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Identification of glycated sites in ovalbumin under freeze-drying processing by liquid chromatography high-resolution mass spectrometry

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ABSTRACT

The glycation reaction between ovalbumin and D-glucose during freeze-drying was investigated and the mechanism of protection of the protein structure was studied, the precise glycated sites and degree of substitution per peptide (DSP) of each site were determined using liquid chromatography high-resolution mass spectrometry. It was found that lysine residues are the main glycated sites under freeze-drying. K62 and K264 were the most reactive glycated sites in lyophilized ovalbumin, with a DSP close to 80%. The glycated sites were located at the outer surface of the global protein. The unglycated sites were located at the outer surface of the hydrophobic pocket and in the six main strands of the β-sheet. Therefore, the glycation reaction of the protein was occurred in the solvent accessible area. It was hypothesized that few changes occurred in the conformation to disturb the glycated sites under freeze-drying. In particular, the main strands of the *β*-sheet of ovalbumin were more stable. Freeze-drying was a mild process and protected the conformation without extensive denaturation.

1. Introduction

Vacuum freeze-drying, also known as lyophilization, is commonly used to process protein foods and pharmaceuticals to ensure the maintenance of a stable form for distribution and storage as an aqueous formulation (Peters et al., 2016). This process of drying and dewatering comprises a combination of vacuum technology and freezing technology, and removes water via the sublimation of ice crystals in the sample. Some studies have proved that freeze-drying could prevent deterioration and microbiological reactions and give food an excellent quality (Ma, Chen, Zhu, & Wang, 2013; Pei et al., 2013; Stephan, Da Silva, & Bisutti, 2016). Freeze-drying was proved to be a good choice for preventing the structural change in, as well as maintaining, the angiotensin-converting enzyme inhibitor activity of a protein hydrolyzate from fresh-water fish compared with oven-drying (Elavarasan, Shamasundar, Badii, & Howell, 2016).

Unfortunately, often only limited quantities of potential biopharmaceuticals are available after the freeze-drying process. Furthermore, inactivation (Ma et al., 2013), denaturation (Peters et al., 2016) and additional reactions (Rosas et al., 2014) may take place during the manufacturing process, which lasts several days. Therefore, in order to minimize protein denaturation during freeze-drying processing, the careful addition of an effective stabilizer to the formulation, such as sucrose and other disaccharides, has been carried out to prevent denaturation of the protein (Grohganz, Lee, Rantanen, & Yang, 2013) or enzyme (Heljo, Jouppila, Hatanpää, & Juppo, 2010; Peters, Leskinen, Molnár, & Ketolainen, 2015). The additive was added to freeze-dried protein formulations to provide a stable matrix structure and protect the proteins from stress involved in the process, and to prevent damage to the conformation during dehydration processing (Grohganz et al., 2013). Therefore, the interaction between the protein and the additive is important for resistance to structural changes. Although the degradation, activity, and changes in conformation of proteins were commonly analyzed by methods, such as Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), and enzyme activity analysis, it is also possible to elucidate the mechanism

Abbreviations: AGEs, advanced glycation end products; CD, circular dichroism; CID, collision induced dissociation; DSP, average degree of substitution per peptide molecule; DTT, DL-dithiothreitol; ETD, electron transfer dissociation; FTIR, Fourier Transform Infrared Spectroscopy; FTICR-MS, Fourier-transform ion cyclotron resonance mass spectrometry; HCD, high-energy C-trap dissociation; L-Oval, lyophilized ovalbumin; *N*-Oval, native ovalbumin; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; MS/MS, tandem mass spectrometry; MS, mass spectrometry.

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by which the additive prevents structural change in the protein and maintains its activity via analyzing the interaction and reactions between the protein and the additive.

The mechanism that protects proteins from conformational changes has attributed to the high viscosity of the glassy state that prevents the proteins from unfolding. In this case, the additive could replace water in the formation of hydrogen bonds when the protein is dehydrated (Allison, Dong, & Carpenter, 1996). Therefore, the solvent-accessible surface of the protein is the main area that interacts with the additive. On the other hand, the stability of the protein to lyophilization depends on a number of factors including temperature, stress and time. Therefore, changes in the protein structure and reactions between several formulation components may occur during the dehydration processing. Some studies have indicated that differences in the protein secondary structure, at different cooling rates, could be detected in sucrose-lysozyme samples at a sucrose:lysozyme ratio of 1:1 (Peters et al., 2015). The Maillard reaction is a non-enzymatic reaction, between reducing sugars and amino acids, which commonly occurs in the natural environment. Therefore, it was hypothesized that when a reducing sugar molecule replaces water during dehydration processing, the reducing sugar molecule may react with the amino group of a residue to catalyze the Maillard reaction and caused glycation modification of the protein. However, there is no information about the effects of freeze-drying on the glycation reaction and the precise glycated sites of proteins and reducing sugars.

Glycation is the first step of the Maillard reaction, of which the extent is the main evaluable index for assessing the degree of closeness between the amino groups of the residue and the reducing sugar. Although the extent of the Maillard reaction can be determined by measuring the absorbance of the products (Guan et al., 2010; Thomsen et al., 2012), the free amino acid groups (Wong, Day, & Augustin, 2011), the fluorescence intensity (Yeboah, Alli, & Yaylayan, 1999) and the size of the molecule by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Liu, Zhao, Zhao, Ren, & Yang, 2012), these methods do not indicate the detailed glycated sites of the protein or analyze the interaction between the protein and the reducing sugar (Huang, Tu, Wang, Zhang, Shi, et al., 2013). In recent years, liquid chromatography high-resolution mass spectrometry has provided the greatest combination of simultaneous accuracy in the measurement of mass, resolution, and sensitivity. Moreover, it has also enabled the unambiguous characterization of peptides with various modifications. Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) allows a detailed analysis of the nature and extent of protein glycation in terms of the mass shift and the relative abundance of the glycated peptides (Huang, Tu, Wang, Zhang, Shi, et al., 2013; Wang et al., 2013; Zhang et al., 2014). However, the precise glycated sites must be determined by high-energy C-trap dissociation (HCD), and electron transfer dissociation (ETD) (Singh, Zampronio, Creese, & Cooper, 2012), which are important for evaluating the interaction between the amino acid and sugar.

The purpose of this study was to identify the glycated sites and their extent in ovalbumin that was dried by freeze-drying, and also investigate the mechanism of the protection of the protein conformation by the additive.

2. Materials and methods

2.1. Chemicals and materials

Ovalbumin (Grade V, A-5503), D-glucose, and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). DL-Dithiothreitol (DTT) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). All other reagents used were of analytical reagent grade. Ultrapure water from a water purification system (Millipore; Billerica, MA) was used throughout this study.

2.2. Freeze-drying

Freeze-drying was performed with an LGJ freeze-dryer (Yataikerong Scientific; Beijing, China). Ovalbumin (0.1 g) and an equal mass of D-glucose were dissolved in 2 ml distilled water at pH 7.0, and then the mixture was frozen at −80 °C for 10 h. A drying temperature of −50 °C was maintained for 48 h, maintaining a vacuum of 50 mTorr throughout the freeze-drying cycle. After the completion of drying, the vials were capped under vacuum.

The lyophilized sample (0.2 g of a mixture of lyophilized ovalbumin [L-Oval] and glucose) was dissolved in 200 μl distilled water and filtered by a Centricon centrifugal filter unit (3000 Da cutoff), (Millipore; Bedford, MA) to remove salts and free glucose. The concentration of the protein was adjusted to 1 mg/ml.

2.3. Sample digestion

Native oval (*N*-Oval) and L-Oval were digested according to the method of Wang et al. (2013), with little modification. The sample (1 mg/ml) was resuspended in 6 M urea, and 5 μl of the reducing reagent (100 mM DTT) was added per 100 μl of the solution. The sample was incubated at 95 °C for 5 min and then cooled in an ice bath.

A samples solution (2 μl, 1 mg/ml) was added to a 500 μl centrifuge tube containing 78 μl of 50 mM ammonium bicarbonate solution and 20 μl of 100 mM DTT. Then, 100 μl of 2 mg/ml pepsin solution was used to hydrolyze the protein sample in a buffer solution (pH 2.2). After digestion by pepsin for 5 min, 40 μl of the sample solution was injected into a C18 column (2 cm × 100 μm 5 μm). The reaction was then quenched by the addition of 2 μl of 50% trifluoroacetic acid.

2.4. Analysis by HPLC HCD/ETD MS/MS

The column effluent was injected into an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific; Waltham, MA, USA) for analysis by tandem mass spectrometry (MS/MS) to identify protein glycated sites. Positive ions were used to detect isolates. Twenty fragment maps (MS/MS scans) showing the mass-to-charge ratio of the polypeptide and polypeptide fragments were collected at each full scan. The ions detected in the precursor ion scanning were further subjected to HCD and ETD fragmentation to detect the fragment ions. Dynamic exclusion was enabled with exclusion duration of 90 s.

To further compare the glycation extent of each peptide, the average degree of substitution per peptide molecule (DSP) of ovalbumin was calculated according to the formula:

$$DSP = \frac{\sum_{i=0}^{n} i \times I(peptides + i \times glucose)}{\sum_{i=0}^{n} I(peptides + i \times glucose)}$$

where I is the sum of the intensities of the peptides, and i is the number of glucose units attached to the peptide in each glycated form.

2.5. Statistical analysis

The data are expressed as the mean ± standard deviation. The analysis was performed using Origin-Pro 8.0 (OriginLab Corp.; Northampton, MA). The values of DSP ± standard deviation were determined from three separate experiments. Statistical data were determined based on a two-tailed *t*-test using standard deviations.