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LC-Orbitrap MS analysis of the glycation modification effects of ovalbumin during freeze-drying with three reducing sugar additives

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ABSTRACT

In general, the reducing sugars were extensively utilized as additives to prevent denaturation and inactivation during the freeze-drying process. However, different additives have unequal protective effects on the protein conformation. To evaluate the mechanism of protection the protein structure using different additives in the freeze-drying process, the glycated sites and degree of substitution per peptide (DSP) of each site were investigated by LC-Orbitrap MS. We found that the ovalbumin that was supplemented with ribose and then lyophilized (R-oval) have the highest extent of glycation modification. K227 was the most reactive glycated site in R-Oval, with a DSP of 0.83. The ovalbumin that was supplemented with lactose and then lyophilized (L-Oval) have the lowest degree of glycation, and K227 did not undergo the glycation reaction. It was hypothesized that the order impact of different additives on protection of the protein conformation were lactose > galactose > ribose during the freeze-drying process.

1. Introduction

Freeze-drying method is widely utilized in various industries such as food, medicine, bio-engineering, and plays a significant role in improving the stability of protein-based drugs (Tang & Pikal, 2004). The process of lyophilization is completed through freezing technology and vacuum technology, and the water in the sample is removed by the sublimation of ice crystals under vacuum condition. Owing to the lack of liquid water and the low-temperature environment, the quality of the sample is preserved under the freeze-drying process. Most of the microbial reactions that deteriorate the quality of the sample are inhibited under such condition (Ratti, 2001). Moreover, freeze-drying is an excellent method to reduce structural denaturation of proteins and develop recombinant proteins (Wang, 2000). Unfortunately, limited quantities of potential biopharmaceuticals after lyophilization may undergo inactivation, denaturation and other reactions during the manufacturing process (Tu, Zhong, & Wang, 2017). Therefore, to minimize the deterioration of biological products under the freeze-drying process, a suitable additive is necessary to prevent undesirable processes, especially protein denaturation. Examples of suitable additives include galactose, ribose and lactose. These monosaccharides and disaccharides have been used to prevent protein denaturation (Allison, Dong, & Carpenter, 1996; Grohganz, Lee, Rantanen, & Yang, 2013; Heljo, Jouppila, Hatanpää, & Juppo, 2011; Sun, Hayakawa, & Izumori, 2004; Tu et al., 2017). Additives are typically added to lyophilized protein formulations, which provide a strong morphological conformation and protect the stability of the protein crystal structure, and to prevent dehydration process of conformation from causing too much change (Allison et al., 1996). Therefore, the mechanism of interaction between the additive and the protein is essential for us to study. Despite the changes in structure of proteins were frequently researched by methods, such as fluorescence spectra analysis, circular dichroism spectra analysis, and raman spectra, and it is also probable to clarify the mechanism that the reducing sugars prevent conformational change in the sample by studying the interaction between the additive and the protein.

The high viscosity of the glassy state and stable structure are important factors in preventing the protein from unfolding. The spatial

Abbreviations: SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; DSP, average degree of substitution per peptide molecule; DTT, DL-dithiothreitol; ANS, 8-anilino-1-naphthalenesulfonic acid; FTICR-MS, Fourier-transform ion cyclotron resonance mass spectrometry; HCD, high-energy C-trap dissociation; MS, mass spectrometry; CD, circular dichroism; LY-Oval, lyophilized ovalbumin; N-Oval, native ovalbumin; SAL-Oval, the ovalbumin that was supplemented with additive and then lyophilized; G-Oval, the ovalbumin that was supplemented with galactose and then lyophilized; R-Oval, the ovalbumin that was supplemented with ribose and then lyophilized; L-Oval, the ovalbumin that was supplemented with lactose and then lyophilized; Trp, tryptophan; Tyr, tyrosine; LC–HRMS, liquid chromatography and high-resolution mass spectrometry

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structure of the natural protein is maintained by the secondary bond, such as hydrogen bond, which is destroyed after denaturation, after which the protein molecule becomes loose, and the originally curled structure was elongated (Prestrelski, Tedeschi, Arakawa, & Carpenter, 1993). The Maillard reaction is a covalent binding reaction between carbonyl groups of reducing sugars (ribose, glucose, galactose, etc.) and the free amino groups of the protein. It was reported that the additive could replace water when the protein was dehydrated (Allison et al., 1996), which may respond with the amino group of a residue to catalyze the glycation reaction and induced glycation modification of the protein. Thus, the hydrophilic region of the protein is the primary region in which the additive acts. In this region, the additive bound to protein and undergo the glycation reaction, and to protect the conformation of the protein from damaging.

Lactose was selected as control sugar reactant due to the difference in aldose and disaccharide structure. Although both ribose and galactose are aldoses, moreover, ribose was considered to be more reactive than galactose because of the higher content of non-cyclic structure (Bunn & Higgins, 1981; Rondeau & Bourdon, 2011). The effect of the type of reducing sugar on the degree of glycation of the protein is considerable (Sun et al., 2004). Studies have shown that the extent of glycation reaction can be reflected by measuring the content of free amino acid groups (Vigo, Malec, Gomez, & Llosa, 1992), UV (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001), fluorescence intensity (Ajandouz et al., 2001) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Wang & Ismail, 2012). However, there is no detailed information that the effects of the Maillard reaction, including the precise glycated sites and DSP value of each site of the protein and the additive. To date, LC-HRMS has been used to analyze the precise glycated sites and DSP values of protein due to its high quality, high resolution and high sensitivity (Huang et al., 2013). In this research, we used liquid chromatography and high-resolution mass spectrometry (LC-HRMS) to explore the glycation reaction mechanism of different additives with ovalbumin and compare the effects of different additives and ovalbumin on the extent of the glycation and the precise glycated sites under the freeze-drying process. Meanwhile, the relationship between the precise conformational changes and the types of additives were discussed through the 3D structure of ovalbumin. The objective of this study is to propose a new method for the large-scale protection of protein conformation by additives in the process of producing sweet foods under freeze-drying conditions.

2. Materials and methods

2.1. Chemicals and materials

Ovalbumin (Grade V, A-5503), α-Lactose monohydrate, D-Ribose, SDS-PAGE, D-(+)-Galactose and DL-Dithiothreitol (DTT) came from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD. (Shanghai, China). Acetonitrile and Formic acid was obtained from Tianjin reagent company (Tianjin, China). All other reagents used in this experiment were of analytical grade. L-Lysine came from Sigma-Aldrich Co. (St. Louis, USA). Ultrapure water was used throughout this research, which came from an ultrapure water preparation system (Millipore, Billerica, USA).

2.2. Preparation of samples

Ovalbumin and ribose, galactose, lactose were mixed at weight ratios of 1:1 and final concentration of 1 mg/mL with Tris-HCl buffer (pH 6.8, 0.05 M), respectively. LY-Oval as a control sample was fulfilled with no added additive, which dissolved in the same solvent with a concentration of 1 mg/mL. The samples of LY-Oval, G-Oval, R-Oval and L-Oval were frozen at −80 °C for 8 h. The samples were accomplished for 48 h in a LGJ-1 freeze-drying machine (Yataikerong Scientific; Beijing, China) at −50 °C, keeping the vacuum of 50 m Torr through the entire freeze-drying cycle. Then, the samples after lyophilization were filtered with the centricon centrifugal filter unit (10 kDa) to remove salts and free sugars, and then kept at −80 °C until use. N-Oval as the blank control group without any treatment was dissolved in the Tris-HCl buffer (pH 6.8, 0.05 M) to give a final concentration of 1 mg/mL.

2.3. SDS-PAGE

SDS-PAGE was determined according to the method reported by Tu et al. (2015). SDS-PAGE was accomplished by using 5% stacking gel and 12% running gel with a vertical gel electrophoresis unit (Bio-Rad, Richmond, CA, USA). The samples (1.0 mg/mL) were boiled for 10 min and centrifuged at 8000*g* for 3 min before electrophoresis. Sample solution (10 μL) was loaded into each the gel. The current is set to 15 mA. After separation, the protein bands were stained for 1 h with Coomassie Brilliant Blue R-250 (0.2%) solution using 25% methanol and 10% glacial acetic acid solution. The protein bands were decolored with 40% methanol and 10% acetic acid.

2.4. Fluorescence spectra and absorbance analysis

The intrinsic emission fluorescence spectra were measured in triplicate at room temperature using a Fluor photometer (F7000, Hitachi, Tokyo, Japan) according to Zhang et al. (2014). The samples were dissolved with Tris-HCl buffer (pH 6.8, 0.05 M) to give a final concentration of 1.0 mg/mL and added to the matching quartz cuvette. The excitation wavelength is 290 nm, the emission wavelength were recorded from 300 to 460 nm at a constant slit of 2.5 nm.

The absorbance of the sample solution was determined according to the method reported by Ajandouz, Desseaux, Tazi, and Puigserver (2008). To minimize the contribution of tyrosine residues to the emission spectra, the sample solution (1.0 mg/mL) was added to the quartz cuvette and the absorbance values were measured at 295 nm and 420 nm with a Double beam UV Spectrophotometer (U-2910, Hitachi, Tokyo, Japan). Each group was measured in triplicate.

2.5. Surface hydrophobicity (H_0) analysis

The H_0 value of the protein was determined according to Kato's method (Kato & Nakai, 1980). The hydrophobicity of the protein is directly reflected by the fluorescence intensity. Each sample (1.0 mg/mL in 0.05 M Tris-HCl buffer at pH 6.8) was serially diluted with the same buffer to obtain sample concentrations ranging from 1.0 to 0.125 mg/mL. Then, 20 μL of ANS was added to 2 mL of the sample. The H_0 value of the sample was analyzed by fluorescence spectrophotometer (F-4500, BioTek, Vermont, USA) at wavelengths of 390 nm (excitation) and 470 nm (emission), with a constant excitation and emission slit of 5 nm. The hydrophobicity of the protein can be expressed by the relative fluorescence intensity versus the slope of the ovalbumin treated under different conditions (calculated by linear regression analysis). The initial slope of relative fluorescence intensity versus protein concentration was defined as the H_0 value of the protein.

2.6. The secondary structure

A Jasco J815 spectropolarimeter (JASCO International Co., Ltd, Tokyo, Japan) was used to measure the secondary structure of the ovalbumin treated by different additives under stable nitrogen flushing conditions. The path length was 1.0 mm, with a scan speed of 50 nm/min, and the spectrum was measured in the wavelength scope of 190–250 nm. The CD spectra of the samples with 0.1 mg/mL were detected in a Tris-HCl buffer (pH 6.8, 0.05 M) at room temperature. The results were expressed as the mean residue ellipticity in deg cm^2 $dmol^{-1}$. The results of the secondary structures of ovalbumin