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Characterisation of phosvitin phosphopeptides using MALDI-TOF mass spectrometry

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ABSTRACT

Putative phosphopeptides produced from enzyme hydrolysis of phosvitin were identified and characterised using MALDI-TOF/MS. Phosvitin was heat-pretreated and then hydrolysed using pepsin, thermolysin, and trypsin at their optimal pH and temperature conditions with or without partial dephosphorylation. Pepsin and thermolysin were not effective in producing phosphopeptides, but trypsin hydrolysis produced many peptides from phosvitin: 12 peptides, 10 of which were phosphopeptides, were identified from the trypsin hydrolysate. Twelve peptides were also identified from the trypsin hydrolysate of partially dephosphorylated phosvitin, but the phosphate groups remaining with the peptides were much smaller than those from the trypsin hydrolysate of intact phosvitin. This suggested that the phosphopeptides produced from the partially dephosphorylated phosvitin lost most of their phosphate groups during the dephosphorylation step. Therefore, partial dephosphorylation of phosvitin before trypsin hydrolysis may not be always recommendable in producing functional phosphopeptides if the phosphate groups play important roles for their functionalities.

1. Introduction

Phosvitin is the major glycophosphoprotein in egg yolk, which accounts for 60% of the total phosphoproteins and holds about 80% of the total egg yolk phosphorous (Taborsky & Mok, 1967). Egg yolk phosphoproteins consist of phosvitin and phosvettes (Wallace, 1985). Vitellogenin, the hepatically-derived macromolecular lipophosphoprotein in non-mammalian vertebrates, serves as the precursor for lipovitellin and phosvitin. The molecular weights of the minor and major phosvitin are 36 kDa and 40 kDa, respectively (Taborsky & Mok, 1967). A detailed analysis indicated that phosvitin is composed of 217 amino acids with 123 serine, 15 lysine, 13 histidine, and 11 arginine residues. Serine is the major amino acid, which accounts for more than 55% of the total amino acids in phosvitin, and many of the serines are arranged in clusters of up to 15 consecutive residues (Byrne et al., 1984). Phosphorylation is one of the major post transitional modifications of proteins: in vertebrates, 89.96% of phosphorylation occurs in serine and 9.99% occurs in threonine residue (Mann et al., 2002).

Almost all the serine residues in phosvitin are phosphorylated, and thus phosvitin molecule has extremely strong metal binding capacity and inhibits the bioavailability of metal ions (Byrne et al., 1984). Fragmentation of phosvitin to small peptides using proteolytic enzymes can increase the bioavailability of calcium and iron because the enzymatically hydrolysed peptides inhibit the formation of insoluble calcium phosphates or iron phosphates, which helps the absorption of calcium and iron in guts (Choi, Jung, Choi, Kim, & Ha, 2005). An interesting feature of phosphopeptides is their ability to form soluble organophosphate salts. The phosphorylated serine moiety of the phosphopeptides play the major role in binding divalent metal ions such as Ca, Mg, Zn, Cu, Fe etc. (Hansen, Sandström, Jensen, & Sorensen, 1997; Kitts, 2005; Li, Tomé, & Desjeux, 1989), and promoted intestinal absorption of

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calcium, minerals and other trace minerals (Konings, Kuipers, & Huis in't Veld, 1999). Mellander (1947) first reported that phosphopeptides derived from casein (CPP) enhanced the calcification of bones. The absorption of iron in the gastrointestinal tract is low because iron forms heavy molecular weight ferric hydroxide in the guts (Derman et al., 1977). However, in the presence of CPP, enhanced iron availability and iron absorption in the gastrointestinal system has been observed. Therefore, phosvitin is an attractive substrate to produce functional phosphopeptides for various nutraceutical applications as antioxidant or carriers for metal ions, which can be useful in the food and pharmaceutical industries (Kitts, 1994; Kitts & Weiler, 2003). However, the enzymatic hydrolysis of natural phosvitin is extremely difficult because almost all the serine residues in phosvitin are phosphorylated. In the natural phosvitin, the negative charges of the phosphate group surround the phosvitin molecule and prevent enzymes from access to peptide bonds (Gray, 1971; Mecham & Olcott, 1949). Therefore, certain pretreatments that can open phosvitin structure are necessary before enzyme treatment.

With the recent advancement of mass spectrometry (MS), almost all the traditional techniques for amino acid sequencing and molecular characterisation have been replaced by the mass spectrometry. The high sensitivity, resolution and mass accuracy have resulted in mass spectrometry as a major tool in proteomics, especially in phosphopeptide analyses (Griffin, Goodlett, & Aebersold, 2001; Mann et al., 2002). In order to produce functional phosphopeptides with antioxidant and mineral binding activities, hydrolysing phosvitin to smaller peptides (<3 kDa) are desirable. Along with the exploration of functional characteristics of the peptides produced, identification and characterisation of the peptides in the hydrolysates are essential.

The objective of present study was to produce phosphopeptides from phosvitin using heat pretreatment and enzyme hydrolysis, and report the preliminary characterisation of the phosphopeptides produced using Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF/MS).

2. Materials and methods

2.1. Materials

Trypsin Type I from bovine pancreas (E.C. 3.4.21.4; ~15,500 U/mg protein), pepsin from porcine gastric mucosa (E.C. 3.4.23.1; 3,220 U/mg) and thermolysin-Type X from *Bacillus thermoproteolyticus rokko* (E.C. 3.4.24.27; 50–100 U/mg), alkaline phosphatase from bovine liver (E.C. 3.1.3.1; 10 U/mg), acetonitrile (ACN), HPLC grade water, α-cyano-4-hydroxy-cinnamic acid (CHCA), and formic acid were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Phosvitin used was prepared from chicken egg yolk using the method of Ko, Nam, Jo, Lee, and Ahn (2011). Phosvitin (10 mg/mL) was dissolved in distilled water and heated at 100 °C for 60 min to improve hydrolysis before use.

2.2. Partial dephosphorylation of phosvitin

Partially dephosphorylated samples of phosvitin were prepared from phosvitin using alkaline phosphatase. The phosvitin (10 mg/mL) was mixed with the same volume of 200 mM sodium phosphate buffer (pH 10) at an enzyme/substrate ratio of 1:50 (w/w) and incubated at 37 °C for 24 h in a shaker water bath. Alkaline phosphatase was deactivated by placing the sample in a boiling water bath for 10 min. The sample was dialyzed for 24 h at 4 °C and then lyophilized (FreeZone Freeze Dryer, Labconco Corp., Kansas City, MO, USA).

2.3. Enzymatic hydrolysis of phosvitin

The phosvitin and partially dephosphorylated phosvitin were digested using trypsin, pepsin or thermolysin. The pH of phosvitin solutions was adjusted using 1 N HCl or 1 N NaOH prior to addition of the protease. Temperature and pH conditions for each enzyme were as follow: Trypsin at 37 °C and pH 8.0; pepsin at 37 °C and pH 2.0; and thermolysin at 68 °C and pH 6.8. The enzyme/substrate ratio was 1:100 (w/w) in all cases. Digestions were carried out for 24 h in a shaker water bath (C7 – New Brunswick Scientific, Edison, NJ, USA). The enzymatic digestion was arrested by keeping the sample for 10 min in a boiling water bath. The resulting hydrolysates were lyophilized.

2.4. SDS–PAGE electrophoresis

The enzymatic hydrolysate of phosvitin was dissolved in distilled water at 1 mg protein/mL. Sample (10 μl) was mixed with 40 μl of Laemmli sample buffer solution under reducing conditions, heated at 95 °C in a block heater for 5 min, and then 10 μl of sample was loaded on the Mini-PROTEIN Tetra cell (Bio-Rad Laboratory Inc.). Ten percent SDS–PAGE gel and Coomassie Brilliant Blue R-250 (Bio-Rad) containing 0.1 M aluminium nitrate staining solution were used for analysis the proteins and peptides containing phosphorus from the modified Coomassie Blue method (Hegenauer, Ripley, & Nace, 1977). After destaining over-night, the proportion of each peptide bands was calculated by converting the density of each band in the gel picture using the ImageJ software (NIH, Bethesda, MD, USA) as the percent of the total gel density.

2.5. MALDI-TOF/MS analysis

The lyophilized hydrolysate was dissolved in distilled water (10 mg/mL) and centrifuged at 3000*g* for 10 min. The supernatant was collected, filtered through a 0.45 μm Millipore Millex-FH filter (Billerica, MA, USA), passed through a C_{18} ZipTip Pipette Tip (Millipore, Billerica, MA, USA) to remove some of the salts from the sample, and then deposited on a stainless steel MALDI plate using the dried droplet method (Cohen & Chait, 1996; Hillenkamp, Karas, Beavis, & Chait, 1991). A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 70% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) was prepared for use as matrix. Matrix and sample were mixed in 1:1 (v:v) ratio, dropped on the MALDI plate, and rinsed with 1 μl of 0.1% after drying the droplets. Mass spectra were acquired using a Bruker Microflex Linear TOF Mass Spectrometer (Bruker Optics, Bellerica, MA). Spectra were acquired over the mass range of 600–4000 Da using 50 laser shots in the positive ion mode at a laser power of 25%. Calibration of the mass spectra was achieved using a 1 μm solution of Bradykinin (*m/z* 1060.6) that also contained 1 μm Neurotensin (*m/z* 1672.9).

2.6. Bioinformatics analysis of MALDI data

To generate theoretical peptide digestion patterns, the Protein Prospector MS-Digest software (UC-San Francisco, CA) was used. The software parameters were set to provide digest peptides ranging from 400 to 3000 Da with a maximum number of two missed cleavages, and a variable number of phosphorylation sites. The appropriate protease was selected.

3. Results and discussion

Phosvitin is generally resistant to proteolysis, presumably due to the large number of negative charges present. The SDS–PAGE of