeta potential (D) (mean $\pm$ SD, $n = 3$). Different superscript letters means significant differences between values with different treatment ($p < 0.05$).

indicated that the removal of glycans destabilized TM conformation and glucose glycation disassociated TM, leading to exposure of hydrophobic groups to bind with ANS, thereby enhanced the surface hydrophobicity.

As shown in Fig. 2C, deglycosylation reduced the particle size, while glucose glycation increased the particle size. Specifically, the particle size of TM reduced from 314.38 nm (native TM) to 277.72 nm (TM-DNG), 285.58 nm (TM-DOG), 283.97 nm (TM-βE), 199.32 nm (TM-DNOG) and 197.51 nm (TM-TFMS), while the particle size of TM-G increased to 467.47 nm, which suggested that the size of N-glycans was larger than O-glycans, β-elimination and O-glycosidases had almost the same deglycosylation effect on removal of O-glycans, and both TFMS and de-N&O-glycosylated could efficiently remove glycans. Fig. 2D showed that the zeta potential of the deglycosylated and glucose glycosylated TM samples decreased, while the zeta potential of TM-G got strengthened, which suggested N-glycans and O-glycans might enhance protein zeta potential by the poly-hydroxyl groups on glycans, while removal of glycans would weaken the strength of zeta potential.

3.3. Allergenicity determination

3.3.1. Allergenicity of the deglycosylated and glycosylated TM

The allergenicity of deglycosylated and glucose glycosylated TM was confirmed by western blot using sera from shrimp food allergy patients. In Fig. 3A, compared with native TM (lane 1), the deglycosylated TM exhibited stronger immune response: TM-TFMS (lane 6) $\approx$ TM-DNOG (lane 5) $>$ TM-DNG (lane 2) $>$ TM-βE (lane 4) $\approx$ TM-DOG (lane 3) $>$ native TM (lane 1), which might be due to the exposure of

interior epitopes of TM, while TM-G got significantly weaker immune response ($p < 0.05$) due to the masked epitopes by glucose residues. The ELISA data further confirmed the western blot results. As shown in Fig. 3B, the ELISA had similar changes with western blot: TM-DNOG $\approx$ TM-TFMS $>$ TM-DNG $>$ TM-βE $\approx$ TM-DOG $>$ native TM $>$ TM-G, which suggested the deglycosylated TM got higher allergenicity while the glucose glycosylated TM had stronger allergenicity. Previous literatures had similar findings on other glycosylated allergen. Nakamura et al. (2005) suggested the lysine content and allergenicity of squid tropomyosin reduced after glycation. Bu, Luo, Lu, and Zhang (2010) indicated that after Maillard reaction with glucose, the free amino groups of whey protein got decreased and led to the reduction of allergenicity.

3.3.2. The allergenicity of *in vitro* gastrointestinal digests

As to gastric digestion (Fig. 3C), the absorbance (OD₄₅₀) of digests reduced, suggesting the epitopes of TM were hydrolyze-destroy by pepsin. However, digests still had a relatively high absorbance after 60 min gastric digestion, indicating pepsin could not completely destroy TM epitopes, which might be resulted from the low content of hydrolysis sites (1.82% phenylalanine and 2.01% tyrosine in TM, Table 2). As to deglycosylation, the absorbance of digests also decreased with the same digestion time, while the allergenicity of digests changed as follows: native TM $>$ TM-DOG $\approx$ TM-βE $>$ TM-DNG $>$ TM-DNOG $\approx$ TM-TFMS, which suggested the deglycosylated TM could be more likely to be hydrolyzed, this might be due to that: (1) the deglycosylation removed the masking effects of glycans on TM epitopes; (2)

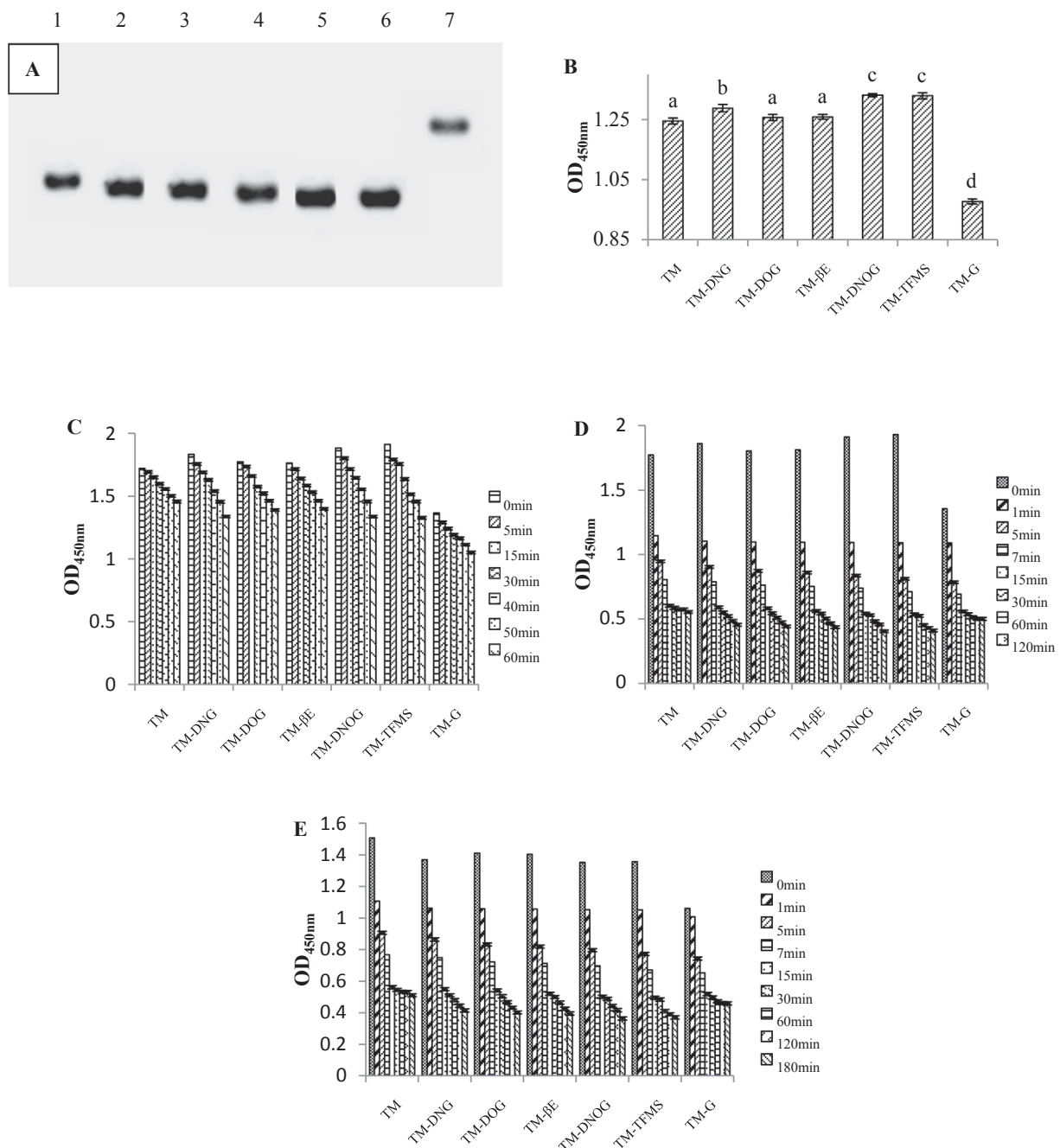


Fig. 3. The western blot (A) and ELISA (B) of TM, the allergenicity of simulated gastric digests (C), simulated intestinal digests (D) and sequential simulated gastric and intestinal digests (E) of deglycosylated and glycosylated TM (mean $\pm$ SD, $n = 3$). Different superscript letters means significant differences between values with different treatment ($p < 0.05$). (Note: lane 1, TM; lane 2, de-N-glycosylated TM; lane 3, de-O-deglycosylated TM; lane 4, β -eliminated TM; lane 5, de-N&O-deglycosylated TM; lane 6, TFMS deglycosylated TM; lane 7, glucose glycosylated TM.)

the deglycosylation loosened TM conformation to make pepsin easier to contact with TM to be hydrolyzed (Yu et al., 2012). After deglycosylation, TM epitopes could be more likely to be hydrolyzed, led to digests with lower allergenicity. As to TM-G, glucose occupied part of epitopes with glycation sites, which blocked the accessibility of pepsin to TM, resulting in gastric digests with relatively higher allergenicity.

As to intestinal digestion (Fig. 3D), the absorbance of digests got reduced quickly as intestinal digestion went further, indicated that most TM epitopes could be destroyed due to the high TM accessible sites of trypsin (10.03% arginine and 10.97% lysine, Table 2). Also, as to the deglycosylated TM, the absorbance of digests also reduced, which might be resulted from that deglycosylation induced conformational alterations, with more hydrolysis sites exposed to enhance the

accessibility to trypsin. While the intestinal digests still had allergenicity even the intestinal digestion time lasted to 3 h. As to TM-G, glucose residues could mask the accessibility of TM to trypsin, resulting in intestinal digests with relatively higher allergenicity.

Compared with the gastric digests (Fig. 3C) and intestinal digests (Fig. 3D), Fig. 3E showed that the gastrointestinal digests had the lowest allergenicity, this might be due to that the pepsin and trypsin could more efficiently hydrolyze proteins than single pepsin/trypsin, and resulted in fewer allergic peptides in digests, led to lower allergenicity. After gastric digestion, there were still allergic peptides with epitopes in gastric digests. Subsequent tryptic digestion of the gastric digests significantly hydrolyzed the remained epitopes, resulted in lower allergenicity. Thus, although TM could be harder hydrolyzed by

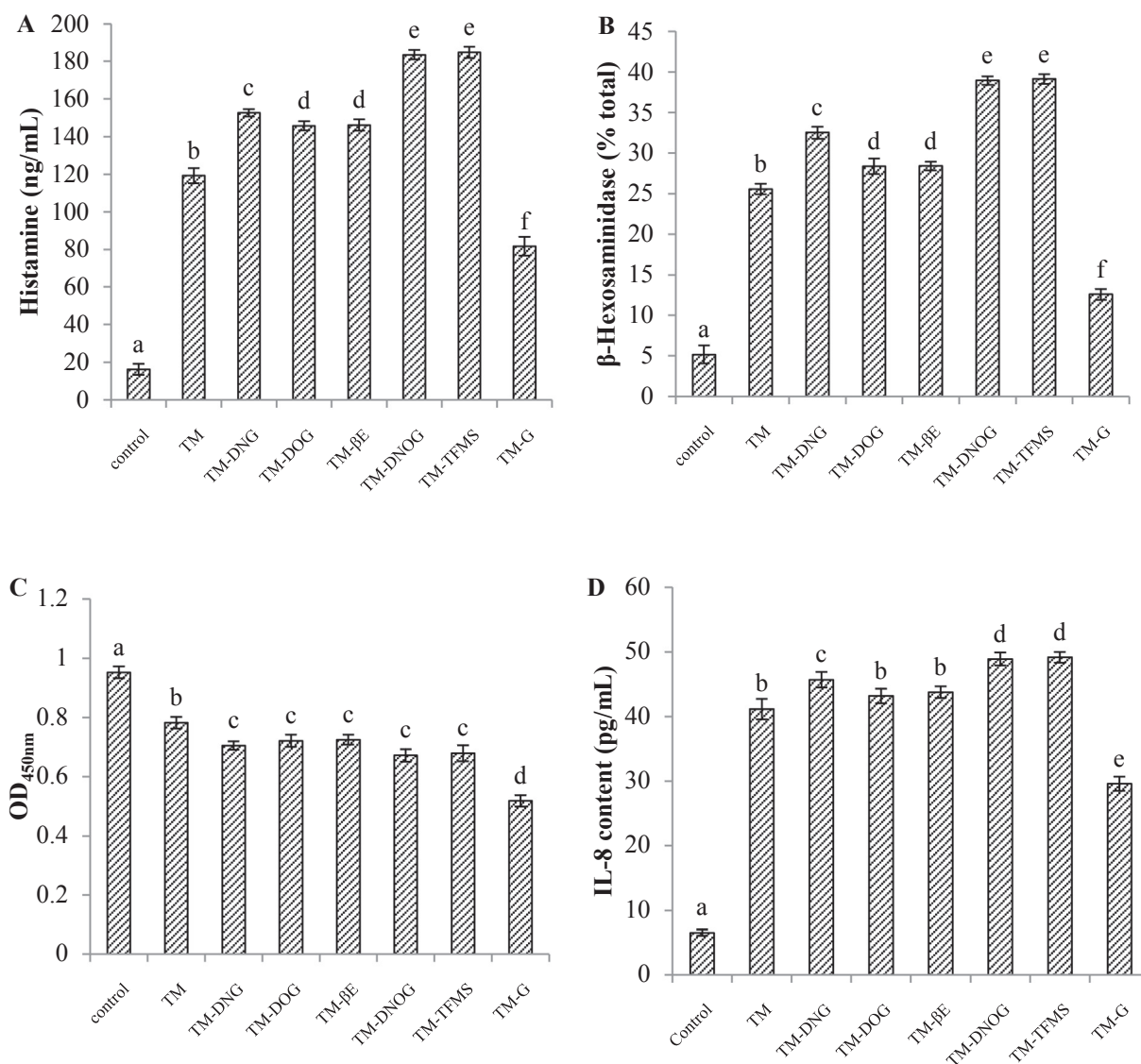


Fig. 4. Effects of deglycosylation and glucose glycation of TM on the histamine release of KU812 cells (A), degranulation of KU812 cells (B), proliferation of Caco-2 (C) and IL-8 secretion of Caco-2 (D). (Note: Different superscript letters means significant differences between values with different treatment ($p < 0.05$).)

gastric digestion, the subsequent hydrolysis actions of trypsin were efficacious to hydrolyze the remained epitopes in gastric digests (Chicón, Belloque, Alonso, & López-Fandiño, 2008). In addition, the deglycosylation could cause conformational instability of TM, resulted in more hydrolysis sites of TM exposed, higher digestibility and reduced allergenicity. As to TM-G, glucose residues masked the epitopes with glycation sites and reduced the accessibility of trypsin to TM, resulting in TM-G digests with relatively higher allergenicity than the digests of deglycosylated TM.

3.4. Human cell sensitization by the deglycosylated and glucose glycated TM

3.4.1. Human basophil cell line (KU812)

To determine the histamine contents, KU812 cell supernatants were collected after activation by deglycosylated TM and glycated TM, and measured by histamine ELISA kit. In Fig. 4A, compared with the control (16.23 ng/mL, PBS activated), the histamine release had a sharp increase after the challenge of native TM (119.31 ng/mL, $p < 0.05$), while compared to native TM, the activation ability of deglycosylated TM became stronger, and TM-TFMS (184.87 ng/mL) $\approx$ TM-DNOG (183.59 ng/mL) $>$ TM-DNG (152.68 ng/mL) $>$ TM-βE (146.15 ng/mL)

mL) $\approx$ TM-DOG (145.79 ng/mL) $>$ native TM (119.31 ng/mL), which suggested the deglycosylated TM had higher basophil activation ability and led to higher release of histamine. This might be due to that: (1) glycans on TM could mask the surface spatial space of epitopes, to block the binding between IgE and allergen, after deglycosylation, glycans were removed and the protein parts could be more available to IgE; (2) deglycosylation led to the exposure of interior epitopes to surface, which could be more accessible to bind IgE, leading to stronger basophil activation and more secretion of histamine. As to TM-G, the release of histamine decreased significantly, which might be due to that glucose occupied TM epitopes, reduced the affinity of TM to IgE, resulted in weaker activation of basophil.

The degranulation of stimulated KU812 cells was determined by the content of β-hexosaminidase. In Fig. 4B, compared with the control (5.18%, PBS activated), the release of β-hexosaminidase also increased and TM-TFMS (39.14%) $\approx$ TM-DNOG (38.95%) $>$ TM-DNG (32.52%) $>$ TM-βE (28.42%) $\approx$ TM-DOG (28.38%) $>$ native TM (25.57%), which suggested the deglycosylated TM had higher basophil degranulation ability and led to increase of β-hexosaminidase. This might be due to that glycans blocked the interaction between IgE and allergen, after deglycosylation, the protein parts could be more accessible to IgE, and deglycosylation contributed to the exposure of interior

epitopes to protein surface, TM could be more accessible to bind IgE, leading to more severe degranulation. As to TM-G, the degranulation weakened significantly, which might be due to that glucose residues masked TM epitopes, blocked the affinity of TM to IgE, resulted in weaker basophil degranulation.

3.4.2. Human colon epithelial cell (Caco-2)

Caco-2 could be used to analyze the biological activity of food allergen to human intestine epithelial cells, reflected in proliferation of cell and release of cytokines. As shown in Fig. 4C, compared with the absorbance (OD₄₅₀) of the control (0.952, PBS activated), the proliferation decreased after the challenge of native TM (0.782), while compared to native TM, the proliferation of deglycosylated TM became weaker and native TM (0.782) > TM-βE (0.725) ≈ TM-DOG (0.721) > TM-DNG (0.705) > TM-DNOG (0.682) ≈ TM-TFMS (0.679), which suggested the deglycosylated TM inhibited Caco-2 proliferation. As to TM-G, the glycosylated TM also exerted inhibitive effects on Caco-2 proliferation. Teodorowicz et al. (2013) found the peanut 7S globulin (Ara h 1) glycosylated by glucose exerted inhibition on Caco-2 proliferation.

Intestinal epithelial cells could influence the immune system by cytokine release (such as IL-8). IL-8 was an important mediator of epithelial immune responses that influenced the activation of basophils. The secretion of IL-8 was measured by human IL-8 ELISA kit. In Fig. 4D, compared with the control (6.51 pg/mL, PBS activated), the release of IL-8 from Caco-2 activated by deglycosylated TM got promoted, and TM-TFMS (49.15 pg/mL) ≈ TM-DNOG (48.89 pg/mL) > TM-DNG (45.68 pg/mL) > TM-βE (43.75 pg/mL) ≈ TM-DOG (43.19 pg/mL) > native TM (41.15 pg/mL), which suggested the deglycosylated TM exhibited activation and led to increase of IL-8. This might be due to that deglycosylation contributed to the exposure of interior epitopes to protein surface, more epitopes were accessible to Caco-2 cell, leading to more severe release of IL-8. As to TM-G, the IL-8 secretion reduced significantly, which might be due to that glucose masked TM epitopes, decreased the affinity of TM to cell, resulted in weaker IL-8 secretion. Teodorowicz et al. (2013) also found the peanut 7S globulin (Ara h 1) glycosylated by glucose possessed suppression on the IL-8 release of Caco-2.

4. Conclusions

TM had both N-glycans and O-glycans. Deglycosylation increased the Asn and Ser contents of TM, and glucose glycation decreased the lysine contents of TM. After deglycosylation and glycation, the α-helix reduced, while other structures got promoted. Deglycosylation enhanced TM allergenicity, while glycation reduced the allergenicity of TM. As to the deglycosylated and glucose glycosylated TM, gastric digestion partly weakened the allergenicity of digests, intestinal digestion efficiently cleaved TM and lowered the digests' allergenicity, while the sequential gastric and intestinal digestion effectively hydrolyzed TM and reduced the allergenicity of digests. Deglycosylation could relax TM conformation, promote TM digestibility, and induce digests with less allergenicity. After deglycosylation, the deglycosylated TM possessed stronger activation of basophil (KU812), while TM-G weakened KU812

activation. Deglycosylation exerted inhibition on Caco-2 proliferation and increase of IL-8 release.

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