

Deciphering Changes in the Structure and IgE-Binding Ability of Ovalbumin Glycated by α -Dicarbonyl Compounds under Simulated Heating

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ABSTRACT: As a complex reaction, biological consequences of the Maillard reaction (MR) on dietary proteins need to be deciphered. Despite previous studies on the structural and antigenic properties of ovalbumin (OVA) by MR, associated changes induced by specific MR intermediates and their downstream products are largely unknown. This study focused on the impacts of glycation by α -dicarbonyl compounds (α -DCs), intermediates of MR and precursors of advanced glycation end-products (AGEs), on the structural and IgE-binding properties of ovalbumin (OVA) under simulated heating. Methylglyoxal (MGO), glyoxal (GO), and butanedione (BU) were selected as typical α -DCs to generate glycated OVA with different AGE-modifications (AGE-Ms). The results showed that reactions between OVA and α -DCs generated OVA-AGE with various degrees of modification and conformational unfolding, and the reactivity of α -DCs followed the order GO > MGO > BU. Depending on the precursor type, the levels of 10 specific AGEs were verified, and the amounts of total AGEs increased with heating temperature and α -DC dosage. Compared to native OVA, glycated OVA showed reduced IgE-binding levels but with sRAGE-binding ligands, the extent of which was associated with the contents of total AGEs and N ϵ -carboxymethyllysine, and changes in certain protein conformational structures. High-resolution mass spectrometry further identified different AGE-Ms on the Lys and Arg residues of OVA, confirming variations in the glycation sites and their associations with the immunoreactive epitopes of OVA under different conditions.

KEYWORDS: ovalbumin, advanced glycation end-products, dicarbonyls, protein conformations, antigenicity

INTRODUCTION

Heat treatment is one of the most commonly used methods during industrial food processing and home cooking. During heat treatments, the Maillard reaction (MR), also known as the nonenzymatic glycation, can readily occur between reducing sugars and the amino groups of proteins, peptides, and free amino acids.¹ Despite improvements in the flavor, texture, and shelf life of food products, MR leads to the formation of a large quantity of MR products (MRPs), including undesired “glycotoxins,” such as acrylamide, 5-hydroxymethylfurfural, and advanced glycation end-products (AGEs).² Specifically, AGEs are a group of heterogeneous and nonreactive terminal products generated in the “advanced” stage of MR. In the early and middle stages of glycation, rearrangement of the Amadori products and fragmentation of sugars via retro-aldolization results in the formation of α -dicarbonyl compounds (α -DCs), including methylglyoxal (MGO), glyoxal (GO), butanedione (BU), and 3-deoxyglucosone. Lipid peroxidation is another main source of α -DCs, especially for GO, MGO, and BU.^{3,4} These MR intermediates are highly unstable and are prone to react with the side chain groups of protein active residues leading to AGEs formation.^{5,6} Previous studies have observed that the condensation, cross-linking, and covalent conjugation with AGEs have the potential to modify structures of proteins to change their physicochemical and nutritional characteristics.^{7,8} In this scenario, deciphering the structural and

biological consequences of AGE-modified food proteins has generated a surge of interest.

Despite previous studies on the structural changes of dietary proteins by MR, associated changes induced by specific MR intermediates and their downstream products are largely unknown. Moreover, previous studies have documented the presence of α -DCs in a wide range of heat-processed foods, which can be up to 180 mg/day, as estimated in a German population.^{9,10} Due to the greater reactivity of α -DCs, it can be speculated that glycation reactions would occur between food proteins and α -DCs in the heating process. Only a few studies have reported the structural modifications of milk proteins glycated by α -DCs, including bovine serum albumin (BSA), β -casein, and β -lactoglobulin.^{7,11–14} Nonetheless, relevant information on the structural profiles of α -DC-glycated proteins from other food sources is quite limited.

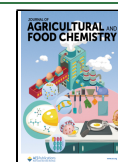
Egg albumins are widely used as food ingredients due to their high nutritional value and desirable functional properties. However, the application of egg proteins in the food industry

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has been restricted due to a growing prevalence of egg allergies worldwide.¹⁵ Ovalbumin (OVA), a 45 kDa protein with 386 amino acids, is the most predominant protein in avian egg white (54%, w/w),¹⁶ and the major egg allergen that causes IgE-mediated food allergic reactions especially in children.^{17,18} It has been accepted that the antigenicity of OVA can be changed by food manufacturing procedures, which can mask, destroy or disclose the epitopes of food allergens through structural modifications, leading to changes in the IgE-binding levels of modified OVA and possible allergenicity.^{19,20} During common heat treatments such as pasteurization and steaming, undoubtedly, the free amino group of OVA can be glycated by reactive compounds (e.g., α -DCs) as a consequence of MR. Although previous work has reported the effect of MR on the structural and IgE-binding properties of OVA, to our knowledge, no studies have yet focused on the associated changes induced by α -DCs and the formed AGEs.

Understanding the reactions between α -DCs and food proteins would provide new insights into the biochemical consequences of MR and heat-processed foods, thus benefiting human health. Therefore, this study aims to investigate the role of α -DC-mediated glycation on the structural and IgE-binding properties of OVA under simulated heating. GO, MGO, and BU, three typical α -DCs, were selected as glycation reagents, thereby generating different types of OVA-AGE modifications. Multiple spectroscopic, chromatographic, and immunological methods were applied to unveil changes in protein structures in association with the IgE-binding capability. A high-resolution mass spectrometry was also employed to provide more detailed information on the glycation sites and types of AGE-modifications (AGE-Ms).

MATERIALS AND METHODS

Materials and Chemicals. OVA ($\geq 98\%$), MGO (40% aqueous solution), GO (40% aqueous solution), BU ($\geq 99\%$), and HPLC-grade ammonium formate, formic acid (FA), and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AGEs standards and isotopically labeled internal standards: *N*-carboxyethyllysine (CEL, $\geq 95\%$) and *N*-carboxymethyllysine (CML, $\geq 95\%$) were purchased from Cayman Chemical (MI, USA); methylglyoxal-hydroimidazolone isomers [MGO-H1 acetate salt ($\geq 96\%$), MGO-H2 acetic acid salt ($\geq 94\%$), MGO-H3 TFA salt ($\geq 99\%$)], glyoxal-hydroimidazolone isomers (GO-H1, $\geq 98\%$), pyralline ($\geq 99\%$), argpyrimidine TFA salt ($\geq 99\%$), glyoxal-lysine dimer (GOLD acetate salt, $\geq 98\%$), methylglyoxal-lysine dimer (MOLD acetate salt, $\geq 95\%$) CML-d4 ($\geq 99\%$), CEL-d4 ($\geq 95\%$), MGO-H1-d3 acetate salt ($\geq 98\%$), GO-H1-13C2 ($\geq 98\%$), and GOLD-15 N2 acetic acid salt ($\geq 94\%$) were bought from Iris Biotech GmnH (Marktredwitz, Germany). O-Phthalaldehyde (OPA), sodium dodecyl sulfate (SDS), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 8-anilino-1-naphthalenesulfonic acid (ANS), and 3,3',5,5'-tetramethylbenzidine (TMB) solution were obtained from Shanghai Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE serum was a product of Novus Biologicals (Littleton, CO, USA). Human soluble receptor for AGE (sRAGE) recombinantly produced in *E. coli* was purchased from Biovendor (Brno, Czech Republic; cat# RD172116100), and the mouse anti-human RAGE antibody was a product of R&D systems (MN, USA; cat# MAB11451). An HRP-labeled goat anti-mouse antibody was bought from Abcam (cat# ab6789). Ultrapure (UP) water used in this study was purified from a Millipore Milli-Q Advantage A10 Water Purification System (Bedford, MA, USA). All other chemicals and reagents used were of analytical grade unless otherwise specified.

Ten sera samples from hen's egg-allergic individuals were obtained from PlasmaLab International (Everett, WA, USA), an FDA-licensed

blood collection institution (information shown in Table S1). The use of these human sera was approved by the Zhejiang Gongshang University Ethics Review Committee and no additional consent was required by law.

Glycation Treatments. Glycation of OVA was performed in sodium phosphate buffer (PBS; 10 mM, pH 7.4) containing 5 mg/mL of native protein and 1 or 10 mM of GO, MGO, and BU, respectively.^{12,13} In order to simulate common heat treatments such as pasteurization and steaming, each mixed solution was sealed in glass vials and incubated in a water bath at 65 °C for 30 min or at 95 °C for 15 min, individually.¹⁷ Native OVA heated in the absence of α -DCs served as respective control. After treatments, samples were centrifuged at 5000g for 10 min, 4 °C, and the supernatants were dialyzed against UP water in 3.5 kDa dialysis bags at 4 °C for 48 h to remove any unreacted α -DCs and salts. The samples were then lyophilized and stored at -20 °C for further experiments (labeled as GO-/MGO-/BU-OVA for samples incubated with GO, MGO, and BU, respectively).

Measurements of the Free Amino Group and Free Thiol Group. The content of free amino group was measured by a modified OPA method.¹² In brief, the OPA reagent was freshly prepared and diluted to 50 mL with UP water as a working solution. Then, 100 μ L of sample solution (5 mg protein/mL, in 10 mM PBS, pH 7.4) was mixed with 2 mL of OPA reagent and incubated at 40 °C for 3 min. The absorbance was recorded at 340 nm with a SpectraMaxi3x microplate reader (Molecular Devices, San Jose, CA, USA). L-Leucine was used as a standard and the results were calculated as a percentage relative to the free amino content of native OVA.

The level of free thiol group was measured by a DTNB method according to Zhao et al.,²¹ with minor modifications. Briefly, 0.5 mL of sample solution (5 mg protein/mL, in 10 mM PBS, pH 7.4) was combined with 50 μ L of 10 mM DTNB and 1 mL of 10 mM PBS containing 2.0% SDS and 3.0 M urea (pH 6.5). The mixture was incubated in the dark at room temperature for 30 min before reading for absorbance at 412 nm. The loss of -SH groups during glycation was expressed as a percentage relative to the -SH group level of native OVA.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Reducing SDS-PAGE was performed to evaluate changes in the molecular weight (MW) of glycated OVA. Each sample was diluted with 4 $\times$ Laemmli loading buffer and heated in boiling water for 5 min. Then, the samples (10 μ g protein/lane) were electrophoresed with 5% stacking gel and 12% separating gel in a Mini Protean II system (Bio-Rad Laboratories, Richmond, CA, USA). A broad-range MW marker (6.5–200 kDa) was applied in each gel to estimate the band size. After electrophoresis, the gels were stained with Coomassie blue R-250 and decolorized with 40% methanol, 10% acetic acid in UP water. Visualization of protein bands was acquired in a FluorChem HD2 System (Protein Simple, Santa Clara, CA, USA).

Quantification of AGEs. Fluorescent AGEs Formation. The formation of total fluorescent AGEs was assessed by measuring their fluorescent intensities with a microplate reader. The excitation wavelength was 350 nm, and the emission spectrum was recorded from 400 to 500 nm.²² Results are expressed as arbitrary units.

Quantification of 10 AGEs by UHPLC-QqQ-MS/MS. Quantification of 10 typical AGEs in glycated OVA was conducted by an isotope dilution UHPLC-QqQ-MS/MS method as previously described by Poojary et al.²³ and Cheng et al.,²⁴ with some modifications. In brief, 10 mg of glycated protein was reduced by sodium borohydride (1 M in 0.1 M NaOH) and hydrolyzed with 3 mL of 6 M HCl under nitrogen at 110 °C for 24 h. Afterward, the samples were subjected to vacuum drying to remove HCl and water. The hydrolyzed samples were reconstituted in 5 mL of UP water and filtered through a 0.22 μ m membrane. Then, 100 μ L filtrate was taken, appropriately diluted, and spiked with each internal standard (stock solution at 1 μ g/mL prepared in UP water) to a final concentration of 100 ng/mL. The mixtures were ready for subsequent LC-MS/MS analysis. Previous studies have demonstrated that some AGEs, e.g., MG-Hs and G-Hs, are not stable and may undergo degradation and transformation processes during acid hydrolysis.^{25,26} To confirm this effect in our

samples, we tested the reliability of the method in a similar food matrix as previously described.^{23,24} The method showed 85.9–111.5% recovery with an intraday/interday precision within 10%, confirming that possible degradation/transformation of AGEs during acid hydrolysis would not affect the precision of our results (data not shown).

Quantitative analysis of AGEs was performed using a SCIEX ExionLC UHPLC system (AB Sciex, MA, USA) coupled online to a SCIEX QTRAP5500 mass spectrometer (AB Sciex, MA, USA). Chromatographic separation was carried out on a Kinetex C18 column (100 × 3.0 mm, 2.6 μm, Phenomenex, CA, USA) with mobile phase A (5 mM ammonium formate and 0.1% FA in water) and mobile phase B (5 mM ammonium formate in ACN) at a flow rate of 0.12–0.22 mL/min. The gradient elution program was as follows: 0–3 min, 2% B (0.12 mL/min); 3–6 min, 4% B (0.22 mL/min); 6–9 min, 95% B (0.20 mL/min); 9–10 min, 95% B (0.20 mL/min); 10–10.2 min, 2% B (0.12 mL/min); 10.2–14 min, 2% B (0.12 mL/min) held for equilibration. The injection volume was 3 μL, and the column temperature was set at 40 °C. Positive electrospray ionization (ESI) in multiple reaction monitoring mode was applied to monitor the parent and fragment ions of each target analyte. The optimized ESI conditions were as follows: spray voltage, 0.55 kV; source temperature, 500 °C; curtain gas (N₂), 30 psi; ion source gas 1 and 2, 50 psi; and optimized collision energy and declustering potential for each analyte (Figure S1). System control and data analysis were performed by AB Sciex Analyst software (version 1.6.2; AB Sciex, MA, USA). AGEs were quantified by establishing the standard curve of each compound based on the isotope dilution calibration method as previously indicated.⁶ Duplicate measurements were conducted for each sample.

Protein Conformational Changes. Surface Hydrophobicity (*H₀*). The surface hydrophobicity of glycated OVA was determined according to the method of Yang et al.²⁷ Each sample (1.0 mg/mL in 10 mM PBS, pH 7.4) was serially diluted to obtain protein concentrations from 0.2 to 1.0 mg/mL. Then, 20 μL of ANS solution (8 mM, in 10 mM PBS, pH 7.4) was combined with 4 mL of each sample solution. Afterward, the fluorescence intensity was recorded with a fluorescence spectrophotometer at an excitation wavelength of 390 nm and emission spectra within 400–600 nm. The slope of the fluorescence intensity vs protein concentration was defined as the surface hydrophobicity value (*H₀*).

UV Absorption and Intrinsic Fluorescence Spectroscopy. The UV absorption spectrum was analyzed by a UV-2600 spectrophotometer (Shimadzu, Japan). The OVA samples (1 mg protein/mL, in 10 mM PBS, pH 7.4) were scanned from 250–400 nm at a scan rate of 800 nm/min. The intrinsic fluorescence spectrum was acquired with a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan). Each sample (1 mg protein/mL) was excited at 280 nm and the emission wavelength was ranged from 300 to 500 nm. The excitation and emission slit widths were 5 nm.

Circular Dichroism (CD) Spectroscopy. Changes in the secondary structure of glycated OVA were evaluated by CD spectroscopy on a Jasco J-1500 spectropolarimeter (Jasco Corp., Japan). Prior to analysis, each sample was diluted in UP water to obtain a protein concentration of 0.2 mg/mL. The CD spectra were acquired in the far-UV region of 190–260 nm at a scanning speed of 100 nm/min. The path length of the quartz cuvette was 1 mm. Data are expressed as the mean residue ellipticity (θ) in deg. cm² dmol^{−1}. The secondary structural composition was calculated using CD pro software with the SELCON3 and CDSSTR computational methods.

Measurements of IgE-Binding Ability. Indirect Competition Enzyme-Linked Immune Absorbent (icELISA) Assay. The IgE-binding ability of glycated samples was first evaluated by icELISA.²⁸ In brief, a 96-well ELISA plate was coated with 100 μL of native OVA overnight at 4 °C (20 μg protein/mL, in 10 mM PBS, pH 7.4; conditions optimized by a checkerboard titration procedure). After washing and blocking with 3% BSA for 2 h at 37 °C, the plates were incubated with 100 μL of pooled serum (1:50, v/v) and 100 μL of each glycated sample (20 μg protein/mL) for 1.5 h at 37 °C. Afterward, the plates were washed and incubated with HRP goat anti-

human IgE serum (1:5000, v/v) for 1 h at 37 °C. Following repeated washing, 100 μL of TMB solution was added for color development in the dark for 30 min at 37 °C, after which 50 μL of 2 M sulfuric acid was used to terminate the reaction. The absorbance was recorded at 450 nm. The IgE-binding ability was calculated as the inhibition rate (%) using the following formula: inhibition rate (%) = $[1 - \text{OD}_{\text{inhibitor}} / \text{OD}_{\text{blank}}] \times 100\%$, where OD_{inhibitor} is the absorbance in the presence of samples, and OD_{blank} is the absorbance of the uninhibited serum control. The final results were expressed as a percentage relative to the IgE-binding level of untreated OVA.

Dot Blotting. A dot-blot assay was further performed to evaluate changes in the IgE-binding levels of glycated OVA, according to the method of Lin et al.,²⁹ with slight modifications. In brief, 3 μL of each sample (1 mg protein/mL, in 10 mM PBS, pH 7.4) was spotted on an Immobilon PVDF membrane (Millipore Corporation, MA, USA). The membrane was left to dry at room temperature and blocked for 2 h in 3% BSA in phosphate-buffered saline with Tween 20 (PBST). The membranes were washed and incubated with pooled serum (1:20, v/v) for 1.5 h at 37 °C, followed by incubation with HRP goat anti-human IgE serum (1:5000, v/v) for an additional 1 h. After repeated washing, the immunoreactive spots were developed by ECL reagent (Biosharp, Hefei, China) and visualized on a FluorChem HD2 System. The intensity of each spot represents the amount of specific IgE bound to the sample.

sRAGE-Binding Assay. A competition ELISA-based RAGE binding assay was used to assess the binding between glycated OVA and human sRAGE as described by Liu, Teodorowicz, Wichers, Boekel, and Hettinga,³⁰ with some modifications. The specificity was detected by establishing competition between a known high-affinity ligand coated on the plate and the possible ligand formed in glycated OVA. The ELISA plates were coated with BSA-AGE (100 μg/well, in 1.5 mM sodium carbonate buffer, pH 9.6) and kept overnight at 4 °C. BSA-AGE, a potent RAGE ligand, was prepared by incubating BSA (50 mg/mL) with 0.5 M glucose in 0.2 M PBS (pH 7.4) at 50 °C for 4 days, a rapid method simulating the conventional 60-day preparation method.³¹ The plates were washed and blocked with 3% BSA in PBST for 2 h at room temperature. Each glycated sample (1 mg protein/mL) was preincubated with sRAGE (27.4 nM) in 1.5% BSA/PBST for 45 min at 37 °C. After blocking, the plates were washed and added with each premixed solution (100 μL/well), followed by a 1 h incubation at 37 °C. The plates were repeatedly washed and incubated with mouse anti-RAGE antibody (1 μg/mL) for another 1 h at 37 °C. Afterward, the detection antibody (HRP goat anti-mouse antibody; 1:5000, v/v) was added and incubated for 30 min at room temperature. After washing, TMB substrate was added for coloring and the reaction was terminated by 2 M H₂SO₄. Absorbance was read at 450 nm with 620 nm as a reference. The inhibition rate was calculated with the following equation: $[(\text{OD}_{\text{sRAGE}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{sRAGE}}] \times 100\%$, where OD_{sample} represents the absorbance of the sample well, and OD_{sRAGE} is the signal of the wells containing sRAGE but without any competition agent.

Analysis of Glycation Sites by High-Resolution Mass Spectrometry. The structural modification sites in glycated OVA were determined by nano LC–MS/MS analysis according to Dong et al.⁷ In brief, 50 μg protein samples were reduced by dithiothreitol and alkylated with iodoacetamide to reduce disulfide bridges. Then, the samples were subjected to proteolysis by chymotrypsin (Promega, WI, USA; enzyme to substrate ratio, 1:40, w/w) at 37 °C for 2 h. After digestion, the peptides were desalted on a self-priming desalting column, and the eluates were collected, vacuum-dried, and reconstituted in sample solution (0.1% FA, 2% ACN in water) for further analysis.

An Ultimate 3000 system (ThermoFisher Scientific, USA), connected to a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) with an ESI nanospray source was used for the identification of glycated peptides and glycation sites. Fractionation of peptide mixtures was performed on an Acclaim PepMap RSLC C18 column (150 μm × 150 mm, 1.9 μm, Thermo Fisher) coupled to an Acclaim PepMap 100 RP-C18 guided

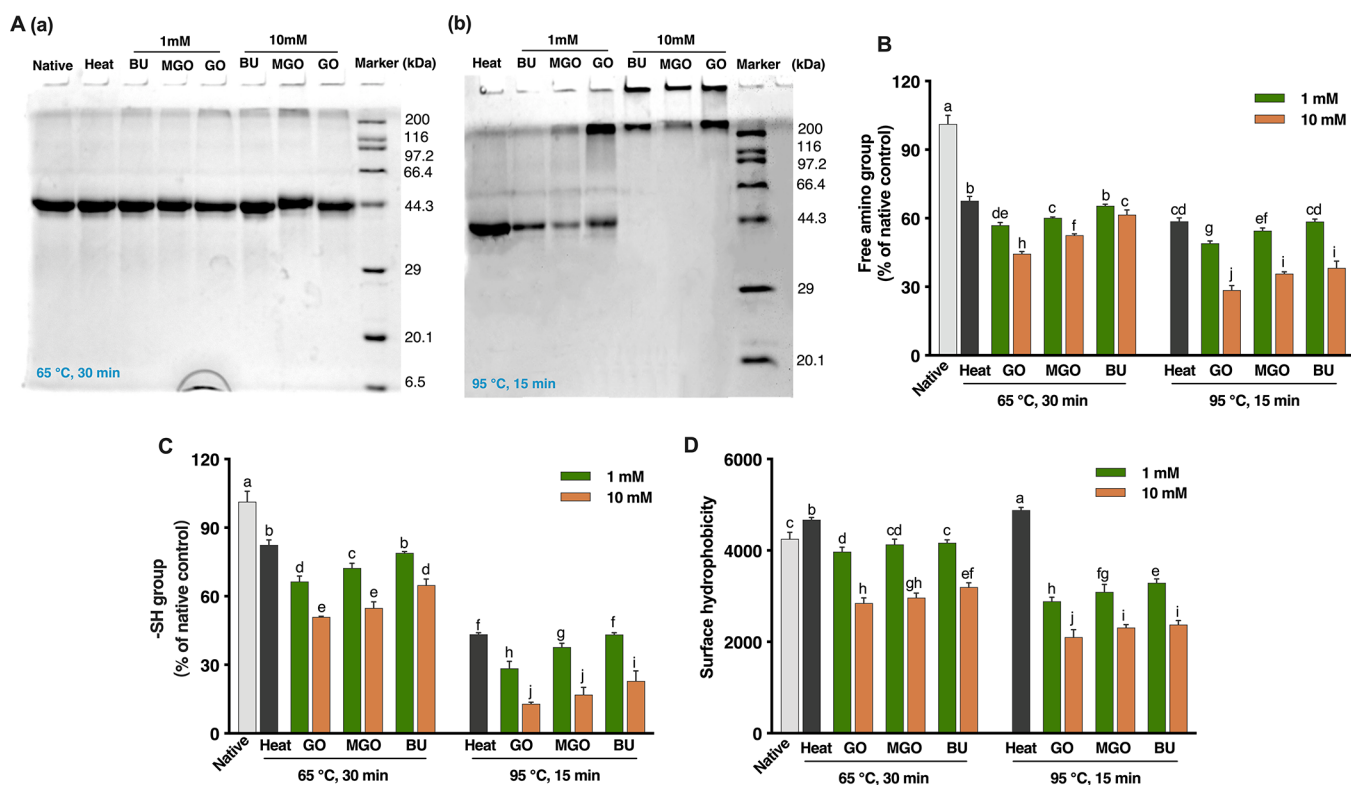


Figure 1. (A) SDS–PAGE profile: (a) 65 °C, 30 min, (b) 95 °C, 15 min, (B) free amino group content, (C) –SH group content, and (D) surface hydrophobicity of OVA glycosylated by different α -dicarbonyls under simulated heating. Values represent the mean $\pm$ SD from three independent replicates. Bars with different letters indicate statistical difference ($p < 0.05$, Tukey's test). OVA, ovalbumin; GO, glyoxal; MGO, methylglyoxal; BU, butanedione.

column (300 μ m $\times$ 5 mm, 5 μ m, Thermo Fisher). The elution program was set as follows: mobile phase A, 0.1% FA and 2% ACN in water; mobile phase B, 0.1% FA in 80% ACN; flow rate, 600 nL/min; and an elution gradient of 4% B at 0 min, increased to 8% B at 2 min, increased to 28% B at 45 min, then increased to 40% B at 55 min, and increased to 95% B at 56 min, and finally, the column was equilibrated with 95% B for 10 min and returned to 4% B prior to the next analysis. All MS spectra were acquired in data-dependent mode. The MS1 and MS2 spectra were acquired in positive mode with a full scan MS: the parent ion scan range was 300–1800 m/z with a resolution of 70,000, and the daughter ion scans started from 100 m/z , where the 20 most abundant ions per MS scan were selected and fragmented by higher-energy collisional dissociation with a resolution of 17,500. The raw MS files were analyzed and searched against the UniProt protein database using Byonic software (Protein Metrics, CA, USA). Some search parameters for the peptide sequence and glycation site mapping were as follows: the accession number of P01012 (OVA), enzyme chymotrypsin, maximum missed cleavage of 3, precursor ion mass tolerance of 20 ppm, and MS/MS tolerance of 0.02 Da. Variable modifications, including oxidation of Met, carbamidomethylation of Cys, acetylation of Lys, and MGO/GO/BU (dehydrated or not)-mediated modifications of Arg and Lys residues were set as previously described.^{7,32,33}

Statistical Analysis. All experiments were conducted in at least triplicates unless otherwise noted and data were reported as the mean $\pm$ standard deviation (SD). Statistical analysis was conducted with one-way analysis of variance (one-way ANOVA) using Duncan's multiple range test. A P value < 0.05 was considered statistically significant. IBM SPSS Statistics Version 22.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS AND DISCUSSION

The Degree of OVA Modification by α -DCs. α -DCs are highly reactive compounds derived from MR and oil

peroxidation, which can react with proteins to form downstream MRPs, i.e., AGEs. In this study, the dosages of α -DCs were 1 mM to simulate the level of daily consumed dicarbonyls,⁹ and 10 mM to amplify the consequences of glycation derived from α -DCs, as previously described.^{7,12,13} SDS–PAGE was first applied to evaluate changes in the molecular size of OVA peptide chains after glycation under simulated heating (Figure 1A). In the absence of α -DCs, compared to native OVA, heating at 95 °C, but not 65 °C, induced broadening of the protein band, indicating the formation of aggregates at steaming temperature, which was in line with previous reports.¹⁷ In the presence of α -DCs, treatments at 65 °C did not lead to obvious changes in the MW of the OVA band except for a slight upshift in the MGO-treated sample at 10 mM (Figure 1A-a). In comparison, at 95 °C, modifications by 10 mM α -DCs revealed significant increments in the MW of OVA and the products even failed to pass through the stacked gels; in contrast, in 1 mM-treated groups, only GO was able to generate crosslinked molecules with higher MW which retained in the upper part of the resolving gel (Figure 1A-b). It has been reported that α -DCs can strongly promote protein polymerization, which is attributed to the rapid degradation of intermediate products to form highly crosslinked MRPs.⁷

To further assess the degree of protein glycation, changes in the contents of free amino and –SH groups were evaluated. With the proceeding of MR, α -DCs degraded from reducing sugars and oil peroxidation could react with the ϵ -NH₂ and guanidine groups of Lys and Arg residues, and the proteins are prone to be grafted with carbonyl/aldehyde compounds, resulting in a loss of their free amino contents.¹ In parallel,

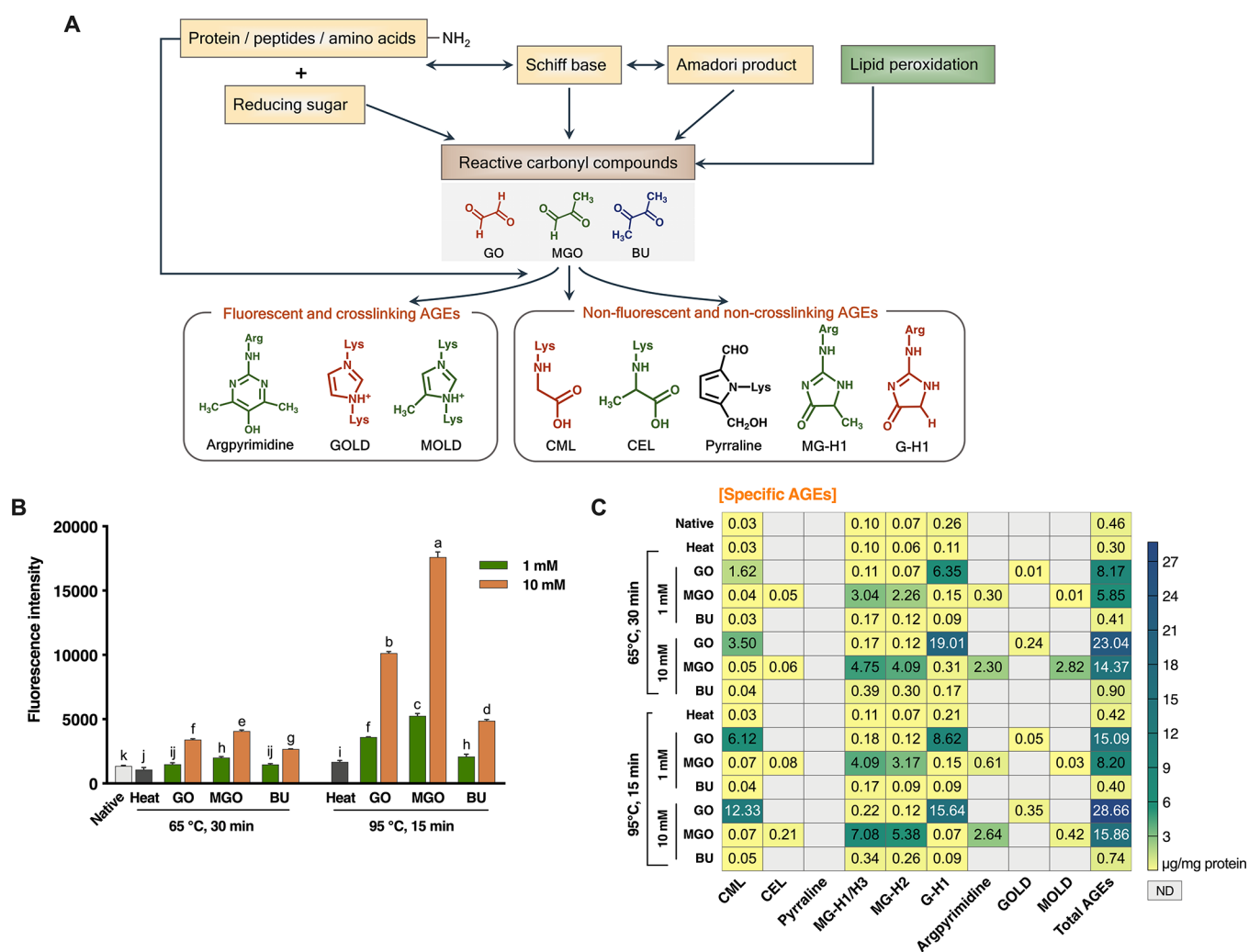


Figure 2. (A) AGEs chemistry and examples of α -dicarbonyls and generated AGEs discussed in this study, (B) total fluorescent AGEs, and (C) quantification of 11 specific AGEs in OVA glycated by different α -dicarbonyls. Values represent the mean $\pm$ SD from three independent replicates for (B) and two independent replicates for (C) (the SD values of (C) are given in Figure S4). Bars with different letters indicate statistical difference ($p < 0.05$, Tukey's test). AGEs, advanced glycation end-products; OVA, ovalbumin; GO, glyoxal; MGO, methylglyoxal; BU, butanedione; ND, not-detected.

there are one cystine disulfide (Cys73–Cys120) and four Cys sulfhydryls (Cys11, Cys30, Cys367, and Cys382) in the polypeptide chain of OVA.³⁴ During MR, the thiol group of Cys residues can be readily oxidized to form disulfide bonds, which consequently leads to protein aggregation and dysfunction.³⁵ As shown in Figure 1B,C, compared to native OVA, glycated OVA suffered various reductions in their free amino and –SH group levels, and this effect was particularly evident in 10 mM GO-treated samples at 95 °C. Meanwhile, heat treatments alone showed a reduction in both free amino and –SH groups but to a lesser extent than the samples incubated with α -DCs. At the same dosage and heating temperature, decreases in the free amino and free thiol groups followed the order GO- > MGO- > BU-OVA > heated control. This is in line with the trend observed in β -casein and β -lactoglobulin glycated by GO, MGO, and BU,¹³ and corroborated well with the reactivity of these α -DCs. As explained by Meade and Gerrard, the lower reactivity of MGO and BU compared to GO can be ascribed to the electron donation and steric hindrance effect of the methyl group next to the carbonyl carbons.³⁶ Overall, these results clearly showed that reactions occurred between OVA and typical α -DCs

generated glycated products with various MWs and degrees of modification.

Generation of AGEs in α -DCs-Glycated OVA. MR generates fluorescent and nonfluorescent AGEs with cross-linked and non-crosslinked structures, yielding heterogeneous products with various compositions.¹ As illustrated in Figure 2A, different AGEs can be formed through the reactions between the ϵ -NH₂ group of Lys or the guanidine group of Arg with different AGE precursors (i.e., α -DCs). In brief, CML and CEL can be produced from the reactions of GO and MGO with Lys, respectively. Modifications of Arg with GO and MGO would generate MGO-derived hydroimidazolones (MG-Hs) and GO-derived hydroimidazolones (G-Hs), respectively. In parallel, two molecules of MGO reacted with Arg to form argpyrimidine. Subsequently, rearrangement and cross-linking of GO and MGO with Lys will eventually cause the formation of GO-lysine dimer (GOLD) and MGO-lysine dimer (MOLD), respectively.^{1,37,38} The reactions between BU and the side chain group of Arg are reported to generate hydroimidazolone compounds with structures similar to those of the MGO- and GO-derived hydroimidazolones.^{13,39}

Total fluorescent AGEs were first determined to provide an overview of the levels of fluorescent AGEs formed in glycosylated OVA (Figure 2B). After α -DC exposure, compared to native and heated controls, significantly higher levels of fluorescent AGEs were generated in the proteins. A higher α -DC dose and heating temperature greatly contributed to the formation of fluorescent AGEs, and the maximum fluorescence intensities followed the order of MGO- > GO > BU-OVA under each treatment condition. This is inconsistent with the above-mentioned reactivity of α -DCs, which could be explained by the formation of specific fluorescent products (e.g., argpyrimidine and MOLD) in MGO-induced groups, which will be discussed in the following parts.

An isotope dilution-based UHPLC–MS/MS method was further employed to establish the quantification of those aforementioned AGEs (detailed information is shown in Figure S1). The results were given as a heatmap in Figure 2C. Clearly, a precursor-dependent pattern of AGEs formation was observed in glycosylated OVA treated with different α -DCs. The GO-induced OVA generated high levels of GO-derived AGEs, namely, CML, G-H1, and GOLD. In comparison, the MGO-treated counterparts primarily produced high amounts of MGO-related products, i.e., CEL, MG-H1/3, MG-H2, argpyrimidine, and MOLD. The BU-treated products generally had low levels of MG-H1/3, MG-H2, G-H1, and CML. Pyrroline, a pyrrole derivative containing the *N*-amino group of Lys, was nondetectable in all samples. Similar to the results of fluorescent AGEs, heating at boiling temperature with a higher α -DC dosage led to the formation of superfluous AGEs, especially in GO- and MGO-treated groups. The amounts of total AGEs formed in GO-, MGO-, and BU-modified OVA at 95 °C were 1.2–1.8 times, 1.1–1.4 times, and 0.8–1.0 times higher, respectively, than those exposed at 65 °C. These results clearly showed that, depending on the precursor type, the cooking temperature had different effects on the contents of AGEs formed in OVA. When discussing the role of α -DCs in AGEs formation during food processing, attention should be given to both key factors, i.e., the presence of specific precursors and the (re)heating conditions.⁷

Protein Conformational Structural Changes. Through far-UV CD spectroscopy, the secondary structural compositions of AGE-modified OVA (referred to OVA-AGE) were determined and are tabulated in Table 1. Native OVA exhibits a typical α -helix (54.9%) and random coil (21.7%) enriched structure, consistent with previous reports.^{18,40} Heat treatments alone induced transformation from the α -helices to β -elemental structures, particularly at 95 °C. With the addition of α -DCs, increases in the contents of β -sheets and random coils at the cost of α -helices were observed, in comparison to the nearly unchanged levels of β -turn structure. Of note, changes in the secondary structure of OVA were much more significant at 95 °C than at 65 °C, and GO-modified OVA showed the greatest extent of structural alterations, followed by MGO- and BU-glycosylated counterparts. It has been reported that glycation of proteins tends to induce an intrinsic instability of the molecules and facilitates their transformation to the unfolded state with β -sheet propensity.⁴¹ Similar minimization of the α -helix on expansion of the β -sheet was previously noticed in OVA glycosylated with monoses^{18,42} and dextrans.⁴³

The surface hydrophobicity value (H_0) was further determined and is presented in Figure 1D. As a parameter indicative of the number of hydrophobic groups on the protein surface, changes in H_0 can be correlated with the stability,

Table 1. Secondary Structural Compositions of OVA Glycosylated by Different α -Dicarbonyls^a

	sample	α -helix	β -sheet	β -turn	random coil
	native OVA	54.9 $\pm$ 0.3 ^a	5.9 $\pm$ 0.3 ^j	16.9 $\pm$ 0.4 ^b	21.7 $\pm$ 0.2 ^j
65 °C, 30 min					
	heated OVA	53.8 $\pm$ 0.4 ^{ab}	7.3 $\pm$ 0.5 ⁱ	17.8 $\pm$ 0.2 ^{ab}	21.4 $\pm$ 0.2 ^j
1 mM	OVA + GO	50.3 $\pm$ 0.9 ^c	8.9 $\pm$ 0.1 ^h	17.2 $\pm$ 0.8 ^{ab}	22.7 $\pm$ 0.4 ⁱ
	OVA + MGO	53.3 $\pm$ 0.6 ^b	7.7 $\pm$ 0.4 ⁱ	16.8 $\pm$ 0.2 ^b	22.5 $\pm$ 0.5 ⁱ
	OVA + BU	54.4 $\pm$ 0.7 ^{ab}	7.4 $\pm$ 0.3 ⁱ	17.4 $\pm$ 0.6 ^{ab}	21.7 $\pm$ 0.2 ^j
10 mM	OVA + GO	44.5 $\pm$ 0.6 ^e	11.4 $\pm$ 0.3 ^f	17.9 $\pm$ 0.4 ^{ab}	25.7 $\pm$ 0.3 ^g
	OVA + MGO	45.1 $\pm$ 1.2 ^e	10.6 $\pm$ 0.4 ^g	17.8 $\pm$ 0.2 ^{ab}	25.1 $\pm$ 0.4 ^g
	OVA + BU	48.3 $\pm$ 0.7 ^d	9.8 $\pm$ 0.2 ^g	17.1 $\pm$ 0.6 ^{ab}	23.7 $\pm$ 0.3 ^h
95 °C, 15 min					
	heated OVA	36.8 $\pm$ 0.4 ^f	17.1 $\pm$ 0.4 ^e	18.3 $\pm$ 0.3 ^{ab}	27.9 $\pm$ 0.6 ^f
1 mM	OVA + GO	29.2 $\pm$ 0.6 ^h	18.9 $\pm$ 0.4 ^{cd}	18.0 $\pm$ 0.2 ^{ab}	33.0 $\pm$ 0.4 ^c
	OVA + MGO	33.2 $\pm$ 0.4 ^g	18.6 $\pm$ 0.3 ^{cd}	17.2 $\pm$ 0.6 ^{ab}	30.4 $\pm$ 0.4 ^d
	OVA + BU	36.3 $\pm$ 0.5 ^f	18.1 $\pm$ 0.6 ^d	16.7 $\pm$ 0.3 ^b	28.8 $\pm$ 0.2 ^e
10 mM	OVA + GO	25.2 $\pm$ 0.6 ^j	20.3 $\pm$ 0.6 ^a	18.6 $\pm$ 0.4 ^a	36.2 $\pm$ 0.3 ^a
	OVA + MGO	27.1 $\pm$ 0.4 ⁱ	19.8 $\pm$ 0.7 ^{ab}	17.9 $\pm$ 0.4 ^{ab}	35.3 $\pm$ 0.3 ^b
	OVA + BU	28.9 $\pm$ 0.4 ^h	19.2 $\pm$ 0.3 ^{bc}	18.1 $\pm$ 0.6 ^{ab}	33.6 $\pm$ 0.2 ^c

^aValues represent the mean $\pm$ SD from three independent replicates. Different letters suggested significant difference within the same column ($p < 0.05$, Tukey's test). OVA, ovalbumin; GO, glyoxal; MGO, methylglyoxal; BU, butanedione.

conformation, and functionality of protein molecules. Additionally, hydrophobic force was involved in the specific paratope–epitope interactions between an antibody and antigen in the allergen recognition process.⁴⁴ Glycation with α -DCs decreased the H_0 level of OVA in a dose-dependent manner, whereas heat treatments alone enhanced the H_0 value of OVA. Under the same treating condition, again, the H_0 level of glycosylated OVA ranked in the order GO- < MGO- < BU-OVA < heated control. These results correspond well with previous studies showing that MR significantly decreases the H_0 value of OVA.^{18,45} The carbonyl compounds covalently linked to the amino acid residues would introduce an increased steric hindrance of certain hydrophobic groups and mask some hydrophilic groups such as Lys and Arg residues; in comparison, thermal treatment itself may cause unfolding of the protein structures to expose buried hydrophobic groups to the polar solvent, thereby showing an elevated H_0 value.^{18,45}

To acquire more detailed information on the conformational and microenvironmental changes of glycosylated OVA, UV absorption and intrinsic fluorescence spectroscopies were applied (Figure 3). The UV absorption spectrum mainly accounts for the light absorption by the chromophores of Trp and Tyr residues, and perturbation in the protein structure can be reflected in the shape and intensity of absorption peaks. As seen in Figure 3A, compared to native OVA, heated OVA exhibited a slight increase in the absorbance intensity at 280

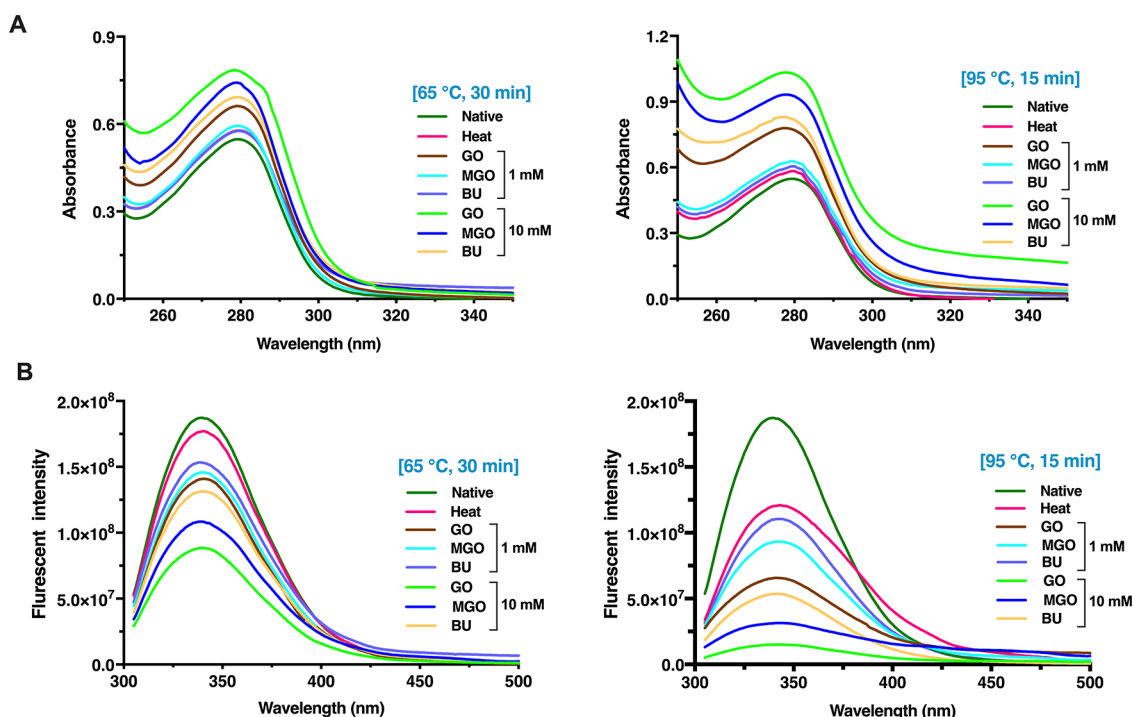


Figure 3. Effect of glycation derived from α -dicarbonyls on the (A) fluorescence emission spectra and (B) UV absorption spectra of OVA under simulated heating. OVA, ovalbumin; GO, glyoxal; MGO, methylglyoxal; BU, butanedione. Values represent the mean from three independent replicates.

nm; α -DC-glycation of proteins further exacerbated this growing effect of the maximum absorbance intensity, especially at higher treatment temperatures and α -DC doses. The hyperchromicity of OVA was increased by 89.3, 70.6, and 51.7% in GO-, MGO-, and BU-treated OVA, respectively. These data demonstrated that the glycation process unfolded the structure of proteins to expose more residues with aromatic groups on the molecular surface, in agreement with previous observations.^{7,18,46}

The intrinsic fluorescence spectrum was closely related to the polarity of the microenvironment near Trp, Tyr, and Phe residues along with their specific interactions, which can be used to evaluate changes in the conformations of proteins.⁴⁷ Generally, when excited at 280 nm, untreated OVA presented a maximum fluorescence emission at approximately 347 nm (Figure 3B). As glycation would promote Trp loss and fluorescence quenching, the fluorescence intensities of glycated OVA displayed significant reductions to various degrees compared to the native control, as expected. Similar to the CD and UV spectra, the GO-decorated samples showed the largest decline in the maximum fluorescence intensities, followed by MGO- and BU-treated counterparts. A small blueshift in the λ_{max} (from 342 to 340 nm at 65 °C; and from 347 to 341 nm at 95 °C) was also noted in glycated samples, implying changes in the polarity of the environment of Trp residues. The quenching effect of α -DCs on OVA fluorescence can be result from, on the one hand, the progressive exposure of Trp residues to the hydrophilic solvent during protein unfolding and, on the other hand, the generation of crosslinked AGEs with modified accessibility and microenvironments of the key reactive residues.^{12,18}

Glycated OVA Shows Lower IgE-Binding Levels but with sRAGE-Binding Ligands. As OVA can be modified by α -DCs upon heating, resulting in OVA-AGE conjugates with

various degrees of modification and conformational structures, we next questioned that whether these α -DC-mediated changes would impact the antigenicity of OVA. The IgE-binding ability assay serves as a prerequisite for evaluating the antigenicity of a modified allergen. In doing so, both iELISA and dot-blotting assays were applied, using serum samples from egg white-allergic individuals. As shown in Figure 4A, compared to native OVA, OVA subjected to heating alone displayed about 20 and 50% reductions in the IgE-binding level at 65 and 95 °C, respectively, in agreement with previous studies.^{17,48} In glycated samples, notable decreases in the IgE-binding capacity were observed. Again, the IgE-binding level of OVA-AGE increases in the order GO- < MGO- < BU-OVA at each treatment. Compared to native OVA, the highest reduction in the antigenicity was in the 10 mM GO-induced group, where the binding level was reduced by 45% at 65 °C and 72% at 95 °C, respectively. Similar trends were noticed in the immune-dot-blot assay (Figure 4B): native OVA presented the strongest intensity of blotting points, while GO-glycated OVA at 10 mM had the weakest points in the dot under both heating conditions.

Although previous work has explored the impacts of MR on the IgE-binding ability of OVA by using various reducing sugars, this is the first study where α -DCs, intermediates of MR, are directly used for glycation treatment to investigate their effects on immunogenic epitopes. Compared to monoses, the reactivity of α -DCs is much stronger,³ and by comparing different dicarbonyls, we are able to interpret the role of different types of AGE-Ms. The above results clearly showed that glycation by α -DCs could potentially attenuate the IgE-binding level of OVA, but the mitigation efficiency depended on the precursor type and exposure conditions. In general, the failure to recognize OVA by specific IgE in glycated proteins can be ascribed to the destruction or masking of epitopes by

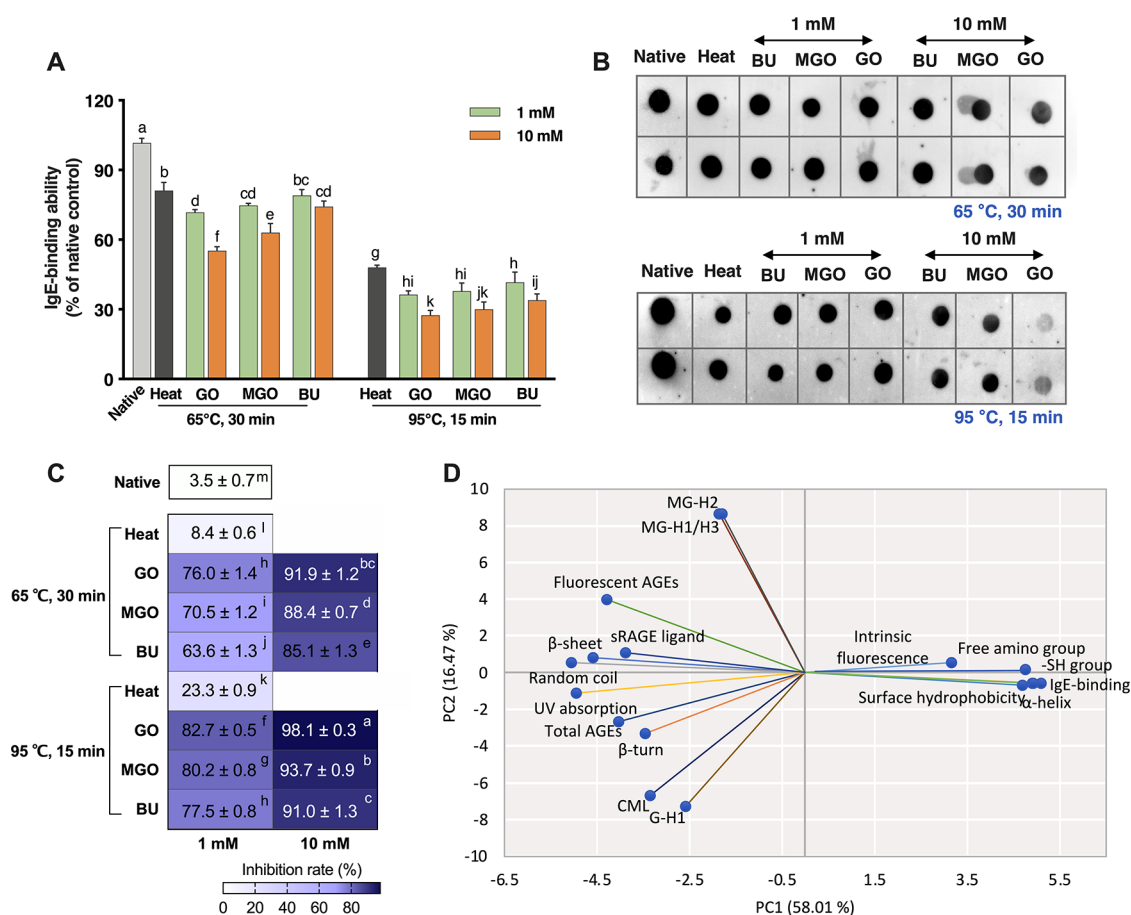


Figure 4. Changes in the IgE-binding ability of glycated OVA, as tested by (A) icELISA and (B) dot blotting, (C) heatmap showing the sRAGE-binding capacity of glycated OVA, and (D) PCA showing associations between investigated variables. PC1 (x) vs PC2 (y) was presented. Values represent the mean $\pm$ SD from three independent replicates. Bars/values with different letters indicate statistical difference ($p < 0.05$, Tukey's test). A representative image is shown of the blotting membranes. OVA, ovalbumin; RAGE, receptor for advanced glycation end-product; GO, glyoxal; MGO, methylglyoxal; BU, butanedione.

covalent modification and condensation between reactive compounds and amino acid residues of OVA, as explained in several previous reports.^{18,27}

To further elucidate the factors that contributed to the hypoantigenic properties of OVA-AGE, principal component analysis (PCA) was applied. The score plot of PC1 vs PC2 is presented in Figure 4D, and the eigenvalues of principal components (PCs) and factor loading are given in Table S2. The first five PCs explained 95.2% of the total variability, and PC1 and PC2 represented 58.0 and 16.5% of the total variation, respectively. The IgE-binding ability can be best explained by PC1, which positively correlated with the degree of protein modification (free amino and -SH groups), H_0 value, α -helix content, and intensity of protein intrinsic fluorescence, but negatively correlated with the contents of β -sheets, β -turns, random coils, UV absorption intensity, and levels of AGEs formed (e.g., total and fluorescent AGEs). The amounts of CML, but not other AGEs, were found to be related to the IgE-binding capacity of OVA-AGE. It has been demonstrated that the glycation process would induce protein aggregation, leading to a loss of accessibility of some IgE epitopes. In addition, partial unfolding of the OVA structure, as reflected by reduced α -helix on expansion of β -sheets and random coils, would also cause destruction of some conformational IgE epitopes.⁴⁹ Overall, these results manifested that discrepancies in the IgE-binding level of samples can be

explained by the reactivity of different α -DCs, various degrees of protein modifications, and conformations of the final products.

Despite a reduction in the IgE-binding capacity of OVA following glycation, MR has long been criticized for the generation of health-hazardous AGEs, which are capable of interacting with AGE receptors (e.g., RAGE) in vivo to activate downstream pathways related to oxidative stress, inflammation, and adaptive immune responses.^{1,6} In this study, we next sought to see, accompanying the lower IgE-binding ability, whether sRAGE-binding ligands were generated in the glycated OVA. As portrayed as a heatmap in Figure 4C (OD data shown in Figure S2), native OVA was almost unable to bind sRAGE; in contrast, up to 92 and 98% inhibition rates were obtained for 10 mM GO-glycated OVA at 65 and 95 °C, respectively. The binding ability between sRAGE and α -DC-modified OVA ranged in the order GO- > MGO- > BU-OVA > heated control. The inhibition rate of sRAGE-binding was also found to correlate with the amounts of total AGEs formed ($r = 0.596$, $p = 0.02$, Pearson correlation analysis) and, as expected, the IgE-binding ability of glycated samples ($r = -0.544$, $p = 0.02$). Previous studies have shown that dicarbonyl compounds are highly reactive molecules that induce greater AGEs formation during MR than reducing sugars, thus providing a stronger capability to bind sRAGE.³⁰ In this regard, it has been speculated that the effects of nonenzymatic

Table 2. Glycation Sites and Structures Identified in OVA Treated by Different α -Dicarbonyls (10 mM)^a

glycation sites	65 °C, 30 min			95 °C, 15 min		
	GO	MGO	BU	GO	MGO	BU
Lys residues						
K17	CML	CEL		CML	CEL	
K20	CML	CEL		CML	CEL, CML, pyrraline	
K62			CEL			
K93	CML			CML	CEL	
K123	CML	CEL		CML	CEL, CML, pyrraline	
K182	CML	CEL		CML	CEL, CML, pyrraline	
K187	CML			CML	CEL	
K190	CML	CEL		CML, CEL	CEL, CML	
K207	CML, CEL	CML, CEL, pyrraline	CML, CEL	CML, CEL	CEL, CML, pyrraline	CML, CEL
K227				CML, CEL	CEL	
K229		CEL		CML	CEL, CML, pyrraline	
K264	CML			CML		
K278	CML, CEL	CML, CEL, pyrraline	CML	CML, CEL	CEL, CML, pyrraline	
K280	CML, CEL	CEL		CML, CEL, pyrraline,	CEL, CML, pyrraline	
K287	CML	CEL		CML, CEL	CEL, CML, pyrraline	
K291	CML			CML	CEL, pyrraline	
K323						
K370	CML	CEL		CML	CEL	
Arg residues						
R59			G-DHs			
R85	G-DHs	MG-Hs, THP		G-Hs, G-DHs	MG-Hs, MG-DHs, RPYR, DHP, THP	
R105	G-DHs, G-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR	MG-Hs	G-Hs, G-DHs, MG-Hs	MG-Hs, MG-DHs, RPYR, DHP, THP	MG-Hs
R111	G-DHs, G-Hs, MG-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR	MG-Hs	G-Hs, G-DHs	MG-Hs, MG-DHs, RPYR, DHP, THP	MG-Hs
R127	G-DHs, G-Hs, THP	MG-DHs, MG-Hs, DHP, THP, RPYR	MG-Hs, G-DHs	G-Hs, G-DHs, THP MG-Hs	MG-Hs, MG-DHs, RPYR, DHP, THP	MG-Hs DHP, THP
R143	G-DHs, G-Hs, MG-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR	MG-Hs, MG-DHs	G-Hs, G-DHs, THP, MG-Hs, RPYR	MG-Hs, MG-DHs, RPYR, DHP, THP, G-DHs	MG-Hs
R159				G-DHs	MG-Hs, THP	-
R200	G-DHs, G-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR, G-Hs, G-DHs	MG-Hs, MG-DHs	G-Hs, G-DHs	MG-Hs, MG-DHs, RPYR, DHP, THP, G-Hs	MG-Hs
R219				G-DHs	MG-Hs, DHP, THP	
R277	G-DHs, G-Hs, MG-DHs	MG-DHs, MG-Hs, DHP, THP, RPYR, G-Hs, G-DHs	MG-Hs, MG-DHs, G-DHs	G-Hs, G-DHs, THP MG-DHs, RPYR, MG-Hs	MG-Hs, MG-DHs, RPYR, DHP, THP, G-Hs, G-DHs	MG-Hs
R285	G-DHs, G-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR	-	G-Hs, G-DHs, THP	MG-Hs, MG-DHs, RPYR, DHP, THP	-
R360	G-DHs, G-Hs, MG-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR	MG-Hs	G-Hs, G-DHs, MG-Hs	MG-Hs, MG-DHs, RPYR, DHP, THP	MG-Hs
R382	G-DHs, G-Hs, MG-DHs, MG-Hs	MG-DHs, MG-Hs, DHP, THP, RPYR	MG-DHs	G-Hs, G-DHs, MG-Hs, MG-DHs	MG-Hs, MG-DHs, RPYR, DHP, THP	MG-Hs

^aOVA, ovalbumin; GO, glyoxal; MGO, methylglyoxal; BU, butanedione; CML, *N* ϵ -carboxymethyllysine; CEL, *N* ϵ -carboxyethyllysine; MG-Hs, MGO-hydroimidazolone isomers; MG-DHs, MGO-derived dihydroxyimidazolines; GO-Hs, GO-hydroimidazolone isomers; GO-DHs, GO-derived dihydroxyimidazolines; RPYR, argpyrimidine; DHP, dihydropyrimidine; THP, tetrahydropyrimidine.

glycation on food allergens go beyond IgE binding. On the one hand, the glycation structures may affect the recognition, uptake, and processing of food allergens by antigen-presenting cells (e.g., dendritic cells);^{50,51} on the other hand, AGEs formed during MR would interact with AGE receptors expressed in tissues and some immune cells to promote immune responses and cytokine secretion.^{52,53} Here, our data highlighted the presence of sRAGE-binding ligands in hypo-antigenic OVA-AGE, which might impact their behaviors involved in immunogenicity and allergenicity in vivo. Future studies such as the uptake and processing of glycated OVA by DCs, subsequent impacts on T cell polarization and the role of AGE-RAGE signaling pathways warrant further investigation.

Glycation Sites Reveal Modification Types of α -DC-Glycated OVA. To further comprehend the patterns of AGE-Ms in glycated OVA, high-resolution Q-Orbitrap-MS/MS was applied to investigate the glycation sites and structures of OVA at the molecular level. Chymotrypsin was selected for digestion due to its high efficiency in cleaving peptide bonds, thus providing higher sequence coverage than trypsin (data not shown). The following variable modifications were set: CEL (+58 Da), CML (+72 Da), pyrraline (+108 Da), MG-Hs (+54 Da), MGO-derived dihydroxyimidazolines (MG-DHs, +72 Da), G-Hs (+40 Da), GO-derived dihydroxyimidazolines (G-DHs, +58 Da), argpyrimidine (+80 Da), dihydropyrimidine (DHP, +126 Da), and tetrahydropyrimidine (THP, +144

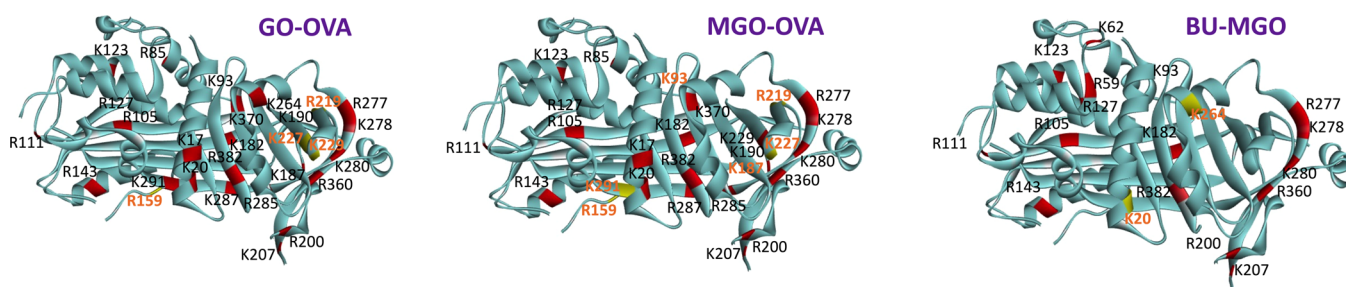


Figure 5. Ribbon diagram of OVA showing the number and location of modified sites in different glycosylated OVA (10 mM). The glycosylated sites were labeled in red for those residues identified at both temperatures, and yellow for those occurred only at 95 °C, 15 min (bold labels).

Da).^{7,32,33,54} It should be noted that some AGE-Ms were not able to be quantified in Figure 2C due to the unavailability of commercial standards. These nonquantified AGE-Ms were considered in this section, in order to have a global proteomic screening of chemical modifications in OVA-AGE.

The peptide sequences were first confirmed by searching the peptide precursor fragmentation in the database, and the glycosylated peptides were mapped by comparing the mass shift between the same glycosylated and unglycosylated sequences through *b* and *y* ion analysis. Figure S3 presents examples of the MS/MS spectra of glycosylated peptides, which are K₁₈₂GLWEKAF (Figure S3a) with different modifications of CML and CEL on K182 and R₂₀₀VTEQESKPVMY (Figure S3b) with different modifications of MG-Hs and argpyrimidine on R299. The location and types of modification in different OVA-AGE (10 mM) are summarized in Table 2. There are total 20 Lys (K) and 15 Arg (R) residues in OVA, and the Lys residues are modified in three types, including CML, CEL, and pyrroline, while the Arg residues are modified in seven types, namely, MG-Hs, MG-DHs, G-Hs, G-DHs, argpyrimidine, DHP, and THP. The pyrroline can be identified here but cannot be quantified in Figure 2C, which can be ascribed to the high limit of detection of the established method. As proteomic is a high-resolution approach, it is able to identify some AGE-Ms that cannot be detected in the quantification method.

Similar to the results of AGEs contents (Figure 2C), in GO-glycosylated OVA, CML was the main type of AGE-Ms on Lys residues, and G-Hs and G-DHs were the main kinds of AGE-Ms on Arg residues. In comparison, for MGO-induced proteins, CEL and MGO-(di)hydroimidazolones (i.e., MG-Hs and MG-DHs) were the major AGE-Ms identified on Lys and Arg residues, respectively; in contrast, CML, G-Hs, and G-DHs were less often detected in MGO-OVA, which was in line with the results of AGEs quantification. It was also found that heating at steaming temperature (95 °C, 15 min) induced the occurrence of new glycosylation sites (e.g., K187 and R159) and more types of AGE-Ms (e.g., K123 and R143), confirming that heat temperature had a powerful influence on the modification pattern. Figure 5 presents the ribbon diagram of OVA showing the number and location of modified residues in different glycosylated OVA. Taking the 65 °C-treated groups as examples, GO-OVA and MGO-OVA had 24 and 21 glycosylation sites, respectively, in comparison to only 12 glycosylation sites identified in the BU-OVA.

Regarding associations between the identified glycosylation sites and the antigenicity of OVA-AGE, on the one hand, previous work using egg-allergic patients' sera has identified several IgE-binding epitopes of OVA, with amino acid residues (AA) of AA125–134, AA141–154, AA159–172, AA164–176,

AA188–198, AA326–336, and AA370–385.⁵⁵ Here, we identified that GO/MGO-glycosylated OVA contained numerous modification sites, including R127, R143, K190, K370, R382, all of which fell within the sequences of the IgE-binding epitopes, thus providing a masking effect on the epitope recognition. On the other hand, the modification sites at different locations would also disturb tertiary structures of OVA, which would influence the accessibility of some linear and conformational epitopes leading to a reduced IgE–epitope interaction.

In conclusion, this study systematically explored the structural and IgE-binding changes in OVA glycosylated by different α -DCs during common heat treatments. The results revealed that, depending on the precursor type, OVA-AGE exhibited various degrees of modification, conformations, and amounts of AGEs. The ability of OVA-AGE to bind specific IgE and sRAGE correlated with the levels of specific AGEs and the extents of protein structural unfolding. Compared to BU, GO and MGO presented a greater reactivity to modify OVA, highlighting their roles in the generation of OVA-AGE to induce relevant biochemical consequences. It is worth noting that, a lower IgE-binding ability does not guarantee hypo-allergenicity in vivo. In view of this, changes in the allergenicity and metabolism of OVA-AGE, including their recognition by the immune system, sensitization mechanism, and metabolic fates remain to be elucidated by further animal studies, which are already underway in our research lab.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c06939>.

Information of the hen's egg-allergic human sera samples; eigenvalues of the principal components and loading factors; detailed information of the retention time and MS/MS parameters of AGE standards and their isotope-labeled internal standards; the sRAGE-binding capacity of OVA glycosylated by different α -dicarbonyls (OD data); and quantification of 11 specific AGEs in OVA glycosylated by different α -dicarbonyls (PDF)

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Notes

The authors declare no competing financial interest.

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