



Isolation and allergenicity evaluation of glycated α -lactalbumin digestive products and identification of allergenic peptides

Xu-mei Wang^{a,*}, Zong-cai Tu^{a,b,c,*}, Yun-hua Ye^{b,c}, Guang-xian Liu^d, Yue-ming Hu^a, Hui Wang^{a,*}

^a State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China

^b National R&D Branch Center for Conventional Freshwater Fish Processing, Jiangxi Normal University, Nanchang, Jiangxi 330022, China

^c Engineering Research Center of Freshwater Fish High-value Utilization of Jiangxi Province, Jiangxi Normal University, Nanchang, Jiangxi 330022, China

^d Institute of Food Science and Technology, Jiangxi Academy of Agricultural Sciences, Nanchang 330200, China

ARTICLE INFO

Keywords:

α -Lactalbumin

Glycation

In-vitro gastroduodenal digestion

UPLC HCD-MS/MS

Allergenicity

Allergenic peptides

ABSTRACT

This study aimed to isolate and evaluate the allergenicity of glycated α -lactalbumin (ALA) digestive products and identify its allergenic peptides. The digestive products of native-, alone glycated- and ultrasound-assisted glycated ALA (ALA-D, ALA-gal-D, 100ALA-gal-D) were isolated into three fractions (F1, F2 and F3). High-resolution mass spectrometry showed that the digestion-resistant peptides of F2 and F3 mainly distributed in amino acid sequence (AA) 25–31, AA32–53, AA40–53, AA54–60, AA80–90, AA94–104. The allergenicity of the three fractions of glycated ALA was lower than that in ALA-D, indicating glycation of ALA could indeed reduce its allergenicity after digestion. Furthermore, most fractions isolated from high glycation-degree ALA had the lowest allergenicity. The IgG/IgE binding abilities of synthesized peptides indicated that AA94–104 firstly identified by us embodied the strongest allergenicity and might be the potential allergenic peptide. This will provide a theory for preparing hypoallergenic products based on the identified allergenic peptides.

1. Introduction

Scientific evidence confirms that α -lactalbumin (ALA) has the ability to provide 50% of essential amino acids (Heine, Klein & Reeds, 1991; Davis, Harris, Lien, Pramuk & Trabulsi, 2008). However, it is also the major allergen in cow's milk that causes food allergies (Mousan & Kamat, 2016). ALA is a globular protein composed of 123 amino acid residues and stabilized by four disulfide bonds, including cysteine₆-cysteine₁₂₀, cysteine₆₁-cysteine₇₇, cysteine₇₃-cysteine₉₁ and cysteine₂₈-cysteine₁₁₁ (Tu, Xu, Ren & Zhang, 2020; Negue, Miclo, Girardet, Campagna, Mollé & Gaillard, 2006). The tertiary structure of ALA consists of four α -helices (amino acid sequences [AA] 5–11, AA23–34, AA86–98 and AA105–110), three short 3_{10} helices (AA18–20, AA77–80 and AA115–118) and three-stranded antiparallel β -pleated sheets (AA41–44, AA47–50 and AA55–56) (Negue, Miclo, Girardet, Campagna, Mollé & Gaillard, 2006). Although human ALA and bovine ALA are 74% homologous, bovine ALA can cause allergic reactions such as allergic rhinitis and atopic dermatitis, especially on infants due to their immature digestive system (Untersmayr & Jensen-Jarolim, 2008; Meulenbroek

et al., 2014).

The allergic epitope of a food allergen provides the theoretical basis for understanding food allergy, and allergic epitopes are typically divided into two categories: Linear and conformational epitopes (Li, Yuan, He, Gao & Chen, 2015). Linear epitopes are considered more important since they are more stable during gastroduodenal tract digestion than conformational epitopes (Liu & Sathe, 2018). Studies show that the IgG linear epitopes of ALA are AA7–18, AA37–46, AA51–61, AA52–54, AA56–59, AA63–72, AA74–76, AA81–90, AA89–108 and AA92–99. The IgE linear epitopes of ALA includes AA1–16, AA5–18, AA13–26, AA17–58, AA41–46, AA47–58, AA55–60, AA59–94, AA62–72, AA74–76, AA85–90, AA92–99, AA93–102, and AA99–108 and AA109–123 (Maynard, Jost & Wal, 1997; Adams, Barnett, Walsh, Pearce, Hill & Howden, 1991; Järvinen, Chatchatee, Bardina, Beyer & Sampson, 2001; Li et al., 2016). Moreover, recent work by Ogirodowczyk et al. (2021) indicated that AA69–79 may show particularly strong sensitizing properties.

Glycation is successfully used as a promising method to reduce allergen allergenicity (Zhang, Li, Xiao, Nowak-Węgrzyn & Zhou, 2020).

* Corresponding authors at: 235 East Nanjing Road, Nanchang, Jiangxi, China.

E-mail addresses: tuzc_mail@aliyun.com (Z.-c. Tu), wanghui00072@aliyun.com (H. Wang).

<https://doi.org/10.1016/j.foodchem.2022.133185>

Received 4 October 2021; Received in revised form 27 April 2022; Accepted 6 May 2022

Available online 10 May 2022

0308-8146/© 2022 Elsevier Ltd. All rights reserved.

Correspondingly, the glycation degree is directly associated with the allergenicity of a protein allergen (Li, Liu, Ye, Yang & Tu, 2019). The cavitation effect of ultrasound can unfold the tertiary structure of the protein, and promote glycation (Liu et al., 2017; Liu et al., 2018). Therefore, ultrasound combined with glycation is an effective method to reduce the allergenicity of ALA. Our previous study has shown that ultrasound-assisted glycation can reduce the allergenicity of ALA during digestion compared with native digested ALA. In digestive products of ultrasound-assisted glycated ALA, undigested glycated ALA may remain intact and continue to play the role of masking linear epitopes (Wang, Tu, Ye, Liu, Wang & Hu, 2021). Moreover, digestive products remain highly allergenic, which may explain why certain digestion-resistant peptides in the digestive products lead to allergenicity. However, no research has been conducted on the allergenicity evaluation of the fractions isolated from the digestive products of ultrasound-assisted glycated treated ALA and the identification of allergenic peptides.

The objective of this study was to evaluate the allergenicity of different fractions isolated from glycated ALA after *in-vitro* gastroduodenal digestion and to identify the allergenic peptides. The different fractions of the glycated ALA digestive products were isolated by Sephadex G-25 gel filtration and characterized by high-performance liquid chromatography. Enzyme-linked immunosorbent assay (ELISA) and degranulation assay were used to detect the allergenicity of these isolated fractions. The digestion-resistant peptides were identified and characterized by ultra-performance liquid chromatography system coupled to tandem mass spectrometry (UPLC HCD-MS/MS). Finally, to identify allergenic peptides of ALA, four typical digestion-resistant peptides were synthesized and their allergenicity was characterized.

2. Materials and methods

2.1. Materials

ALA ($\geq 85\%$, L6010) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (D131214) was obtained from Aladdin (Shanghai, China). 3, 3', 5, 5'-tetramethylbenzidine (TMB) two-component substrate solution (PR1210), pepsin (P8160), trypsin (T8150) and α -chymotrypsin (YZ-1134007) were supplied by Solarbio (Beijing, China). Goat anti-rabbit IgG antibody horseradish-peroxidase (HRP) conjugate (BL003A) was bought from Biosharp (Shenzhen, China). Sera from individuals (10) allergic to cow's milk (specific IgE levels ranged from 10.774 to ≥ 100 kU/mL) and normal human sera (specific IgE levels < 0.35 kU/mL) was purchased from Plasma Lab International (Everett, WA, USA). Goat anti-human IgE antibody HRP conjugate (A9667) was bought from Biocheck (San Francisco, USA). Human ELISA kits from Meimian (Jiangsu, China) were used to measure the levels of β -hexosaminidase, interleukin-6 and histamine. Standards such as L-hydroxyproline, oxidized glutathione, bacitracin, aprotinin and cytochrome C were chromatographic grade and obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). RPMI 1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were supplied by Gibco (Waltham, MA, USA). All other chemicals utilized were analytical grade.

2.2. Preparation of glycated ALA

Glycated ALA was prepared according to the previous methods (Wang, Tu, Ye, Liu, Wang & Hu, 2021), that is, ALA was pretreated by ultrasound and then glycation with D-galactose. Ultrasound pretreatment of ALA (1 mg/mL) was carried out at an intensity of 100 W/cm^2 for 10 min. D-galactose was mixed with ultrasound-pretreated ALA at a proportion of 1:1 (w/w) and then lyophilized. Samples were glycated at 55°C for 3.5 h (74.41% relative humidity, saturated NaCl solution). Following glycation, free D-galactose and salt were removed by ultrafiltration centrifugal tube (3 kDa cut off; Merck Millipore, Darmstadt, Germany). Samples were stored at -20°C until used. Native ALA was

named ALA as the control group, alone glycated ALA was named ALA-gal and ultrasound-assisted glycated ALA was named 100ALA-gal. The glycation degree was $\text{ALA} < \text{ALA-gal} < 100\text{ALA-gal}$ (Wang, Tu, Ye, Liu, Wang & Hu, 2021).

2.3. Infant digestion model *in-vitro*

Digestion of ALA, ALA-gal and 100ALA-gal was performed as reported previously to better mimic the digestive environment of the infant gastroduodenal tract (Dupont et al., 2010; Cattaneo, Stuknytė, Masotti & De Noni, 2017). In detail, the gastric digestion was performed with 0.15 mol/L of NaCl and a 22.75 U/mg sample of pepsin. The duodenal digestion mix included a 3.45 U/mg sample of trypsin, 0.04 U/mg sample of α -chymotrypsin and 2 mmol/L of bile salts. The pH of gastric and duodenal digestion was 3.0 and 7.0, respectively. The digestion was stopped by 4 mmol/L of 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride. ALA, ALA-gal, and 100ALA-gal were taken after 120 min of gastric digestion and 120 min of duodenal digestion. ALA, ALA-gal, and 100ALA-gal after gastroduodenal digestion were named ALA-D, ALA-gal-D, and 100ALA-gal-D, respectively.

2.4. Purification by gel filtration chromatography

The gel filtration chromatography purification was carried out as previously described (Shao, Zhang, Liu & Tu, 2021). 2 mL of each gastroduodenal digested sample (50 mg/mL) was filtered through a nylon filter membrane (0.45 μm) before being loaded onto the Sephadex G-25 gel chromatography column (16 mm $\times$ 80 cm). Deionized water was used as the mobile phase to complete the elution at a constant flow rate of 0.4 mL/min. 3.2 mL of the eluate was automatically collected via an automatic fraction collector (QiTe, Shanghai, China). The UV absorbance was measured at 220 nm using an ultraviolet detector (HD-21-88, QiTe, Shanghai, China). For subsequent analysis, three fractions were collected according to the elution peak for each sample and then freeze-dried.

2.5. High-performance liquid chromatography

Each fraction isolated from ALA-D, ALA-Gal-D and 100ALA-Gal-D was analyzed via HPLC (L-2000, Hitachi, Japan) to evaluate the molecular weight (MW) distribution, using the method described previously, with slight modifications (Wang, Ye, Tu, Hu, Wang & Huang, 2021).

2.6. Sample desalting

Each isolated fraction of samples was acidified with 0.1% of trichloroacetic acid (200 μL) and then desalted by Pierce C18 tips (Thermo Fisher Scientific, Waltham, MA). These C18 tips were activated by 50% of acetonitrile (100 μL , 2 times) and then equilibrated by 0.1% of trichloroacetic acid (100 μL , 3 times). To be well bound to C18 tips, these fractions of samples were slowly aspirated and dispensed for 10 cycles. Desalting was conducted through two washes with 0.1% trichloroacetic acid. The desalted samples were eluted with 50% of acetonitrile (50 μL) and collected for further analysis.

2.7. UPLC HCD-MS/MS analysis

Desalted samples were subjected to UPLC system (EASY-nLC 1000, Thermo Fisher Scientific, USA). The UPLC was connected to a C18 pre-column (Acclaim PepMap 100, 100 $\mu\text{m} \times 2 \text{ cm}$, Thermo Fisher Scientific, USA). After the pre-column reaction, an analytical column (Acclaim PepMap RSLC, 75 $\mu\text{m} \times 15 \text{ cm}$, Thermo Fisher Scientific, USA) was used. The mobile phase was composed of solution A (2% acetonitrile/0.1% formic acid in water) and solution B (2% water/0.1% formic acid in acetonitrile). Samples were eluted by stepwise gradient elution

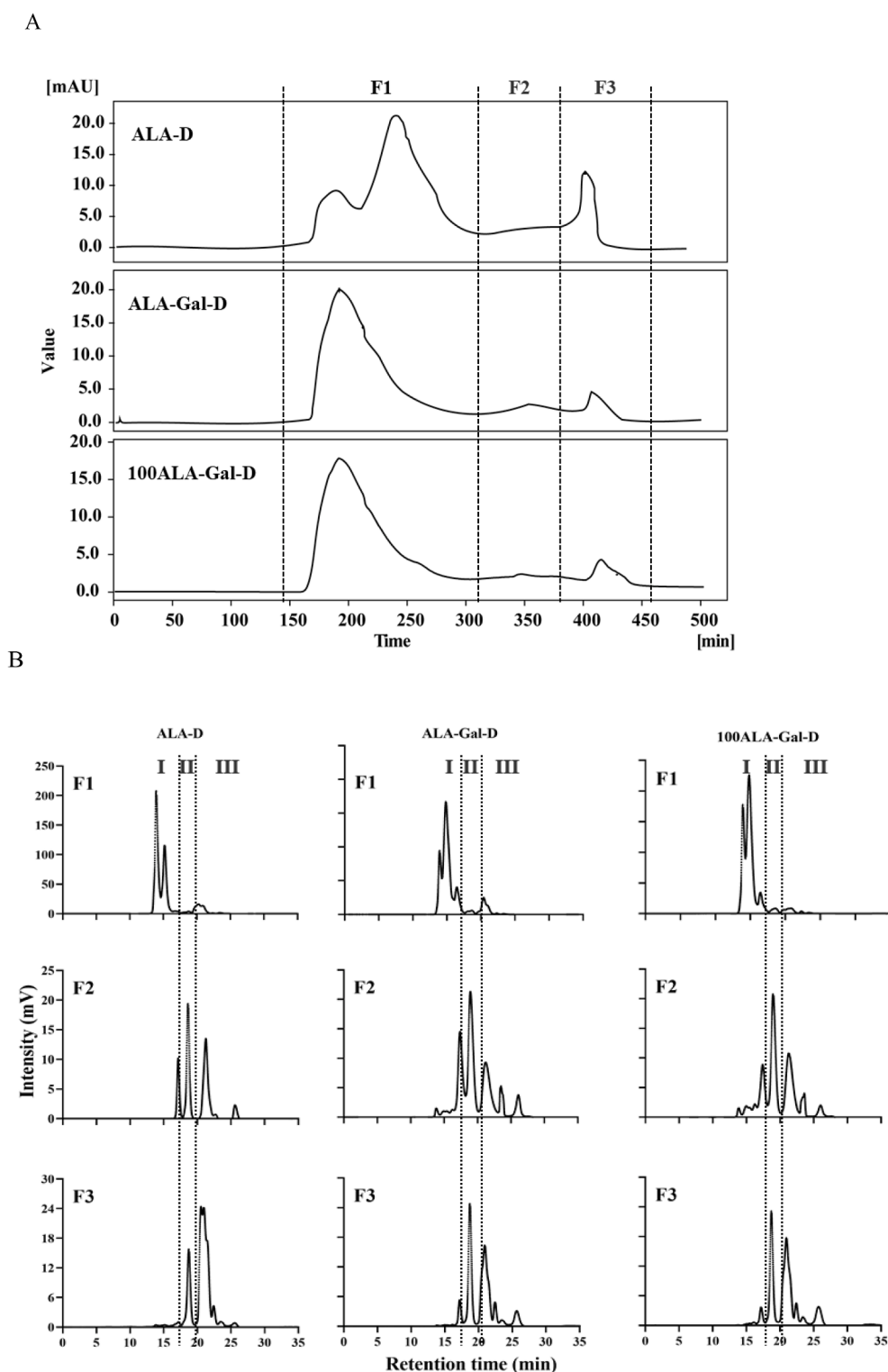


Fig. 1. Isolation of ALA-D, ALA-Gal-D and 100ALA-Gal-D by Sephadex G-25 gel chromatography (A). The HPLC chromatography of F1, F2 and F3 of ALA-D, ALA-Gal-D and 100ALA-Gal-D (B).

following the step: 5% solution B (0 min); 10% solution B (4 min); 20% solution B (50 min); 35% solution B (20 min); and 95% solution B (6 min). The tandem mass spectrometry analysis was referred to the previous method (Ma et al., 2003). Mass spectrometer (Orbitrap Q-Exactive HF, Thermo Fisher Scientific, USA) was used with automatic switching between MS and MS/MS scans using the top-20 method. The scan range was 350–1650 m/z . Peptide fragmentation was performed via higher-

energy collision dissociation (HCD) at the energy of 27 V.

2.8. Indirect competitive ELISA

The IgG/IgE binding rates of each fraction (F1, F2 and F3) of ALA-D, ALA-Gal-D and 100ALA-Gal-D, as well as the synthesized peptides were measured by using indirect competitive ELISA described previously

Table 1

The relative areas of parts I, II and III of peptide fractions (F1, F2 and F3) from HPLC chromatograms (A) and the molecular weights of parts I, II and III are > 3 kDa, 1–3 kDa and < 1 kDa, respectively. The digestion-resistant peptides of F2 and F3 determined by LC-MS/MS (B).

A									
Fraction (%)	ALA-D			ALA-gal-D			100ALA-gal-D		
	I	II	III	I	II	III	I	II	III
F1	80.45	2.53	17.01	85.59	2.46	11.95	92.70	3.78	4.11
F2	0.38	49.67	49.95	5.24	58.01	36.75	9.15	51.33	39.52
F3	1.41	19.76	78.83	0.44	38.98	60.58	0.65	36.02	63.33
B									
Peptide	Mass	Length	ppm	m/z	Start	End			
F2 in ALA-Gal-D									
PEWVCTTF	1100.4307	7	2.1	551.2238	25	31			
AIVQNNDSTEYGL	1444.6447	13	−0.2	723.3295	40	52			
IVQNNDSTEYGL	1351.6256	12	1.3	676.821	41	52			
FLDDDLT	819.3651	7	2.7	820.3745	80	86			
FLDDDLTDDIM	1333.536	11	0	667.7753	80	90			
KILDKVGINY	1161.6758	10	1.3	581.8459	94	103			
KILDKVGINYW	1347.755	11	2	674.8861	94	104			
ILDKVGINY	1055.5627	9	−0.4	528.7885	95	103			
ILDKVGINYW	1235.655	10	0.7	618.8352	95	104			
LDKVGINYW	1106.576	9	0.5	554.2956	96	104			
KVGINYW	878.465	7	1.5	879.4736	98	104			
F3 in ALA-Gal-D									
QINNKIW	914.4974	7	1.7	915.5062	54	60			
ILDKVGINYW	1241.642	10	−0.3	621.8281	95	104			
LDKVGINY	920.4967	8	1.8	461.2565	96	103			
LDKVGINYW	1106.576	9	1.9	554.2963	96	104			
DKVGINYW	1007.4713	8	−0.1	504.7428	97	104			
KVGINYW	892.4443	7	−0.2	447.2293	98	104			
F2 in 100ALA-Gal-D									
AIVQNNDSTEYGL	1444.6447	13	1.8	723.3309	40	52			
IVQNNDSTEYGL	1373.6075	12	1.1	687.8118	41	52			
IVQNNDSTEYGLF	1520.6759	13	1	761.346	41	53			
FLDDDLTDDIM	1333.536	11	0.3	667.7755	80	90			
DDDLTDDIM	1051.4016	9	1.5	526.7089	82	90			
KILDKVGINY	1143.6652	10	1.9	572.8409	94	103			
ILDKVGINY	1034.5648	9	0.8	518.2901	95	103			
ILDKVGINYW	1241.642	10	−0.4	621.828	95	104			
LDKVGINY	902.4861	8	2.2	452.2513	96	103			
KVGINYW	910.4548	7	1.4	456.2354	98	104			
F3 in 100ALA-Gal-D									
HTSGYDTQAIVQ	1340.5973	12	0.3	671.3062	32	43			
DTQAIVQNNDSTEY	1613.7169	14	2.3	807.8676	37	50			
AIVQNNDSTEYGL	1444.6447	13	1.8	723.3309	40	52			
AIVQNNDSTEYGLF	1591.713	14	2.1	796.8655	40	53			
IVQNNDSTEYGL	1373.6075	12	−0.2	687.8109	41	52			
IVQNNDSTEYGLF	1520.6759	13	0.2	761.3454	41	53			
QINNKIW	897.4708	7	1.9	449.7435	54	60			
FLDDDLTDDIM	1333.536	11	0.9	667.7759	80	90			
LDDDLTDDIM	1186.4675	10	1.8	594.2421	81	90			
ILDKVGINY	1015.5702	9	0.8	508.7928	95	103			
ILDKVGINYW	1235.655	10	−1	618.8342	95	104			
DKVGINYW	993.4919	8	1.6	497.754	97	104			
KVGINYW	892.4443	7	−0.2	447.2293	98	104			

(Wang, Ye, Tu, Hu, Wang & He, 2021). In addition, 100 µL/well of ALA (2 µg/mL) was transferred to a 96-wells microtiter plate at 4°C, coating for 12 h. Then 1 g of fish gelatin was dissolved in 100 mL of carbonate buffer solution (CBS); the resulting solution was used to block these wells. Next, 50 µL of each fraction/synthesized peptides (8 µg/mL) and 50 µL of rabbit sera (laboratory self-made, 1: 8000)/human sera (1: 20) were mixed and reacted in each well for 1 h. After that, 100 µL of goat anti-human IgG (1: 5000)/IgE (1:800) antibody HRP conjugate was used to incubate for 1 h. After the TMB solution coloring for 15 min, sulfuric acid solution was immediately used to terminate the reaction. The IgG/IgE binding rates were calculated as the following equation: The IgG/IgE binding rates (%) = $(OD_{\max} - OD_s) / (OD_{\max} - OD_0) \times 100\%$, wherein $OD_{\max}$ is the OD value of samples replaced by CBS, OD_s is the OD value of samples, and OD_0 indicates the OD value of phosphate buffered saline containing 0.05% Tween-20 (PBST) instead of IgG/IgE diluted solution.

2.9. Human basophil leukemia (KU812) cells culture

KU812 cells were cultured according to the previous method (Lu et al., 2022). KU812 cells were provided by iCell Bioscience (Shanghai, China) and were maintained in RPMI-1640 medium including fetal bovine sera (15%, v/v) and penicillin/streptomycin (1%, v/v). KU812 cells were cultured at 37 °C, in atmosphere of 5% CO₂ and then sub-cultured every 3–4 days.

2.10. KU812 cell degranulation assay

To express FcεRI on the surface of KU812 cells, KU812 cells (1×10^6 cells/well) were sensitized by sera from individuals (10) allergic to cow's milk (50 µL) for 24 h on a 24-well sterile cell culture plate with a round hole and flat bottom (Meng, Li, Wang, Gao, Yang & Chen, 2016). Following sensitizing, 50 µg/well samples were added to incubate for 4 h. After that, cells were centrifuged and the supernatant was collected.

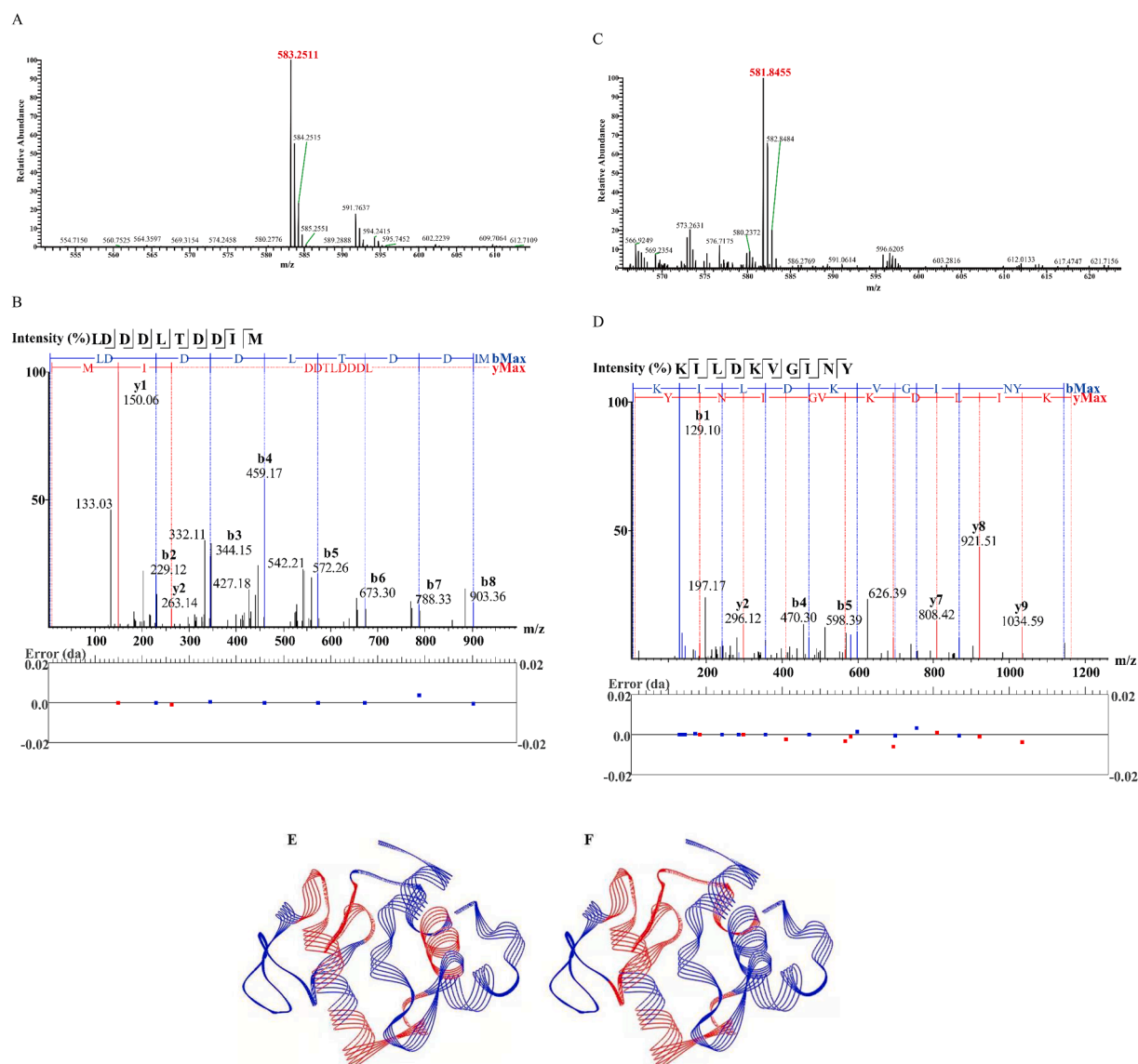


Fig. 2. MS (A, C) spectra of the digestion-resistant peptides of LDDDLTDDIM (AA 81–90) and KILDKVGINY (AA 94–103). HCD-MS/MS spectra (B, D) of LDDDLTDDIM (AA 81–90) and KILDKVGINY (AA 94–103). The digestion-resistant peptides of F1 (E) and F2 (F) in the 3D structure of ALA. PDB code is 1F6R.

The degranulation assay was analyzed by measuring the release of β -hex, IL-6 via the ELISA method, and carried out according to the manufacturer's instructions.

2.11. Peptide synthesis

Peptides were synthesized by GL Biochem Co. Ltd. (Shanghai, China). Using solid-phase organic synthesis and Fmoc protected amino acid strategy, the peptide was assembled, cleaved and purified. The sequence of the synthetic peptides was further reconfirmed by LC-Q-TOF-MS/MS. The synthesized peptide was desalted and the purities of the four peptides were all above 98%.

2.12. Statistical analysis

Data are expressed as means $\pm$ standard deviation. Data were analyzed statistically by repeated measures using SPSS 26.0 procedures. $p < 0.05$ indicated significantly different. All data were processed using GraphPad Prism 8.

3. Result and discussion

3.1. Purification of glycosylated ALA digestive products

Isolation of ALA-D, ALA-Gal-D and 100ALA-Gal-D was performed by Sephadex G-25 gel chromatography column. These elution profiles were divided into three fractions according to different retention time and displayed in Fig. 1A. The three fractions were labeled as fractions 1, 2 and 3 (F1, F2 and F3) with the retention time of 150–300 min, 310–390 min and 400–450 min, respectively. Moreover, the molecular weight of F1, F2 and F3 were in the following the order: F1 > F2 > F3. It could be seen from Fig. 1A that F1 in ALA-D contained two elution peaks, whereas only one elution peak was discovered in F1 of ALA-Gal-D and 100ALA-Gal-D. Results suggested that glycation might modify the primary structure of ALA, which was reflected in the modification of basic amino acids in ALA, thereby masking some enzymatic hydrolysis sites and hindering the digestion of digestive enzymes, resulting in affecting the isolation efficiency of Sephadex G-25 gel chromatography column (Zhao et al., 2017). Therefore, to facilitate comparison of the differences among ALA-D, ALA-Gal-D and 100ALA-Gal-D, the first two elution peaks of ALA-D were divided into F1 in our study.

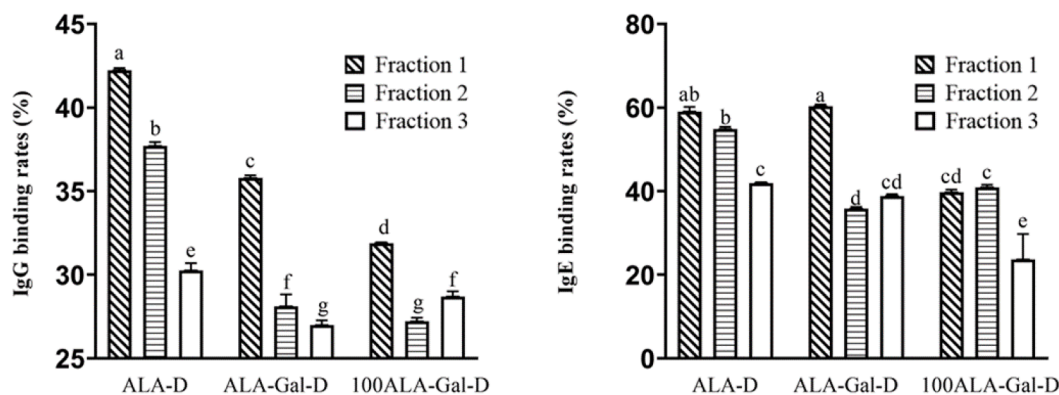


Fig. 3. The IgG/IgE binding rates of F1, F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D. Different letters above the bars mean significant differences ($p < 0.05$).

3.2. Molecular weight (MW) distribution of digestive products

The HPLC chromatograms of F1, F2 and F3 of ALA-D, ALA-Gal-D and 100ALA-Gal-D are shown in Fig. 1B. In general, samples with a long retention time were accompanied by low molecular weight (Shao, Zhang, Liu & Tu, 2020). These chromatographic profiles were divided into parts I, II and III. Two predominant chromatographic peaks (part I) between 13 and 17 min appeared in F1 of ALA-D, ALA-Gal-D and 100ALA-Gal-D. In comparison to part I of F1, parts II and III of F1 (ALA-D, ALA-Gal-D and 100ALA-Gal-D) showed a weaker intensity of chromatographic peaks between 17 and 23 min, indicating that F1 of ALA-D, ALA-Gal-D and 100ALA-Gal-D embodied more peptides with high molecular weight, which was consistent in the results of Sephadex G-25 gel filtration profiles. The peak intensity of parts II and III of F2 in ALA-D, ALA-Gal-D and 100ALA-Gal-D were enhanced within 17–23 min, and the intensity of peaks distributed in part I was weakened. Among F1, F2 and F3, the peak intensity of part I became weaker, whereas the peak intensity of parts II and III became stronger. In all, when it comes to F1, F2 and F3, the peak intensity of part I became weaker, while the peak intensity of parts II and III became stronger. The results further indicated that the molecular weight of F1, F2 and F3 followed the order of $F1 > F2 > F3$.

The standard equation $\log(w) = -0.1474t + 5.9507$ was created to quantify the molecular weight of F1, F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D. These peaks were divided into parts I, II and III, and the molecular weights of them are > 3 kDa, 1–3 kDa and < 1 kDa, respectively. As shown in Table 1A, the molecular weight of F1 in ALA-D, ALA-Gal-D and 100ALA-Gal-D were mainly distributed in > 3 kDa ($>80\%$), and the molecular weight of F2 and F3 were mainly in parts II and III (1–3 kDa and < 1 kDa). Furthermore, the content of part III in F3 was increased, compared with F2. Glycation might hinder the act of digestion, thereby generating less low molecular weights of peptides (Pinto, Léonil, Henry, Cauty, Carvalho & Bouhallab, 2014). Taking F1 of ALA-D, 100ALA-Gal-D for example, part I in F1 of ALA-D was 80.45%, however, it was 92.10% in F1 of 100ALA-Gal-D. Besides, part III was 17.01% in F1 of ALA-D, which was 12.90% more than F1 of 100ALA-Gal-D. Our previous study showed that the glycation degree of ultrasound-assisted glycosylated ALA was higher than that of ALA glycosylated alone (Wang, Tu, Ye, Liu, Wang & Hu, 2021). Expectedly, the content of peptides > 3 kDa (part I) of F1, F2 and F3 in 100ALA-Gal-D was larger than that of ALA-Gal-D. Therefore, the results showed that the act of the digestion of ALA with a high degree of glycation was weak.

3.3. Identification of digestion-resistant peptides

To further research the allergenicity mechanism of each fraction and identify the allergenic peptides, the digestion-resistant peptides were

investigated. Given that the molecular weight of F1 was > 3 kDa, only F2 and F3 were analyzed by HPLC-MS/MS. The MS and MS/MS spectra of peptides LDDDLTDDIM (AA81–90) and KILDKVGINY (AA94–103) are shown in Fig. 2A–D. The MS spectra showed the m/z of AA 81–90 was 583.2511. The MS/MS spectra indicated that peptide AA 81–90 was identified according to a series of b and y ions. Similarly, the m/z of AA 94–103 was 581.8455, and a series of b and y ions of AA 94–103 was well matched. Other peptides shown in Table 1B were identified in the same way. As shown in Table 1B, a total of 11 and 10 digestion-resistant peptides were identified from F2 in ALA-Gal-D and 100ALA-Gal-D, respectively, and identified peptides of F3 in ALA-Gal-D and 100ALA-Gal-D were 6 and 13. These digestion-resistant peptides of F2 mainly distributed in AA 25–31, AA 40–53 (40–52, 41–52, 41–53), 80–90 (80–86, 80–90, 82–90), AA 94–104 (94–103, 94–104, 95–103, 95–104, 96–103, 96–104, 98–104). When it comes to F3, we found that the digestion-resistant peptides mainly included AA 32–53 (32–43, 37–50, 40–52, 40–53, 41–52, 41–53), 54–60, 80–90 (80–90, 81–90, 82–90), 94–104 (94–103, 95–103, 95–104, 96–103, 96–104, 97–104, 98–104). Notably, AA 32–53 (32–43, 37–50, 40–52, 40–53, 41–52, 41–53) and 80–90 (80–90, 81–90, 82–90) were only discovered in F3 of 100ALA-Gal-D. Fig. 2E–F shows the digestion-resistant peptides identified by HPLC-MS/MS in the 3D structure of ALA. It suggested that the digestion-resistant peptides were mainly located on the α -helix of ALA regardless whether they were in F2 or F3 (Soman et al., 2000).

Jarvinen et al. (2001) found that the IgG linear epitopes of ALA included AA 7–18, AA 51–61 and AA89–108. The digestion-resistant stable peptides such as AA54–60 and AA 94–104 were located in the IgG linear epitopes AA51–61 and AA 89–108. Li et al. (2016) identified AA 37–46, AA 52–54, AA 56–59, and AA 63–72, AA 74–76, AA 81–90, AA 92–99 as the IgG linear epitopes of ALA. The IgG linear epitopes AA 37–46 and AA 81–90 were intact in the digestion-resistant peptides AA 37–50 and AA80–90 identified by in this research. The digestion-resistant peptides such as AA81–90 were completely distributed on the previously reported IgG linear epitope (AA81–90). Similarly, the digestion-resistant peptides we identified were also distributed on some previously reported IgE linear epitopes.

3.4. The IgG/IgE binding abilities

The IgG/IgE binding rates of F1, F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D were analyzed by using the method of indirect competitive ELISA and shown in Fig. 3. Each fraction (F1, F2 and F3) of ALA-D, ALA-Gal-D and 100ALA-Gal-D was allergenic. However, the IgG-binding rates of F1 were higher than that of F2 and F3. This might be that F1 contained a large number of digestive products with the molecular weight of > 3 kDa, which still retained strong allergenicity (Jiménez Saiz, Benedé, Miralles, López Expósito, Molina & López Fandiño, 2014).

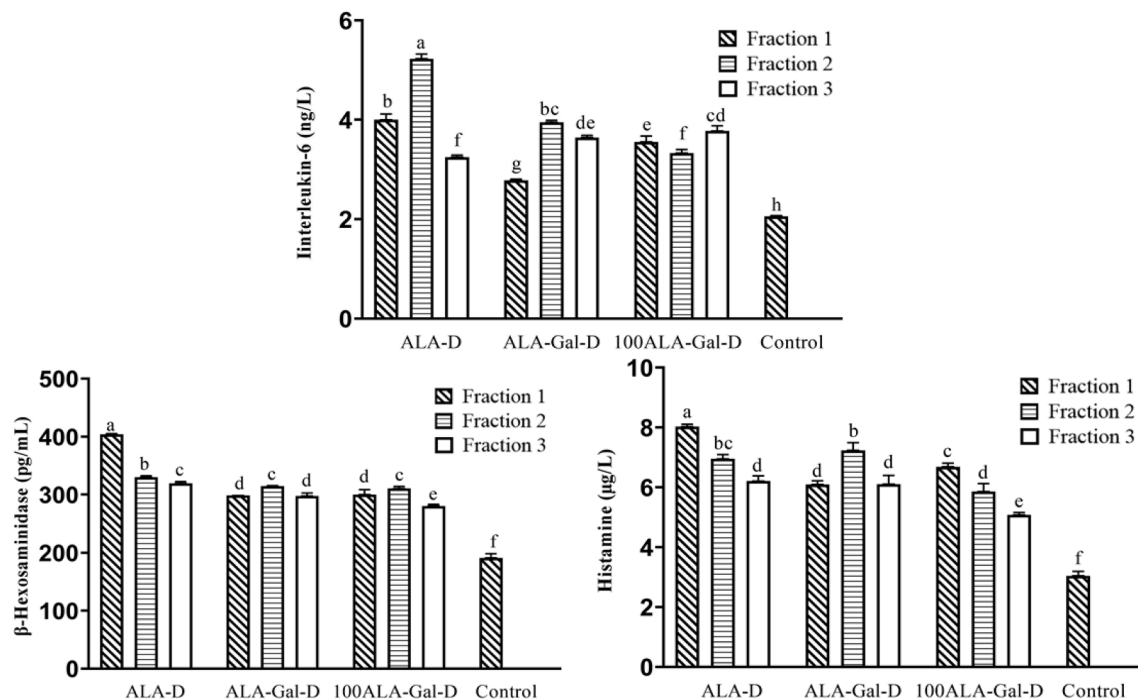


Fig. 4. Degranulation of KU812 cells after treatment with F1, F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D. Different letters above the bars mean significant differences ($p < 0.05$).

In addition, the digestion-resistant peptides in the digestive products also had a certain allergenicity. Here we observed that the IgG binding rates of ALA-D and ALA-Gal-D were $F1 > F2 > F3$. However, for 100ALA-Gal-D, the order of the IgG binding rates was: $F1 > F3 > F2$. The IgG binding rates of F1, F2 and F3 in ALA-D were 42.23%, 37.70% and 30.25%, respectively, and that in ALA-Gal-D shifted to 35.78%, 28.11% and 26.99%, which were lower than that in ALA-D. The same phenomenon was found between ALA-D and 100ALA-Gal-D. The possible reason was that glycation reduced the allergenicity of ALA, which was reflected in reducing the allergenicity of each fraction isolated from the digestive products of glycated ALA. Compared with ALA-D, F1 and F2 of 100ALA-Gal-D with lower IgG binding rates were observed when that of ALA-Gal-D was 35.78% and 28.11%. The results showed that the IgG binding rates of F1 and F2 decreased with the deepening of ALA glycation. For F3, the IgG binding rates of the three samples were 30.25%, 26.99% and 28.68%. The IgG binding rate of F3 in 100ALA-Gal-D was larger than that of ALA-Gal-D perhaps because AA37-50 and AA80-90 were only detected in F3 of 100ALA-Gal-D. Some studies have shown that AA37-46 and AA81-90 are IgG linear epitopes of ALA (Li et al., 2016).

Fig. 3 shows that the IgE binding rates of F1, F2 and F3 of ALA-Gal-D and 100ALA-Gal-D were lower than that of ALA-D. In the digestive products of glycated ALA, F1 and F3 of 100ALA-Gal-D had lower IgE binding rates than ALA-Gal-D. The IgE binding rates of F1 and F3 in 100ALA-Gal-D were the lowest, indicating that further deepening of glycation in ALA might have decreased the IgE binding rates of the isolated fractions. However, the IgE binding rates of F2 in 100ALA-Gal-D (40.93%) were higher than that in ALA-Gal-D (35.75%), demonstrating that the former was easier to bind with the IgE antibodies than the latter. In general, the IgE binding rates of F2 in ALA-Gal-D and 100ALA-Gal-D were always less than that of F2 in ALA-D. These results indicated that F1 exhibited low IgG/IgE binding rates that seemed dependent on the glycation degree.

3.5. Degranulation assay analysis

The release of interleukin-6, β -hexosaminidase and histamine of F1,

F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D are illustrated in Fig. 4. Compared with the control group, each fraction (F1, F2 and F3) of ALA-D, ALA-Gal-D and 100ALA-Gal-D could cause the release of interleukin-6 (Gazme, Rezaei & Udenigwe, 2020). The results indicated that the digestion-resistant peptides identified in each fraction might remain allergenicity. Glycation treatment decreased the interleukin-6 release of F1 and F2 in ALA-Gal-D and 100ALA-Gal-D. However, the interleukin-6 release of F3 in ALA-Gal-D and 100ALA-Gal-D was increased, compared with ALA-D. The interleukin-6 release of F2 in ALA-Gal-D was 3.94 ng/L, which was 0.31 ng/L higher than that in 100ALA-Gal-D. It should be noted that the lowest interleukin-6 release was discovered in F2 of 100ALA-Gal-D, which was characterized by a higher degree of glycation. The interleukin-6 release increased by 0.78 ng/L in cells exposed to F1 of 100ALA-Gal-D as compared with F1 of ALA-Gal-D. However, no significant difference was found in the interleukin-6 release between F3 in ALA-Gal-D and 100ALA-Gal-D ($P > 0.05$).

The β -hexosaminidase release of F1, F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D showed alike tendencies to that of interleukin-6. Glycation decreased the β -hexosaminidase release of each fraction (F1, F2 and F3) in ALA-Gal-D and 100ALA-Gal-D. For the β -hexosaminidase release of F1 and F2, no difference was observed between ALA-Gal-D and 100ALA-Gal-D ($P > 0.05$). However, the β -hexosaminidase release of F3 in 100ALA-Gal-D was the lowest, and opposite results were observed in the release of interleukin-6, which might be that F3 in ALA-Gal-D promoted the release of β -hexosaminidase, compared with F3 in 100ALA-Gal-D.

The release of histamine was well corresponding to the release of interleukin-6 and β -hexosaminidase. Except that F2 and F3 in ALA-D and ALA-Gal-D had no significant difference in the release of histamine, F1, F2 and F3 in the glycated ALA (ALA-Gal-D and 100ALA-Gal-D) decreased the release of histamine compared with ALA-D ($P > 0.05$). The fractions (F2 and F3) isolated from higher degree glycation of ALA (100ALA-Gal-D) presented lower histamine release, excluding F1 which displayed an opposite phenomenon between ALA-Gal-D and 100ALA-Gal-D.

In general, each isolated fraction still could cause cell degranulation.

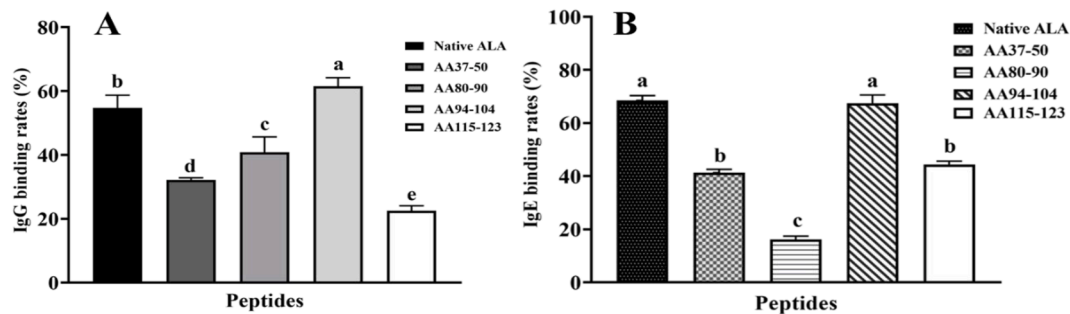


Fig. 5. The IgG/IgE binding rates of native ALA, peptides AA37-50, AA80-90, AA94-104 and AA115-123. Different letters above the bars mean significant differences ($p < 0.05$).

The three fractions (F1, F2 and F3) of glycosylated ALA could inhibit the release of β -hexosaminidase and histamine, indicating that glycosylation of ALA could indeed reduce the allergenicity of ALA after digestion. Moreover, it should be noted that the ability of each fraction to induce degranulation was varied. However, the IgG/IgE binding abilities and degranulation assay indicated that in general, the higher glycosylation degree of ALA led to lower allergenicity of most fractions isolated.

3.6. The IgG/IgE binding rates analysis of synthetic peptides

In view of the fact that the three fractions (F1, F2 and F3) still maintained allergenicity, which might be related to the retention of allergenic potential of digestion-resistant peptides. We found that the glycosylated forms of ALA mainly produced the digestion-resistant peptides including DTQAIVQNNDSTEY (AA37-50), KFLDDDLTDDIM (AA80-90), KILDKVGINYW (AA94-104). Peptide LDQWLCEKL (AA115-123) was used to be a control group. To identify the allergenic peptides, the four peptides were synthesized to study their allergenicity. Fig. 5A shows the IgG binding rates of the native ALA and the four synthesized peptides. It was observed that the four peptides were all allergenic, and their IgG binding rates of them mainly ranged from 22.55% to 61.45%. In comparison with native ALA, the IgG binding rate of AA 94-104 was increased by 6.74% whereas AA115-123 showed the lowest value. The order of the IgG-binding rates was AA94-104 > AA80-90 > AA37-50 > AA115-123. The strong allergenic potential of AA94-104, AA80-90 and AA37-50 (except AA115-123) has been attributed to the previously reported IgG linear epitopes, such that AA81-90 and AA37-46 maintained integrity on them (Li et al., 2016). Notably, AA94-104 has been first identified and embodied the strongest allergenicity.

Fig. 1B shows the IgE-binding abilities of the four peptides. The IgE binding rate of native ALA was 68.55%, and that of AA94-104 was 67.46%. However, no significant difference ($P > 0.05$) was found between them. The result indicated that AA94-104 still held strong IgE binding ability which was comparable to that of native ALA. The IgE binding rates were 41.27%, 16.18% and 44.36% in AA37-50, AA80-90 and AA115-123, respectively. In general, The IgE binding abilities order of the four peptides was AA94-104 > AA115-123 > AA80-90 > AA37-50, showing the highest IgE binding abilities of AA94-104 and the lowest IgE binding abilities of AA37-50. Jarvinen et al. (2001) identified four IgE epitopes from ALA (AA1-16, AA13-26, AA47-58 and AA93-102), of which the peptide AA94-104 identified by us had a high degree of coincidence with AA93-102. Li et al. (2016) located six IgE epitopes of ALA from phage display peptide libraries, and IgE epitopes AA41-46, AA85-90 and AA92-99 overlapped with peptides AA37-50, AA80-90 and AA94-104 that we identified, respectively. Therefore, the synthetic peptides overlapped with the IgE epitopes, which might explain why they still had the ability to bind to IgE. The IgG/IgE binding rates explained why the four peptides still remained allergic, and AA94-104 might be the peptide with the most allergenic potential.

4. Conclusion

In this work, ALA-D, ALA-gal-D and 100ALA-gal-D were isolated into three fractions (F1, F2 and F3) by Sephadex G-25 gel chromatography column. The molecular weight of F1, F2 and F3 followed the order of F1 > F2 > F3. Peptides distributed in AA25-31, AA32-53, AA40-53, AA54-60, AA80-90 and AA94-104 were identified as the digestion-resistant peptides of F2 and F3. The IgG/IgE binding rates of ALA-Gal-D and 100ALA-Gal-D fractions (F1, F2 and F3) were lower than that in ALA-D. Degranulation assay after treatment with F1, F2 and F3 in ALA-D, ALA-Gal-D and 100ALA-Gal-D indicated that glycosylation of ALA could indeed reduce the allergenicity of ALA after digestion. Furthermore, higher glycosylation degree of ALA led to lower allergenicity of most fractions isolated. The analysis of IgG/IgE binding abilities of the four synthesized peptides indicated that AA94-104 might be the peptide with the most allergenic potential. The study provides the theoretical basis for the preparation of hypoallergenic products based on the identified allergenic peptides.

CRediT authorship contribution statement

Xu-mei Wang: Methodology, Data curation, Writing – original draft, Writing – review & editing, Validation. **Zong-cai Tu:** Funding acquisition, Project administration, Resources. **Yun-hua Ye:** Visualization. **Guang-xian Liu:** Visualization. **Yue-ming Hu:** Visualization. **Hui Wang:** Conceptualization, Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by the China Postdoctoral Science Foundation (No.2020M682109) and the Excellent Youth Foundation of Jiangxi Province (No. 20192BCBL23026).

References

- Adams, S. L., Barnett, D., Walsh, B. J., Pearce, R. J., Hill, D. J., & Howden, M. (1991). Human IgE-binding synthetic peptides of bovine β -lactoglobulin and α -lactalbumin. In vitro cross-reactivity of the allergens. *Immunology and Cell Biology*, 69(3), 191–197.
- Cattaneo, S., Stuknytė, M., Masotti, F., & De Noni, I. (2017). Protein breakdown and release of β -casomorphins during in vitro gastro-intestinal digestion of sterilized model systems of liquid infant formula. *Food Chemistry*, 217, 476–482.
- Davis, A. M., Harris, B. J., Lien, E. L., Pramuk, K., & Trabulsi, J. (2008). α -Lactalbumin-rich infant formula fed to healthy term infants in a multicenter study: Plasma essential amino acids and gastrointestinal tolerance. *European Journal of Clinical Nutrition*, 62(11), 1294–1301.

- Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., ... Mackie, A. R. (2010). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Molecular Nutrition and Food Research*, 54(6), 767–780.
- Gazme, B., Rezaei, K., & Udenigwe, C. C. (2020). Effect of enzyme immobilization and in vitro digestion on the immune-reactivity and sequence of IgE epitopes in egg white proteins. *Food and Function*, 11(7), 6632–6642.
- Heine, W. E., Klein, P. D., & Reeds, P. J. (1991). The importance of α -lactalbumin in infant nutrition. *Journal of Nutrition*, 121(3), 277–283.
- Järvinen, K., Chatchatee, P., Bardina, L., Beyer, K., & Sampson, H. A. (2001). IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology*, 126(2), 111–118.
- Jiménez Saiz, R., Benedé, S., Miralles, B., López Expósito, I., Molina, E., & López Fandiño, R. (2014). Immunological behavior of in vitro digested egg-white lysozyme. *Molecular Nutrition and Food Research*, 58(3), 614–624.
- Li, J., Liu, J., Ye, Y. H., Yang, P., & Tu, Z. C. (2019). Reduced IgE/IgG binding capacities of bovine α -lactalbumin by glycation after dynamic high-pressure microfluidization pretreatment evaluated by high resolution mass spectrometry. *Food Chemistry*, 299 (10), Article 125166.
- Li, X., Yuan, S. L., He, S. F., Gao, J. Y., & Chen, H. B. (2015). Identification and characterization of the antigenic site (epitope) on bovine β -lactoglobulin: Common residues in linear and conformational epitopes. *Journal of the Science of Food and Agriculture*, 95(14), 2916–2923.
- Li, X., Yuan, S. L., Huang, M. J., Gao, J. Y., Wu, Z. Y., Tong, P., ... Chen, H. B. (2016). Identification of IgE and IgG epitopes on native Bos d 4 allergen specific to allergic children. *Food and Function*, 7(7), 2996–3005.
- Liu, C. Q., & Sathe, S. K. (2018). Food allergen epitope mapping. *Journal of Agricultural and Food Chemistry*, 66(28), 7238–7248.
- Liu, G. X., Tu, Z. C., Yang, W. H., Wang, H., Zhang, L., Ma, D., ... Li, X. (2018). Investigation into allergenicity reduction and glycation sites of glycated β -lactoglobulin with ultrasound pretreatment by high-resolution mass spectrometry. *Food Chemistry*, 252, 99–107.
- Liu, J., Tu, Z. C., Shao, Y. H., Wang, H., Liu, G. X., Sha, X. M., ... Yang, P. (2017). Improved antioxidant activity and glycation of α -lactalbumin after ultrasonic pretreatment revealed by high-resolution mass spectrometry. *Journal of Agricultural and Food Chemistry*, 65(47), 10317–10324.
- Lu, Q. L., Zuo, L. L., Wu, Z. H., Li, X., Tong, P., Wu, Y., ... Yang, A. S. (2022). Characterization of the protein structure of soymilk fermented by *Lactobacillus* and evaluation of its potential allergenicity based on the sensitized-cell model. *Food Chemistry*, 366, Article 130569.
- Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty Kirby, A., & Lajoie, G. (2003). PEAKS: Powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 17(20), 2337–2342.
- Maynard, F., Jost, R., & Wal, J. (1997). Human IgE binding capacity of tryptic peptides from bovine α -lactalbumin. *International Archives of Allergy and Immunology*, 113(4), 478–488.
- Meng, X. Y., Li, X., Wang, X. K., Gao, J. Y., Yang, H., & Chen, H. B. (2016). Potential allergenicity response to structural modification of irradiated bovine α -lactalbumin. *Food and Function*, 7(7), 3102–3110.
- Meulenbroek, L. A. P. M., Den, H. J. C. F., Lebens, A. F. M., Knulst, A. C., Bruijnzeel-Koomen, C. A. F. M., Garssen, J., ... Van Hoffen, E. (2014). Characterization of T cell epitopes in bovine α -lactalbumin. *International Archives of Allergy and Immunology*, 163(4), 292–296.
- Mousan, G., & Kamat, D. (2016). Cow's milk protein allergy. *Clinical Pediatrics*, 55(11), 1054–1063.
- Negue, M. A., Miclo, L., Girardet, J. M., Campagna, S., Mollé, D., & Gaillard, J. L. (2006). Proteolysis of bovine α -lactalbumin by thermolysin during thermal denaturation. *International Dairy Journal*, 16(10), 1157–1167.
- Ogrodowczyk, A. M., Dimitrov, I., & Wróblewska, B. (2021). Two faces of milk proteins peptides with both allergenic and multidimensional health beneficial impact-integrated in vitro/in silico approach. *Foods*, 10(1), 163.
- Pinto, M. S., Léonil, J. L., Henry, G. L., Cauty, C., Carvalho, A. N. F., & Bouhallab, S. D. (2014). Heating and glycation of β -lactoglobulin and β -casein: Aggregation and in vitro digestion. *Food Research International*, 55, 70–76.
- Shao, Y. H., Zhang, Y., Liu, J., & Tu, Z. C. (2020). Influence of ultrasonic pretreatment on the structure, antioxidant and IgG/IgE binding activity of β -lactoglobulin during digestion in vitro. *Food Chemistry*, 312, Article 126080.
- Shao, Y. H., Zhang, Y., Liu, J., & Tu, Z. C. (2021). Investigation into predominant peptide and potential allergenicity of ultrasonicated β -lactoglobulin digestion products. *Food Chemistry*, 361, Article 130099.
- Soman, K. V., Midoro-Horiuti, T., Ferreón, J. C., Goldblum, R. M., Brooks, E. G., Kurosky, A., ... Schein, C. H. (2000). Homology modeling and characterization of IgE binding epitopes of mountain cedar allergen Jun a 3. *Biophysical Journal*, 79(3), 1601–1609.
- Tu, Y. Q., Xu, Y., Ren, F. Z., & Zhang, H. (2020). Characteristics and antioxidant activity of Maillard reaction products from α -lactalbumin and 2'-fucosyllactose. *Food Chemistry*, 316, Article 126341.
- Untersmayr, E., & Jensen-Jarolim, E. (2008). The role of protein digestibility and antacids on food allergy outcomes. *Journal of Allergy and Clinical Immunology*, 121 (6), 1301–1308.
- Wang, X. M., Tu, Z. C., Ye, Y. H., Liu, G. X., Wang, H., & Hu, Y. M. (2021). Mechanism on the allergenicity changes of α -lactalbumin treated by sonication-assisted glycation during in vitro gastroduodenal digestion. *Journal of Agricultural and Food Chemistry*, 69(24), 6850–6859.
- Wang, X. M., Ye, Y. H., Tu, Z. C., Hu, Y. M., Wang, H., & He, C. Y. (2021). Investigation of the mechanism underlying the influence of mild glycation on the digestibility and IgG/IgE-binding abilities of β -lactoglobulin and its digests through LC orbitrap MS/MS. *LWT-Food Science and Technology*, 139, Article 110506.
- Wang, X. M., Ye, Y. H., Tu, Z. C., Hu, Y. M., Wang, H., & Huang, T. (2021). Mechanism of the reduced IgG/IgE binding abilities of glycated β -lactoglobulin and its digests through high-resolution mass spectrometry. *Journal of Agricultural and Food Chemistry*, 69(12), 3741–3750.
- Zhang, Z. Y., Li, X. M., Xiao, H., Nowak-Węgrzyn, A., & Zhou, P. (2020). Insight into the allergenicity of shrimp tropomyosin glycosylated by functional oligosaccharides containing advanced glycation end products. *Food Chemistry*, 302, Article 125348.
- Zhao, D., Le, T. T., Larsen, L. B., Li, L., Qin, D., Su, G. Y., & Li, B. (2017). Effect of glycation derived from α -dicarbonyl compounds on the in vitro digestibility of β -casein and β -lactoglobulin: A model study with glyoxal, methylglyoxal and butanedione. *Food Research International*, 102, 313–322.