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Analytical Methods

Identification and quantification of the phosphorylated ovalbumin by high resolution mass spectrometry under dry-heating treatment

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ABSTRACT

The specific phosphorylation sites and degree of phosphorylation (DP) at each site are directly related to protein's structure and functional properties. Thus, characterizing the introduced phosphate groups is of great importance. This study was to monitor the phosphorylation sites, DP and the number of phosphorylation sites in P-Oval achieved by dry heating in the presence of pyrophosphate for 1, 2 and 5 days by using Fourier transform ion cyclotron mass spectrometry (FTICR MS). Two phosphorylation sites were found in natural ovalbumin, but the number of phosphorylation sites increased to 8, 8 and 10 after dry-heating phosphorylation for 1, 2 and 5 days, respectively. In addition, dual-phosphorylated peptides were detected for samples without extensive heating. The phosphorylation sites were found to be mainly on Ser residues, which could be the preferred phosphorylation site for dry heating in the presence of pyrophosphate.

1. Introduction

Goal-directed modification of food proteins has been a popular research area to eliminate the undesirable properties, or to enhance or protect the desired functional and nutritional properties. Glycation, succinylation, acylation, enzymatic modification and phosphorylation are the widely practical modification methods of proteins (Li et al., 2009; Ross & Bhatnagar, 1989). Among them, phosphorylation has been proven to be an efficient method to modify the functional properties of food proteins, such as calcium phosphate-solubilizing ability, gelling properties, water absorption capacity, emulsifying properties, foaming properties, and thermal stability (Li, Enomoto, Hayashi, Zhao, & Aoki, 2010). Phosphorylation of food proteins can also present much effect on the structure and physiological function of proteins in biological systems (Hata, Higashiyama, & Otani, 1998). Can-peng Li (Li et al., 2009) has carried out some studies on the phosphorylation of food proteins by dry heating in the presence of pyrophosphate and found that phosphorylation could improve the thermal stability (Li et al., 2005), emulsifying property (Lv & Chi, 2012), foaming property (Hayashi et al., 2009), calcium phosphate-solubilizing ability (Li, Salvador, Ibrahim, Sugimoto, & Aoki, 2003) and digestibility (Li, Ibrahim, Sugimoto, Hatta, & Aoki, 2004) of egg white protein and ovalbumin. In addition, phosphorylation can also improve the exchange reaction between the sulfhydryl and disulfide groups and surface hydrophobicity of ovalbumin, and reduce the anti-ovalbumin antibody response of egg white protein (Li et al., 2005) and ovalbumin (Enomoto et al., 2009). Although many researchers have studied the chemical and physical properties of phosphorylated proteins, few studies have explored specific protein structural alterations after phosphorylation.

Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) is characterized by excellent resolution, high sensitivity and simultaneous mass measurement accuracy (Bruce, Anderson, Wen, Harkewicz, & Smith, 1999). It is unparalleled for the specific structure analyzing of the protein and modified protein through the mass increase. Recently, the combination of Liquid chromatography coupled to FTICR MS and Linear Ion Trap Quadrupole Mass Spectrometer (LTQ-MS) has proven to be an efficient tool for identifying the conformational changes, exactly glycation sites and glycation degree at each site of modified proteins, especially for glycated proteins. By using this method, Huang et al.

Abbreviations: FTICR MS, Fourier transform ion cyclotron mass spectrometry; MALDI, matrix-assisted laser desorption ionization; IMAC, immobilized metal affinity chromatography; CID, collision induced dissociation; DTT, DL-Dithiothreitol; DSP, average degree of substitution per peptide molecule; P-Oval, phosphorylated ovalbumin.

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(Huang et al., 2015) detected 9 glycated peptides and 12 glycation sites from ovalbumin glycated with glucose. Another paper reported that 10, 11 and 11 glycation sites were identified from glycated bovine serum albumin pretreated with DHPM at 50, 100 and 200 MPa, respectively (Huang et al., 2013a). However, applying FTICR MS coupled to LTQ MS in the characterization of specific phosphorylation sites and extent of phosphorylation at each site in phosphorylated protein remains lacking.

Many food proteins are naturally phosphorylated, including casein in milk and ovalbumin in egg white. Ovalbumin, an important ingredient in egg white protein, is a globular, acidic protein consisting of 385 amino acid residues with a molecular weight of 45 kDa (Huntington & Stein, 2001). Its structure and conformation predominantly determine the functional properties (foaming and gelling) in food processing. In natural ovalbumin, phosphorylation has been found on two of the serine residues, namely Ser 68 and Ser 344 (Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981), however little information about the exactly phosphorylation sites and degree has been reported. Therefore, this work aims to determine all the possible phosphorylation sites and phosphorylation degree at each site of ovalbumin induced under dry heating by using FTICR MS and LTQ MS. Phosphorylated ovalbumin (P-Oval) was prepared by dry heating in the presence of pyrophosphate for 1, 2 and 5 days, respectively. Following, P-Oval was digested with pepsin, and subjected to mass spectrometry analysis.

2. Materials and methods

2.1. Chemicals and material

Ovalbumin (Grade V, A-5503), sodium pyrophosphate and porcine stomach pepsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents were of analytical grade. Ultrapure water from a water purification system (Millipore, Bedford, MA, USA) was used throughout this study.

2.2. Preparation of P-Oval

Ovalbumin was phosphorylated according to the method of Li et al. (Li et al., 2003). Ovalbumin (10 g) was dissolved in 100 mL pH 4.0, 0.1 M sodium pyrophosphate buffer, and the pH was adjusted using 1 N HCl. The ovalbumin solution was then lyophilized and incubated at 85 °C for 1, 2, and 5 days, respectively. The P-Oval was dissolved, and the free sodium pyrophosphate was removed by zip-tip.

2.3. Reducing reaction and digestion in solution

A DL-Dithiothreitol (DTT) reducing reaction was performed to reduce the disulfide bonds in ovalbumin before digestion and analysis by mass spectrometry. A 10 μL sample (1 mg/mL) was added to a 500 μL centrifuge tube containing 100 μL of 6 M urea and 5 μL of the reducing reagent. After 1 h of incubation at room temperature, 20 μL of iodoacetamide was added, and the mixture was incubated for another 1 h at room temperature. Then, 20 μL of the reducing reagent was added to consume any unreacted iodoacetamide, and the mixture was stored in a 4 °C refrigerator before hydrolysis. Finally, 775 μL of ultrapure water was added to dilute the urea concentration to preserve pepsin activity.

The urea concentration was reduced by diluting the reaction mixture, and the samples were hydrolyzed using 1% pepsin (w/w) in pH 2.5 buffer solutions according to Wang et al. (Wang et al., 2013). After 5 min of digestion, 40 μL sample was injected into a 1.0 mm i.d. × 50 mm peptide column (Micro-Tech Scientific Inc.).

2.4. LC FTICR MS analysis and peptide identification

A Shimadzu HPLC with two LC-10AD pumps was used to generate a fast gradient at a flow rate of 20 μL/min optimized for the best sequence coverage. The solvent A and solvent B used to separate the peptides were 5% acetonitrile in H_2O, 0.1% formic acid (FA) in 95% acetonitrile, respectively. After desalting with 2% solvent B for 5 min, the peptides were eluted with a gradient elution program of follows: 5–15% B, 0–9 min; 15–30% B, 9–18 min; 30–50% B, 18–19 min; 50–95% B, 19–20 min, then the eluent was returned to 5% B for 5 min of equilibrium. The effluent was infused into FTICR MS. Peptides were identified by a combination of accurate masses. The mass error threshold was set at 2 ppm, and the actual error for each peptide was reported in the "Δppm" column. Thirty-two fractions were collected for phosphorylated peptide identification. Each fraction was subjected to LTQ Mass Spectrometer (Thermo Scientific, Waltham, MA) for MS^n analysis.

To further compare the phosphorus content of each peptide, the average degree of substitution per peptide (DSP) was calculated according to the following formula (Kislinger et al., 2003; Thomsen et al., 2012):

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

where I is the sum of the intensities of every phosphorylated peptide, and i is the number of phosphorus units attached to the peptide in each phosphorylated form.

2.5. Data analysis

Data evaluation was determined using Origin Pro 8.0 (OriginLab Corp., Northampton, MA). DSP ± standard deviations were determined from three separate experiments. Statistical data were determined based on a two-tailed t-test using standard deviations.

3. Results and discussion

3.1. Peptide mapping

Ideally, peptides of 8–20 amino acids are desired for successful sequence determination, and therefore, it is essential to select a suitable protease that can generate small peptides, particularly containing only one phosphorylation site. Examining the phosphorylation sites with multiple different proteases or with a double digestion can often improve overall sequence coverage. However, it also involves more invalid fragment which would bring more additional and complex work to do. Pepsin is the most efficient at cleaving peptide bonds between hydrophobic residues with most of the hydrolysate in the range of 1000–3000 Da, which is ideal for the subsequent MS analysis. Thus, pepsin was used to cut the protein at defined sites to generate small peptides subjected to tandem mass spectrometry (MS/MS) to measure the peptide mass and fragment ion masses.

Phosphorylated peptide mapping is performed by matching these data to theoretical spectra derived from a sequence database. A number of issues complicate phosphorylated peptide identification due to the characteristics of the phosphate moiety. Because the phosphate moiety is highly labile, it is often released during fragmentation, resulting in insufficient sequence information on the peptide backbone with fewer high-confidence peptide matches. Many phosphorylation sites appear to have low occupancy, with only a small fraction of the peptide molecules phosphorylated (Olsen et al., 2010; Wu et al., 2011). In this case, enrichment of the phosphorylated peptides prior to mass spectrometric analysis is often required (Choi, Lee, Jun, & Park, 2011).