

Observation of the structural changes of α -lactalbumin induced by ultrasonic prior to glycosylated modification

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Abstract

Bovine α -lactalbumin (BLA) was treated by ultrasonic at 150 W/cm² for different times and subsequently glycosylated with mannose by dry-heating. Molecular weight, intrinsic fluorescence spectra, glycation sites and degree of modified BLA were observed. The proteinaceous high molecular weight components were formed after ultrasonic prior to glycosylated modification, while the conformational changes were obvious. Prior to ultrasonic pretreatment, K62, K114, and K122 of BLA were identified. After treated by ultrasound at 150 W/cm² for 5, 10, 15, and 20 min, the sites were increased to four, four, five, and five, respectively. All glycosylated sites of modified BLA exhibited a higher degree of substitution per peptide (DSP) values compared to native BLA. Ultrasonic at 150 W/cm² for 20 min revealed the most significant change in the BLA structure. Therefore, conformational changes, the intensified glycation site, and DSP value were responsible for the structural changes of BLA.

Practical applications

BLA is suitable as an ingredient for infant nutrition in food, and has immune-modulating, antioxidant, antibacterial, and antitumor activity etc. This study revealed that the structural changes of BLA induced by ultrasonic prior to glycosylated modification. It will be beneficial to understand the mechanism of the functional changes of modified BLA. Ultrasonic prior to glycosylated modification will be more likely to develop a practical technology to modify protein in the food industry, and improve the functional characteristics of food, such as produce hypo-allergenic cow's milk in future.

KEYWORDS

glycosylated modification, glycation sites, structural changes, ultrasonic, α -lactalbumin

1 | INTRODUCTION

Bovine α -lactalbumin (BLA), one of the model Ca²⁺ binding proteins that carries out diverse functions in the food and biology, as an ingredient of the function and nutrition (Liu, Tu, Shao, et al., 2017) and an ingredient for infant nutrition in the food. The BLA possesses a broad range of the functional properties, such as antioxidant (Liu, Tu, Shao, et al., 2017), immune-modulating (Liu, Tu, Liu, et al., 2018), antibacterial (Sedaghati, Ezzatpanah, Boojar, & Ebrahimi, 2018), or antitumor activity (Fang, Zhang, Wu, Fan, & Ren, 2016), and so on. Chemical

and physical methods are most popular modified technology capable of improving the structural and functional properties of protein (Liu, Tu, Yang et al., 2018; Yang, Tu, Wang, Li, & Tian, 2017). Glycation is a chemical method, which occurs between amino and carbonyl compounds, and affected by intrinsic and extrinsic parameters (Huang et al., 2013; Thomsen, et al., 2012), such as properties of reagents, reaction temperature, reaction time and water activity. These factors could change the structural properties of proteins to improve the functional properties. In addition, BLA is supplemented to infant formulae which undergo the glycation reaction, then may modulate its functionality.

Recently, high-intensity ultrasonication, a physical method, which has been used to improve food properties due to it can maintain a food's inherent nutritional ingredients (AnaCristina & Mar, 2010). The functional properties of food were changed by high-intensity ultrasonication produce the acoustic cavitation, and fluid particles oscillations, etc. (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). In addition to it caused the unfolding of proteins, leading to improve the glycation (Zhang et al., 2014). Recently, two or more methods is effective to improve functional properties of proteins (Hirofumi et al., 2007; Li, Sun, Ma, Jin, & Sheng, 2018), because single modified method cannot achieve a satisfactory result. For example, high-intensity ultrasonic-assisted chemical reactions have been used in the modification of proteins (Chen, Chen, Wu, & Yu, 2016; Liu, Tu, Zhang, et al., 2018; Zhao et al., 2016). Compared with a single glycation modification, high-intensity ultrasonic assistance could effectively shortened reaction times, along with improved food properties to a satisfactory result. Liu, Tu, Wang, Zhang, Sha et al. (2017) and Liu, Tu, Zhang, et al. (2018) reported that the different ultrasonic powers, times, temperatures, and aldose sugars can affect the physiochemical properties of bovine serum albumin. Previously, our laboratory demonstrated that BLA was pretreated by ultrasonic power and subsequently glycation with glucose by dry-heating, the antioxidant activity and glycation reaction were intensified (Liu, Tu, Shao, et al., 2017), but the structural changes of the treated BLA by different ultrasonic time-assisted glycation modification are not studied, especially for the glycation sites.

Conventional spectrometry, such as fluorescence intensity (Yeboah, Alli, & Yaylayan, 1999), ultraviolet spectrometry, and circular dichroism (Kerensa, Voragen, Hamer, & Jongh, 2004), has been used to evaluate the glycation degree and the structure of the modified protein. But, in these technologies, the glycation degree of modified protein at the molecular level could not be characterized owing to the change in extent of glycation which was revealed at the protein level. High-resolution mass spectrometry has recently emerged as a very powerful tool to characterize amino acid sequencing of modified proteins (Bondarenko, Second, Zabrouskov, Makarov, & Zhang, 2009), especially for glycation beta-lactoglobulin (Yang, Tu, Wang, Zhang, et al., 2017) and ovalbumin (Liu, Tu, Wang, Zhang, Huang, et al., 2017). Therefore, it was employed to characterize structural changes of the modified BLA which undergo different ultrasound time assisted with glycation modification at the molecular level.

In this work, BLA was treated by different ultrasonic time-assisted glycation modification, aiming to research the structural changes of modified BLA. The first, size exclusion chromatography (SEC) and intrinsic fluorescence emission spectroscopy were used to monitor primary and tertiary structure changes of modified BLA. Additionally, mass spectrometry was used for determining the glycation site, included its numbers and location, and glycation extent, which reflected in the degree of substitution per peptide (DSP) value. Results of this research can reveal the crucial role of high-intensity ultrasonic in changing the structure of proteins, and providing the information for application of high-intensity ultrasonic food industry.

2 | MATERIALS AND METHODS

2.1 | Reagents

BLA (L6010, Type III, $\geq 85\%$), mannose (M2069), and trypsin (T8003) were from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents used were of analytical grade or higher.

2.2 | Preparation of BLA samples

Twenty milligrams of α -lactalbumin (BLA) was dissolved in 20 ml of PBS (50 mM, pH 7.4). Three milliliters of BLA solution were split into 5-ml glass bottle which were immersed in the ice bath, and BLA was treated with sonicator (Misonix Qsonica Q700, USA), 20 kHz, microtip probe (1/8 in. = 3 mm) with a 9 s on and 1 s off pulsation at ultrasonic intensity of 150 W/cm² for 0, 5, 10, 15, and 20 min, respectively. Then, 3.0 mg of mannose (Man) was added to the solution. After freeze dry, ultrasonicated BLA-Man complex were dry-heated at 55°C and 79% relative humidity (saturated potassium chloride solution) for 3 hr. Then salt and the unreacted mannose were removed by a centrifugal filter (3,000 Da cutoffs, Millipore, MA). All samples were diluted by ultrapure water to get a concentration of 5.0 mg/ml. Native BLA was named BLA. Glycated BLA by ultrasound at 150 W/cm² for 0, 5, 10, 15, and 20 min was named BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min, respectively.

2.3 | Size exclusion chromatography

A previously reported setup and method (Liu, Tu, Shao, et al., 2017) were used for the SEC analysis.

2.4 | Intrinsic fluorescence spectroscopy

The intrinsic fluorescence emission spectra were tested by previous report (Liu, Tu, Wang, Zhang, Sha, et al., 2017) with modification. One milligram/milliliter of the glycated BLA was recorded from 300 to 400 nm (both at a constant slit of 5 nm) with excitation at 290 nm, and phosphate buffer solution was used as blank.

2.5 | HPLC-CID/FTICR-MS/MS analysis

About 0.5 mg of modified BLA samples was added into 25 μ l of ammonium bicarbonate stock solution and 25 μ l of trifluoroethanol, following by adding 1.0 μ l of 200 mM DTT with incubation for an hour at 60°C. Then the addition of 4.0 μ l of 200 mM iodoacetamide, and incubation for an hour at 25°C, following by adding 1.0 μ l of 200 mM DTT with incubation for an hour at 25°C. To dilute and adjust pH, 300 μ l of water and 100 μ l ammonium bicarbonate stock solution were added. About 4.0 μ l of 1 mg/ml trypsin was added and incubated overnight at 37°C. At last, the digestion was stopped by adding of 10% formic acid (1.0 μ l, FA). After digestion, 100 μ l of sample was

injected into a C18 column (4.0 μm , 2.1 $\times$ 50 mm, Waters, MA) for high-performance liquid chromatography, of which the flow rate was 0.2 ml/min. Mobile phase included the solvent A (0.1% FA in H_2O) and solvent B (100% acetonitrile containing 0.1% FA). After desalting for 5 min with 5% B, the peptides were eluted with a gradient of 5%–50% B for 40 min, 60%–80% B for 3 min, and 80% B for 2 min. The effluent was infused into Fourier transform ion cyclotron resonance-Mass spectrometry (7T-SolariX, Bruker Daltonics, MA) and the peptides were fragmented by CID. Finally, the peptides and glycosylated sites of sample were identified according to MS data, which were processed using DataAnalysis, BioTools, and SequenceEditor (Bruker Daltonics, Billerica, MA).

3 | RESULTS AND DISCUSSION

3.1 | Size exclusion chromatography

SEC diagrams for BLA, BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min was displayed in Figure 1. SEC analysis of all samples confirms that the protein exists as a dimer and monomer. The elution time (monomer) of BLA-5min, BLA-10min, BLA-15min, and BLA-20min shifted to around 19.5, 19.4, and 19.3 min, which were much shorter than that of BLA and BLA-Man at 20.6 and 19.8 min, separately. These strongly show that glycosylated modification can induce the formation of proteinaceous high molecular weight components, and ultrasonic pretreatment promoted the increase. It is worth noticing that the glycation between polypeptide backbone of BLA and mannose thus was increasing the molecular weight of BLA.

3.2 | Intrinsic fluorescence emission spectroscopy

Intrinsic fluorescence emission spectra of BLA, BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min were revealed in the Figure 2.

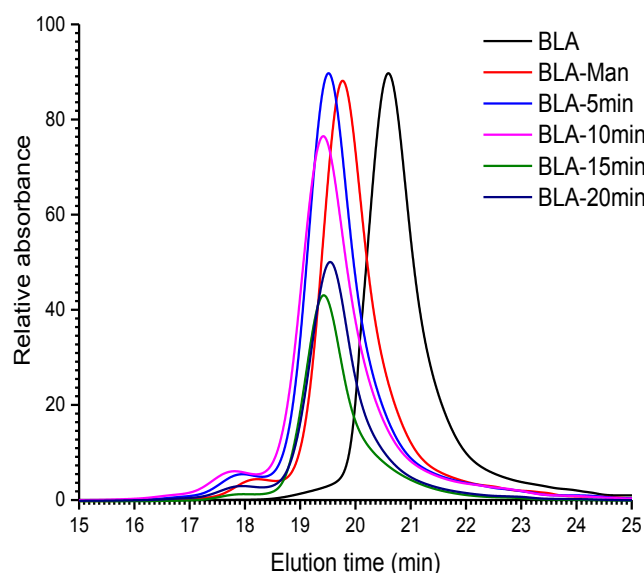


FIGURE 1 Size exclusion chromatography (SEC) for analyses BLA, BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min

The intensity of treated α -La was gradually reduced as compared to BLA, particularly for BLA-20min, which indicates different ultrasonic time-assisted glycation modification affected the conformational structure of BLA. It may result from the exposure of Tryptophan in more hydrophilic surroundings (Huang et al., 2012), solvent relaxation (Dalsgaard, Nielsen, & Larsen, 2010), and more quenching agents which were produced in the BLA-Man complex. Moreover, it has been reported that mannose bound to BLA induced the shielding effect (Kobayashi et al., 2001). Dry-heating for glycation induced conformational changes of BLA around the Tryptophan. More importantly than all of that, Tryptophan can donate a proton to the substance. The result of the present study is consistent with the previous published reports (Liu, Tu, Shao, et al., 2017).

3.3 | Glycation site determination

In the Figure 3a, the glycation sites in glycosylated peptide 59–79 of BLA-20min was analyzed. The m/z peak of native peptide 59–79 was 648.7585⁴⁺, whereas that of glycosylated peptide was 689.2644⁴⁺. The m/z shift of this peak was 40.5059 Da, confirming that the peptide 59–79 was glycosylated by a molecule of Man. To better identify the location of the glycation site, CID/FTICR-MS/MS should be used. Figure 3b presents CID/FTICR-MS/MS of peptide 59IWC(carbamidomethyl)KDDANPHSSNIC(carbamidomethyl)NISC(carbamidomethyl)DK⁷⁹ of BLA-20min with m/z of 689.2644⁴⁺. K62 was modified with mass difference between the b3 and b5 ions, or between the y17 and y19, indicating that K62 was glycosylated with Man in the peptide 59–79. When a peptide of proteins was conjugated with two molecules of mannose, the mass increase will be 324.1056 Da. For example, the m/z peaks of native peptide 63–94 was 763.7024⁵⁺ (Figure 4a), while the corresponding m/z peaks of dual-glycosylated peptide 63–94 were 796.3124⁵⁺ and 828.5172⁵⁺. The m/z differences of two peaks were 32.6100 and 32.2048 Da. Similarly, Figure 4b presents

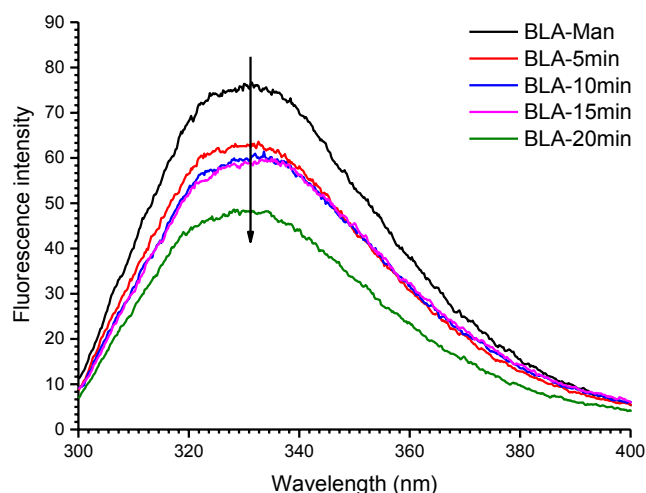


FIGURE 2 The intrinsic fluorescence spectra of BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min

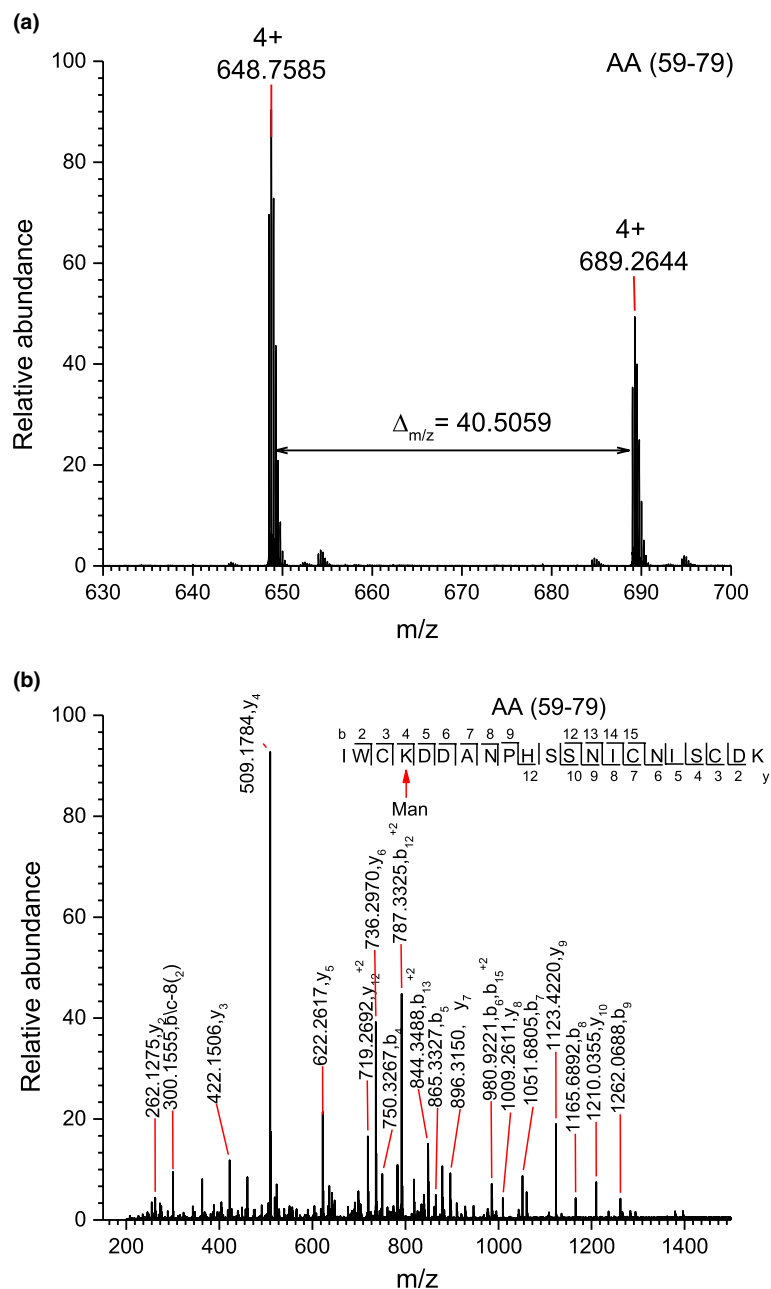


FIGURE 3 Determination of glycation sites in glycated peptide AA59-79 of BLA-20min. (a) Mass spectrum of peptide AA59-79 with one glycation site. (b) CID/FTICR MS/MS spectrum of mono-glycated peptide with m/z of 648.7585⁴⁺. The sequence of each peptide is shown in the top of figure. The mass differences are exhibited with numbers and arrows. The determined glycation sites are highlighted by red arrows and indicated by an arrow with symbol, in which “Man” mean glycation site is added by one mannose molecules. The b and y ions are indicated by the numbers and lines

the dual-glycated peptides ⁶³DDQNPHSSNIC(carbamidomethyl)NISC(carbamidomethyl)DKFLDDDLTDDIMC(carbamidomethyl)VKK⁹⁴ of U-LA-20min with m/z of 828.5172⁵⁺ which were identified to be K79 and K93. According to the m/z shift of ⁷⁹FLD DDLTDDIMC(carbamidomethyl)VKK⁹⁴, ¹⁰⁹ALC(carbamidomethyl)SEKLDWLC(carbamidomethyl)EKL¹²³, ¹¹⁵LDWLC(carbamidomethyl)EKL¹²³ of U-LA-20min, K93, K114, and K122 were also determined by CID/FTICR-MS/MS, respectively. Figures 3 and 4 provide more direct evidence that CID/FTICR-MS/MS can measure the number and location of glycated sites clearly.

As showed in Table 1, the glycation peptide and glycation site of BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min were summarized. BLA-Man contains three sites (K62, K114, and K98). We used ultrasonic time at 5 and 10 min to pretreat BLA, K62, K93, K114, and K122 of BLA which were glycated. When the ultrasonic time increased to 15 and 20 min, a new glycated site of K79 was found to be glycated. The increase of glycation sites was probably with ultrasonic pretreatment, which could loose the structure of BLA, cause the molecular unfolding, and expose more reactive sites (Stanic-Vucinic et al., 2012). There was no differences in

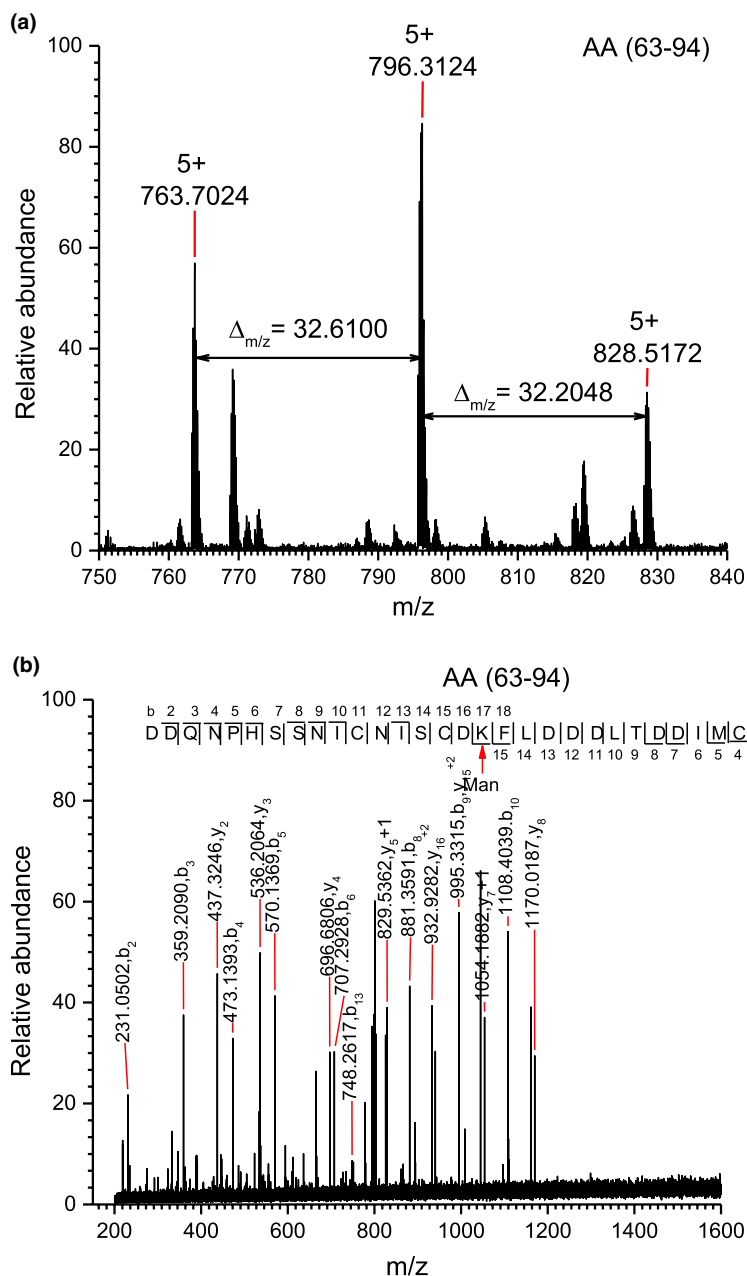


FIGURE 4 Determination of glycation sites in glycated peptide AA63-94 of BLA-20min. (a) Mass spectrum of peptide AA63-94 with two glycation sites. (b) CID/FTICR MS/MS spectrum of dual-glycated peptide with m/z of 828.5172⁵⁺. The sequence of each peptide is shown in the top of figure. The mass differences are exhibited with numbers and arrows. The determined glycation sites are highlighted by red arrows and indicated by an arrow with symbol, in which “Man” mean glycation site is added by two mannose molecules. The *b* and *y* ions are indicated by the numbers and lines

the glycation site of BLA-5min and BLA-10min, the similar phenomenon was observed in the BLA-15min and BLA-20min, suggesting that protein–protein interactions were happened by the increase of ultrasonic time, then suppress the glycation reaction, finally did not increase the glycation sites of BLA. We suggested that the fragmentation pathway of CID/FTICR that was used in the mass spectrometry analysis, which may be destroyed the post-translational modifications of proteins or modified group of peptides (Wells & Mcluckey, 2005).

3.4 | Degree of substitution per peptide value

DSP value was calculated based on previous report (Liu, Tu, Shao, et al., 2017), which reflected in the extent of glycation. Figure 5a shows the DSP value for the AA59-79, AA79-94, AA63-94, AA109-123, and AA115-123 of BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min. All glycated peptides of BLA-5min, BLA-10min, BLA-15min, and BLA-20min exhibited a higher DSP values than that of BLA-Man. For instance, K62 of BLA-Man with DSP value close to 0.18, but, ultrasound pretreatment increased

TABLE 1 Summary of the glycosylated peptides in the BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min

Sample	Peptide location	Glycosylated peptide (m/z)	Δ ppm	Sequence ^a	Glycosylated site
BLA-Man	59–79	689.2652 ⁴⁺	0.35	(K)IWC*KDDQNPHSSNIC*NISC*DK(F)	K62
	109–123	685.9642 ³⁺	3.65	(K)ALC*SEKLDQWLC*EKL	K114
	115–123	683.8103 ²⁺	–1.69	(K)LDQWLC*EKL	K122
BLA-5min	59–79	689.2648 ⁴⁺	–2.03	(K)IWC*KDDQNPHSSNIC*NISC*DK(F)	K62
	79–94	663.9726 ³⁺	–5.46	(K)FLDDDLTDDIMC*VKK(I)	K93
	109–123	685.9672 ³⁺	–2.24	(K)ALC*SEKLDQWLC*EKL	K114
	115–123	683.8054 ²⁺	1.14	(K)LDQWLC*EKL	K122
BLA-10min	59–79	689.3104 ⁴⁺	–2.02	(K)IWC*KDDQNPHSSNIC*NISC*DK(F)	K62
	79–94	663.9705 ³⁺	0.15	(K)FLDDDLTDDIMC*VKK(I)	K93
	109–123	685.9720 ³⁺	–1.00	(K)ALC*SEKLDQWLC*EKL	K114
	115–123	455.8794 ³⁺	0.87	(K)LDQWLC*EKL	K122
BLA-15min	59–79	689.0455 ⁴⁺	1.60	(K)IWC*KDDQNPHSSNIC*NISC*DK(F)	K62
	79–94	663.9721 ³⁺	–0.31	(K)FLDDDLTDDIMC*VKK(I)	K93
	63–94	828.3568 ⁵⁺	–3.17	(K)DDQNPHSSNIC*NISC*DKFLDDDLTDDIMC*VKK(I)	K79, K93
	109–123	685.9720 ³⁺	8.67	(K)ALC*SEKLDQWLC*EKL	K114
	115–123	455.8693 ³⁺	3.48	(K)LDQWLC*EKL	K122
BLA-20min	59–79	689.2644 ⁴⁺	3.67	(K)IWC*KDDQNPHSSNIC*NISC*DK(F)	K62
	79–94	663.9726 ³⁺	–0.76	(K)FLDDDLTDDIMC*VKK(I)	K93
	63–94	828.5172 ⁵⁺	4.08	(K)DDQNPHSSNIC*NISC*DKFLDDDLTDDIMC*VKK(I)	K79, K93
	109–123	685.9664 ³⁺	3.80	(K)ALC*SEKLDQWLC*EKL	K114
	115–123	683.8039 ²⁺	3.48	(K)LDQWLC*EKL	K122

^aC* refers to carbamidomethyl.

their DSP value to 0.25, 0.29, 0.45, and 0.46 in BLA-5min, BLA-10min, BLA-15min, and BLA-20min separately. Interestingly, the DSP value of other glycosylated peptides was improved. This is due to the structural changes of modified BLA which undergo ultrasound treatment, thus, gaining more accessibility to the glycation, as had been previously demonstrated (Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Velickovic, 2013).

3.5 | Ultrasonic prior to glycosylated modification effect on the structural changes of BLA

The ϵ -NH₂ of the Lys, and guanidine groups of Arg in the protein can be glycosylated by the reducing sugars (Tagami, Akashi, Mizukoshi, Suzuki, & Hirayama, 2015). Ultrasound pretreatment could accelerate the course of the glycation between polypeptide backbone of BLA and mannose (Figure 1). According to the data in Figure 2, ultrasonic prior to glycosylated modification induced the conformational changes of BLA around the Trp residues. As mentioned before, ultrasonic pretreatment can affect the glycation of BLA. For this study, K79 and K93 were identified in the glycosylated BLA after ultrasound pretreatment (Figure 5b). Our earlier study shows that glycosylated BLA mainly occurred on Lys (Liu, Tu, Liu, et al., 2018). It is of interest to point out that glycosylated site and DSP values could

be altered by conformational structure of modified BLA via ultrasound. The conformational changes of ultrasonicated BLA need to be further confirmed by using hydrogen/deuterium exchange mass spectrometry. In our previous study, we found that performed a similar study on BLA glycation by different ultrasonic power, for two pretreatments, the glycation sites and DSP values are remarkably increased. Despite the similarity, the location and number of glycosylated sites were different. For example, six sites (K62, K94, K98, K108, K114, and K122) were glycosylated by glucose in the sample ultrasonicated at 150 W/cm² for 15 min (Liu, Tu, Shao, et al., 2017). In current study, five sites (K62, K79, K93, K114, and K122) were glycosylated by mannose in the same ultrasonic treatment condition. This is probably a result of the difference between glucose and mannose, because the conformational changes have some effect on the glycation reactivity (Ana Irene, Gabriela, & Luz, 2008; Ruud, Schols, & Harry, 2011). The different ultrasonic power and time induced different heating effects, acoustic cavitation etc., which generate the powerful shock waves and the transient higher temperature and pressure to radiate BLA solution, leading to the structural changes of BLA, thereby improving the glycation reactivity (Suslick et al., 1999). Therefore, different ultrasonic pretreatment condition can affect the structural properties of BLA, including the part of conformational changes, the increased glycation sites, and DSP values.

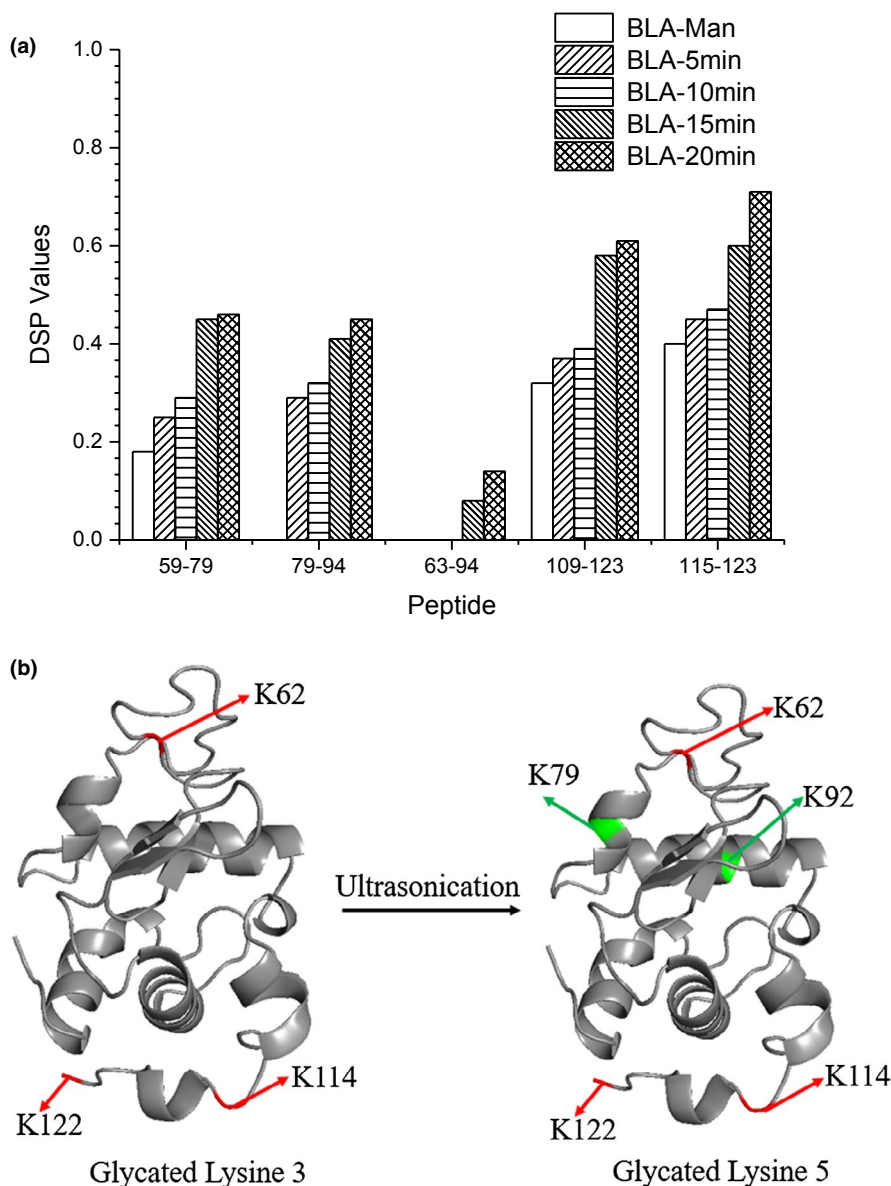


FIGURE 5 The average degree of substitution per peptide molecule (DSP) value of glycation sites of BLA-Man, BLA-5min, BLA-10min, BLA-15min, and BLA-20min (a). The schematic diagram of the glycation site changes of BLA before and after ultrasonic. Red, glycation sites on lysine in native BLA; green, additional glycation sites on lysine in ultrasonicated BLA (b)

4 | CONCLUSIONS

In this study, the molecular weight, glycation sites, and DSP values of BLA were increased by different ultrasonic time-assisted glycation modification, but much greater decrease of the intrinsic fluorescence emission also observed. These results were attributed to ultrasonic prior to glycation modification significantly changed the structure of BLA. Interestingly, comparison of previous and current results obtained using high-resolution mass spectrometry made clear that glycation site of BLA was affected by ultrasonic conditions and carbohydrate types, including ultrasonic power and time. However, some factors that influence the ultrasonic effect, such as ultrasonic condition, the type of environment and solution, further study needs to be implemented to control the consistency of ultrasonic effect in the treating processes.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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