



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.

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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, Hata, & 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 glycosylated 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 glycosylated peptides and 12 glycosylation sites from ovalbumin glycosylated with glucose. Another paper reported that 10, 11 and 11 glycosylation sites were identified from glycosylated 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 H₂O, 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 MSⁿ 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):

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

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).

When multiple phosphorylation sites are presented in a single peptide, it is difficult to identify the specific site of each modification. In addition, the sequence-specific protease commonly used in proteomic analysis (trypsin or endopeptidase lysC) can also hamper phosphorylation site identification.

Stefanowicz et al. (Stefanowicz, Kijewska, Kluczyk, & Szewczuk, 2010) has used pepsin to digest the glycation protein and identified the glycation site by LC FTICR MS with considerably digestion time (36 h) and relatively high temperature (22 °C) that may be cause further change of the protein modification. In our previous work, the pepsin and trypsin were also selected to digest the glycation ovalbumin and analyzed the glycation site by LC FTICR MS to identify and quantify the glycation content (Huang et al., 2013b). However, in order to minimise the further change of the protein structure in the digestion procedure, we improved the digestion procedure with pepsin digestion in an ice bath for as short as 5 min (Wang et al., 2013).

When basic residues, such as lysine and arginine, are not evenly distributed in the protein, the peptides generated by proteolysis will not be ideal for subsequent mass spectrometric analysis. In this work, we used pepsin, a non-specific protease, to perform protein digestion. The peptic peptides were subjected to LC FTICR MS to determine the phosphorylation sites and their content (see Table 1 in Data in Brief).

3.2. Identification and phosphorus content of each phosphorylated peptide

The peptic peptides were detected by LC FTICR MS after 5 min of digestion of natural ovalbumin. Therefore, the phosphorylated peptides can be directly determined from the mass difference induced by phosphorylation. Theoretically, if a peptide is phosphorylated by one phosphate, the corresponding m/z peaks with 1, 2 and 3 charges will display m/z increases of 79.9663, 39.9826 and 26.6549, respectively (Li et al., 2009; Stensballe, Andersen, & Jensen, 2001). For the dual- and tri-phosphorylated peptides, the mass increases will be equal to 159.9327 and 239.8990 Da, respectively. FTICR MS routinely offers a mass accuracy of <2 ppm, which allows us to determine the phosphorylated peptides directly based on accurate mass. Under the conditions, dry-heating was used for phosphorylation, the only possible cause of mass shift of 79.9663 Da or its multiples will be the addition of one or more phosphates. In fact, when tandem MS (MS/MS) is applied to these peptides, the mass addition of 79.9663 Da is confirmed to be phosphorylation (see Figs. 1–4 in Data in Brief).

For peptide 366–385 (m/z 741.7251³⁺), a peak with m/z of 768.3817³⁺ was emerged after 1 day of incubation (see Fig. 1 in Data in Brief). The m/z difference of these two peaks was 26.6564, equivalent to a mass shift of 79.9695 Da, indicating that this peptide was

modified by one molecular equivalent of HPO₃. Extended incubation of this peptide significantly increased the relative intensity of the phosphorylated peptide peak. For peptides 41–59 and 217–241 (m/z of 732.4160³⁺ and 921.46³⁺), peaks with mass shifts of 79.9677 and 79.9668 Da (m/z of 759.0725³⁺ and 948.1231³⁺), respectively, appeared after 1 day of incubation (see Figs. 2 and 3 in Data in Brief). The intensity of the phosphorylated form (759.0725³⁺ and 948.1231³⁺) was increased after 2 days of incubation. In addition, a third peak with m/z of 785.7261³⁺ and 974.7782³⁺ emerged. Compared to the mono-phosphorylated peak, the newly emerged peaks underwent a further m/z change of 26.6564 (equivalent to a mass shift of 79.9695 Da), indicating that these two peptides were modified by an additional phosphate molecule. Thus, two molecules of phosphate were added to these two peptides to form dual-phosphorylated peptides after 2 days of dry heating. No tri-phosphorylated form of this peptide was found when both peptides were subjected to further incubation (5 days); however, the intensity of the mono-phosphorylated and dual-phosphorylated form was further increased. Similarly, another example for a peptide 142–157 with m/z of 929.9910²⁺ exhibited a m/z increase of 39.9826 after 1 day of incubation, indicating that mono-phosphorylation of this peptide occurred. The dual-phosphorylated form of this peptide was present after 2 days of incubation (see Fig. 4 in Data in Brief).

The mass spectrometry results demonstrated that the peptides exhibited varying degrees of phosphorylation. Some of the peptides showed faster phosphorylation rates within 5 days of incubation in the presence of pyrophosphate. For example, peptide 41–59 was dual-phosphorylated with 2 days of incubation. In contrast, other peptides, such as peptide 366–385, remained mono-phosphorylated after 5 days of incubation. As shown in Table 1, all the phosphorylated peptides and their corresponding masses in a time course. Four other peptic peptides, including 60–70, 148–159, 217–222 and 337–350, were found to be mono-phosphorylated rapidly within 1 day of incubation. However, several peptic peptides, including 100–105 and 101–107, were mono-phosphorylated only after 5 days of incubation.

To further understand the relative phosphorylation reactivity of ovalbumin during dry-heating in the presence of pyrophosphate, the DSP (the average degree of substitution per peptide as compared with the phosphorus content of each peptide) of each phosphorylation peptide was calculated (see Fig. 5 in Data in Brief) and shown in Fig. 1. Ovalbumin is a mixture of three types of proteins. The dual-phosphorylated form (pI 4.75) represents 86.9%, compared to 12.5% for the mono-phosphorylated form (pI 4.89) and 0.96% for the unphosphorylated form (pI 4.94) (Kitabatake, Ishida, & Doi, 1988). In this work, the phosphorus contents of peptide 60–70 calculated from the LC FTICR MS spectra of P-Oval by dry heating incubation for 1, 2 and 5 days were 85.21%, 87.64% and 88.42% compared

Table 1
Ovalbumin phosphorylated peptides.

No.	m/z	St.	End	Δ ppm	Sequence	1d	2d	5d
1	732.4160 ³⁺	41	59	1.79	(M)VY ⁴² LGAKDS ⁴⁸ T ⁴⁹ RT ⁵¹ QINKVRF(D)	759.07 ³⁺	759.07 ³⁺ , 785.72 ³⁺	759.07 ³⁺ , 785.72 ³⁺
2	589.2911 ²⁺	60	70	1.17	(F)DKLPFGDS ⁶⁸ IE(A)	628.35 ²⁺	628.35 ²⁺	628.35 ²⁺
3	646.3889	100	105	1.31	(F)S ¹⁰⁰ LAS ¹⁰³ RL(Y)	–	–	726.38
4	793.4565	101	107	–0.21	(S)LAS ¹⁰³ RLYA(E)	–	–	873.4580
5	929.9894 ²⁺	142	157	0.20	(A)RELINS ¹⁴⁷ WVES ¹⁵¹ QT ¹⁵³ NGII(R)	969.97 ²⁺	969.97 ²⁺ , 1009.95 ²⁺	969.97 ²⁺ , 1009.95 ²⁺
6	708.8586 ²⁺	148	159	1.07	(S)WVES ¹⁵¹ QT ¹⁵³ NGIIR(V)	748.85 ²⁺	748.85 ²⁺	748.85 ²⁺
7	710.3662	217	222	1.025	(L)FRVAS ²²¹ M(A)	790.45	790.45	790.45
8	921.4662 ³⁺	217	241	1.39	(L)FRVAS ²²¹ MAS ²²⁴ EKMKILELP F ²³⁴ AS ²³⁶ GT ²³⁸ MS ²⁴⁰ M(L)	948.12 ³⁺ , 974.78 ³⁺	948.12 ³⁺ , 974.78 ³⁺	948.12 ³⁺ , 974.78 ³⁺
9	658.8255 ²⁺	337	350	1.85	(E)AGREVVG ³⁴⁴ AEAGVD(A)	698.8078 ²⁺	698.8078 ²⁺	698.8078 ²⁺
10	741.7251 ³⁺	366	385	1.62	(L)FCIKHIAT ³⁷³ NAVLFFGRCVS ³⁸⁴ P(–)	768.38 ³⁺	768.38 ³⁺	768.38 ³⁺

The phosphorylated amino acid residues are colored in gray.

The possible phosphorylation sites are indicated by sequence number.

* Naturally phosphorylated amino acids Ser68 and Ser344.

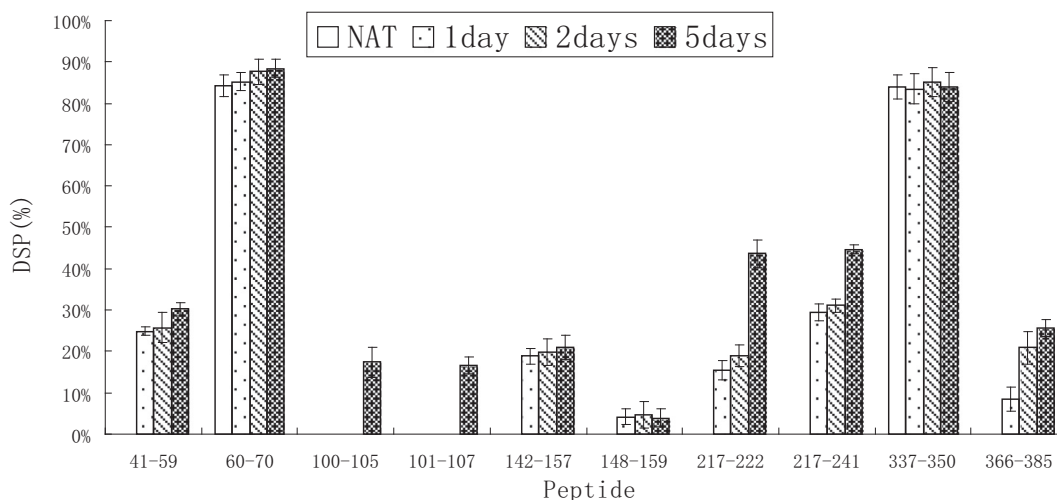


Fig. 1. DSP of phosphorylated peptides of natural and P-Oval after incubation for 1, 2 and 5 days.

with natural ovalbumin of 84.21%. The corresponding values for peptide 337–350 were 83.50% 85.20% and 83.92% compared with natural ovalbumin of 83.89% (Fig. 1). Therefore, the calculated average phosphorylation extent of natural ovalbumin was approximately 84.05%, which is consistent with the ratio reported based on Kitabatake's measurements (Kitabatake et al., 1988). The measured phosphorus contents of these two peptides were highly similar to the natural phosphorylation extent in ovalbumin, implying that the natural phosphorylation sites Ser 68 and Ser 344 were not further phosphorylated by dry heating in the presence of pyrophosphate. Li et al. (Li et al., 2009) concluded that a typical phosphorylation protein was not readily phosphorylated by dry

heating in the presence of pyrophosphate because there was a stronger repulsive interaction between the phosphate group of the peptide (negative charge) and the $\text{H}_3\text{P}_2\text{O}_7^-$ ion (negative charge). This repulsion could explain why the DSPs of peptides 60–70 and 337–350 remained unchanged in P-Oval. However, the other 8 phosphorylated peptides exhibited varying DSP values. The DSP values in a time course allow us to determine not only the extent but also the rate of the phosphorylation. The DSP values in Fig. 1 illustrate that peptides 41–59, 142–157, 217–222, 217–241, and 366–385 contain more reactive amino acid side chains for phosphorylation.

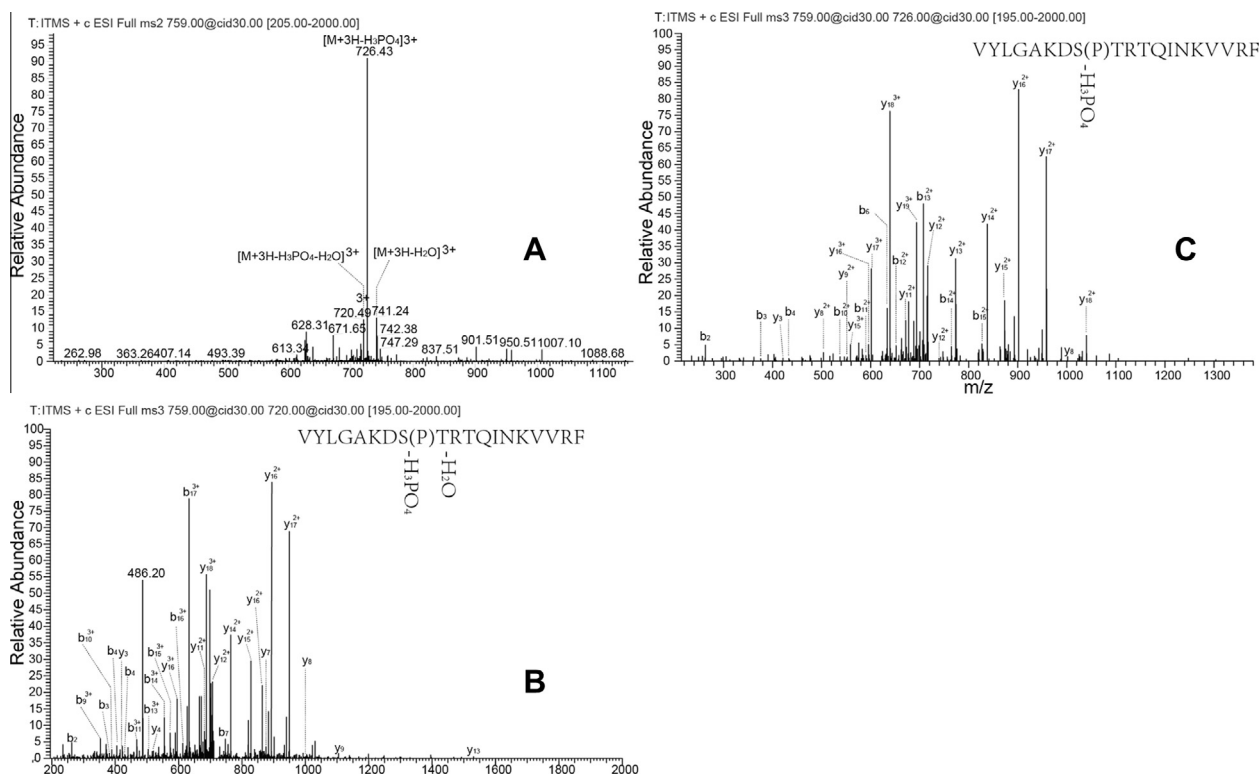


Fig. 2. The CID MS/MS of the mono-phosphorylated peptide 41–59 ($^{41}\text{VYLGAQDSTR TQINKVVR}^{59}$) at m/z 759.0708 $^{3+}$. The sequence of each peptide is shown on top of the spectrum. Mainly, b and y ions are shown in mass spectra. (A) CID of m/z 759.0708 $^{3+}$ giving an abundant ion at m/z 720.49 $^{3+}$ and m/z 726.43 $^{3+}$ due to neutral loss of 116 and 98 mass units; (B) CID MS 3 spectrum of the peptide 41–59 by selecting the ion peak with m/z of 720.49 $^{3+}$ for secondary MS/MS (C) CID MS 3 spectrum of the peptide 41–59 by selecting the ion peak with m/z of 726.43 $^{3+}$ for secondary MS/MS.

A closer examination of ovalbumin structure revealed that the phosphorylation under dry-heating condition was structure dependent. Ser 103 belongs to β -strand 2 (residue 98–109), which is deeply embedded in the core of the protein. This region is also involved in a disulfide bond between Cys 74 and Cys 121. These two factors hinder the reaction of Ser 103 with phosphate. In contrast, Ser48 (peptide 41–59) and Ser 221 (peptide 217–241) are located on the surface of the protein, which allows them to react more rapidly with phosphate. The structure-dependent characteristics of phosphorylation rate under dry-heating conditions are highly similar to our previous finding for Maillard-type glycation under dry-heating conditions (Huang et al., 2013a; Zhang et al., 2014). This result is understandable, as both are non-enzymatic heat-driven reactions occurring under highly similar conditions.

3.3. Identification of the phosphorylation sites

As shown in Table 1, the peptides phosphorylated by dry heating in the presence of pyrophosphate after incubation for 1, 2 and 5 days, respectively. For peptides with a single possible phosphorylation site, the phosphorylation sites can be directly noted. For example, peptide 217–222 was found to be phosphorylated at the Ser 221 residue, which is the only amino acid residue that can be phosphorylated. For peptides containing more than two possible phosphorylation sites, an additional step, tandem mass spectrometry is required for accurate site determination. For example, peptide 142–157 contains three possible phosphorylation sites (Ser 147, Ser 151, and Thr 153), thus the exact phosphorylation sites cannot be determined solely from the accurate mass.

3.4. Identification of the phosphorylation sites by NL triggered MSⁿ

To locate the exact phosphorylation sites, especially for phosphorylated peptides with multiple possible phosphorylation sites, collision induced dissociation MS/MS (CID MS/MS) and CID MS/MS/MS were performed. This method can produce extensive fragment ions for large peptides and small peptides (Wu, Hühmer, Hao, & Karger, 2007). Although the electron transfer dissociation MS/MS (ETD MS/MS) is always used for pinpointing phosphorylation sites, facile cleavage of the PO₄-PO₃ bonds in the peptide was shown. Furthermore, in some cases, ETD MS/MS analysis of the abundant precursor ions mainly resulted in the formation of the charge reduced product and associated neutral losses, leading to a very low relative abundance of the most abundant sequence ions due to the use of supplemental activation (Smith et al., 2010). In this work, CID MS/MS analysis of the phosphorylated peptides was herein performed.

The CID MS/MS of the phosphorylated peptide VYLGAQDSTRT-QINKVVR with m/z 759.0708³⁺ was found to accompany a number of neutral losses, including 3H₂O (−54 u), H₃PO₄ (−98 u), and HPO₄ + H₂O (−116 u), yielding intense ions at m/z 741.24³⁺, 726.43³⁺, and 720.49³⁺ in the spectrum (Fig. 2). When the ion peak of m/z 741.24³⁺ was subjected to further fragmentation (MS³), only additional neutral loss was observed without useful sequence information. However, the other two ion peaks yielded fruitful sequence information on the peptide when subjected to MS³ (Fig. 2). The CID MS³ spectrum clearly confirmed the sequence, and the phosphorylation site was determined to be Ser48.

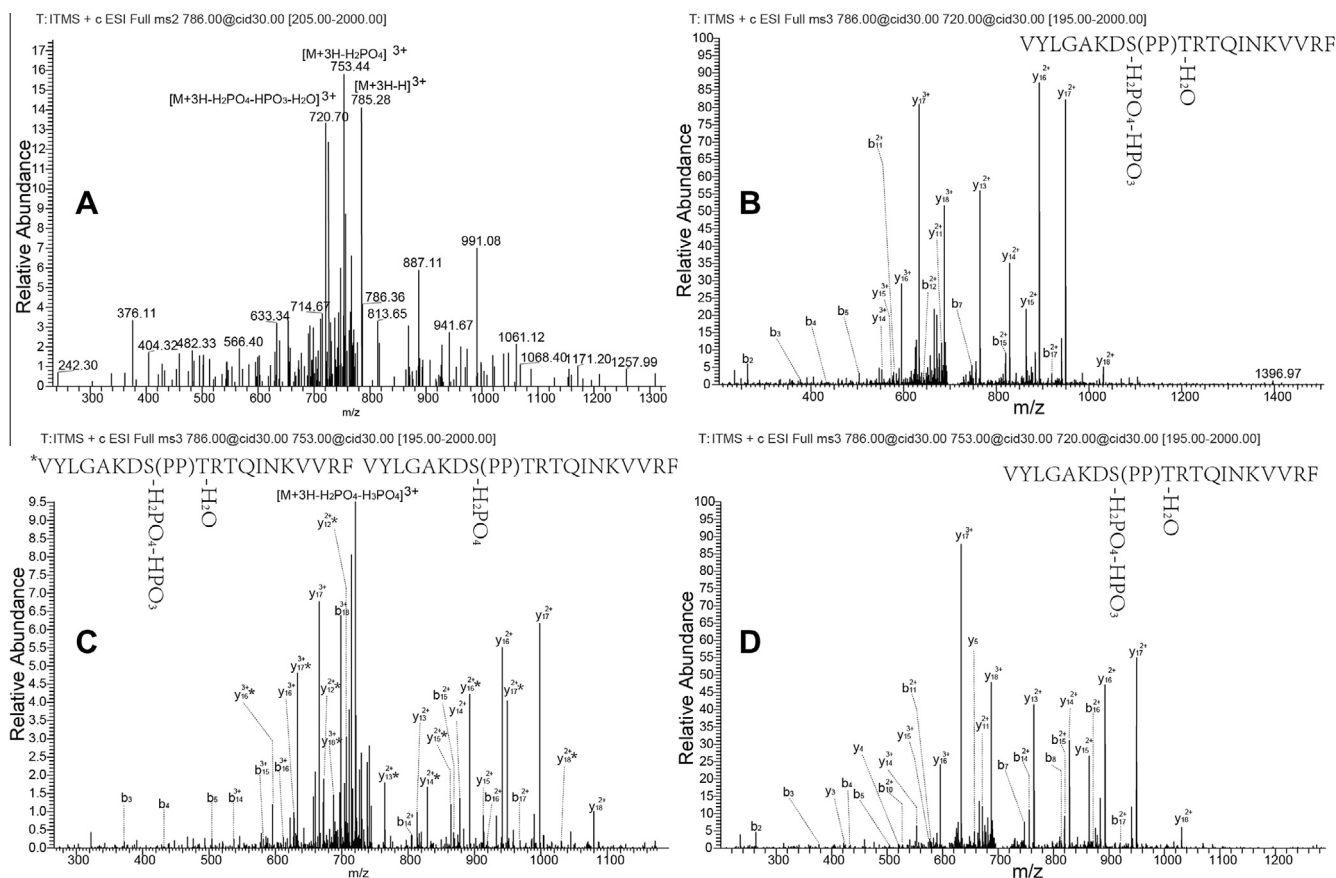


Fig. 3. The CID MSⁿ of the dual-phosphorylated peptide 41–59 (⁴¹VYLGAQDSTRTQ INKVVR⁵⁹) at m/z 785.7263³⁺. The sequence of each peptide is shown on top of the spectrum. Mainly, b and y ions are shown in the mass spectra. (A) CID of m/z 785.7263³⁺ giving an abundant ion at m/z 753.44³⁺ and m/z 720.70³⁺ due to neutral loss of 97 and 195 mass units; (B) CID of m/z 720.70³⁺ losing double phosphated group and one water from Tyr giving the daughter ion spectrum that is used for phosphorylation site identification; (C) CID of m/z 753.44³⁺ losing phosphated group giving the daughter ion spectrum that is used for phosphorylation site identification. (D) CID of m/z 720.70³⁺ losing the second phosphated group and one water from Tyr giving the daughter ion spectrum that is used for phosphorylation site identification.

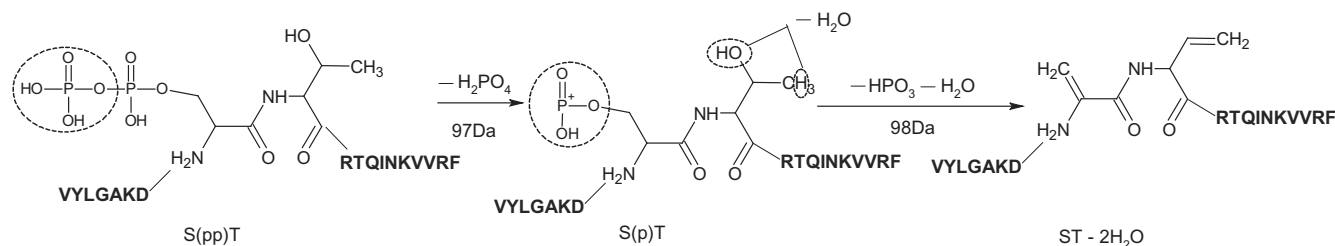


Fig. 4. Structure and obtained fragment ions of the phosphorylated peptide 41–59 (⁴¹VYLGAKDSTRTQINKVVRF⁵⁹) at m/z 753.44³⁺ (SpT) and m/z 720.70³⁺ (ST-2H₂O) due to neutral loss of 97 and 195 mass unit.

Similarly, in the CID MS spectra of the dual-phosphorylated peptide 41–59 at m/z 785.7263³⁺, losses of H (–1 u), H₂PO₄ (–97 u), and H₂PO₄ + HPO₃ + H₂O (–195 u) from the precursor ion appear conspicuously and are more intense than the remainder. We successfully identified the phosphorylation sites of the dual-phosphorylated peptide with m/z of 785.7263³⁺ by CID MS³ (Fig. 3). Interestingly, the neutral loss of H₄PO₃ + H₂O (–117 u) derived from the loss phosphate moiety from phosphor-Ser, and dehydration from the neighbor amino acid (Thr), which was in agreement with a previous report (Moon, Shin, & Kim, 2009). Fig. 4 shows that the structure and obtained fragment ions of the phosphorylated peptide at m/z 753.44³⁺ and m/z 720.70³⁺ were due to neutral losses of 97 and 195 mass units. We confirmed that both phosphates were added to the amino acid residue Ser 48, with no phosphorylation at Tyr 42. Li et al. (Li et al., 2009) reported that some phosphate groups were grafted into the protein as linkages bound to the phosphate groups at the ends of phosphodiester, such as ADP or ATP in PP-Dex under these conditions. However, no trace of phosphodiester linkage was observed in P-Oval under these conditions. Interestingly, the new phosphorylation linkage was identified as an O-PP linkage at Ser48 based on the CID MSⁿ spectrum of peptide 41–59.

All identified phosphorylation sites under dry-heating conditions for 5 days of incubation were found to be on serine residues, including Ser 48, Ser 103, Ser 147, Ser 151, Ser 221, Ser 224 and Ser 384 (Table 1). The amino acid residues that can be phosphorylated are Ser, Thr, Tyr, Arg, and Lys. Li et al. (Li et al., 2009) reported that Lys and Arg were not phosphorylated in dry heating with pyrophosphate. The reason for why phosphorylation is not found on the Tyr and Thr residues remains under investigation in our laboratory. Nevertheless, the preferred phosphorylation under the dry-heating conditions may provide an approach for the preparation of protein with phosphorylation on serine.

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