Journal of Functional Foods 18 (2015) 190–197

Available online at www.sciencedirect.com

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Preparation and characterization of phosphopeptides from egg yolk phosvitin

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ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form 4 July 2015
Accepted 7 July 2015
Available online

Keywords:
Phosvitin
Phosphopeptides
Hydrolysis
Identification
LC–MS/MS

ABSTRACT

Egg yolk phosvitin is a highly phosphorylated protein. Its derived phosphopeptides may help improve bone and dental health, in addition to its antioxidant and anti-inflammatory activities. However, the structural information of these peptides was poorly characterized. In this study, we aimed to prepare phosvitin phosphopeptides by enzymatic hydrolysis and characterize the peptide sequences and phosphorylation by LC–MS/MS. Phosvitin was partially dephosphorylated by 0.2 M NaOH prior to pancreatin hydrolysis. The degree of hydrolysis was 12.9% and the peptide recovery was 6.1% (based on egg yolk). The N/P value of 3.9 was comparable or lower than purified casein phosphopeptides, which ranged from 6 to 18. After anion exchange chromatography, 32 peptides were identified from three phosvitin domains: AEFGTEPDAKTSSSSSASSTATSSSSSASSPN (PV 1–34), KPMDEEENDQV (PV 37–47) and SGHLEDDSSSSSSSVLSKIWG (PV 190–211). These peptides contain up to 10 phosphate groups.
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1. Introduction

Chicken egg yolk phosvitin, with a molecular weight of 37–45 kDa (Abe, Itoh, & Adachi, 1982), is the most phosphorylated protein in nature since 124 (123 serine and 1 threonine residues) out of total 217 amino acid residues are monoesterified with phosphate (Byrne et al., 1984; Clark, 1985; Grogan, Shirazi, & Taborsky, 1996). Phosvitin is derived from the multi-domain precursors of vitellogenin, which is synthesized in the liver of vertebrates under the stimulation of oestrogen and later cleaved into phosvitin, lipovitellin and other minor proteins (Finn, 2007). Due to a large amount of negatively charged phosphoserine residues, phosvitin exhibits strong metal chelating ability, which is believed to carry and provide metal ions during the embryo development (Taborsky, 1983).

Phosvitin is usually considered nutritionally negative due to its strong affinity to metal ions and resistance to proteolytic digestion (Grogan et al., 1996). It is reported that 95% of the iron in egg yolk is bound to this protein but only 30% is available for intestinal absorption (Greengard, Mendelso, & Sentenac, 1964; Morris & Greene, 1972). Animal experiment proved that both phosvitin and egg yolk proteins could inhibit calcium, magnesium and iron absorption (Ishikawa, Tamaki, Arihara, & Itoh, 2007). In contrast, the phosphorylated fragments derived from bovine milk casein digests, known as casein phosphopeptides (CPPs), have been suggested to enhance vitamin D independent bone calcification in rachitic children (Mellander, 1950; Mellander & Isaksson, 1950). CPPs have been extensively studied for its potential applications in bone health (Heaney, Saito, & Orimo, 1994; Teucher et al., 2006; Tsuchita, Goto, Yonehara, & Kuwata, 1995). The phosphoserine

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http://dx.doi.org/10.1016/j.jff.2015.07.007
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residues in CPPs are considered to play a key role in promoting calcium absorption (Meisel et al., 2003). CPPs were reported to increase both bone mass density and the renal tubular reabsorption rate, suppress the bone mass resorption, or facilitate the transportation of insolubilized calcium in small intestines (Matsui, Yano, Awano, Harumoto, & Saito, 1994; Mora-Gutierrez, Farrell, Attaie, McWhinney, & Wang, 2007; Parkinson & Cransberg, 2004; Tsuchita et al., 1995).

However, compared with casein which contains less than 15 phosphoserine residues and 3 phosphoserine residues in a consecutive run (Swaisgood, 1992), phosvitin could be another attractive protein source for preparing bioactive phosphopeptides since it contains 123 phosphoserine residues and 113 of them are consecutive (Grogan et al., 1996). Phosvitin phosphopeptides (PPPs) were reported to exhibit antioxidant, anti-inflammatory and calcium-absorption promoting ability *in vitro* and *in situ* (Feng & Mine, 2006; Jiang & Mine, 2000; Katayama, Ishikawa, Fan, & Mine, 2007; Katayama, Xu, Fan, & Mine, 2006; Xu Katayama, & Mine, 2007; Young, Nau, Pasco, & Mine, 2011; Choi, Jung, Choi, Kim, & Ha, 2005). However, the potential use of phosvitin as functional food ingredient is hampered by lack of a scalable method of extraction; previous methods of phosvitin extraction involved either organic solvents or non-food compatible chemicals, and the procedure of extraction is lengthy and tedious, resulting in low purity and yield (Ren & Wu, 2014). We recently developed a scalable method of extraction without using organic solvents or non-food compatible chemicals with a yield of 2.3 g/100 g yolk solids and a purity of 88.0% (Ren & Wu, 2015), which are comparable to previous results (Jung, Ahn, Nam, Kim, & Jo, 2013; Lee, Abeyrathne, Choi, Suh, & Ahn, 2014). There is limited knowledge available on structural characterization of PPPs. Young et al. (2011) identified thirteen peptide sequences from tryptic hydrolysate of phosvitin, but only two were phosphorylated; Samaraweera et al. (2014) partially characterized 10 phosphopeptides from phosvitin tryptic hydrolysate by comparing the determined peptide molecular weight (MALDI/MS data) with the theoretical peptides molecular weight predicted by Protein Prospector MS-Digest software, but not from de novo sequencing from MS/MS data. The objectives of this study were to prepare PPPs by pancreatic digestion and characterize the peptide sequences and phosphorylation by liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Chemicals

Glycine, Precision Plus Protein™ Dual Xtra Standards, sodium dodecyl sulphate (SDS), and precast gels (12% Tris–HCl) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Deionized distilled water was prepared from a Barnstead water purification system (Thermo Scientific, Asheville, NC, USA). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Nepean, ON, Canada).

2.2. Enzymatic hydrolysis

Phosvitin for enzymatic digestion was prepared from fresh egg yolks as previously reported (Ren & Wu, 2015). Partial dephosphorylation was performed by dissolving phosvitin into 0.2 M NaOH solution at 5% (w/w) at room temperature (25 °C) and magnetically stirring for 0.5 h according to Jiang and Mine (2000) with slight modifications. After pH was adjusted to 8.0 with 3 M HCl, pancreatin was added at a ratio of 1/50 (enzyme/phosvitin, w/w). The choice of enzymes and conditions was adopted from our unpublished results. Incubation was carried out at 40 °C for 3 h. The pH and temperature were controlled by a heating circulator LAUDA A103 (LAUDA-Brinkmann, LP, Lauda-Koenigshofen, Baden-Württemberg, Germany) and the enzyme was inactivated by increasing the temperature to 95 °C and holding for 15 min. Hydrolysate was centrifuged at 10 000 *g* for 30 min at 4 °C and the supernatant obtained was then lyophilized for the following assay. The yield was defined as the amount of protein in the hydrolysate produced from one gram yolk solids. The recovery was calculated as a percentage of amounts of protein in the hydrolysate to the total protein in the starting egg yolk (w/w). The degree of hydrolysis (DH) was determined as described by Alder-Nissen (1979). Briefly, protein hydrolysate was dissolved in 1% sodium dodecyl sulphate (SDS) and the free amino groups will be determined spectrophotometrically at 340 nm.

2.3. Anion exchange chromatography of phosvitin hydrolysate

Gradient elution was applied to fractionate phosvitin hydrolysate. Buffer A was 0.05 M Tris–HCl at pH 8.0 and buffer B was 1.0 M NaCl in buffer A. Phosvitin hydrolysate was prepared at 10 mg/mL with buffer A and filtered through 0.45 μm membrane (Millipore, Billerica, MA, USA). A HiPrep 16/10 Q FF anion exchange column (GE Healthcare, Piscataway, NJ, USA) was attached to an AKTA explorer 10S system (GE Healthcare, Piscataway, NJ, USA) and equilibrated with buffer A for five column volumes (CV). The elution was performed by a gradient from 100% buffer A to 100% buffer B over 10 CV at a flow rate of 2 mL/min. The elution was monitored at 220 nm. Fractions were collected and lyophilized. Before LC–MS/MS, fraction P-4 was dissolved in Milli-Q water and desalted by eluting from a Superdex peptide 10/300 GL column (GE Healthcare, Piscataway, NJ, USA) using Milli-Q water as running buffer. Elution was performed with the same FPLC system and monitored at 215 nm. The protein/peptide fractions were collected and lyophilized for LC–MS/MS analysis.

2.4. Nitrogen and protein determination

Nitrogen content was determined by using the Leco-N nitrogen instrument (Model FP-428, Leco Corporations, St. Joseph, MI, USA). The crude protein content was calculated as $N \times 6.25$.

2.5. Phosphorus determination

Phosphorus content was determined by using a malachite green phosphate assay kit (Bioassay Systems, Hayward, CA, USA) as described by manufacturer's manual.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Shapiro, Vinuela, and Maizel (1967). Samples were prepared at 4 mg/mL. Gels were

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