

The mechanism of the reduction in allergenic reactivity of bovine α -lactalbumin induced by glycation, phosphorylation and acetylation

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ABSTRACT

Bovine α -lactalbumin (α -Lac) allergy is a common health problem. This study assesses the allergenic reactivity and the structural properties of α -Lac after protein modification (glycation, phosphorylation and acetylation) by ELISA, cells experiment and high-resolution mass spectrometry. Three modified methods significantly reduced the IgE/IgG-binding capacity, and the release of histamine and interleukin-6, and changed the conformational structure of α -Lac. α -Lac was glycosylated at K13, K16, K94, K98, and K108, phosphorylated at Y18, S22, Y103, and S112, and acetylated at K13, T33, S34, T38, S47, K62, S69, S70, K108, and K114, respectively, leading to masking the linear epitopes of α -Lac. Therefore, the decrease of allergenic reactivity of α -Lac induced by glycation, phosphorylation and acetylation depends upon not only the shielding effect of their modified sites, but also the change of conformational structure. This study confirmed that protein modification was a promising method for decreasing the allergenic reactivity of allergic proteins.

1. Introduction

Bovine α -lactalbumin (α -Lac), is a simple model Ca^{2+} binding protein, because of its unique amino acid composition, can be used as a component of infant formulas, modulate neurological function, and a therapeutic agent with applications in conditions or diseases (Layman, Lönnerdal, & Fernstrom, 2018; Permyakov & Berliner, 2000). However, its use in infant formulas was limited due to potential allergy, which causes about 30–35% IgE-mediated cow's milk allergy (Qasba, Kumar, & Brew, 1997).

Recently, researchers have increased their scrutiny of protein allergies, and multiple studies have been employed to alter the allergenic reactivity of α -Lac, for example, heat treatment (Bu, Luo, Zheng, & Zheng, 2009), protein modification (Ming et al., 2014; Li, Luo, & Feng, 2011), ultrasonic (Tammineedi, Choudhary, Perezalvarado, & Watson, 2013), γ -irradiation (Meng et al., 2016), and combination therapies such as ultrasonic-assisted with glycation (Liu et al., 2018a), and glycation combined with phosphorylated modification (Enomoto et al., 2009). For these methods, protein modification is a sound process that could affect the physicochemical and functional characteristics of protein (Gruener & Ismond, 1997; Ruud, Yvonne, Wierenga, Schols, &

Harry, 2011). Liu et al. demonstrated that succinylated soy protein exhibited a high stability (Wan, Liu, & Guo, 2018). According to Franzen succinylated and acetylated soy protein exhibited improved functional properties in terms of emulsifying activity, and foaming capacity (Franzen & Kinsella, 1976). Another study showed that glycation combined with phosphorylated modification could reduce the anti- α -Lac antibody response (Enomoto et al., 2009). As we know, allergy epitopes of allergic protein are classified into linear epitopes and conformational epitopes. Järvinen et al. reported that α -Lac has four IgE binding regions (specific sequences of amino acids 1–16, 13–26, 47–58 and 93–102) and three IgG binding regions (specific sequences of amino acids 7–18, 51–61 and 89–108) (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). These regions contain one or more reactive groups, such as lysine, arginine, serine, threonine and tyrosine residues etc., which can be modified to change the protein structure.

Glycated modification occurs in the lysine and arginine of α -Lac, to mask the part of the linear epitopes, thereby reducing its allergenic reactivity (Liu et al., 2018a). The usual modified sites for the phosphorylated modification on proteins are the serine, threonine and tyrosine of protein (Alverdi et al., 2005), acylation occurred in the serine and threonine

Abbreviations: α -Lac, bovine α -lactalbumin; IgE/IgG, immunoglobulin E/immunoglobulin G; IL-6, interleukin-6; Gal, galactose; SP, sodium pyrophosphate; AA, acetic anhydride; ELISA, enzyme-linked immunosorbent assay; DH-Lac, native α -Lac subjected to dry heat; HPLC, high performance liquid chromatography; HCD/ETD MS/MS, high-energy C-trap/electron transfer dissociation mass spectrometry/mass spectrometry

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residues (Mukherjee, Hao, & Orth, 2007). A crucial research question of this study was thus whether a similar result can also be observed with phosphorylated and acetylated modification. Although glycation, phosphorylation and acetylation also have been widely used to improve functional characteristics of protein, there has been no reported comprehensive comparison of their effect on the allergenic reactivity of α -Lac. Moreover, previously our laboratory found that the IgE/IgG-binding capacity of α -Lac was partly weakened after ultrasonic-assisted with glycation, because of it change the multi-structure and epitopes of α -Lac (Liu et al., 2018a). But more interestingly, an effective way to reduce the mediator release from basophils/mast cells and eliminate the allergenic reactivity of α -Lac that was treated by protein modification is rare. And since our previously reported methods was worth considering. What is not yet clear is the impact of phosphorylation and acetylation on the allergenic reactivity of α -Lac. α -Lac is supplemented to infant formulae which undergo the glycation, phosphorylation and acetylation, then may modulate its functionality.

The main purpose of this study is to gain an understanding of the mechanism of the reduction in allergenic reactivity of the modified α -Lac by glycation, phosphorylation and acetylation. The IgE/IgG-binding capacity, the histamine and interleukin-6 release of modified α -Lac were evaluated by ELISA and RBL-2H3 cell model, respectively. The multi-structural changes, the number and location modified sites of α -Lac were characterized by fluorescence spectrometry, and mass spectrometry, which would reflected in changing the linear epitopes and conformational epitopes of α -Lac. Studies on the mechanism and functional characteristics of milk whey proteins by protein modification has guiding significance for improving the nutritional value and biological functions of milk in the food industry, and will also be helpful for designing the optimal nutritional performance of milk product.

2. Materials and methods

2.1. Materials

Bovine α -lactalbumin (L6010, Type III, $\geq 85\%$), galactose (G0625), goat anti-human IgE-horseradish peroxidase (HRP) conjugate (A9667) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sodium pyrophosphate (SP), acetic anhydride, and goat anti-rabbit IgG-HRP conjugate (SE131) were obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China). ELISA kits for histamine and interleukin-6 (IL-6) were purchased from MeiMian Systems (Jiangsu, China). The rat mast cell lines RBL-2H3 were obtained from the Chinese Academy of Sciences. Rabbit antisera were prepared using a previously reported protocol (Liu et al., 2018a). Human antisera were from Plasma Lab International (Everett, WA, U.S.A.). The milk protein-specific IgE levels of five human antisera were 6.71, 9.977, 17.1, 43.6 and $> = 100$ kUA/L respectively.

2.2. Sample preparation

Native α -Lac was dissolved in double distilled water to concentration of 1.0 mg/mL. Then, 3 mg of galactose (Gal) and sodium pyrophosphate (SP) was dispersed in 3 mL of α -Lac solution, respectively. Two samples were lyophilized to powder, followed by incubation at 55 °C and 65% relative humidity (saturated potassium chloride solution) for 3 h. The reaction was stopped in an ice bath, and then the samples was filtered using Centricon (Millipore) centrifugal filters with 3000 Da to remove unreacted Gal and salts. 1.0 mg/mL of native α -Lac was adjusted to pH 8.0 with 1 M NaOH. Acetic anhydride (AA, 2 mL) was added slowly with constant stirring at 37 °C for 1 h. The pH was maintained 8.0 by the addition of 1 M NaOH during the reaction. After acetylation, pH of the reaction solution was adjusted to 4.0 by the addition of 2 M HCl, the precipitates were collected by centrifugation at the speed of 3500 r/min for 10 min, then washed twice by double distilled water. Lastly, the sample was filtered using Centricon (Millipore) centrifugal filters with 3000 Da to remove salts. The concentrations of all samples were diluted into 5.0 mg/mL for future use.

Native α -Lac was named N-Lac. Native α -Lac subjected to dry heat under the same glycation conditions was named DH-Lac. The modified samples with glycation, phosphorylation and acetylation were named Gal-Lac, SP-Lac, AA-Lac, respectively.

2.3. IgE/IgG-binding capacity

The IgE/IgG-binding capacity of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac was estimated with inhibition ELISA assays. Human antisera (prepared by mixing the sera of 5 patients at the same volume) and rabbit antisera were applied to study the IgE/IgG-binding capacity. The experimental methods and detailed steps were performed, as previously reported (Liu et al., 2018a).

2.4. Allergenicity in RBL-2H3 cells

The rat mast cell lines RBL-2H3 were cultured in RPMI-1640 medium supplemented with FBS in a 5% CO₂-95% humidity incubator at 37 °C. The cells (5×10^5 cells) were inoculated in 24-well culture plates, then human antisera (prepared by mixing the sera of 5 patients at the same volume) IgE from milk-allergic sufferers was added to the plates. After 24 h, the cells were stimulated by 50 μ g/well test samples for 4 h. Finally, the release of histamine and interleukin-6 were detected though the reported methods (Kuehn et al., 2010; Lv et al., 2019).

2.5. Intrinsic fluorescence emission spectroscopy

The intrinsic emission fluorescence spectra of the test samples (0.1 mg/mL, with 50 mM PBS, pH 7.4) were obtained using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, LTD, Tokyo, Japan).

2.6. Surface hydrophobicity

Surface hydrophobicity (H_0) of all the test samples was determined by ANS assay (Liu et al., 2018b). The fluorescence intensity of test sample was measured at 390 nm (excitation) and 470 nm (emission) using a Hitachi F-7000 fluorescence spectrophotometer. The initial slope of fluorescence intensity versus protein concentration plot was used as the index of H_0 .

2.7. Identification of the modified sites

The test sample was digested with pepsin, the detailed steps were performed according to our reported methods (Liu et al., 2017). After digestion, the peptides were separated with ultimate 3000RSLCnano high-performance liquid chromatography (HPLC, Thermo Fisher Scientific) using a RP-C18 column, and then the column effluent was performed by Thermo Fisher Orbitrap Fusion Mass Spectrometer. Positive ions were used to detect isolates. The mode was used to acquire MS/MS spectra. Detection mode: scanning range of 350–1800 m/z , primary mass spectrum resolution of 60,000 at 200 m/z , AGC target of 4×10^5 , maximum IT 50 ms, dynamic exclusion of 60 s. The m/z of polypeptides and polypeptide fragments was obtained according to the following methods: cycle time of 3 s, MS2 activation type of high-energy C-trap/electron-transfer dissociation (HCD/ETD), isolation window of 1.6 m/z , secondary resolution of 15,000 at 200 m/z , normalized collision energy of 30 eV. Raw file was executed by our previous method for data analysis (Liu et al., 2017).

2.8. Statistical analysis

The experiments were carried out in triplicate and the results were presented as mean value $\pm$ standard deviation (SD). The analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL) and Origin-Pro 2016 (OriginLab Corp., Northampton, MA).

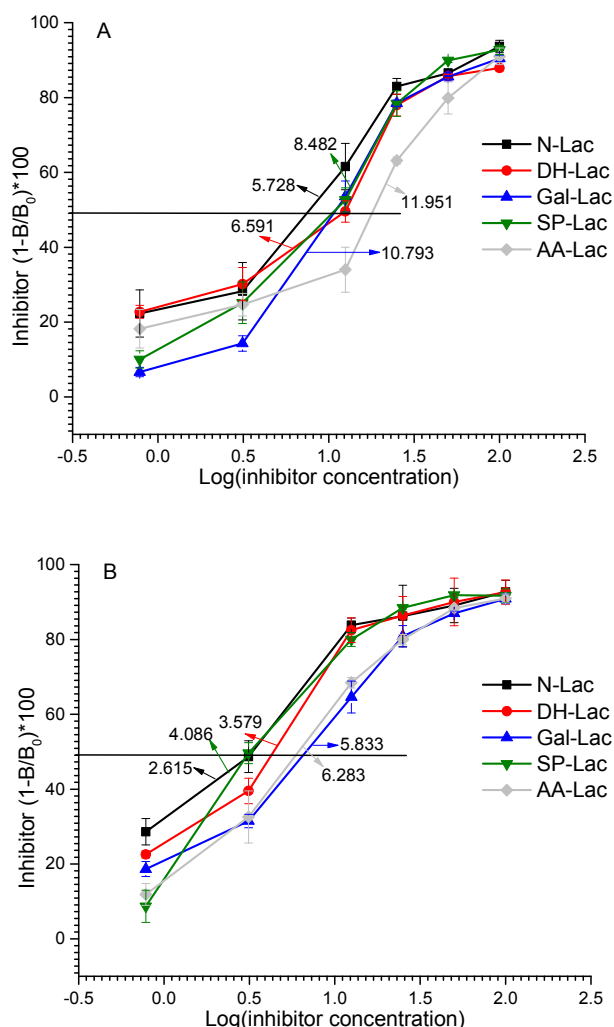


Fig. 1. The IgE (A) and IgG (B) binding ability of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac was performed by inhibition ELISA. IC_{50} : the concentration of inhibitors that causes a 50% inhibition of antibody binding ($\mu\text{g/mL}$). Anti- α -Lac rabbit pooled sera or Anti- α -Lac patients' pooled sera (50 μL /well) were incubated separately with 0.78125, 3.125, 12.5, 25, 50, 100 $\mu\text{g/mL}$ (50 μL /well) of the corresponding modified α -Lac as inhibitors.

3. Results and discussion

3.1. Analysis of the IgE/IgG-binding capacity

As shown in Fig. 1, the IC_{50} value of native α -Lac was increased by dry heating and further increased by glycation, phosphorylation and acetylation. The IC_{50} value of DH-Lac, Gal-Lac, SP-Lac and AA-Lac respectively shifted to 6.591, 10.793, 8.482 and 11.951 $\mu\text{g/mL}$, much higher than that of N-Lac, which was 5.728 $\mu\text{g/mL}$ (Fig. 1A). Data in this chart can be compared with those in Fig. 1B, which showed a similar phenomenon, the IC_{50} values of DH-Lac, Gal-Lac, SP-Lac and AA-Lac were 1.37-, 2.23-, 1.56-, and 2.40-times that of N-Lac (2.615 $\mu\text{g/mL}$). We all know that lower IC_{50} value implies higher IgG/IgE-binding capacity, thus, these results indicated that the IgG/IgE-binding capacity of DH-Lac, Gal-Lac, SP-Lac and AA-Lac were reduced significantly, and that means their allergenic reactivity was reduced. In the study by Liu et al., glycated α -Lac was found to have a lower IgG/IgE- capacity compared with N-Lac (Liu et al., 2018a), similar results here was also observed in the Fig. 1. The reduction in the IgG/IgE-binding capacity of the DH-Lac, Gal-Lac, SP-Lac and AA-Lac were considered to be attributed to shielding of the linear epitopes and destruction of the conformational epitopes by dry heating (Morisawa et al., 2009), glycation (Enomoto et al., 2009), phosphorylation (Enomoto et al., 2007)

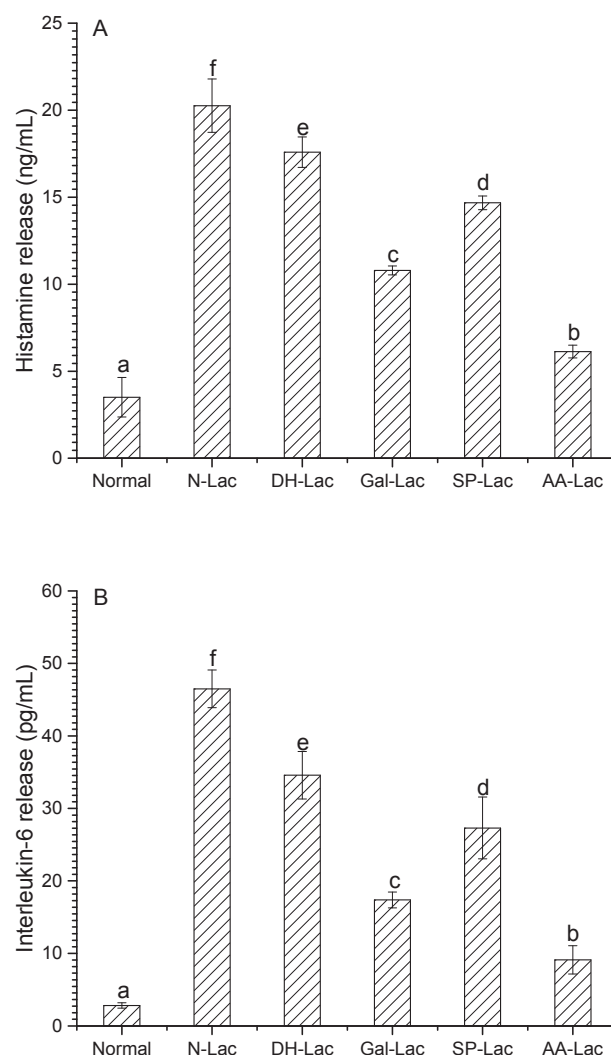


Fig. 2. Effects of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac on the release of histamine (A) and Interleukin-6 (B) from the rat mast cell lines RBL-2H3 cell. Letters (a-f) in the bars mean significantly different ($p < 0.05$).

and acetylation respectively. The IgG/IgE-binding capacity of the modified α -Lac was observed in the order acetylated α -Lac > glycated α -Lac > phosphorylated α -Lac, suggesting that acetylation could destroy the structure of α -Lac effectively, so as to notably reduce the IgG/IgE-binding capacity. Moreover, there was a significant positive correlation between the mediators release from basophils/mast cells and the occurrence of type I allergic reactions (He, Zhang, Zeng, Chen, & Yang, 2013). In order to better confirm the reduction in the allergenic reactivity of DH-Lac, Gal-Lac, SP-Lac and AA-Lac, the level of histamine and interleukin-6 was appropriate to evaluate the allergenic reactivity using a cell model.

3.2. The impact of the test sample on IgE sensitized RBL-2H3 cells

The effects of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac on the release of histamine and interleukin-6 (IL-6) were shown in Fig. 2. The results showed that α -Lac was treated by dry-heating, glycation, phosphorylation and acetylation showed an insignificant affect the histamine and IL-6 release compared to native α -Lac ($p < 0.05$). The histamine release were 17.59, 10.79, 14.60, and 6.13 ng/mL when α -Lac was processed by dry-heating, glycation, phosphorylation and acetylation, respectively (Fig. 2A). Fig. 2B shows a similar phenomenon, the IL-6 release of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac were 46.49, 34.58, 17.37, 27.30, and 9.11 ng/mL, separately. These

results indicated that the histamine and IL-6 release of allergenic α -Lac treated by glycation, phosphorylation and acetylation is lower than that of N-Lac. Glycation can decrease the release of mediators related to allergic symptoms (Yang et al., 2018; Han et al., 2018). From this, we suggest phosphorylation and acetylation could also induce the similar results. The possible explanation is that the response of RBL-2H3 cells to Gal-Lac, SP-Lac and AA-Lac, which alters IgE epitopes on the surface of α -La, hindering the basophil degranulation. Combine the results shown in Figs. 1 and 2, allergenic reactivity of the modified α -Lac undergo glycation, phosphorylation and acetylation was significantly reduced, which reflected in the decreased IgE/IgG-binding capacity, the release of histamine and IL-6 from RBL-2H3 cells. To understand the relationship between allergenic reactivity and structural changes was investigated using conventional spectroscopy and high-resolution mass spectrometry in the subsequent experiments.

3.3. Intrinsic fluorescence emission spectroscopy and surface hydrophobicity

The Trp fluorescence spectra of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac are analyzed to evaluate the conformational changes of α -Lac. As shown in Fig. 3A, when excited at 290 nm, the fluorescence intensity (FI) of DH-Lac and Gal-Lac reduced, it was considered to be due to the shielding effect (Kastrup Dalsgaard, Holm Nielsen, & Bach Larsen, 2007), and solvent relaxation (Kobayashi et al., 2001). This result was consistent with previous reports on glycosylated α -Lac (Liu et al., 2017), and β -lactoglobulin (Chen et al., 2016). However, the opposite trend was observed in the FI of SP-Lac and AA-Lac, suggesting that phosphorylation and acetylation damaged the hydrophobic interactions within α -Lac molecules (Lakkis & Villota, 1992), and induced more exposure of Trp residues to solvent (Enomoto et al., 2009). It could be inferred from intrinsic fluorescence emission spectroscopy results that glycation, phosphorylation and acetylation may have more significant effects on the conformational structure of α -Lac.

From the data in Fig. 3B, the surface hydrophobicity (H_0) of native α -Lac and DH-Lac showed no significant changes ($p > 0.05$). When α -Lac was modified by Gal, SP and AA, the H_0 declined from 53.01 (N-Lac) to 45.33 (Gal-Lac), 14.86 (SP-Lac), and 14.39 (AA-Lac), respectively. Previous studies led some researchers to conclude that glycosylated modification reduced the H_0 of proteins (Chen et al., 2016; Yang et al., 2017), who reported that the masking of some hydrophobic groups induced by covalent binding of amino acids to saccharides. Phosphorylation and acetylation are expected to achieve similar results even worse. On the other hand, the cationic groups can be bind with ANS, such as Lys, Arg and Ser residues, these amino acids by modified also led to the decrease of H_0 of α -Lac.

3.4. Location and number of the modified sites determination

In theory, a peptide of α -Lac was glycosylated, phosphorylated and acetylated by one molecule of galactose (Gal), sodium pyrophosphate (SP), and acetic anhydride (AA) respectively, then the corresponding m/z peaks will appear mass shift 162.0528, 79.9663, and 42.0106. The results, as shown in Fig. 4A, indicated that native peptide 104–117 (sequence of WLAHKA-LCSEKLDQ) was m/z of 425.4710⁴⁺, the corresponding m/z peak of glycosylated peptide had an increase in m/z of 44.5130. The m/z peak of peptides 9–18 (sequence of FRELKDLKGY) and 91–103 (sequence of CVKKILDKVGINY) was 423.5710³⁺ and 388.2607⁴⁺, while new peak of the glycosylated peptide were 531.6060³⁺ and 469.2506⁴⁺, respectively (Figure S1A). It has been clearly shown that the peptides 9–18, 91–103 and 104–117 were mono-glycosylated or dual-glycosylated induced by Gal. The m/z peaks of native peptide 1–25, 13–40, 58–82 and 95–121 was 965.7934³⁺, 1040.1071³⁺, 1031.7984³⁺, and 826.5643⁴⁺, however, phosphorylation increased the m/z value to 992.4770³⁺, 1066.4742³⁺, 1058.7845³⁺, 866.4093⁴⁺ (as showed in Fig. 4B and S1B), respectively. This indicated that these peptides 1–25, 13–40, 58–82 and 95–121 were phosphorylated by one or two

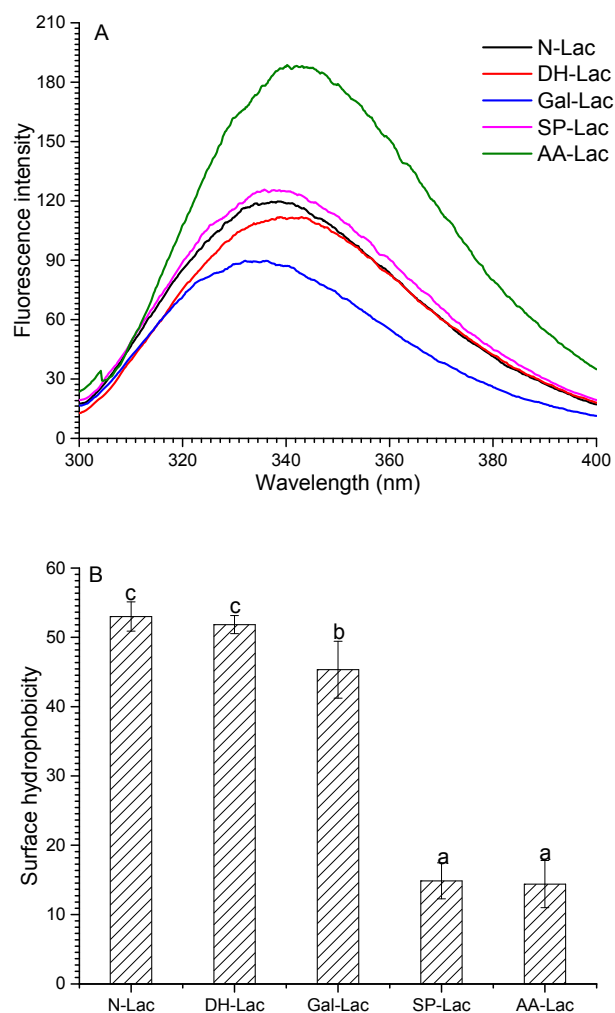


Fig. 3. The intrinsic fluorescence spectra (A) and surface hydrophobicity (B) of N-Lac, DH-Lac, Gal-Lac, SP-Lac and AA-Lac. Letters (a-c) in the bars mean significantly different ($p < 0.05$).

molecules of phosphate group. Moreover, Fig. 4C and S1C shows the m/z peaks of native peptide 8–14, 29–40, 30–40, 40–49, 61–71, 104–112 and 111–117 was 453.8081²⁺, 664.7977²⁺, 614.3145²⁺, 545.7488²⁺, 672.3068²⁺, 543.2445²⁺, and 440.1659²⁺, whereas the new peaks with the m/z values at 474.7602²⁺, 706.8016²⁺, 635.2738²⁺, 566.7595²⁺, 714.2786²⁺, 564.2866²⁺, 461.2024²⁺, and 601.7823²⁺ had an increase in m/z of shift 20.9521, 42.0009, 20.9603, 21.0107, 41.9718, 21.0421, and 21.0365 separately. Therefore, these peptides attached one or more molecules of acetyl group.

Subsequent HPLC-HCD/ETD-MS/MS was performed to identify accurately the number and location of the modified sites K13, Y18, K16, S22, T33, S34, T38, S47, K62, S69, S70, K94, K98, Y103, K108, S112 and 114 (Table 1). For example, the glycosylated peptide ¹⁰⁴WLAHKA-LC(carbamidomethyl)SEKLDQ¹¹⁷ with a peak at m/z 465.9840⁴⁺. K108 was obtained by the complete matching of c and z fragment ions according to Fig. 5A, confirming that K108 was glycosylated by Gal. Similarly, K13, K16, K94 and K98 were glycosylated by ETD-MS/MS as showed in Figure S1A. The phosphorylated sites Y18, S22, S69, Y103, and S112 of SP-La, and acetylated sites K13, T33, S34, T38, S47, K62, S69, S70, K108, and K114 of AA-La were identified through the matching of b and y fragment ions from HCD-MS/MS (Fig. 5B, 5C, S1B and S1C). Therefore, the modified peptides and sites of Gal-Lac, SP-Lac and AA-Lac can be identified by HPLC-HCD/ETD-MS/MS clearly.

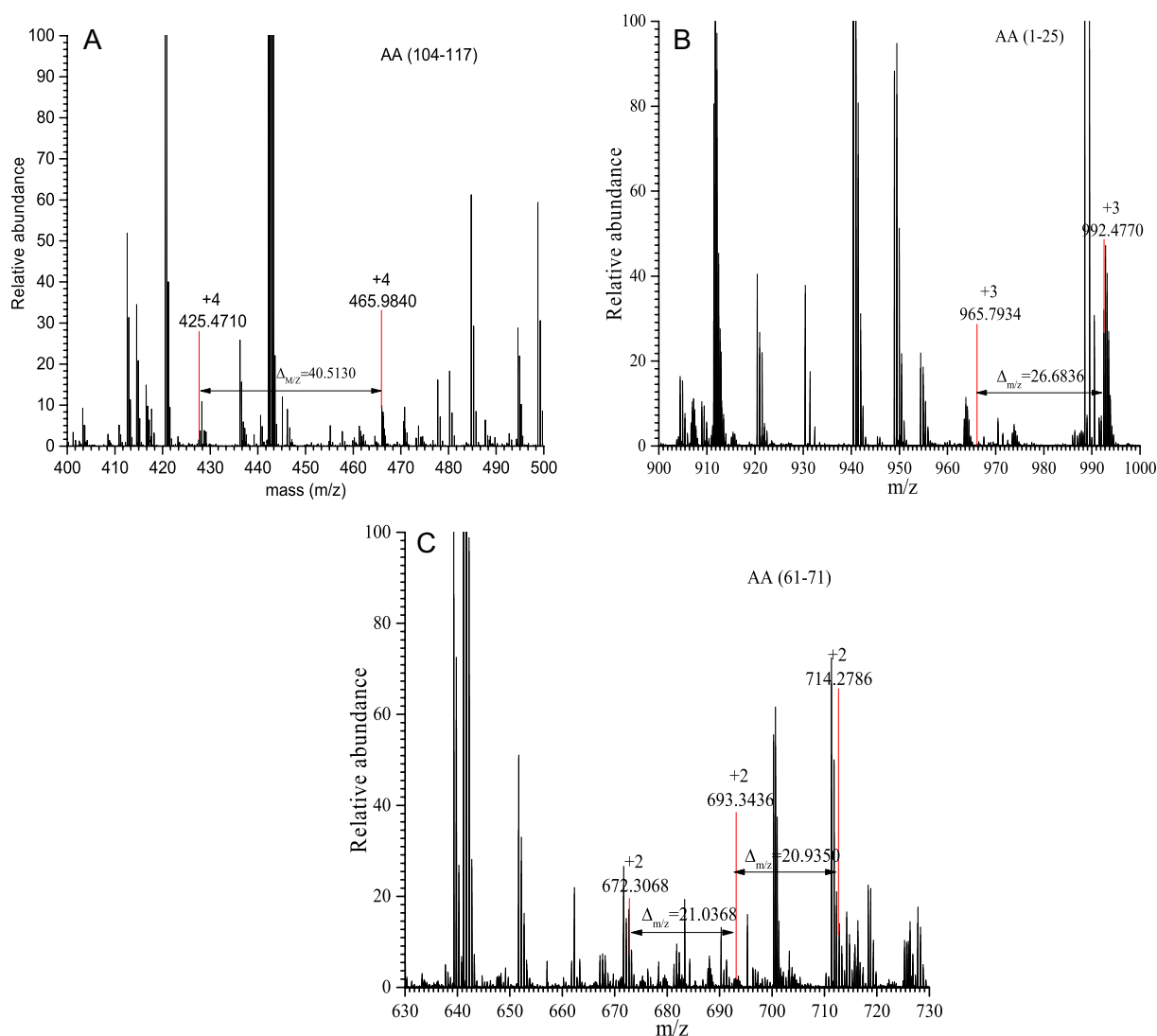


Fig. 4. Mass spectra for the un-modified peptides. (A) peptide 104–117 at m/z 425.4710⁴⁺, (B) peptide 1–25 at m/z 965.7934³⁺, (C) peptide 61–71 at m/z 672.3068²⁺. The determined peptides are labelled by residue numbers. The m/z differences between modified and un-modified peptides are indicated above the arrows.

Table 1

Summary of the modified peptides and sites in the Gal-Lac, SP-Lac and AA-Lac.

Sample	Peptide location	m/z Modified peptide	Δm ppm	Sequence ^a	Modified site
Gal-Lac	9–18	531.6060 ³⁺	1.12	(V)FRELKDLKGY(G)	K13, K16
	91–103	469.2506 ⁴⁺	1.07	(M)C*VKILDKVGINY(W)	K94, K98
	104–117	465.9840 ⁴⁺	1.04	(Y)WLAHKALC*SEKLDQ(W)	K108
SP-Lac	1–25	992.4770 ³⁺	5.51	EQLTKC*EVFRELKDLKGYGGVSLPE(W)	S22
	13–40	1066.4742 ³⁺	1.41	(L)KDLKGYGGVSLPEWVC*TTFTSGYDTQA(I)	Y18
	58–82	1058.7845 ³⁺	0.32	(N)KIWC*KDDQNPHSSNIC*NISC*DKFLD(D)	S69
	95–121	866.4093 ⁴⁺	2.60	(K)ILDKVGINYWLAHKALC*SEKLDQWLC*E(K)	Y103, S112
AA-Lac	8–14	474.7602 ²⁺	4.71	(E)VFRELKD(L)	K13
	29–40	706.8016 ²⁺	1.47	(C)TTFTSGYDTQA(I)	T33, T38
	30–40	635.2738 ²⁺	2.18	(T)TFHTSGYDTQA(I)	S34
	40–49	566.7595 ²⁺	3.37	(I)AIVQNNDSTE(Y)	S47
	61–71	714.2786 ²⁺	1.65	(W)C*KDDQNPHSSN(I)	K62, S69, S70
	104–112	564.2866 ²⁺	2.60	(Y)WLAHKALC*S(E)	K108
	111–117	461.2024 ²⁺	5.03	(L)C*SEKLDQ(W)	K114

^a C* refers to carbamidomethyl.

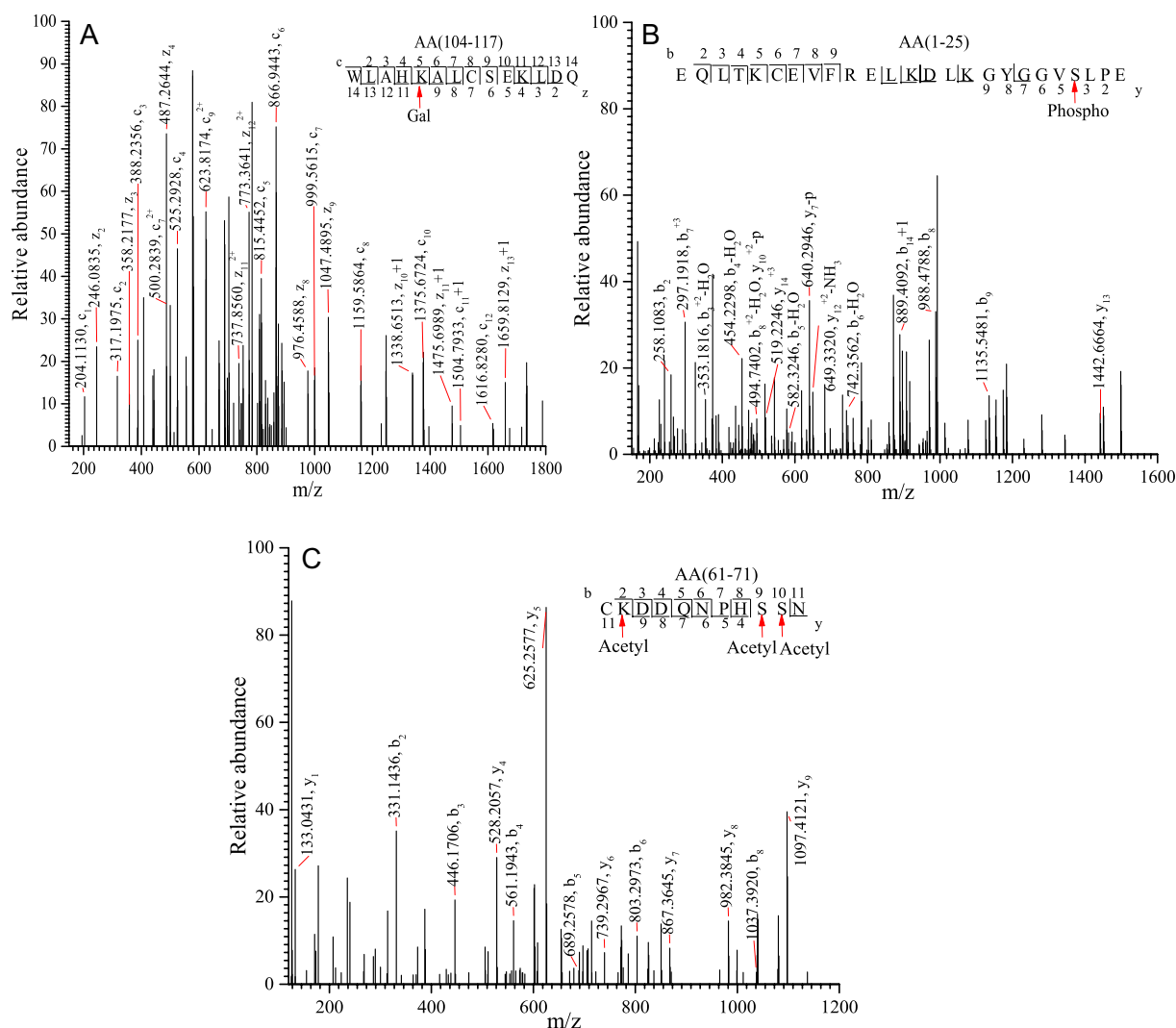


Fig. 5. The HCD/ETD MS/MS spectra of the modified peptides. (A) the glycated peptide 104–117 (WLAHKALCSEKLDQ) with m/z of 465.9840⁴⁺, (B) the phosphorylated peptide 1–25 (EQLTKCEVFRELKDLKGYGGVSLPE) with m/z of 992.4770³⁺, (C) the acetylated peptide 61–71 (CKDDQNPHSSN) with m/z of 714.2786²⁺. The sequence of per peptide is depicted on the top of the spectrum. The identified the modified sites are indicated by a line with Gal, Phospho, and Acetyl. The c and z or b and y ions are shown by the numbers and lines.

3.5. Mechanism of the reduction in the allergenic reactivity of α -Lac by glycation, phosphorylation and acetylation

In the current study, protein modification methods, including glycation, phosphorylation and acetylation, notably reduced the IgG/IgE-binding capacity of α -Lac and the release of histamine and interleukin-6 from RBL-2H3 cells, which could be attributed to its structural changes (mainly illustrated by the conformational epitopes and linear epitopes). These structural changes of α -Lac were discussed in subsequent sections to comparative studies on the mechanism of the reduction in allergenic reactivity of α -Lac induced by glycation, phosphorylation and acetylation.

We all know that the presence of both conformational and linear epitopes on α -Lac molecules (Tanabe, 2007). After the modification, intrinsic fluorescence emission spectroscopy (Fig. 3A) and surface hydrophobicity (Fig. 3B) analyses confirmed that the conformational structure of α -Lac was significant changes, leading to the destruction of conformational epitopes of α -Lac, ultimately cause the reduction in allergenic reactivity, which reflected in the reduction in the IgE/IgG-binding capacity (Fig. 1) and the release of allergic mediators of basophils (Fig. 2). The probable epitopes of α -Lac reported by Järvinen suggests that the peptide 1–16, 13–26, 47–58, and 93–102 as IgE-binding epitopes, and peptide 7–18, 51–56, and 89–108 as IgG-binding

epitopes (Järvinen et al., 2001). Moreover, the sequence 17–58, 109–123, and (6–10):S–S(115–123) of native α -Lac have also been confirmed to be specific response to IgE and larger peptides confirmed the importance of conformational epitopes (Maynard, Jost, & Wal, 1997). These epitopes contain one or more lysine, serine, threonine and tyrosine residues, and the glycation, phosphorylation and acetylation results in modification of the epitopes with an obvious reduction in allergenic reactivity of α -Lac. For instance, five lysine residues (K13, K16, K94, K98, and K108) were glycated by Gal (Table 1), and results in the change of linear epitopes, finally leading to a lower allergenic reactivity compared with native α -Lac and the dry-heating alone (Figs. 1 and 2). α -Lac was reacted with SP through the phosphorylation, five phosphorylated sites (Y18, S22, S69, Y103, and S112) were confirmed in this study (Table 1). The four sites (Y18, S22, Y103, and S112) have similarly been found for its ability to masking the linear epitope, and cause the reduction in allergenic reactivity. As shown in Figs. 1 and 2, the reactivity of glycated α -Lac were declined compared to that of phosphorylated α -Lac, which was attributed to the fact that phosphate group and Gal could have different molecular weight and modify different sites, causing the masking of the different epitopes, leading to the difference of allergenic reactivity. The most striking observation to emerge from the data analysis was the minimum allergenic reactivity

was detected in the acetylated α -Lac compare to the glycated α -Lac and phosphorylated α -Lac, owing to it has the most acetylated sites, including K13, T33, T38, S34, S47, K62, S69, S70, K108, and K114 (Table 1), which can effectively affect the epitopes. These data indicated that the reduction in allergenic reactivity of α -Lac was correlated to the type, number and location of the modified sites. Thereby, a current observational data also demonstrated that glycation, phosphorylation and acetylation can significantly reduce allergenic reactivity of α -Lac by causing the changes of allergy epitopes.

4. Conclusion

In this investigation, glycation, phosphorylation and acetylation significantly reduced the allergenic reactivity and changed the multi-structure of α -Lac, which reflected in reduction in the IgE/IgG-binding capacity, and the release of histamine and interleukin-6. The decrease in the allergenic reactivity of α -Lac depends upon not only the shielding effect of the linear epitope found to be caused by the modified sites, including glycated sites, phosphorylated sites and acetylated sites, but also the change of conformational epitopes, which reflected in the difference of the modified sites, and the changes of intrinsic fluorescence intensity and surface hydrophobicity. However, the potential use of modified α -Lac by protein modification in reducing the risk of suffering from α -Lac allergy remains experimental but the hypoallergenic products continued research. Furthermore, the modification could also produce the reaction products, they can affect the safety of protein. The products of α -Lac modification, such as α -dicarbonyl compounds and advanced glycation end products (AGEs), need to be measured to fully ensure the safety of α -Lac during food processing.

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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.

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