Preparation of Novel Functional Oligophosphopeptides from Hen Egg Yolk Phosvitin

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Novel hen egg phosvitin phosphopeptides (PPP) with molecular masses of 1−3 kDa were prepared from tryptic hydrolysis following partial alkaline dephosphorylation. The phosvitin treated with various NaOH concentrations (0.05−0.4 N) resulted in different dephosphorylated proteins ranging from 17.5 to 96.3% of phosphate retention. The protein was digested into 10−20 amino acid peptides with trypsin. Calcium-binding properties of PPP were compared with those of commercial casein phosphopeptides in vitro. The PPP with 35% phosphate retention is shown to be effective for enhancing calcium binding capacity and inhibiting the formation of insoluble calcium phosphate. The results provide potential novel functional oilgophosphopeptides as nutraceuticals.

Keywords: *Phosvitin; phosphopeptides; CPP; egg yolk; nutraceuticals; calcium enhancer*

INTRODUCTION

In the egg yolk, phosvitin is a principal phosphoprotein with a molecular mass of 35 kDa, containing ~10% phosphorus and 6.5% carbohydrates. The proposed name "phosvitin" indicates both its high phosphorus content and its source in the egg yolk (Mecham and Olcott, 1949; Taborsky and Mok, 1967). Phosvitin is derived from a large precursor molecule, vitellogenin, which is formed in the liver of oviparous vertebrates under estrogen induction, and it contains 123 serine residues, which account for 57.7% of the total amino acid residues (Byrne et al., 1984). As nearly all of the serines are monoesterified with phosphate, phosvitin acts as a metal chelator participating in dynamic equilibria involving a multicomponent system of metals (Taborsky, 1983).

Early experiments indicated that proteins containing phosphorylated amino acid residues were resistant to protease activity; consequently, it was thought that phosvitin might not be as rapidly digested by trypsin and pepsin as were proteins containing little or no phosphorus (Mecham and Olcott, 1949). Goulas et al. (1996) reported that tryptic digestion of phosvitin resulted in a large peptide fragment (Gln 49−Arg 212) and a small one (Ala 1−Arg 35). The core of the protein remained largely intact upon digestion with proteolytic enzymes. The reason might be that the negative charge of the phosphate group renders neighboring peptide bonds insensitive to tryptic action, but an attempt to neutralize the charge effect by saturating the protein with iron did not enhance fragmentation. It was assumed that the formation of a properly constituted enzyme−substrate complex might be blocked by polynuclear complexes (Gray, 1971).

Ninety-five percent of the iron is present in the yolk and is bound to phosvitin. It is in the ferric form (Greegard et al., 1964). However, it has been demonstrated that the relative biological value of this iron is only 30% (Morris and Greene, 1972). The studies by Taborsky (1980) indicated that additional binding of iron by phosvitin prevented this protein from assuming a β-sheet conformation. The significance of this observation is that native phosvitin has a very stable conformation. Iron once bound may not be easily released (Albright et al., 1984). Therefore, phosvitin can be considered nutritionally negative due to its resistance to proteolytic actions and low bioavailability of iron. In contrast, it is already proven that phosphorylated fragments of casein, casein phosphopeptides (CPP), increase the bioavailability in intestinal calcium and its retention by the body. Vitamin D is not required for phosphopeptide-induced changes in calcium metabolism (Mykkanen and Wassermann, 1980; Sato et al., 1986). These biologically active peptides can also form organophosphate salts with trace elements such as Fe, Mn, Cu, and Se and, hence, have wide applications in nutraceuticals (West, 1986; Meisel and Schlimme, 1990). It has been confirmed that the key function to form a soluble complex with calcium exists in the phosphoserine moieties (Gerber and Jost, 1986; Sato et al., 1986). However, the effects of the number of phosphorylated serines and the sizes of peptides on the formation of calcium complexes have not yet been fully understood. In addition, the calcium-binding bahavior of phosphopeptides from phosvitin has not been investigated, partly because so far little progress has been made in preparing small phosphopeptides from egg yolk phosvitin. Although there is increasing public awareness of the health benefits of phosphopeptides in the prevention of osteoporosis, the only source for the peptides is CPP from milk casein. It could be valuable to develop new functional bioactive peptides derived from egg yolk phosvitin by controlling the phosphate residues in the peptides if peptides with appropriate amino acid chain length and number of phosphoryl groups were obtained. It is also of great interest to study the structure−function relationship of such biologically active peptides derived from food proteins. This paper deals with the preparation of phosphopeptides from highly phospho-

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rylated phosvitin and its characterization as to calcium-binding properties.

MATERIALS AND METHODS

Materials. Trypsin (EC 3.4.21.4; 11000 units/mg), chymotrypsin (EC 3.4.21.1; 42 units/mg), pepsin (EC 3.4.23.1; 3900 units/mg), 2,4,6-trinitrobenzenesulfonic (TNBS; 5% w/w), and phosvitin were purchased from Sigma Chemical Co. (St. Louis, MO). Fresh eggs were obtained from the University of Guelph's Arkell Poultry Farm. Due to the large quantities required, phosvitin was also isolated from egg yolk. CPP II was obtained from Meiji Seika Co., Ltd., Tokyo, Japan.

Preparation of Phosvitin. Hen egg yolk phosvitin was isolated according to the method of Losso and Nakai (1994) with the following modifications. Twelve egg yolks were diluted with 1 L of cold Milli-Q water at pH 5.0 and stirred at 4 °C for 6 h. The precipitate was collected by centrifugation at 70000*g* (Beckman L8-M, rotor type Ti 45, Palo Alto, CA) and 15 °C for 60 min. The pellet was dissolved and stirred in 400 mL of 0.05 M NaCl for 4 h, and then the suspension was centrifuged at 110000*g* and 15 °C for 30 min. The lipid fraction in the collected precipitate was extracted with 400 mL of hexane/ethanol (3: 1) at 4 °C for 6 h and filtered through a Whatman No. 4 filter paper. The phosvitin in the cake was extracted with 200 mL of 1.74 M NaCl overnight at 4 °C. The filtrate was dialyzed against Milli-Q water at 4 °C for 48 h and then lyophilized.

Alkaline Dephosphorylation of Phosvitin. Phosvitin (50 mg) was dissolved in 2 mL of 0.1, 0.2, 0.3, and 0.4 N NaOH, respectively, and incubated at 37 °C for up to 24 h. After a given time, the pH was dropped to 7.0 by the addition of 1 N HCl. After overnight dialysis against Milli-Q water, the sample was lyophilized and kept at −30 °C until use.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). The SDS−PAGE was performed according to the method of Hegenauer et al. (1977) with 12.5% (w/w) polyacrylamide gel. Gels were first stained by using the modified Coomassie blue method for phosphoproteins and followed by the normal Coomassie blue procedure. After overnight destaining in a solution containing acetic acid/methanol/water (1:8:12, v/v/v), gels were scanned on a Sharp JX-330 scanner (Sharp Electronics, Tokyo, Japan).

Determination of Phosphorus Content. The alkaline dephosphorylated phosvitin samples were eluted with Milli-Q water on a PD-10 column filled with Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) to remove free phosphate. The initial 5.5 mL of eluate was collected. Phosphorus content was determined according to the method of Bartlett (1959), and untreated phosvitin was used as a control.

Preparation of Proteolytic Digests of Phosvitin. Lyophilized native and alkaline-dephosphorylated phosvitins (each 200 mg) were suspended in 6 mL of Milli-Q water, and the pH of the solution was adjusted to 8.0 with 0.1 N NaOH prior to the addition of either trypsin or chymotrypsin or to pH 2.5 with 0.1 N HCl prior to the addition of pepsin (Goulas et al., 1996). Incubation was maintained at 37 °C for up to 24 h, and the pH was kept constant by the addition of 0.25 N NaOH. The enzyme/substrate ratio was 1:50 (w/w) for all three enzymes. All enzymatic reactions were stopped by adjusting the pH to 5. The isolation of phosphopeptides was carried out by using the two different methods. The tryptic digestion of phosvitin was centrifuged at 5900*g* and 10 °C for 15 min. The clear supernatant was made up to a final 0.25% (w/w) of $BaCl_2$ concentration by adding a 10% (w/w) $BaCl_2$ solution (Manson and Annan, 1971). After an equal volume of cold absolute ethanol had been added, the solution was centrifuged at 5000*g* for 30 min. The precipitate was dissolved in 5 mL of a solution containing 60 mg of EDTA and then lyophilized. The preparation of phosphopeptides for calcium-binding determination was also performed as follows. The tryptic digests were centrifuged as described previously, and the supernatant was microfiltrated through a 0.45 μm membrane and then ultrafiltrated on a 1 kDa cutoff Diaflo membrane (Amicon, Beverly, MA) with Milli-Q water three times. The retenate was collected and lyophilized. The yield of smaller fragments (molecular mass < 1 kDa) was <3% of total digests.

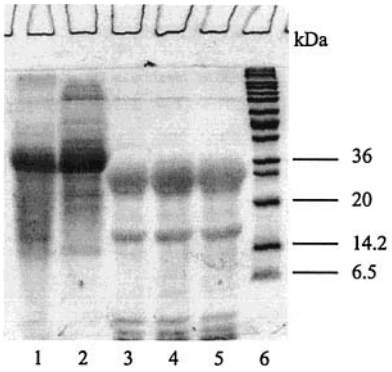

Figure 1. SDS−PAGE pattern of trypsin-digested phosvitin: (lane 1) native phosvitin (Sigma); (lane 2) native phosvitin (our procedure); (lanes 3, 4, and 5) phosvitin treated with trypsin for 1, 5, and 24 h, respectively; (lane 6) molecular marker.

Amino Acid Analysis. The samples were hydrolyzed in vacuo with 200 μL of 5.7 N HCl at 105 °C for 24 h and then dried and dissolved in 500 μL of buffer system and ninhydrin. Amino acid analysis was carried out with a Beckman System Gold amino acid analyzer (Palo Alto, CA) equipped with postcolumn ninhydrin detection, and the flow rate was 0.6 mL/min.

Determination of Amino Groups with TNBS. Determination of amino groups was carried out according to the procedure of Fields (1972) with the following modifications. The sample containing amino groups was added to 0.5 mL of 0.2 M phosphate buffer, pH 8.2, and the volume was made up to 1.0 mL by Milli-Q water. Then, 1 mL of 0.1% (w/w) TNBS solution was added, and the solution was rapidly mixed. After 2 h at 40 °C, the reaction was stopped by adding 2.0 mL of 0.1 M NaH_2PO_4, which contained 1.5 mM sulfite, and the absorbency at 420 nm was determined. A blank was also prepared as control, and lysine-HCl (3−15 μg) was used as a standard. Determination should be carried out with great care; otherwise, a high spreading of the results from repeated analysis might be observed. Because of a nuisance of formation of CO_2, it is recommended to use buffer systems such as borate (if sugars are not present) or phosphate instead of carbonate. During incubation, the test tubes and the incubator must be covered with aluminum foil because the blank reaction is accelerated by exposure to light (Adler-Nissen, 1979).

Analysis of Soluble Calcium from Phosphate Precipitation. The analysis was performed basically according to the method of Sato et al. (1991) with the following modifications. Various concentrations up to 600 mg/L of phosphopeptides drived from ultrafiltrated membrane that had been demineralized with Chelex-100 (Bio-Rad, Hercules, CA) (Sato et al., 1991) were mixed with 5 mM $CaCl_2$ and 20 mM sodium phosphate buffer (pH 8.0). The mixture was stirred at 22 °C for 30 min, and the pH was maintained at 7.80 with a pH-stat. Calcium contents of the supernatant after 0.45 μm microfiltration were determined by atomic absorption spectroscopy with a Varian SpectrAA-300 (Melbourne, Australia).

RESULTS AND DISCUSSION

Proteolytic Digestion of Phosvitin. Native phosvitin was incubated with pepsin, trypsin, and chymotrypsin, respectively. The SDS−PAGE pattern in Figure 1 indicated that phosvitin was cleaved by trypsin into two large distinct peptides and three small ones. The large peptide with a molecular mass of 28 kDa may be equivalent to a 164 amino acid residue long segment of Gln49−Arg212 (Goulas et al., 1996). It was difficult to

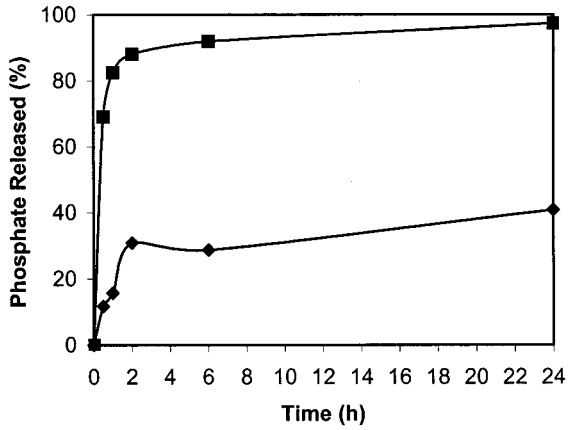

Figure 2. Time course of dephosphorylation of phosvitin with 0.1 N NaOH (◆) and 0.3 N NaOH (■).

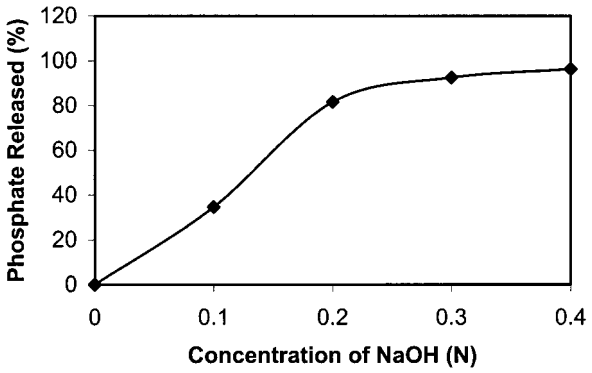

Figure 3. Relationship between degree of dephosphorylation of phosvitin and concentration of NaOH. Incubation of phosvitin with NaOH was carried out at 37 °C for 3 h.

obtain smaller peptide fragments by tryptic action. The SDS−PAGE patterns of peptic and chymotryptic digestion showed similar results (data not shown).

Alkaline Dephosphorylation of Phosvitin. The results of dephosphorylation of phosvitin by alkaline treatment are shown in Figure 2. After dramatic dephosphorylation during the initial 1−3 h, the time course of dephosphorylation reached a plateau. The released phosphate neutralized the pH of 0.1 N NaOH solution, and further dephosphorylation of the protein did not proceed after 2 h, whereas it proceeded continuously with 0.3 N NaOH. Thus, 3 h of incubation time was chosen for the following experiments. After alkaline dephosphorylation, 31 and 88% of the original phosphate were released from phosvitin after incubation with 0.1 and 0.3 N NaOH for 2 h, respectively. Gradual loss of solubility was observed as increasing hydrophilic phosphate groups were removed from native phosvitin. The effect of sodium hydroxide on the dephosphorylation of native phosvitin was further investigated by incubating phosvitin with various concentrations of NaOH at 37 °C for 3 h, and the results are shown in Figure 3. The data showed that 34.6, 81.6, 92.5, and 96.3% of phosphate residues were released from native phosvitin by treatment with 0.1, 0.2, 0.3, and 0.4 N NaOH for 3 h, respectively. Figures 2 and 3 indicate that dephosphorylation was NaOH concentration- and time-dependent. With the above information, we tried to prepare partially dephosphorylated phosvitin with suitable phosphate group contents. No lysinoalanine was observed within 4 h of treatment with up to 0.3 N NaOH on the basis of lysine measurement (data not shown).

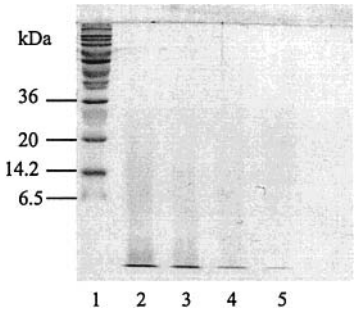

Figure 4. SDS−PAGE pattern of tryptic digestion of alkaline-dephosphorylated phosvitin: (lane 1) molecular marker; (lanes 2, 3, 4, and 5) tryptic digestion following treatment with 0.1, 0.2, 0.3, and 0.4 N NaOH, respectively. The tryptic digestion was carried out at 37 °C for 4 h.

Tryptic Digests of Alkaline-Dephosphorylated Phosvitin. Figure 4 shows electrophoretic patterns of tryptic digested phosvitin for 4 h following alkaline dephosphorylation. In contrast to the SDS−PAGE pattern of Figure 1, Figure 4 shows that only one visible band is found at the bottom of running gel. It can be estimated that the molecular mass of the tryptic digestion should be <3 kDa, and there would not be a big difference among their molecular sizes among different treatments with NaOH. It has been well-known that of the 123 serine residues in native phosvitin, 80 are located in a core section (residues 56−154) that consists of runs of up to 14 serines alternated with the basic amino acids arginine and lysine and, occasionally, asparagine residues (Byrne et al., 1984). This protein is also known to undergo a conformational transition toward a β-sheet structure at low pH, which results in making a cleavage site less accessible (Taborsky, 1968). Negative charge and polynuclear complexes of phosphate groups have been shown to render neighboring peptide bonds insensitive to tryptic action. During alkaline dephosphorylation, the conformation of the core might change so as to increase susceptibility to attack by trypsin due to the unmasking of peptide bonds specifically vulnerable to proteases. Therefore, small peptide segments, phosvitin phosphopeptides (PPP), could be prepared by proteolytic action. It is interesting to evaluate how the degree of phosphorylation and the chain length of peptides can affect their biological availability related to the formation of calcium complexes and enhance their adsorption. Here, we prepared three different phosvitins with 65, 35, and 17.5% phosphate retention under controlled incubation with 0.05, 0.1, and 0.15 N NaOH at 37 °C for 3 h, respectively.

Determination of Chain Lengths of Phosvitin Phosphopeptides. In this study, we prepared PPP according to two different methods. One was isolation by adding 10% $BaCl_2$ solution to the tryptic hydrolysate supernatant. These samples were used only for amino acid analysis and chain length (CL) determination, not for calcium-binding experiments, due to the difficulty of the removal of the barium ion from the precipitate. The other was retentate of tryptic digestion by ultrafiltration (UF) with a 1 kDa cutoff membrane.

The total free primary amino groups in PPP was determined by using the TNBS method, which is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines. The total amino acids including lysines in PPP were determined by amino acid analysis. As there are two primary amino

Table 1. Average Chain Length of Phosphopeptides from Tryptic Hydrolysates[a]

	sample I	sample II	sample III
UF	22.8	20.0	17.1
$BaCl_2$ precipitation	13.1	9.6	11.8

[a] Samples I−III are PPP from phosvitin with 65, 35, and 17.5% phosphate retention, respectively.

groups in a mole of lysine, the average chain length of PPP was calculated as follows:

$$CL = M_{aa}/(M_{pa} - M_l) \quad (1)$$

In the above equation, CL is the peptide chain length, M_{aa} (nmol/mg) is the total amino acid content, M_{pa} (nmol/mg) is the total primary amino group content, and M_l (nmol/mg) is the total lysine content in PPP.

It is noteworthy that in the TNBS method there is a considerable difference between the different proteins with respect to the actual value of the *slope* and *intercept* of this relationship, although it is generally assumed that a linear relationship between color intensity and the concentration of α-amino groups exists (Adler-Nissen, 1979). The results summarized in Table 1 indicate that CL of PPP from the UF procedure is larger than that of $BaCl_2$ precipitation. The average CL of the $BaCl_2$ fraction of sample II was 9.6, whereas that from the UF fraction was 20.0. The $BaCl_2$ method might precipitate phosphopeptides including smaller fragments of <1 kDa, whereas the UF procedure isolated only peptides with molecular masses of >1 kDa. This is because the CL of UF-isolated PPP is larger than that of $BaCl_2$-precipitated ones. The dephosphorylation of phosvitin affected its cleavage by trypsin and resulted in different CLs among samples I−III.

Trypsin is an enzyme that hydrolyzes peptides, amides, esters, etc., at bonds involving the carboxyl group of L-arginine or L-lysine (Barman, 1969). There are 26 arginine and lysine residues all together in a mole of native phosvitin (Byrne et al., 1984). If phosvitin is fully hydrolyzed by trypsin, the average CL will be 8, which is close to that of PPP from the $BaCl_2$ procedure. The data in Table 1 suggest that most peptide bonds susceptible to tryptic action, if not all, are cleaved. As also shown in Table 2, a considerable decrease of serine was found in the samples of UF fraction. The reason for the decrease of the percentage of serine residues in the UF segment is not clear; however, the smaller serine-rich fragment might be lost during the UF process. It was difficult to analyze these small fragments because of their limited amount. Some of these small fragments might not be precipitated with $BaCl_2$ treatment. These fragment should be taken into account for further studies.

Formation of Soluble Complexes with Calcium. The PPP were shown to inhibit formation of insoluble calcium phosphate, as measured by determining the calcium contents of the supernatant during incubation of calcium phosphate mixture with PPP from phosvitin with the remaining phosphate contents of 65 (I), 35 (II), and 17.5% (III). Addition of PPP and CPP to the calcium phosphate suspension caused a pH drop, which meant that the calcium was transformed from phosphate to Ca−PPP or Ca−CPP and H^+ ions were liberated. Because low pH could increase the solubility of calcium phosphate, the pH of the reaction system was kept constant by the addition of 0.1−0.5 N NaOH. The

Table 2. Amino Acid Composition of Phosvitin and Phosphopeptides (Mole Percent)

amino acid	Losso[a]	Byrne[b]	$BaCl_2$ precipitation[c]	UF[d]
Asx	7.07	5.99	9.50	10.67
Thr	2.16	1.84	2.17	2.57
Ser	51.12	56.68	40.84	32.39
Glx	6.90	5.07	7.37	9.20
Pro	1.57	1.38	0.54	4.13
Gly	3.07	2.30	3.64	3.84
Ala	4.16	3.23	4.38	4.87
Val	1.90	1.38	1.57	2.83
Met	0.65	0.46	0.48	0.94
Ile	1.03	0.92	0.45	1.72
Leu	1.91	1.38	1.11	3.92
Tyr	0.58	0.46	0.58	0.96
Phe	1.07	0.46	1.16	1.46
Lys	6.73	6.91	12.54	8.97
His	4.53	5.99	6.66	6.18
Arg	5.19	5.07	7.01	5.36
Trp	0	0.46	*e*	*e*

[a] Determined by amino acid analysis of phosvitin isolated by Lasso et al. (1994). [b] Predicted by nucleotide sequence. (Byrne et al., 1984). [c] $BaCl_2$ precipitation of tryptic hydrolysate from phosvitin with 35% phosphate retention. [d] UF retenate of tryptic hydrolysate from phosvitin with 35% phosphate retention. [e] Tryptophan was not determined.

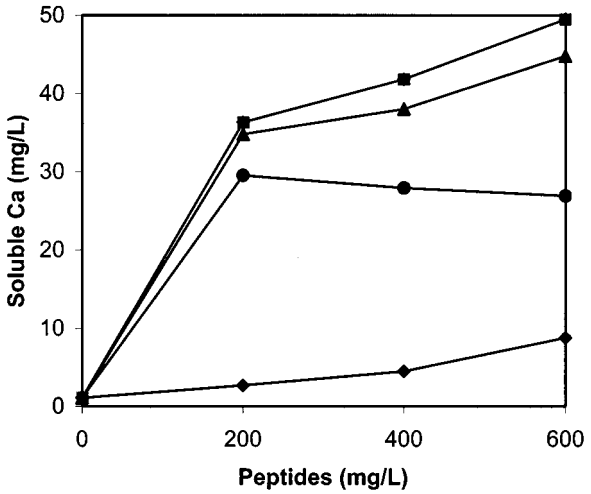

Figure 5. Inhibition of calcium phosphate precipitation with phosphopeptides. A mixture of 5 mM $CaCl_2$, 20 mM phosphate, and various amounts of CPP (●) and PPP from tryptic hydrolysates of phosvitin, with 65 (◆), 35 (■), and 17.5% (▲) phosphate retention, was maintained at pH 7.80 with addition of 0.1 N NaOH and at 22 °C for 15 min.

results in Figure 5 indicate that PPP with different phosphates show different formation abilities with calcium from phosphate precipitation. High soluble calcium contents were observed when calcium phosphate was incubated with PPP (II), PPP (III), and CPP, and among them, PPP (II) showed the highest solubilization ability. The solubility of 36.3 mg/L of Ca was obtained at the concentration of 200 mg of PPP (II). The ability of PPP to prevent the precipitation of calcium phosphate salts confirms the essential role played by the phosphoseryl groups in Ca^{2+}-phosphopeptide interactions as studied with CPP (Gerber and Jost, 1986; Berrocal et al., 1989). It seems that phosphate contents of PPP have a critical effect on calcium binding ability, and solubilization of calcium from phosphate precipitate did not increase with increasing phosphoryl groups in peptides. This implies that there may exist an optimal requirement of phosphoryl groups and/or peptide sizes

for calcium complex formation. CPP obtained from whole bovine casein by tryptic hydrolysis followed by precipitation and chromatography (Juillerat et al., 1989) are composed of the following identified fragments: α_{s1}-CN (43−58), 2P; α_{s1}-CN (59−79), 5P; α_{s2}-CN (46−70), 4P; β-CN (1−28), 4P; β-CN (2−28), 4P; and β-CN (33−48), 1P. With the above data, it can be deduced that a 20 amino acid peptide with 4 phosphates (20% phosphorylation) would be favorable for its physicochemical properties such as formation of soluble complexes with calcium. In this study, PPP with 35% phosphate retention showed satisfactory calcium solubilization capacity from phosphate precipitate. It is noteworthy that the calcium contents of the supernatant were not increased linearly by increasing phosphopeptides as reported (Sato et al., 1991). The mechanism by which PPP can maintain soluble calcium, even in the presence of high concentrations of inorganic phosphate, has not yet been entirely understood.

In conclusion, small phosphopeptides from hen egg yolk phosvitin have been prepared from tryptic hydrolysates following alkaline dephosphorylation. The SDS−PAGE patterns showed that no peptide >3 kDa existed in tryptic hydrolysates, which indicated that most of the lysine and arginine peptide bonds were cleaved because the conformational changes induced by alkaline dephosphorylation made the bonds more susceptible to enzyme attack. Therefore, phosvitin could be digested to 10−20 amino acid peptides with partial dephosphorylation with alkaline treatment (0.1 N NaOH × 3 h) and following tryptic digestion (E/S = 1/50, 37 °C × 4 h incubation). The sample will be filtrated using UF for recovery of PPP. The PPP with 35% phosphate retention proved to be effective for enhancing calcium-binding capacity and inhibiting the formation of insoluble calcium phosphates. The method of very rapid dephosphorylation developed from this research makes it possible to prepare peptides with suitable phosphate groups for calcium absorption and utilization without the formation of lysinoalanine. The phosphopeptides obtained by 35% phosphate retained phosvitin can solubilize more calcium than CPP in this experiment and provide novel functional oilgophosphopeptides as nutraceuticals.

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