

Mechanism on the Allergenicity Changes of α -Lactalbumin Treated by Sonication-Assisted Glycation during *In Vitro* Gastroduodenal Digestion

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ABSTRACT: Physical-assisted chemical modification is effective to reduce the allergenicity of α -lactalbumin (ALA). However, there are few in-depth studies on the allergenicity changes of physical-assisted chemical-modified ALA during digestion. The effect of gastroduodenal digestion on the allergenicity changes of ALA treated by sonication-assisted glycation was assessed. Digestion of both ALA and its glycated forms generated peptide fractions, and intact undigested glycated ALA in the hydrolysates still covalently bound to D-galactose. High-resolution mass spectrometry revealed that a higher glycation degree was discovered in sonication-preprocessed ALA compared to native ALA. Enzyme-linked immunosorbent assay and basophil degranulation showed that sonication-assisted glycation could significantly reduce ALA allergenicity. The allergenicity of both gastric and gastroduodenal hydrolysates was further increased, and the hydrolysates of sonication-assisted glycated ALA showed the lowest allergenicity. The reason could be the shielding effect of the linear epitope found to be caused by a higher glycation degree; although linear epitopes were exposed, D-galactose covalently bound to intact undigested glycated ALA in the hydrolysates retained its masking role. These results indicated that sonication-assisted glycation could be a promising method to prepare immunotherapeutic agents for allergen immunotherapy to achieve the purpose of allergy desensitization.

KEYWORDS: α -lactalbumin, sonication-assisted glycation, allergenicity, high-resolution mass spectrometry, gastroduodenal digestion

INTRODUCTION

Milk, divided into casein and whey protein, has been identified capable of providing nutrients (such as protein and minerals), foaming, emulsifying, etc.^{1,2} Cow milk is marketed, both liquid or solid, including milk beverage, pasteurized milk, and milk powder.³ Cow-milk-based infant formulas are of great significance for babies when breastfeeding is impossible.⁴ However, milk is defined to be among the primary inducers of human food allergy, and allergy to cow milk differs extensively in the clinical manifestation.^{5–7} Individuals with cow milk protein allergy (CMPA) and no consumption of cow milk are shorter than those on a normal diet.⁸ In the allergogram of cow milk, monomeric α -lactalbumin (ALA), accounting for 5% of cow milk and consisting of 123 amino acids to form a globular domain, is the main allergen in whey protein.⁹ The linear and conformational epitopes on ALA are responsible for its severe clinical symptoms.¹⁰

CMPA is an immune-mediated response caused by the ingestion of protein allergens of cow milk. This hypersensitivity is predominately immediate and immunoglobulin E (IgE)-mediated but can be delayed in non-IgE-mediated cases.¹¹ The IgE-mediated hypersensitivity of ALA includes the sensitization, stimulation, and effect phase. After a second interaction with a host that has already been sensitized with ALA, the second intake of ALA will combine with previously attached IgE antibodies that have bound on the surface of mast cells and basophils, leading to degranulation and the release of inflammatory mediators, such as histamine. In the effect phase, these released inflammatory mediators will act on their

receptors present in several organs or tissues of the body, resulting in various clinical symptoms (rhinitis, diarrhea, colic, etc.) of allergy.¹² Numerous methods, including skin prick test, immunoblotting, and enzyme-linked immunosorbent assay (ELISA, the most commonly used), have been adopted in terms of evaluating the allergenicity of food allergens.^{13,14} Moreover, *in vitro* cell evaluation can mimic paths of allergenicity *in vivo*, which is convenient and, in particular, has provided an effective potential.¹⁵

The reduction of the allergenicity of cow milk has been carried out by adopting numerous methods, including physical treatment and chemical processing, such as phosphorylation, glycation, and their combination.^{16–18} In recent years, physical-assisted chemical methods that allow for an effective reduction in foodstuff allergenicity have attracted the interest of researchers.¹⁹ High-intensity sonication, a non-thermal processing technology, used to be applied to extract, has caught the attention to study its effect on food allergens.²⁰ Glycation can cause the free amino groups of alkaline amino acids on protein allergens to covalently bind with reducing sugars, which, in the case of linear epitopes are masked, thus

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reducing the binding capacity of IgG/IgE antibodies. Our previous studies showed the role of sonication in unfolding the advanced structure of food allergens (ALA), which was conducive to glycate with reducing sugars, resulting in reducing its antibody-binding abilities.²¹ However, the allergenicity changes of ALA treated by sonication-assisted glycation are unknown during digestion. *In vitro* simulated digestion provides a replaceable choice because of the difficulties in conducting *in vivo* digestion.²² Simultaneously, the content of IgE antibodies in the blood is not the only reason for the induction of allergic symptoms. Only when the protein allergens bind to more than two adjacent IgE antibodies and make multiple high-affinity IgE receptors (FcεRI) cross-link to form a complex can the inflammatory mediators be released, eventually leading to allergic symptoms.²³ The forming of the complex can be reflected in *in vitro* cell evaluation. Our previous studies lacked the investigation of cell evaluation on the allergenicity changes of ALA treated by sonication-assisted glycation during digestion.²¹

This work aimed to provide an allergenicity change evaluation of ALA treated by sonication-assisted glycation during *in vitro* simulated digestion in infants by indirect-competitive ELISA (icELISA) and cell degranulation. The glycosylated sites in ALA were determined through high-resolution mass spectrometry (MS/MS). Finally, the mechanism of allergenicity changes was analyzed.

MATERIALS AND METHODS

Chemicals and Materials. ALA (L6010, ≥85%, from bovine milk) was bought from Sigma-Aldrich (St. Louis, MO, U.S.A.). *o*-Phthalaldehyde (OPA, P10963) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (ABFH, D131214) were purchased from Aladdin (Shanghai, China). Protease used in the study included pepsin (P8160, from porcine gastric mucosa, with the activity of 1:10 000), trypsin (T8150, from pancreas, with the activity of 1:250), and α-chymotrypsin (YZ-1134007, with the activity of 1:1200), obtained from Solarbio (Beijing, China). The sera pool was prepared with the same amounts of sera of 10 CMPA patients and was bought from Plasma Lab International (Everett, WA, U.S.A.).

Preparation of the ALA- α -Galactose Covalent Complex. The ALA- α -galactose covalent complex was prepared by the method of sonication-assisted glycation. ALA (1 mg/mL) from bovine milk was preprocessed by sonication at the intensity of 100 W/cm². Then, α -galactose (1 mg/mL) was introduced into glycation with native ALA or sonication-preprocessed ALA in the ratio of 1:1 (w/w) and further lyophilized. The lyophilized mixture was glycosylated at 55 °C for 3.5 h (saturated NaCl with the humidity of 74.41%). Samples were stored at -20 °C after removing free α -galactose and salt (ultrafiltration centrifugal tube, 3 kDa cutoff). ALA without any treatment was named ALA; native ALA glycosylated with α -galactose was called ALA-gal; and sonication-preprocessed ALA (100 W/cm²) glycosylated with α -galactose was regarded as 100ALA-gal. ALA, ALA-gal, and 100ALA-gal were called primary samples.

***In Vitro* Simulation of Infantile Gastroduodenal Digestion.** The infantile simulated gastroduodenal digestion of ALA, ALA-gal, and 100ALA-gal, with some adaptations, was prepared as described previously.²² The simulated gastric solution (pH 3.0) with 0.15 mol/L NaCl was prepared when pepsin was 22.75 units/mg of sample. The pH of the simulated gastric solution was readjusted to 7.0 after digesting for 2 h at 37 °C. Then, the simulated duodenal solution was added for further digestion (2 h and 37 °C). The simulated duodenal solution consisted of 3.45 units of trypsin/mg of sample, 0.04 unit of chymotrypsin/mg of sample, and bile salts (2 mmol/L). The digestive behavior was terminated by adding a solution of ABFH (4 mmol/L). ALA, ALA-gal, and 100ALA-gal digested by simulated gastric solution (2 h) were named ALA-G, ALA-gal-G, and 100ALA-gal-G, respectively, and were collectively called gastric hydrolysates. ALA,

ALA-gal, and 100ALA-gal digested by simulated gastric solution (2 h) followed by 2 h of simulated duodenal solution were ALA-D, ALA-gal-D, and 100ALA-gal-D, respectively, and were collectively named gastroduodenal hydrolysates.

Tricine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Tricine SDS-PAGE was elucidated according to a previous study with some modifications.²⁴ The concentrations of separating gel, spacer gel, and stacking gel used were 16.5, 10, and 4%, respectively. The tricine SDS-PAGE gel was imaged by ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, U.S.A.). Image Lab was used to calculate the mobility of the bands that appeared. The mobility was calculated as follows:

$$\text{mobility} = \frac{S}{L}$$

where L means the total length of the gel and S represents the distance of the bands to the well of the gel.

High-Performance Liquid Chromatography (HPLC) Performance. ALA, ALA-gal, 100ALA-gal, and their gastric/gastroduodenal hydrolysates were analyzed using HPLC (L-2000, Hitachi, Japan), equipped with a L-2130 pump, L-2400 ultraviolet (UV) detector, and L-2200 autosampler. A gel chromatographic column XBridge BEH 125 Å SEC (7.8 mm × 300 mm × 3.5 μm) was applied. The samples were filtered by 0.45 μm of syringe-driven filters (Jinteng, Tianjin, China). Solvents A (acetonitrile, 99.9%, HPLC) and B (deionized water with 0.1% trifluoroacetic acid) were used for isocratic elution (40:60, v/v) with the detection time of 60 min. The calibration curve, $\log(w) = -0.1884t + 6.6012$, with $R^2 = 0.9915$, was prepared using standards, such as oxidized glutathione (613 Da), aprotinin (6512 Da), bacitracin (1423 Da), and L-hydroxyproline (131 Da).

Determination of Free Amino Groups. As a result of the effect of the digestive behavior, the content of free amino groups will increase after digestion. The content of free amino groups was measured using OPA solution, prepared according to the previous method.²⁵ A total of 100 μL of ALA, ALA-gal, 100ALA-gal, and their gastric/gastroduodenal hydrolysates was mixed with OPA solution (2 mL) in the dark for 2 min. The standard curve ($y = 1.6414x + 0.0604$, with $R^2 = 0.9997$) was drawn by L-lysine (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) at the wavelength of 340 nm. The grafting degree was calculated as follows:

$$\text{grafting degree (\%)} = \frac{A_0 - A_s}{A_0} \times 100\%$$

where A_0 refers to the content of free amino groups of native ALA and A_s is the content of free amino groups of glycosylated ALA.

Indirect Competitive ELISA. The IgE-binding rates of ALA, ALA-gal, 100ALA-gal, ALA-G, ALA-gal-G, 100ALA-gal-G, ALA-D, ALA-gal-D, and 100ALA-gal-D were measured via icELISA.²⁶ A total of 100 μL/well of native ALA (4 μg/mL) was used to coat a 96-well microplate for 1 h at 37 °C. A total of 250 μL/well of fish gelatin solution (1%, w/v) was applied to block for 1 h. A total of 50 μL of sera of CAM patients (1:20) and 50 μL of samples were incubated for 1 h. After that, 100 μL of goat anti-human IgE-horseradish peroxidase conjugate (1:800, Immunology Consultants Laboratory, Shanghai, China) was added. A total of 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) was added for coloring (Solarbio, Beijing, China). A total of 100 μL of sulfuric acid solution (12 mol/L) was used to terminate the reaction. The calculation formula of IgE-binding rates was

$$\text{IgE-binding rates (\%)} = \frac{OD_n - OD_s}{OD_n - OD_0} \times 100\%$$

wherein OD_n indicates the OD value of samples replaced by phosphate-buffered saline (PBS), OD_s is the OD value of samples, and OD_0 is the OD value of lacking sera of CAM patients.

Analysis by High-Performance Liquid Chromatography–Higher Energy Collisional Dissociation–Tandem Mass Spectrometry (HPLC-HCD-MS/MS). The working condition of high-

performance liquid chromatography (HPLC, Ultimate 3000RSLCnano, Thermo Fisher Scientific, Waltham, MA, U.S.A.) was as follows: The mobile phase consisted of solution A (0.1% formic acid aqueous solution) and solution B (0.1% formic acid acetonitrile solution). The column was equilibrated with 4% B. The samples were loaded into the loading column (Acclaim PepMap100, RP-C18, 100 $\mu\text{m} \times 2\text{ cm}$, Thermo Fisher Scientific, Waltham, MA, U.S.A.) by an automatic injector and then separated by an analytical column (Acclaim PepMap RSLC, 75 $\mu\text{m} \times 15\text{ cm}$, RP-C18, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Separated peptides were analyzed by MS and MS/MS (Orbitrap Fusion, Thermo Fisher Scientific, Waltham, MA, U.S.A.). The detection method is a positive ion. Each full scan was performed, and 100 fragment maps were received. Positive ions were fragmented via HCD with an ionization energy of 30 eV. Finally, dynamic exclusion was performed for 90 s.

KU812 Culture and Degranulation Assay. Human peripheral blood basophilic leukemia cells (KU812 cells) were provided by iCell Bioscience (Shanghai, China). The KU812 cells were cultured by complete cell culture medium, consisting of 80–85% Roswell Park Memorial Institute 1640 (RPMI-1640), 15–20% fetal bovine serum, and 1×10^5 units/L penicillin/streptomycin. The culture conditions were as follows: the cell incubator contained 5% CO_2 (humidified sterilized atmosphere), and the temperature was 37 $^\circ\text{C}$. When the KU812 cells were 10^5 – 10^7 cells/mL, they could be used for the sensitization phase.

The expression of Fc ϵ RI on the KU812 cell surface was incubated with sera of CMA patients overnight and then stimulated by 50 μg /well ALA, ALA-gal, 100ALA-gal, ALA-G, ALA-gal-G, 100ALA-gal-G, ALA-D, ALA-gal-D, and 100ALA-gal-D for 3–5 h. The KU812 cells were centrifuged for 10–15 min (6000 rpm). The supernatant was collected for the degranulation assay. The release of histamine, β -hexosaminidase, and interleukin 6 was measured using commercial human ELISA kits (Meimian, Jiangsu, China).

Statistical Analysis. All assays of every independent experiment were carried out in triplicate. Data were analyzed statistically by repeated measures using SPSS 26.0 procedures ($p < 0.05$ was considered as statistically significant). The linear regression was analyzed by GraphPad Prism 8.

RESULTS AND DISCUSSION

Tricine SDS–PAGE Profile. To preliminarily evaluate the molecular weight of glycosylated ALA (covalent binding between ALA and D-galactose), tricine SDS–PAGE was performed. As shown in Figure 1, ALA (lane 1) showed an obvious band. Both ALA-gal (lane 2) and 100ALA-gal (lane 3) had only one band and migrated slower than ALA, revealing that both ALA and sonication-preprocessed ALA covalently bound with D-galactose. Table 1A shows the mobility and relative molecular mass (RMM) of primary samples, and samples with higher

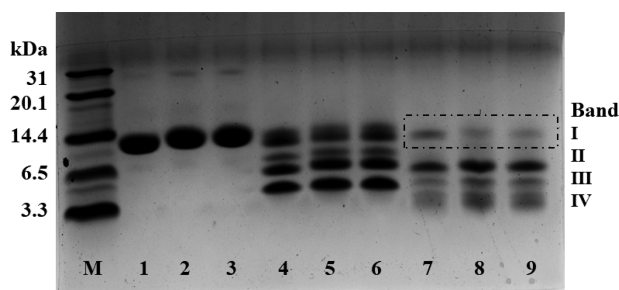


Figure 1. Effect of sonication-assisted glycation on the tricine SDS–PAGE profile of ALA during *in vitro* simulated gastroduodenal digestion in infants. Lane 1, ALA; lane 2, ALA-gal; lane 3, 100ALA-gal; lane 4, ALA-G; lane 5, ALA-gal-G; lane 6, 100ALA-gal-G; lane 7, ALA-D; lane 8, ALA-gal-D; and lane 9, 100ALA-gal-D. The molecular weight of the marker (M) was 3.3, 6.5, 14.4, 20.1, and 31 kDa.

mobility embodied lower RMM. The mobility and RMM of ALA, ALA-gal, and 100ALA-gal were 0.637 (13.60 kDa), 0.622 (14.60 kDa), and 0.618 (15.30 kDa). These results suggested that ALA glycosylated with D-galactose and sonication preprocessing might promote the glycation that happened to ALA.²⁷

The molecular weight of both gastric and gastroduodenal hydrolysates was also analyzed by tricine SDS–PAGE (lanes 4–9 in Figure 1). On the basis of pepsin hydrolytic results, ALA-G, ALA-gal-G, and 100ALA-gal-G were digested to form four bands and mainly distributed between 3.3 and 14.4 kDa. Interestingly, intact undigested bands (band I) were also discovered with decreased intensity compared to the primary samples (lanes 4–6 in Figure 1). As expected, ALA showed partial resistance to pepsinolysis in the immature digestive condition of infants.²⁸ Band I in ALA-gal-G and 100ALA-gal-G showed lower mobility (higher RMM) compared to ALA-G, indicating that the intact undigested glycosylated ALA of gastric hydrolysates, especially sonication-assisted glycosylated ALA, retained its covalently bound D-galactose when the digestion was performed in the presence of pepsin.

After 4 h of gastroduodenal digestion, many peptides between 3.3 and 6.5 kDa were formed and the intact undigested bands with weaker intensity remained. A wide band (band IV) with the molecular weight of ~ 3.3 kDa formed with a large amount during gastroduodenal digestion, which suggested that trypsin and α -chymotrypsin further hydrolyzed to make gastroduodenal hydrolysates smaller after pepsin hydrolysis.²⁹ Band I in the gastroduodenal hydrolysates of glycosylated ALA showed lower mobility compared to that of ALA. However, as a result of the edge effect of tricine SDS–PAGE, the mobility of 100ALA-gal-G did not show a regular change rule after gastroduodenal digestion. In summary, both gastric and gastroduodenal digestion made primary samples from peptide fractions, and D-galactose still covalently bound to intact undigested glycosylated ALA after digestion.

Molecular Weight Distribution of Peptide Fractions.

The representative HPLC chromatograms of the three primary samples (ALA-0, ALA-50, and ALA-100) following the *in vitro* gastric and gastroduodenal digestion are depicted in Figure S1 of the Supporting Information. One predominant chromatographic peak appearing in ~ 13.9 min was observed in all primary samples, corresponding to only one band observed by tricine SDS–PAGE. ALA-G, ALA-gal-G, and 100ALA-gal-G showed two new peaks (peaks 2 and 3) that were not observed in ALA, ALA-gal, and 100ALA-gal. Meanwhile, the relatively strong intensity of peak 1 at ~ 13.9 min appeared in comparison to the other two peaks. We observed seven chromatographic peaks (mainly distributed in 13.9–27 min) with varying intensity, obtained from the degradation after gastroduodenal digestion. Further digestion with trypsin and α -chymotrypsin led to a marked decline in the intensity of peak 1 of gastroduodenal hydrolysates. Interestingly, peaks 1 and 2 in ALA-gal-D and 100ALA-gal-D were not separated obviously in comparison to ALA-D, suggesting that glycation increased ALA protection to hydrolysis during simulated gastroduodenal digestion, affecting the separation efficiency of HPLC.³⁰

The standard equation $\log(w) = -0.1884t + 6.6012$ ($R^2 = 0.9915$) was created to quantify the molecular weight distribution of the peptide fractions obtained from *in vitro* simulated digestion. The peaks were divided into three peptide fractions (I, II, and III) according to different retention times. The molecular weights of peptide fractions I, II, and III are 3.3–14.4, 1–3.3, and <1 kDa, respectively. As shown in Table

Table 1. (A) Mobility and RMM of Primary Samples, Gastric Hydrolysates, and Gastroduodenal Hydrolysates, (B) Relative Areas of Peptide Fractions from HPLC Chromatograms, (C) Content of Free Amino Groups and Grafting Degree of Samples, and (D) Summary of the Glycated Sites and the DSP Values of Glycated ALA^a

A										
band		lane								
		1	2	3	4	5	6	7	8	9
mobility	I	0.637	0.622	0.618	0.620	0.609	0.608	0.608	0.604	0.607
	II				0.666	0.654	0.653	0.693	0.690	0.690
	III				0.699	0.685	0.684	0.730	0.730	0.731
	IV				0.745	0.737	0.736	0.774	0.777	0.778
RMM	I	13.60	14.40	15.30	13.87	14.50	14.80	14.60	14.70	14.79
	II				9.50	10.38	10.48	7.62	7.62	7.62
	III				7.01	7.99	8.06	5.62	5.62	5.58
	IV				5.20	5.50	5.5	4.15	4.05	4.02

B						
fraction	ALA-G	ALA-gal-G	100ALA-gal-G	ALA-D	ALA-gal-D	100ALA-gal-D
I	82.69	91.64	93.04	63.10	65.68	65.79
II	0.10	0.16	0.16	16.36	10.69	10.63
III	17.21	8.20	6.80	20.54	23.63	23.58

C				
samples	grafting degree (%)		free amino groups (mg/mL)	
	primary samples		gastric hydrolysates	gastroduodenal hydrolysates
ALA			0.068 ± 0.002 a	0.113 ± 0.002 a
ALA-gal	38.24		0.054 ± 0.001 b	0.087 ± 0.002 b
100ALA-gal	42.62		0.053 ± 0.000 b	0.083 ± 0.001 c

D					
peptides	m/z	sequences	glycated m/z	glycated sites	DSP (%)
ALA-gal					
11–16	373.2260 ²⁺	(R)ELKDLK(G)	454.2532 ²⁺	K13/K16	80.53 ± 0.004 c
47–58	707.3537 ²⁺	(D)STEYGLFQINN(K)	788.7621 ²⁺	K58	63.76 ± 0.005 d
106–120	582.2908 ³⁺	(L)AHKALCSEKLDQWLC(E)	636.85 ³⁺	K108/K114	17.29 ± 0.009 g
100ALA-gal					
11–16	373.2260 ²⁺	(R)ELKDLK(G)	454.2532 ²⁺	K13/K16	97.86 ± 0.005 a
54–68	926.9419 ²⁺	(F)QINNKIWCKDDQNP(H)	1007.9680 ²⁺	K58/K62	90.99 ± 0.004 b
94–97	488.3084 ¹⁺	(K)KILD(K)	650.8056 ¹⁺	K94	20.49 ± 0.018 f
102–110	558.3037 ²⁺	(I)NYWLAKH(K)	639.6255 ²⁺	K108	61.05 ± 0.011 e

^aLetters a–f indicated significant differences ($p < 0.05$).

1B, the molecular weights of peptide fraction I of gastric hydrolysates distributed in 3.3–14.4 kDa were arrayed in the order: ALA-G (82.69%) < ALA-gal-G (91.64%) < 100ALA-gal-G (93.04%). At the same time, gastric digestion of primary samples resulted in the appearance of the peptide fractions II and III with 1–3.3 and <1 kDa, which was not spotted in tricine SDS–PAGE. The total content of peptide fractions II and III in the gastric hydrolysates of both glycated ALA samples was lower than the gastric hydrolysates of ALA, indicating that glycation could hinder the act of pepsin.³¹

Along with the extension of gastroduodenal digestion, there was a decrease in peptide fraction I with the molecular weight of 3.3–14.4 kDa, among which peptide fraction I identified in 100ALA-gal-D was 65.79% and the highest. Besides, the low content in the total content of peptide fractions II and III was noticed in ALA-gal-D and 100ALA-gal-D. The more peptide fractions in 3.3–14.4 kDa and the fewer peptide fractions in <3.3 kDa of the gastroduodenal hydrolysates of glycated ALA particularly proved that the digestion was aggravated by the action of trypsin and α -chymotrypsin, and the hydrolysis was inhibited as a result of the modification of alkaline amino acids on ALA.³¹

Overall, the degree of glycation is in agreement with the inhibition of enzyme activity, and the gastric/gastroduodenal hydrolysates with <3.3 kDa, which were not found in the tricine SDS–PAGE profile, were characterized from HPLC (Table 1B).

Free Amino Groups. The reduction in free amino groups revealed that alkaline amino acids on ALA happened to glycation. As a result of 3.5 h of glycation, the free amino groups of ALA-gal fell to 0.042 mg/mL (grafting degree of 38.24%) compared to 0.068 mg/mL ALA (grafting degree of 42.62%), whereas 100ALA-gal was lowest where the free amino groups were 0.039 mg/mL (Table 1C). Glycated ALA subjected to the intensity of 100 W/cm² of sonication preprocessing displayed a lower free amino group content when compared to native glycated ALA. The results showed that glycation could reduce the content of free amino groups of ALA, and the cavitation effect of sonication was conducive to unfold the advanced structure of ALA, resulting in a higher degree of glycation,³² which is in agreement with the results obtained from tricine SDS–PAGE. The degree of glycation differs in ALA, which can lead to changes in the hydrolysis of digestive enzymes, especially trypsin, once the hydrolysis sites

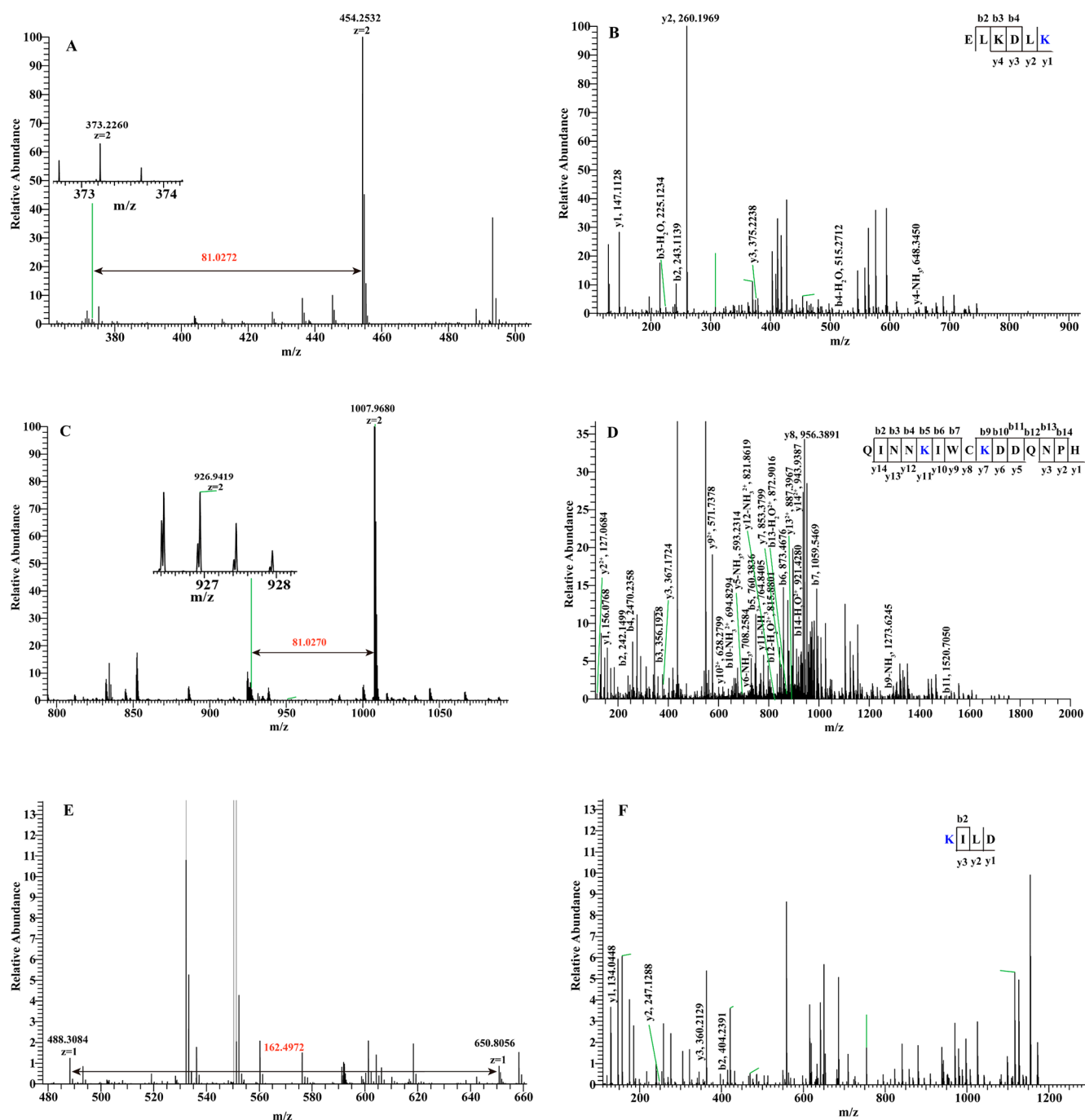


Figure 2. Mass spectrum for the glycated peptides (A) amino acids 11–16 at m/z of 373.2260²⁺, (C) amino acids 54–68 at m/z of 926.9419²⁺, and (E) amino acids 94–97 at m/z of 488.3084¹⁺ of ultrasonic-assisted glycated ALA. Red numbers represent the m/z differences between glycated and unglycated peptides. HCD MS/MS spectrum of the glycated peptides (B) amino acids 11–16 at m/z of 454.2532²⁺, (D) amino acids 54–68 at m/z of 1007.9680²⁺, and (F) amino acids 94–97 at m/z of 650.8056¹⁺. The glycated sites (K) are shown in blue. The b and y ions are indicated by the numbers and lines.

of trypsin and glycation sites are both alkaline amino acids of ALA.

Gastric hydrolysates obtained with the use of pepsin showed increased free amino group content in contrast to primary samples. On the other hand, ALA-gal-G and 100ALA-gal-G displayed a significant reduction in the free amino group content from 0.076 to 0.054 and 0.053 mg/mL, respectively, displaying 0.022 and 0.023 decreases compared to ALA-G. Therefore, we concluded that glycation with D-galactose resulted in relatively high stability to pepsinolysis. The

exposure to trypsin and α -chymotrypsin promoted the hydrolysis of ALA-D, ALA-gal-D, and 100ALA-gal-D, and the rank of the free amino group content was ALA-D (0.113 mg/mL) > ALA-gal-D (0.087 mg/mL) > 100ALA-gal-D (0.083 mg/mL). The obtained results might, furthermore, indicate the participation of trypsin and α -chymotrypsin, which have further digested the gastric hydrolysates. The ability of ALA preprocessed by sonication to glycate with D-galactose protected ALA against gastroduodenal digestion, slowing proteolysis. In the tricine SDS–PAGE analysis, we found

that the gastroduodenal hydrolysates still remained intact in undigested ALA molecules. The results of the free amino group content showed that 100ALA-gal-D had the lowest values, which might be due to covalently bound D-galactose to undigested sonication-assisted glycosylated ALA still remaining after gastroduodenal digestion.

In general, the results were in correlation with the analysis of tricine SDS–PAGE and HPLC, and we obtained the following conclusions: (1) glycosylated ALA had increased stability against digestion, especially in the case of sonication-preprocessed ALA; (2) the peptides distributed in <3.3 kDa were produced after digestion, especially for the gastroduodenal digestion; and (3) the reducing sugars still covalently bound to the intact undigested glycosylated ALA molecules.

Glycation Sites and Glycation Extent Analysis. The mass (MS) and tandem mass (MS/MS) spectra were used to identify the glycosylated peptides and glycation sites in ALA-gal and 100ALA-gal. Pepsin, a kind of non-specific pepsin, is capable of extensively hydrolyzing the peptide bonds among hydrophobic amino acids and, thus, was used for hydrolyzing glycosylated ALA before MS analysis. After pepsin digestion, MS/MS with HCD was performed to identify the glycosylated peptides. In theory, if a peptide is covalently bound with one D-galactose molecule, the corresponding m/z peak of the peptides will increase to 162, 81, and 54 at charges of +1, +2, and +3 after losing a molecule of water.³³ Similarly, when a peptide is covalently bound with two molecules of D-galactose, the m/z peak of the peptide could be 324 Da. The MS spectra of three glycosylated peptides of ALA-gal and 100ALA-gal were revealed in Figure 2.

Panels A and C of Figure 2 illustrate the MS spectra of the peptides ELKDLK (amino acids 11–16) and QINN-KIWCKDDQNPH (amino acids 54–68). The m/z peak of the unglycosylated form of amino acids 11–16 and 54–68 at charge +2 was 373.2260 and 926.9419 and was changed to 454.2532 and 1007.9680 in the glycosylated form, indicating that amino acids 11–16 and 54–68 were in the single glycosylated forms (glycosylated with one D-galactose molecule) with an increase m/z of 81.0272 and 81.0270, respectively. Figure 2E is the MS spectra of the peptide KILD (amino acids 94–97) only discovered in 100ALA-gal, and the m/z peak of amino acids 94–97 at charge +1 was 488.3084. The m/z peak of the single glycosylated form of amino acids 94–97 was 650.8056, with an increasing value of 162.4972 Da, indicating that amino acids 94–97 also glycosylated with one D-galactose molecule. Panels B, D, and F of Figure 2 display the MS/MS spectra of glycosylated peptides amino acids 11–16, 54–68, and 94–97 with the glycosylated m/z peaks of 454.2532²⁺, 1007.9680²⁺, and 488.3084¹⁺. A series of b and y ions and their corresponding neutral loss forms (NH₃ and H₂O) were marked to identify the glycosylated sites, and they matched well with amino acids 11–16, 54–68, and 94–97. Furthermore, Lys13/Lys16, Lys58/Lys62, and Lys94 were the glycosylated sites in amino acids 11–16, 54–68, and 94–97, as identified by the mass difference between y1 and y2, y7 and y8 or y11 and y12, and y3 and y4 (lost), which was the combination of lysine and D-galactose. Other glycosylated peptides shown in Table 1D were identified in the same method.

The m/z peaks of unglycosylated peptides amino acids 47–58, 106–120, and 102–110 were 707.3537²⁺, 582.2908³⁺, and 558.3037²⁺. However, the m/z peaks of the glycosylated peptides amino acids 47–58, 106–120, and 102–110 were 788.7621²⁺, 636.85³⁺, and 639.6255²⁺, separately. The m/z shift of these peptides was 81.4084, 54.5592, and 81.3218 Da separately,

indicating that these peptides glycosylated with one D-galactose. Generally, amino acids 11–16, 47–58, and 106–120 of ALA-gal were glycosylated at Lys13/Lys16, Lys58, and Lys108/Lys114, and amino acids 11–16, 54–68, 94–97, and 102–110 of 100ALA-gal were glycosylated at Lys13/Lys16, Lys58/Lys62, Lys94, and Lys108. Among these glycosylated sites, Lys13, Lys108, and Lys94 were located on the α -helix of ALA. It was worth noting that Lys94 only underwent glycation in 100ALA-gal, which might be due to sonication treatment unfolded the advanced structure of ALA, exposing Lys94, thus promoting its glycation.³² Studies showed that the acidic amino acids or alkaline amino acids near lysine in the primary structure of the protein are beneficial for its glycation.³⁴ Obviously, glycosylated sites, such as K13 and K108, were near Asp14 and His107, also glycosylated.

To know the glycation extent of each peptide, the value of the average degree of substitution per peptide molecule (DSP) was calculated.³² As shown in Table 1D, The DSP value of amino acids 11–16, 47–58, and 106–120 in ALA-gal was 80.53, 63.76%, and 17.29%, whose glycosylated sites were Lys13/Lys16, Lys58, and Lys108/Lys114; 100ALA-gal presented four glycosylated sites, namely, Lys13/Lys16, Lys58/Lys62, Lys94, and Lys108, with DSP values of 97.86, 90.99, 20.49, and 61.05% in relative glycosylated peptides amino acids 11–16, 54–68, 94–97, and 102–110. In general, as the advanced structure of ALA was unfolded by sonication preprocessing, more glycosylated sites were exposed, resulting in a higher DSP value of 100ALA-gal than that of ALA-gal.

IgE-Binding Abilities. Glycation can mask linear epitopes by covalent modification of alkaline amino acids, thus reducing the allergenicity of ALA. Evaluation of the IgE-binding abilities of ALA, ALA-gal, 100ALA-gal, and the obtained gastric/gastroduodenal hydrolysates was performed against the pooled sera of 10 patients (Table S1 of the Supporting Information) diagnosed with CMPA (Figure 3). Analysis of the IgE-binding

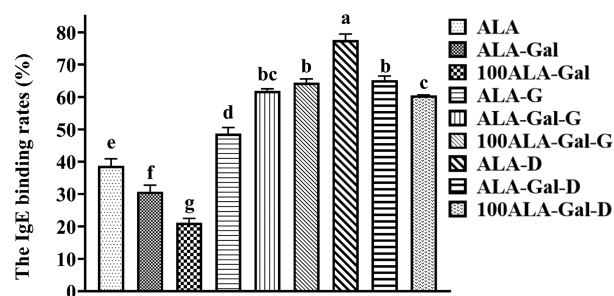


Figure 3. Effect of ultrasonic-assisted glycation on the IgE-binding rates of ALA during *in vitro* simulated gastroduodenal digestion in infants. Letters (a–g) above the bars demonstrate significant differences ($p < 0.05$).

rates of ALA (38.72%), ALA-gal (30.76%), and 100ALA-gal (20.10%) showed that sonication preprocessing-assisted glycation led less IgE antibodies (reduced by 18.62%) to bind to ALA, which was consistent with our previous studies.²¹ The drastically felled IgE-binding abilities of glycosylated ALA might be due to the marked linear epitopes induced by glycation after covalent cross-linking. The gastric digestion of ALA and its glycosylated forms with the use of pepsin increased the ability of IgE to bind to these hydrolysates. We further checked the gastroduodenal hydrolysates obtained with the use of trypsin and α -chymotrypsin, and it was found that higher

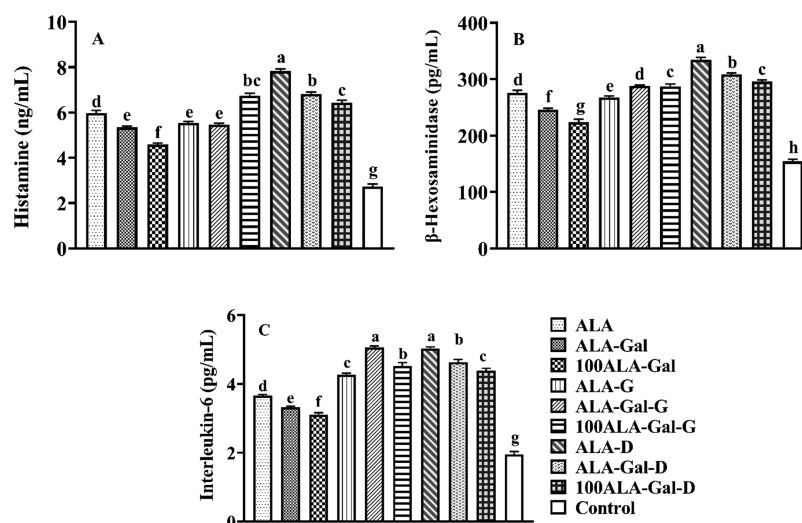


Figure 4. Effect of ultrasonic-assisted glycation on the KU812 cell degranulation of ALA during *in vitro* simulated gastroduodenal digestion in infants. Letters (a–h) above the bars demonstrate significant differences ($p < 0.05$).

IgE-binding rates were discovered in comparison to gastric hydrolysates, with the order of ALA-D > ALA-gal-D > 100ALA-gal-D. The results suggested that the deeper glycation of ALA resulted in lower IgE-binding ability during gastroduodenal digestion ($p < 0.05$), which amounted to 60.50% (100ALA-gal-D). The results showed that, although the allergenicity of all hydrolysates reached higher values after gastrointestinal digestion compared to primary samples, the hydrolysates of glycosylated ALA held lower allergenicity, especially the hydrolysates of glycosylated ALA preprocessed by sonication.

Degranulation of Glycosylated ALA and Its Hydrolysates.

When it comes to more than two Fc ϵ RI reporters cross-link to form a complex, it can induce the degranulation of basophils and the release of inflammatory mediators, resulting in allergic symptoms. To investigate, in-depth, the impact of gastroduodenal digestion of infants on the allergenicity changes of ALA and to mutually authenticate the analysis obtained from the ELISA, the inflammatory mediators (histamine, β -hexosaminidase, and interleukin 6) of ALA, its glycation form, and their hydrolysates were evaluated by performing KU812 degranulation assay. Histamine secretion can cause some pathological effects on human bodies. As shown in Figure 4A, in comparison to the control group (PBS, 2.74 ng/mL) sensitizing KU812 cells, the release of histamine increased apparently after challenged by ALA (5.98 ng/mL). While regarded as glycosylated ALA, the histamine release became weaker and followed the order of ALA (5.98 ng/mL) > ALA-gal (5.35 ng/mL) > 100ALA-gal (4.59 ng/mL), suggesting that the ability of histamine secretion was much weaker in glycosylated ALA, especially the sonication-assisted glycosylated ALA. This might be due to the following: (1) glycation masked the linear epitopes of ALA and hindered the interactions between IgE and linear epitopes of ALA, leading to lower histamine release, or (2) sonication preprocessing promoted the linear epitopes of ALA to glycate, resulting in the lowest release of histamine.

The amount of released histamine of gastric hydrolysates was higher than corresponding primary samples, again indicating that the gastric digestion improved the release of histamine related to allergic symptoms. On the basis of the results of trypsin and α -chymotrypsin hydrolysis, the hydrolysates of glycosylated ALA in the case of sonication preprocessing

effectively impeded stimulation of KU812 cells, which suggested that the inhibitory effect of glycation on cell degranulation during digestion was kept.

β -Hexosaminidase is an important symbol to be considered in cell degranulation as a result of its concentration in the blood increasing significantly when allergic reactions occur. As shown in Figure 4, the β -hexosaminidase release from KU812 cells activated by ALA, ALA-gal, and 100ALA-gal increased in the order of ALA > ALA-gal > 100ALA-gal > PBS, which was in agreement with the histamine release data. A similar phenomenon has been found for β -lactoglobulin, where the glycation carried on, with the subsequent loss of linear epitopes by the modification of alkaline amino acids reducing its allergenicity.³² The β -hexosaminidase release in the gastroduodenal hydrolysates increased and ranged from 334.61 pg/mL (ALA-D) to 308.53 pg/mL (ALA-gal-D) and 295.75 pg/mL (100ALA-gal-D). A similar phenomenon was discovered in the release of interleukin 6. In general, it was expected that the glycation degree oppositely caused the ability of ALA toward allergenicity. Gastroduodenal digestion that led to increased allergenicity might be due to the exposure of the linear epitopes. However, the lowest allergenicity of the hydrolysates of sonication-assisted glycosylated ALA was the reason that the highest amount of intact undigested glycosylated ALA still bound to D-galactose, which kept its ability to mask linear epitopes.

Mechanism Analysis of Allergenicity Changes. The allergenicity of ALA somehow can be reduced by common processing technologies, with glycation long seen as most prospective. This process of glycation to mask some linear epitopes is pointed out by many studies as a potential cause of hypoallergenicity.^{21,32} The number of glycosylated sites and the glycation extent of each glycosylated site are important parameters that give the chance to assess the glycation degree. In our study, we obtained that ALA was glycosylated with D-galactose in the case of dry-state glycation. Especially 100ALA-gal, preprocessed by sonication with the intensity of 100 W/cm², was glycosylated at a relatively high degree compared to ALA-gal.

The results of this study demonstrated that the glycation of ALA employing D-galactose was effective in reducing its ability to cause allergy, which belongs to the patients with CMPA. The lowest allergenicity was observed in the glycosylated form of sonication-assisted ALA, where deep glycation resulted in

masking a great number of reactive linear epitopes. Studies revealed that the potential IgE linear epitopes of ALA are peptides amino acids 1–16, 13–26, 47–58, 93–102, 5–8, 17–58, 59–94, 99–108, 41–46, 55–60, 62–72, 74–76, 85–90, and 92–99.^{35–38} Three glycosylated sites (Lys13/Lys16, Lys58, and Lys108/K114) with high DSP values contained in ALA-gal were located in some of those linear epitopes mentioned above, resulting in lower allergenicity than native ALA subject to no treatment. We applied sonication with the intensity of 100 W/cm² to preprocessed ALA; the addition of the glycosylated site Lys94 on the IgE linear epitopes amino acids 93–102, 59–94, and 92–99 was identified with the DSP value of 20.49%; and the lowest ALA–IgE complexes were formed. The acquired results might confirm that sonication could well expose the advanced structure to favor the glycosylation of ALA and, thus, masked more epitopes reactive with IgE. In our study, increased allergenicity of the gastric/gastrointestinal hydrolysates might correlate with the exposure of the linear epitopes subjected to digestion. Incubation of a pooled sera of CMPA patients with the gastroduodenal hydrolysates revealed that intact undigested glycosylated ALA retained its covalently bound D-galactose to eliminate linear epitope allergenicity (Figure 5).

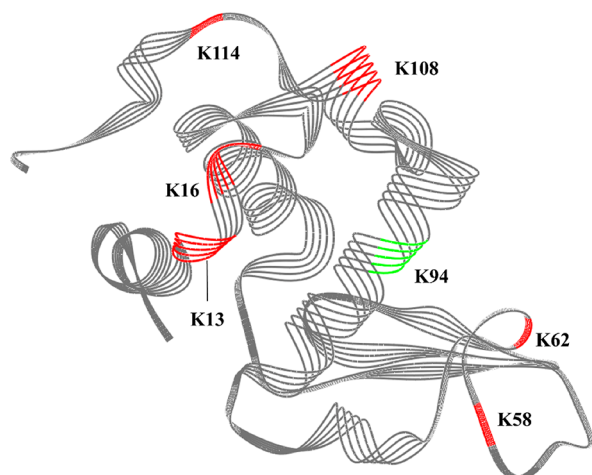


Figure 5. Ribbon diagram of glycosylated ALA. The Protein Data Bank (PDB) code is 1F6R. The framework of ALA is cyan; glycosylated Lys are in red; and Lys94 only discovered in ultrasonic-assisted glycosylated ALA is black.

In conclusion, we suggested that sonication-assisted glycosylation showed a better role, which significantly reduced ALA response to corresponding IgE of sera of CMPA patients. The increased allergenicity of gastric/gastrointestinal hydrolysates might be due to the exposure of the linear epitopes subjected to digestion. Intact undigested glycosylated ALA was responsible for retaining its masking function during ingestion. The in-depth interpretations carried out in our study will allow for the understanding of the allergenicity changes of glycosylated ALA when it comes to digestion, of which glycosylation may be used as an immunotherapeutic agent for allergen immunotherapy to achieve the purpose of allergy desensitization. However, *in vivo* research, such as animal experiments, should be considered in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c02205>.

Information of the sera of CMPA patients (Table S1) and HPLC of ALA, ALA-gal, 100ALA-gal, ALA-G, ALA-gal-G, 100ALA-gal-G, ALA-D, ALA-gal-D, and 100ALA-gal-D (Figure S1) (PDF)

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Notes

The authors declare no competing financial interest.

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