



Cite this: DOI: 10.1039/c9fo01118h

Effect of microbial transglutaminase cross-linking on the quality characteristics and potential allergenicity of tofu

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Microbial transglutaminase (MTGase) has been developed as a new tofu coagulant in recent years due to its good hydrophilicity, high catalytic activity, and strong thermal stability. This study aimed to investigate the effect of MTGase on the physicochemical properties and immunoreactivity of tofu relative to conventional coagulants [brine and glucono- δ -lactone (GDL)]. Structural changes of the MTGase cross-linked soymilk protein were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy, and fluorescence spectroscopy. The IgE-binding capacity of MTGase cross-linked proteins was tested by enzyme-linked immunosorbent assay (ELISA). The physicochemical properties, quality characteristics, and surface microstructures of five different types of tofu were determined by the Kjeldahl nitrogen method, texture analysis, and scanning electron microscopy (SEM). The digestibility of tofu was evaluated *in vitro* by simulated gastrointestinal (GIS) digestion. A cell sensitization experiment was performed *in vitro* to evaluate the capability of tofu digestion products to induce the release of bioactive mediators from human basophil leukemia (KU812) cells. Results indicated that MTGase significantly changed the advanced structure of the soymilk protein. Compared with tofu without MTGase, the composite coagulant tofu containing MTGase exhibited better quality. MTGase improved the water-holding capacity (WHC) of the internal mesh structure and increased the yield of tofu. The digestion products of the composite coagulant tofu, especially the GDL plus MTGase tofu, induced KU812 cells to release fewer bioactive mediators compared with those of MTGase-free tofu. MTGase can not only improve the quality of conventional coagulant tofu but also reduce the potential allergenicity of tofu to a certain extent.

Received 26th May 2019,
Accepted 23rd July 2019
DOI: 10.1039/c9fo01118h
rsc.li/food-function

1. Introduction

Tofu is a widely consumed soybean product in eastern countries because it contains no cholesterol but has many nutrients, such as minerals, vitamins, omega-3 fatty acids, and isoflavones.^{1,2} The typical process of preparing tofu from soybeans includes soaking, grinding, filtration, heating, coagulation, pressing, and gel reforming.³ The choice of coagulant is the most critical step because it affects the texture, rheological properties, microstructure, water-holding capacity (WHC), protein content, and other physicochemical properties of the tofu gel on account of the different functional mechanisms.⁴ Salts (*e.g.* MgCl_2 and CaSO_4) and acids [*e.g.* glucono- δ -lactone

(GDL)] are frequently used coagulants in tofu production. However, conventional tofu has limitations in terms of hardness, flavor, and texture.^{5,6} In recent years, microbial transglutaminase (MTGase), a new coagulant, has gained increasing attention due to the constant pursuit of food quality by consumers.

In addition to tofu quality, an important safety issue has been a research hotspot. Soybean, the raw material of tofu, is one of the eight major allergens announced by the World Health Organization and can lead to symptoms, such as asthma, rhinitis, urticaria, and even shock in sensitive individuals.⁷ The International Union of Immunological Societies identified eight allergenic proteins in soybeans.⁸ Among them, glycinin (10%–12.7% of seed proteins) and β -conglycinin (19.5%–23.1% of seed proteins) are the two main allergens that play a major role in the preparation of tofu.^{9–11} Thus, reducing the allergenicity of allergenic proteins in tofu production has become an urgent issue.

MTGase is an enzyme that catalyzes the transfer reaction among many proteins by cross-linking the amino acid residues

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of glutamine and lysine.¹² In 1998, the Food and Drug Administration approved MTGase (no. GRN 000095) as a “Generally Recognized as Safe” additive.¹³ MTGase has been extensively used in food products that mainly contain proteins in their structure, such as sausages,¹⁴ yoghurt,¹⁵ fish meat,^{16,17} and crab meat,¹⁸ due to its wide range of protein selection, high degree of cross-linking polymerization, calcium-independent activity, and other advantages.^{19,20} The cross-linking reactions catalyzed by MTGase lead to significant alterations in the protein molecules present in food matrices, leading to improved texture, stability, gelation, emulsification potential, and WHC without conferring a negative change in sensory properties or nutritional quality.^{12,21,22} For example, the addition of MTGase into yogurt contributed to increasing the yogurt network strength, WHC, viscosity, texture, and viability of the starter and probiotic bacteria.²³ MTGase improved the texture, color, WHC, elasticity, and thermal stability of the red tilapia surimi gel.²⁴ The addition of MTGase increased the pH and viscosity of milk and decreased the titratable acidity and syneresis in kefir samples.²⁵ As for tofu processing, Chang *et al.*²⁶ found that the addition of MTGase increased the gelation temperature of soymilk and produced a firm and elastic packed tofu with low cooking loss. Guo *et al.*²⁷ revealed that transglutaminase addition significantly improved the tofu texture and produced a dense network composed of relatively even pores. In terms of altering the allergenicity of food proteins, Zhou *et al.*²⁸ found that transamidation of gluten by MTGase reduced the immunogenicity of gluten. Lombardi *et al.*²⁹ indicated that K-C₂H₅ cross-linking *via* MTGase specifically affected gliadin immunogenicity and reversed the inducible inflammatory response in models of gluten sensitivity without affecting other aspects of biological activity. However, our previous research reported that the immunoreactivities of glycinin increased because of MTGase-induced alterations in primary and spatial structures.³⁰

According to the findings mentioned above, these studies mainly focused on the effects of MTGase on the stability, functional properties, and structure of food proteins, but provided little information regarding its potential allergenicity. The main objectives of this paper were to systematically investigate the effect of MTGase cross-linking on the structure of soybean proteins and observe differences in quality characteristics, digestibility, and allergenicity among different coagulant types of tofu (with or without MTGase).

2. Materials and methods

2.1 Materials

Soybean cultivar (Dong Nong 42) was provided by Northeast Agricultural University, China. MTGase (a food additive, the enzyme activity of MTGase solution was 102.3 U mL⁻¹) was purchased from Taixing Yiming Biological Products Co. Ltd (Jiangsu, China). The human peripheral blood basophilic white blood cell line was provided by the Shanghai Institute of Biochemistry and Cellular Sciences, Chinese Academy of

Sciences. Human sera containing a specific IgE antibody against soybean from eight patients were acquired from Chongqing Manuik Technology Co. Ltd (Chongqing, China). The group of patients consisted of six males and two females with specific IgE levels of 5.95, 32.66, 22.4, 10.8, 6.61, 12.60, 30.60, and 12.97 kU L⁻¹. Among the eight patients, five suffered from allergic rhinitis, one had allergic rhinitis and asthma, and two had unknown allergic reactions. Informed consents were obtained from the patients who agreed on using their sera for research on soybean allergenicity. All human sera-use protocols were approved by the University of Nanchang Ethics Committee (Nanchang, China) under the International Ethical Guidelines for Biomedical Research Involving Human Subjects. Goat anti-human specific IgE was acquired from Sigma-Aldrich (St Louis, Mo, USA). Human IgE was purchased from Merck Millipore, USA. All other chemicals used in this research were of analytical grade.

2.2 Preparation of samples

2.2.1 Preparation of MTGase cross-linked soymilk protein.

Soybeans were soaked in distilled water (soybean/distilled water = 1 : 6.5, m/v) for 10 h at room temperature and ground using a Joyoung soymilk machine (C012, Joyoung Co. Ltd, China). The slurry was filtered using gauze to obtain raw soymilk. The raw soymilk was heated in a water bath at 75 °C for 10 min and then at 95 °C for 5 min. The soymilk was defatted by *n*-hexane extraction (soymilk/hexane = 1 : 2, v/v), and the protein was extracted with pH 8.0 Tris-HCl (0.05 M) buffer (soymilk/Tris-HCl buffer = 1 : 1, v/v) at 4 °C for 4 h.

The obtained soymilk protein was treated with MTGase (5 U g⁻¹ protein) after adjusting the protein concentration to the same value. The samples with different pH values (5.0, 6.0, 6.4, 7.0, and 8.0) were incubated with MTGase at 50 °C for 3 h to investigate the effect of pH on the polymerization of the soymilk protein. The samples with pH 6.4 were incubated with MTGase at different temperatures (40 °C, 45 °C, 50 °C, 55 °C, and 60 °C) for 3 h to investigate the effect of temperature on the polymerization of the soymilk protein. The samples with pH 6.4 were incubated with MTGase at 50 °C for different time periods (1, 2, 3, 4, and 5 h) to investigate the effect of time on the polymerization of the soymilk protein. Finally, all the samples were heated at 95 °C for 5 min to inactivate MTGase.

2.2.2 Preparation of single coagulant tofu. Soymilk was prepared using the same method as described in Section 2.2.1. The pH was adjusted to 6.0 after decreasing the temperature of the cooked soymilk to 85 °C. The soymilk was then coagulated with brine (brine/soymilk = 0.5%, w/v) and GDL (GDL/soymilk = 0.33%, w/v) for 30 min, respectively. Then, the samples were immediately transferred to a plastic mold and pressed for 3 h.

Single MTGase tofu was prepared using the same method with a slight difference. After the temperature of soymilk was decreased to 50 °C, soymilk was coagulated with MTGase (5 U g⁻¹ protein) for 3 h. The sample was then heated at 95 °C for 5 min to inactivate MTGase.

2.2.3 Preparation of composite coagulant tofu. The ratio of soybeans to water was adjusted to 1 : 8 to obtain soymilk for

preparation of composite coagulant tofu. After cross-linking with MTGase for 3 h at 50 °C, the enzyme was inactivated as mentioned above. The sample was further coagulated with brine (brine/soymilk = 0.5%, w/v) and GDL (GDL/soymilk = 0.33%, w/v) for 30 min at 85 °C, respectively. The samples were then transferred to a plastic mold and pressed for 3 h.

2.3 Characterization of protein structure

2.3.1 Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the molecular weight (MW) distribution of the soymilk protein and tofu digestion products. SDS-PAGE was performed on a Mini-PROTEAN system (Bio-Rad Laboratories, USA) with 12% acrylamide separating gel and 4% acrylamide stacking gel by using a previously described method.³⁰ Peptides with low MW were analyzed by tricine-SDS-PAGE³¹ with 4% stacking gel, 10% interlayer gel, and 16.5% compact gel. After electrophoresis, the gels were immersed in a fixative solution containing ethanol, acetic acid, and water (4:1:5 v/v/v) for 30 min. The gels were stained with Coomassie Brilliant Blue R-250, destained with a solution composed of 5% methanol and 7.5% acetic acid, and scanned using a SQ-GS800 scanning densitometer (Bio-Rad Laboratories, USA). Band intensity was calculated using Quantity One software (Bio-Rad Laboratories, USA).

2.3.2 Spectroscopy analysis. The secondary structures of the protein samples were determined by Far-UV (190–250 nm) circular dichroism (CD) (MOS450/AF-CD, Bio-Logic, France). The CD spectrum was collected at a protein concentration of 0.2 mg mL⁻¹ by using a 0.1 cm path length quartz cell at 25 °C. The parameters were set as follows: a spectral bandwidth of 1 nm, a scan range of 190–250 nm, and a scan speed of 60 nm min⁻¹. Secondary structure contents were calculated using JASCO secondary structure software.

The tertiary structures of the protein samples (0.2 mg mL⁻¹) were analyzed using a double-beam UV-Vis spectrophotometer (TU-1901, Puxi universal apparatus Co., Ltd, Beijing, China). The parameters were set as follows: an optical path length of 1 cm, a scan range of 200–350 nm, a wavelength interval of 1.0 nm, and a scan speed of 100 nm min⁻¹.

The surface hydrophobicity of the protein samples (0.2 mg mL⁻¹) was assessed using an 8-anilino-1-naphthalenesulfonate (ANS) fluorescent probe method according to Liu *et al.*³² In brief, 5 mL of the protein sample was added to 50 µL of ANS solution (8 µM), mixed, and reacted for 1 h at room temperature in the dark. Fluorescence intensity was measured using a fluorescence spectrometer (F-4500, Hitach, Japan). The parameters were set as follows: an optical path length of 1 cm, a slit width of 5.0 nm, a response time of 2.0 s, an excitation wavelength of 375 nm, an emission wavelength of 400–600 nm, and a scan speed of 1200 nm min⁻¹.

2.4 Assessment of tofu quality

2.4.1 Analysis of physicochemical properties. Tofu yield was recorded and calculated as the weight (g) of tofu obtained from 100 g of dry soybeans. The crude protein content of tofu

was quantified using an Automatic Kjeldahl Nitrogen Meter (K9860, Haineng, China) in accordance with GB 5009.5-2010 with a nitrogen factor of 5.71. Moisture content was determined using a quick halogen moisture meter (HR83, Mettler Toledo, Switzerland). WHC was measured as described by Saffon *et al.*³³ Tofu samples were centrifuged at 8000 rpm and 4 °C for 20 min. WHC was calculated as the weight of seep water relative to the percentage weight of tofu as follows:

$$\text{WHC}(\%) = [(W_0 - W_1)/W_0] \times 100$$

where W_0 is the quantity (g) of tofu before centrifugation and W_1 is the quantity (g) of tofu after centrifugation.

2.4.2 Texture analysis. The texture of tofu was analyzed using a CT3 texture analyzer (Brookfield, USA) by the method reported by Zhang *et al.*³⁴ with some modifications. The tofu samples were cut into small squares (1.0 cm × 1.0 cm × 1.0 cm) and compressed twice to 50% deformation by using a P5 probe, with a trigger force of 5.0 g, a pre-test speed of 1.0 mm s⁻¹, a test speed of 1.0 mm s⁻¹, and a post-test speed of 1.0 mm s⁻¹. Texture parameters including hardness, springiness, cohesiveness, gumminess, chewiness, and resilience were evaluated using Texture Pro-CT software (Brookfield, USA).

2.4.3 Microstructure analysis. The microstructure of the tofu gel was examined using a JSM-6701F scanning electron microscope (SEM, JEOL Ltd, Tokyo, Japan) according to a modified method described by Lee *et al.*³⁵ The tofu samples were cut into slices of approximately 1 mm × 1 mm, then fixed in 2.5% glutaraldehyde for 14 h and rinsed thrice with phosphate buffer (0.1 M, pH 7.4), before stepwise dehydration by 30%, 50%, 70%, 80%, 90%, and 100% ethanol. Then, all of the samples were freeze-dried after ethanol was completely replaced with pure tertiary butanol. Dried samples were mounted on stubs and sputter coated with gold, and imaged at an acceleration voltage of 5 kV under vacuum.

2.5 In vitro gastrointestinal (GIS) digestion

Simulated GIS digestion of tofu was performed using the method described by Minekus *et al.*³⁶ with a few modifications. The protein content of the samples was adjusted to the same value before digestion. In the oral phase, mastication of tofu was simulated by squeezing with glass rods. The samples were suspended in the simulated salivary fluid (SSF) at a ratio of 50:50 (w/v or v/v), then salivary α-amylase was added to achieve 75 U mg⁻¹ protein in the final mixture, and then incubated at 37 °C in a shaker for 2 min. The reactions were terminated by decreasing the pH to 3.5 by adding 1 mol L⁻¹ HCl. In the gastric phase, the oral bolus was mixed with the simulated gastric fluid (SGF) at a ratio of 50:50 (v/v), then pepsin was added to achieve 182 U mg⁻¹ protein in the final digestion mixture, and then incubated at 37 °C in a shaker for 1, 5, 20, 40, and 60 min. The reactions were terminated by increasing the pH to 7.0 by adding 1 mol L⁻¹ NaHCO₃. In the intestinal phase, the gastric chyme was mixed with the simulated intestinal fluid (SIF) at a ratio of 50:50 (v/v) and protease in pancreatin was added to achieve 35 U mg⁻¹ protein in the

final mixture. The solutions were incubated at 37 °C in a shaker for 5, 10, 30, 60, 90, 120 min, and the reactions were then terminated by adding a trypsin–chymotrypsin inhibitor. All protein samples were collected and stored at –20 °C for further analysis.

2.6 Assessment of potential allergenicity

2.6.1 ELISA. The IgE-binding capacity of the cross-linked soymilk protein and tofu digestion products was evaluated by indirect ELISA³⁷ and competitive inhibition ELISA,³⁸ respectively, by using previously described methods with a few modifications. Soybean proteins in carbonate buffer were coated overnight at 4 °C on a 96-well ELISA microplate. Human serum was diluted 15 times for indirect ELISA and 10 times for competitive inhibition ELISA, respectively.

2.6.2 Mediator release assay of human basophil leukemia (KU812) cells. KU812 cells were cultured in accordance with the method reported by Meng *et al.*³⁹ The cells were placed in the complete RPMI 1640 medium containing 2 mM L-glutamine, 100 U mL^{–1} penicillin, 100 µg mL^{–1} streptomycin, and 15% fetal bovine serum (FBS) in a humidified incubator with 5% CO₂ at 37 °C. When the number of KU812 cells reached 2 × 10⁶ cells per mL, human myeloma IgE (0.5 µg mL^{–1}) was added and then incubated for 1 week at 37 °C to stimulate the expression of FcεRI. The cells were then incubated in a 10% human sera pool at 37 °C for another 12 h. Finally, KU812 cells were challenged with 50 µg mL^{–1} GIS digestion products of tofu. Cytokine (β-HEX, histamine and IL-4) levels in KU812 cells were measured using ELISA kits

(Neobioscience, Shenzhen, China) as per the manufacturer's recommendations.

2.7 Statistical analysis

Data were subjected to independent Student's *t*-test using SPSS program (SPSS version 19.0 for Windows, Chicago, IL, USA). A probability value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1 Subunit distribution and IgE-binding capacity of soymilk protein

Fig. 1 shows the MW distribution and IgE-binding capacity of soymilk protein cross-linked under different conditions. The soymilk samples exhibited a typical electrophoresis pattern of soybean proteins, mainly consisting of the α subunit (57–68 kDa), the α' subunit (57–72 kDa), and the β subunit (45–52 kDa) of β-conglycinin and the acidic polypeptide (38 kDa) and the basic polypeptide (20 kDa) of glycinin, which are the main allergens in soybeans.⁴⁰ The electrophoresis bands of these allergic subunits gradually weakened and nearly disappeared after MTGase treatment. Moreover, some macromolecular proteins with MW above 250 kDa were formed on top of the gel (Fig. 1A₁–C₁). The formed polymers were speculated to be intermolecularly cross-linked soybean proteins according to the subunit changes in the proteins, thereby confirming that soybean proteins are good substrates for

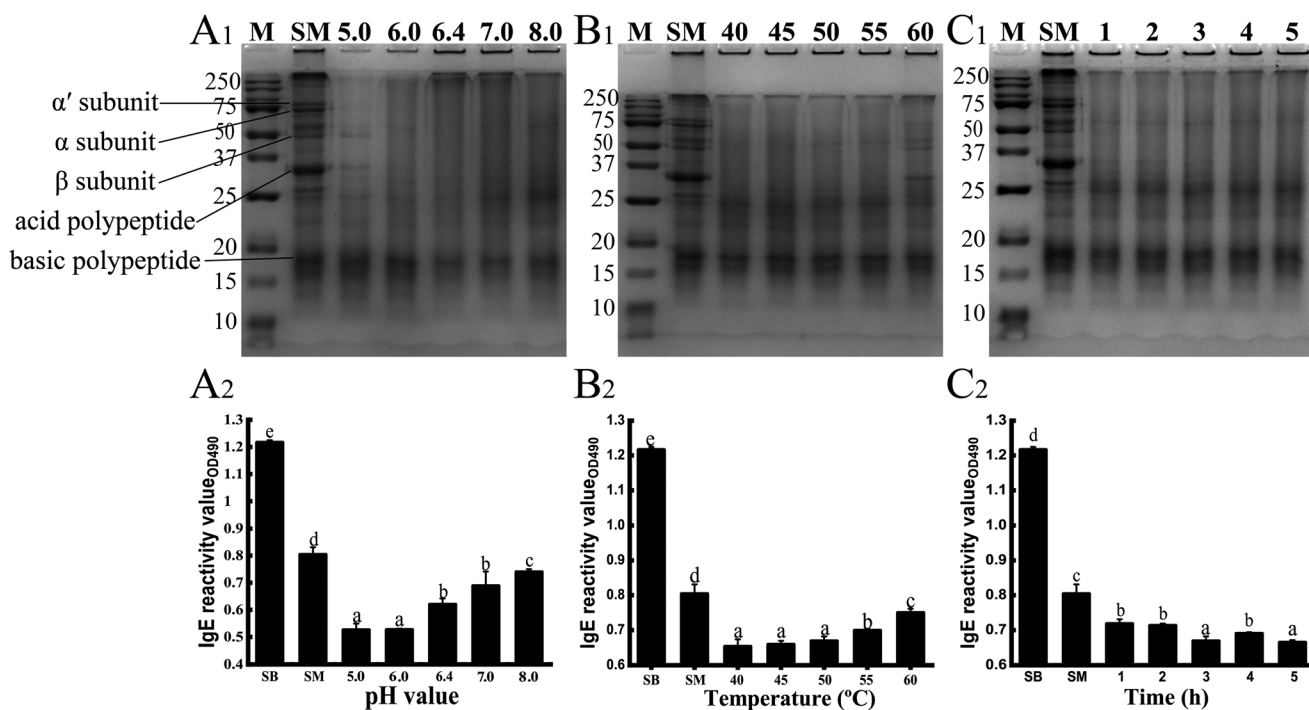


Fig. 1 Effect of MTGase cross-linking conditions (A: pH, B: temperature, C: time) on subunit distribution and the IgE-binding capacity of the soymilk protein. M: Marker, SB: soybean protein, SM: soymilk protein.

MTGase.⁴¹ However, the degree of cross-linking of proteins varied under different conditions possibly because of the mechanism of action of MTGase.¹² Cross-linking also changed the IgE-binding capacity of the soymilk protein (Fig. 1A₂–C₂). The IgE-binding capacity of the soymilk protein after cross-linking significantly decreased, indicating that MTGase cross-linking significantly reduced the potential allergenicity of the protein. The IgE-binding capacity and subunit distribution of the protein samples were closely related, but no regularity was observed.

As shown in Fig. 1A₁, the protein subunit content was the lowest when the cross-linking pH values were 5.0 and 6.0, and the IgE-binding capacity of the cross-linked products was significantly lower than that of the products under other pH conditions (Fig. 1A₂). This finding may be due to the fact that MTGase catalyzes the highest degree of cross-linking of soymilk protein at pH 5.0 and 6.0, when most allergen epitopes were hidden or destroyed. Considering that pH 5.0 is close to the isoelectric point of soybean proteins (pH 4.5 or so),⁴² the soymilk protein accumulated before cross-linking, which is unfavorable for MTGase cross-linking. Therefore, 6.0 is the optimal pH of cross-linking for reducing soymilk protein allergenicity. Fig. 1B₁ shows the band distribution of protein subunits at different cross-linking temperatures. The β -subunit band with a molecular weight of 50 kDa was more obvious when the cross-linking temperature was 60 °C than at other temperatures, indicating that β -conglycinin subunits at 60 °C cannot be efficiently cross-linked. The IgE-binding capacity of the protein after cross-linking also increased with increasing cross-linking temperature. When the cross-linking temperature was 60 °C, the IgE-binding capacity of the cross-linked protein was the highest (Fig. 1B₂), consistent with the electropherogram results. The IgE-binding capacity of the protein was the weakest after cross-linking at 40 °C, 45 °C, and 50 °C. Given the solidification of soymilk at these three temperatures (the results are not shown), 50 °C was considered the optimum for MTGase cross-linking. No significant difference was observed in the cross-linking of the soymilk protein and the distribution of subunits at different cross-linking times (Fig. 1C₁). The IgE-binding capacity of the protein after cross-linking decreased with a prolonged cross-linking time (Fig. 1C₂) and reached the

lowest value after 3 h of cross-linking. However, the IgE-binding capacity of the product unexpectedly increased after the reaction time exceeded 4 h possibly because cross-linking at this time exposed new allergen epitopes. Therefore, 3 h was considered the optimum time for MTGase cross-linking.

3.2 Structural analysis of the cross-linked soymilk protein

The structure of the MTGase cross-linked soymilk protein under optimal cross-linking conditions (pH 6.0, 50 °C, 3 h) is shown in Fig. 2. MTGase catalyzed the intramolecular and intermolecular cross-linking of proteins and the cross-linking between proteins and amino acids, thereby increasing the molecular weight (Fig. 1A₁–C₁) and considerably changing the molecular structure.⁴³ The CD spectrum was recorded to characterize the differences in the secondary structure among the soybean protein, the soymilk protein, and the MTGase-induced soymilk protein (Fig. 2A). From the perspective of interacting forces, the secondary structure of the protein was mainly determined by the amount and balance of chain entanglement, hydrogen bonds, hydrophobic interactions and SS bridges.⁴⁴ The native soybean protein presented a typical α -helix and β -sheet structure. Heat treatment caused the unfolding of the protein structure,⁴⁴ as indicated by the loss of the α -helix and β -sheet structure and the increased number of random coils in the soymilk protein (Fig. 2A). In comparison with the soymilk protein, the numbers of α -helices and β -sheets increased and random coils were lost after MTGase treatment. Hence, the effect of MTGase on the secondary structure of the protein was opposite to heating, suggesting that the MTGase evoked a more folded conformation of soymilk proteins, to establish a three-dimensional network.⁴⁵

The alteration in the tertiary structure of soybean proteins can be characterized on the basis of the UV absorption spectrum and the fluorescence spectrum. In the UV spectrum, the maximum UV absorption peak of the native soybean protein was found at 259 nm (Fig. 2B), indicating that the UV absorption spectrum of the soybean protein was mainly dependent on the side-chain groups of the phenylalanine (Phe) residue. The UV absorption of the soymilk protein increased because the soybean protein structure loosened due to heating, and

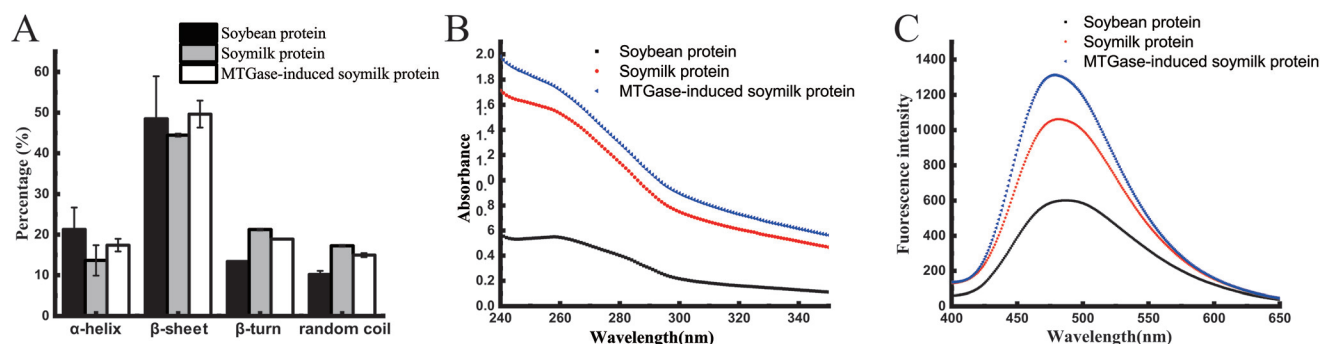


Fig. 2 Structure of MTGase cross-linked products of the soymilk protein. (A) CD spectrum, (B) UV absorption spectrum, and (C) fluorescence spectrum.

more aromatic amino acids were exposed. Given that the substrates of MTGase are glutamine and lysine,⁴⁶ the cross-linking of these two amino acids after MTGase treatment may expose numerous aromatic amino acids, enhancing the UV absorption of the MTGase-induced soymilk protein. Surface hydrophobicity is one of the most important structure-related factors influencing the functional properties of proteins,⁴⁷ such as gelation. Variation in surface hydrophobicity indicates that changes in hydrophobic interactions affect the formation of aggregates and the denaturation of proteins.⁴⁸ The combination of ANS as an effective fluorescent probe with the protein non-polar group can be used to detect the surface hydrophobicity of proteins (Fig. 2C). The hydrophobic group of the native soybean protein is typically located inside the molecule, so the soybean protein shows the lowest fluorescence intensity. Based on the results of CD and UV analyses, heat treatment destroyed the advanced structure of the soybean protein, thereby exposing the hydrophobic amino acids inside the protein and increasing the fluorescence intensity of the soymilk protein. The fluorescence intensity increased further after MTGase cross-linking, indicating that the process enhanced the hydrophobicity of the protein surface. This finding may be attributed to deamidation during cross-linking, which weakens the hydrogen bonds between the proteins and water, reduces the interaction between proteins, and exposes more hydrophobic regions.¹²

3.3 Physicochemical properties of tofu

The results of crude protein content, water content, yield, and WHC of tofu with or without MTGase are presented in Fig. 3. In tofu production, yield is the most important factor.⁴⁹ Compared with conventional single coagulant tofu (GDL tofu and brine tofu), the addition of MTGase increased the yield of tofu (Fig. 3A), which is related to the increase in the WHC (Fig. 3C) and water content (Fig. 3D). WHC is a pivotal index of tofu quality, which is generally considered to be an essential parameter to evaluate gel mouthfeel.⁵⁰ The increase in the WHC was probably due to the fact that MTGase could enhance the cross-linking of the gel network evoked by the formation of intra- and intermolecular cross-links by the γ -glutamyl- ϵ -lysine chain bridge. The improvement of protein structure promotes the enhancement of water-binding capacity and the increase of moisture content.⁵¹ However, the addition of MTGase decreased the crude protein content of tofu (Fig. 3B). This may be attributed to the formation of chain bridges catalyzed by MTGase cross-linking. It was reported that the occurrence of bridging reaction led to the loss of soluble substances.⁵² In particular, single MTGase tofu possessed a significantly lower crude protein content than the other tofu samples but had the highest yield due to its high water content. As such, the amount of MTGase added during the production of MTGase tofu should be controlled to ensure its nutritional value. In

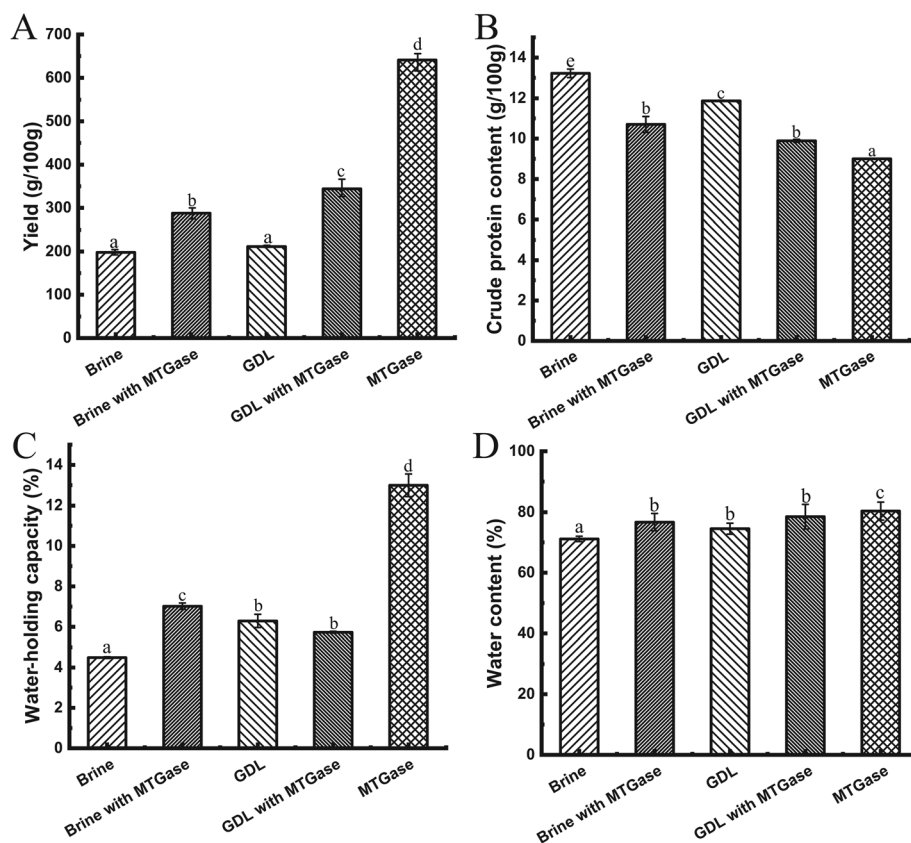


Fig. 3 Analysis of the physicochemical properties of tofu. (A) Yield, (B) crude protein content, (C) water-holding capacity, and (D) water content.

addition, when MTGase was used as the sole coagulant, the tofu was poured into the mold after cross-linking. This process destroyed the formability of the tofu. Therefore, single MTGase is unsuitable for making pressed tofu but is suitable for making filled tofu.

3.4 Texture profile analysis (TPA) of tofu

As a critical indicator of tofu quality, texture analysis possesses an important role in consumer acceptability and quality evaluation.⁵³ The TPA test can simulate textural changes of tofu during the chewing process and it is a useful method for determining tofu quality and consumer acceptability. Table 1 shows the textural properties of tofu with or without MTGase. The hardness of tofu is an indicator of its resistance to destructive forces during processing and application. The addition of MTGase reduced the hardness of conventional tofu, which was different from the findings of Wang *et al.*,⁵⁴ probably due to different amounts of enzyme added. Springiness, defined as the distance to which the sample bounces at the first compression, was closely connected with resilience. The addition of MTGase increased the springiness and resilience of conventional tofu to a certain extent. Cohesiveness and gumminess were indicators of binding intensity between groups inside the tofu. The internal gel bonds of composite coagulant tofu were generally weakened compared with conventional tofu, which was consistent with the results of Wang.⁵⁴ Overall, addition of MTGase to brine or GDL tofu decreased the hardness, chewiness, cohesiveness and gumminess, and increased the springiness and resilience of the samples. When MTGase was used as a single coagulant, the texture of tofu was not analyzed because the sample was too soft. Therefore, when making tofu, adding an appropriate amount of MTGase can reduce the hardness of conventional tofu, and thus products with stronger springiness and better taste can be obtained.

3.5 Microstructure of tofu

Fig. 4 shows that all the obtained tofu gels exhibited a honeycomb structure consisting of protein sheets, consistent with the findings of Lee and Shin.^{35,55} Numerous pores were observed in brine tofu and GDL tofu at a magnification of 100 times. Small pores were detected in GDL plus MTGase tofu and brine plus MTGase tofu at a magnification of 250 times, and the pores were uniformly distributed. The tofu made from the single MTGase coagulant had the smallest pore size, and brine tofu had the largest pore size. The tofu gel without

MTGase possessed a loose network with relatively uneven pores. As such, the gel could not trap water and other soluble substances, possibly explaining the low water content and low yield of brine tofu and GDL tofu (Fig. 3). The structure of tofu containing MTGase was more rigid and had a finer meshed network, smaller pores, and lower porosity compared with the conventional coagulant tofu. Hence, the addition of MTGase can improve the texture of tofu (Table 1), consistent with the results of Gan *et al.*⁵⁶ The presence of such a dense and fine network improved the firmness of the tofu during cold storage. At the end of the experiment, the tofu was placed in a refrigerator at 4 °C. The conventional GDL tofu and brine tofu deteriorated under the same storage conditions, whereas the tofu with MTGase retained its original shape. Therefore, the addition of MTGase can extend the shelf life of tofu.

3.6 Digestibility of tofu

GIS digestion was performed in the SSF, SGF, and SIF to evaluate the effect of cross-linking by MTGase on the digestibility of tofu. The products were analyzed by SDS-PAGE and tricine-SDS-PAGE (Fig. 5). The SDS-PAGE diagram shows the electrophoresis patterns of the digestion products at different residence times in the mouth and stomach (Fig. 5A₁–G₁). The α subunit (57–68 kDa) and α' subunit (57–72 kDa) of β -conglycinin were almost completely degraded after the soybean was digested for 20 min, but the β subunit (45–52 kDa) of β -conglycinin persisted in the gastric digestion stage and showed strong digestive resistance (A₁). With the extension of the digestion time, the digestion degree of the soymilk protein increased obviously after 5 min of digestion in the stomach (B₁). The amount of protein retained in the tofu samples (C₁, D₁, E₁, F₁, G₁) was always higher than that of soymilk (B₁) at the same digestion time. Obvious protein bands were detected at the top of the separation gel in the lane of tofu containing MTGase (D₁, F₁, G₁) due to the accumulation of proteins during production, and their digestibility was slightly weaker than that of conventional tofu (C₁, E₁). This finding may be due to the fact that MTGase promotes the cross-linking of the soybean protein to form an indigestible isopeptide bond. Macierzanka *et al.*⁵⁷ indicated that the addition of MTGase inhibited the digestibility of proteins. Moreover, food supplemented with MTGase exhibits increased satiety, which may be related to the formation of isopeptide bonds.⁵⁸

Table 1 Analysis of tofu texture

Sample	Hardness/g	Springiness/mm	Cohesiveness	Gumminess/g mm	Chewiness/g mm	Resilience
Brine	113.183 ± 1.821	0.871 ± 0.045	0.566 ± 0.048	64.079 ± 1.023	55.833 ± 0.426	0.093 ± 0.012
Brine with MTGase	100.922 ± 1.725	0.985 ± 0.103	0.424 ± 0.032	42.835 ± 0.828	42.176 ± 0.624	0.107 ± 0.021
GDL	76.519 ± 0.803	0.887 ± 0.078	0.533 ± 0.061	40.761 ± 1.018	36.148 ± 0.418	0.080 ± 0.016
GDL with MTGase	64.741 ± 1.036	1.066 ± 0.124	0.447 ± 0.036	37.520 ± 0.926	30.433 ± 0.501	0.092 ± 0.017
MTGase	^a	^a	^a	^a	^a	^a

^a Not detected. Data are expressed as mean ± SD (*n* = 3).

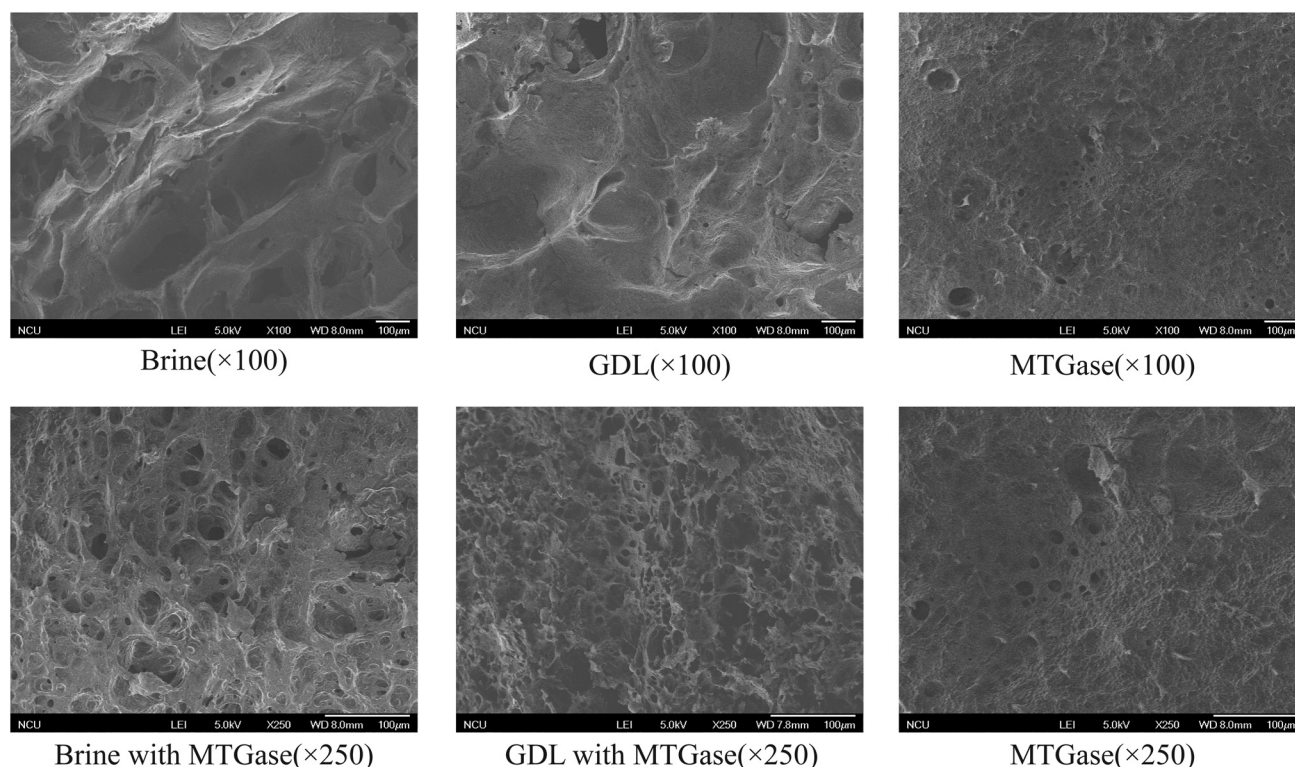


Fig. 4 Analysis of the gel microstructure of tofu.

According to the electrophoresis patterns of the intestinal digestion products (A_2 – G_2), obvious protein subunit residues were still observed in Fig. 5A₂ after the soybean was digested for 120 min. But no significant difference in digestibility was found between soymilk (B_2) and tofu (C_2 – G_2). The MW of all samples was below 50 kDa after digestion for 120 min in the intestine. Hence, the tofu with MTGase can be well digested in the intestine. The isopeptide bonds of proteins present in the tofu with MTGase were degraded by the action of intestinal enzymes. In the study on the digestibility of the soybean protein gel, Rui *et al.*⁵⁹ showed that the lactone gel product had the highest GIS digestibility, followed by the $MgCl_2$ gel, and the MTGase gel had the lowest digestibility. The results slightly different from those in the present study because of differences in the amount of enzyme added. That is, the amount of enzyme added in the Rui experiment is larger than that used in our experiments, leading to the formation of more stable isopeptide bonds between proteins and decrease in digestibility.

3.7 IgE-binding capacity of tofu digestion products

As shown in Fig. 6, the IgE-binding capacity of the gastric digestion products of tofu, especially GDL tofu and MTGase tofu, was significantly lower than that of soybeans. The IgE-binding capacity of the GIS digestion products was lower than that of the gastric digestion products, and the difference was significant. In all experimental groups, the IgE-binding capacity of GIS digestion products of soybean was higher than those of soymilk and tofu. However, no significant change was

found between the digestion products of soymilk and various types of tofu, which may be related to the degree of digestion. As mentioned above, unprocessed soybeans are resistant to digestion, and the protein band remained intact even after intestinal digestion for 2 h. As such, the spatial structure was not significantly damaged, leading to intact allergen structure and epitopes. Eiwegger *et al.*⁶⁰ simulated the crude protein digestion of peanuts *in vitro* and analyzed the potential allergenicity of the digestion products. The small fragments of the crude peanut protein could lead to potential allergenicity even though no intact protein was present after digestion by the stomach and duodenum. Digestive enzymes may partially destroy allergen epitopes but may also expose allergen epitopes within the native protein structure, resulting in an inconspicuous change in potential allergenicity.

3.8 Assessment of potential allergenicity of tofu digestion products in KU812 cells

Basophils express a large amount of IgE Fc receptors ($Fc\epsilon RI$) with high affinity for IgE and are important effector cells involved in type I hypersensitivity caused by food allergy. The main feature of food allergy is an allergen-specific immune response dominated by Th2 cells. The allergen-stimulated Th2-type response can produce a large number of cytokines, such as IL-4, which induces the proliferation and differentiation of specific B cells into plasma cells that secrete IgE.⁶¹ The release of IL-4 from KU812 cells treated with the GIS digestion products of soybean, soymilk, and tofu is presented in

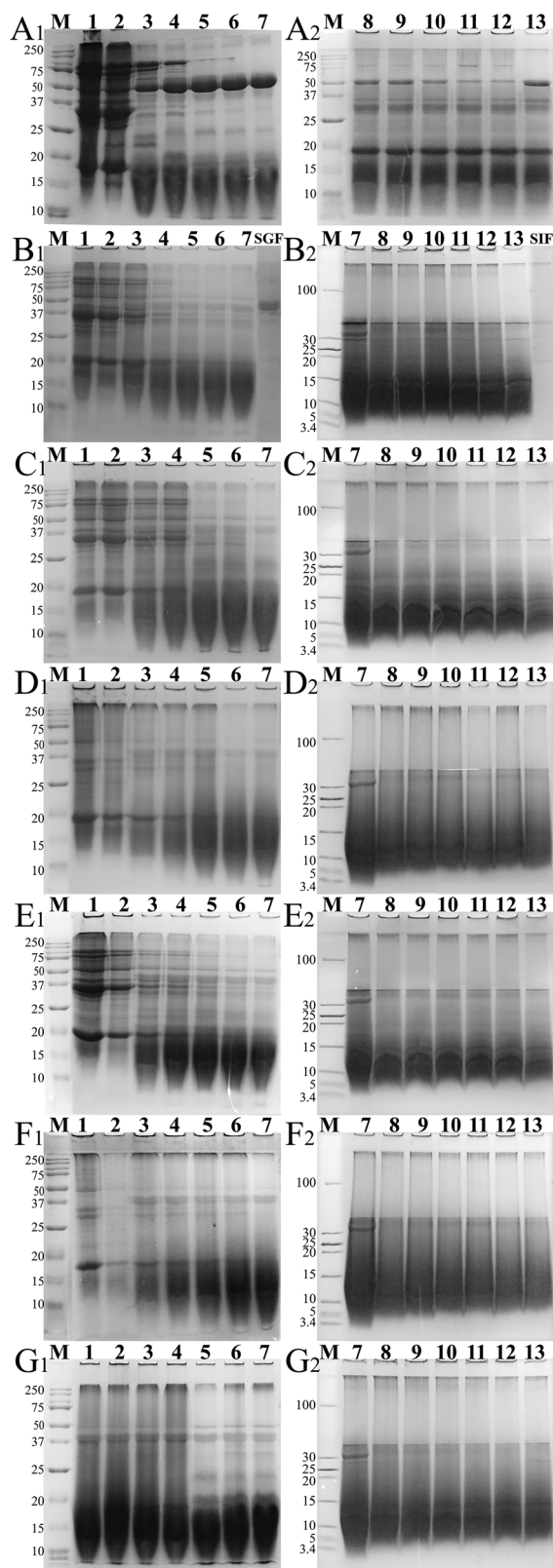


Fig. 5 The electrophoresis profile of tofu GIS digestion products. (A) Soybean, (B) soymilk, (C) brine tofu, (D) brine with MTGase tofu, (E) GDL tofu, (F) GDL with MTGase tofu, and (G) MTGase tofu. 1: undigested; 2: SSF digestion for 2 min; 3–7: SGF digestion for 1, 5, 20, 40, 60 min; 8–13: SIF digestion for 5, 10, 30, 60, 90, 120 min. SGF: simulated gastric fluid; SIF: simulated intestinal fluid.

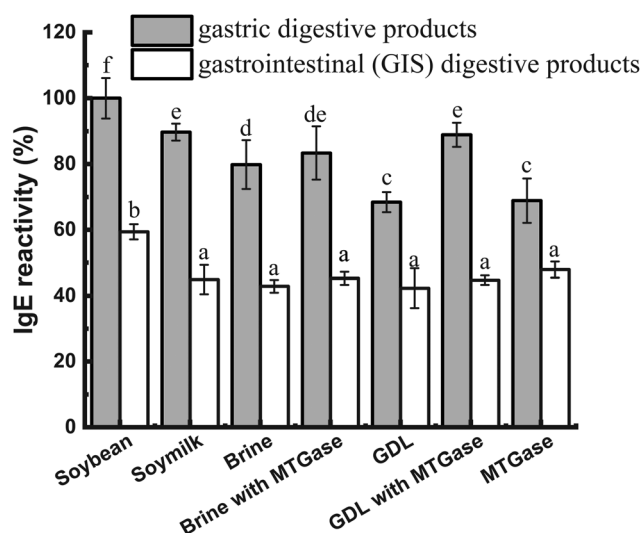


Fig. 6 IgE-binding capacity of tofu digestion products.

Fig. 7A. The amount of released IL-4 induced by soybean was significantly higher than soymilk and tofu, and the amount of IL-4 released from all tofu samples, especially GDL tofu and brine plus MTGase tofu, was significantly lower than that of soymilk. The addition of MTGase reduced the release of IL-4 from brine tofu but increased the release from GDL tofu.

When basophils are activated by different signaling pathways, presynthesis materials (such as histamine and kininogenase) are released into the extracellular environment to play a physiological role. In general, β -HEX is a marker of basophil degranulation. Human blood contains almost no β -HEX under normal circumstances, and the plasma β -HEX concentration will increase significantly only when allergic reaction occurs. Therefore, β -HEX is suitable for the degranulation reaction of basophils to measure the degree of anaphylaxis.⁶² The release rates of β -HEX from the GIS digestion products of soybean, soymilk, and various types of tofu were significantly different (Fig. 7B). The release rate of β -HEX caused by the soybean was significantly higher than the other samples, except for the brine tofu. Meanwhile, the release rate of β -HEX from the GIS digestion products of the conventional single coagulant tofu was higher than that of soymilk. The addition of MTGase could reduce the release of β -HEX induced by the digestion products, but no significant difference was found between the digestion products of single MTGase tofu and soymilk. These results indicate that all samples can induce the degranulation of basophils after GIS digestion, but MTGase can alleviate this process compared with the conventional single coagulant tofu.

Histamine plays an important role in the release of a series of bioactive substances from basophils. Histamine can cause a series of pathological effects on the body, and such effects are directly related to the degree of allergic reactions.⁶³ Histamine release is a characteristic and critical function of basophils. Fig. 7C shows the histamine release from KU812 cells treated with the GIS digestion products of soybean, soymilk, and tofu

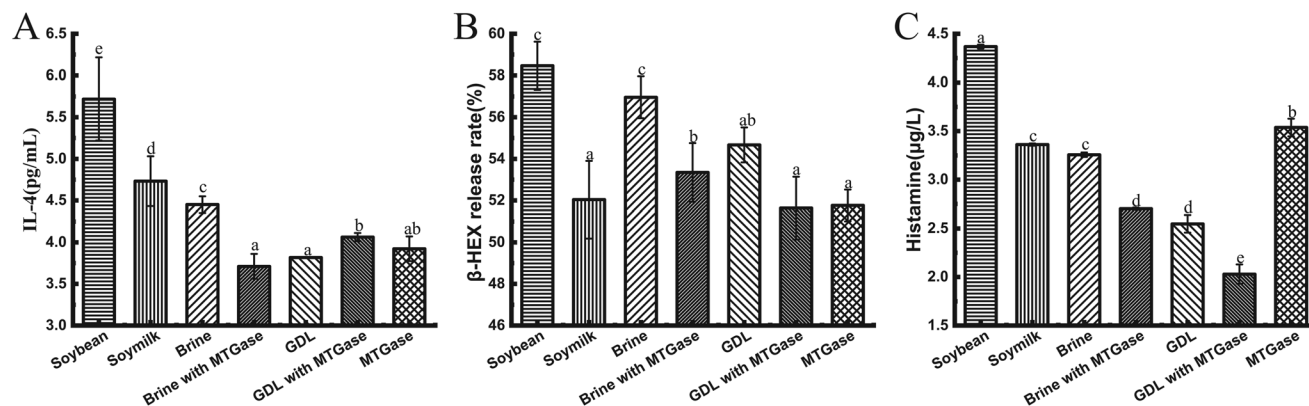


Fig. 7 Release of cytokines (A: IL-4, B: β -HEX, C: histamine) in KU812 induced by tofu digestion products. Data are presented as mean $\pm$ SD.

upon stimulation with specific sera from patients allergic to soybeans. Soybean-induced histamine release is far ahead of soymilk and tofu. The addition of MTGase to the conventional single coagulant tofu significantly reduced the release of histamine, and GDL plus MTGase tofu had the lowest release rate of histamine. However, the release of histamine induced by single MTGase tofu was significantly higher than that of soymilk and other types of tofu. Hence, single MTGase tofu may still have high sensitization.

The results of the IgE-binding capacity of the GIS digestion products differed from those of cell experiments. The possible reason is that although ELISA and KU812 cell activation degranulation model is based on the same principle,⁶⁴ no direct correlation exists between the binding capacity of allergens to IgE and whether or not effector cells are degranulated by allergens.⁶⁵ In addition, the release rates of cytokines slightly differed among different tofu digestion products. That is, the cytokine release rates of single MTGase tofu, brine tofu, and GDL tofu were higher than that of the composite coagulant tofu. Hence, the potential allergenicity of the composite coagulant tofu, especially GDL plus MTGase tofu, was slightly low.

4. Conclusion

The effect of MTGase cross-linking on the quality characteristics and potential allergenicity of tofu was investigated. The optimum cross-linking conditions are as follows: MTGase (5 U g^{-1} soymilk protein) was added to the soymilk with pH 6.0 to cross-linking for 3 h at 50°C . The contents of α -helix and β -turn in the cross-linked proteins increased, the number of random coils decreased, and the UV absorbance and hydrophobicity of the cross-linked products also increased. Compared with conventional single coagulant tofu without MTGase, the addition of MTGase did not only improve the springiness, chewiness, and microstructure of brine tofu and GDL tofu but also increased the yield of tofu and formed a uniform structure. In addition, compared with MTGase-free tofu, the digestion products of the composite coagulant tofu

containing MTGase, especially the GDL plus MTGase tofu, induced KU812 cells to release fewer bioactive mediators. Therefore, MTGase can not only improve the quality of conventional coagulant tofu but also reduce the potential allergenicity of tofu to a certain extent.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are thankful for the support from National Natural Science Foundation of China (No. 31760453, 31460439), National High Technology Research and Development Program of China (863 Program, No. 2013AA102205), and Young Scientist Training Program of Jiangxi Province (No. 20122BCB23006).

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