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Phosvitin Phosphopeptide Preparation Using Immobilised Trypsin and Enhancing Calcium Absorption in Growing Rats

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Abstract

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We demonstrate a new, efficient method for the preparation of phosvitin phosphopeptides using immobilised-trypsin enzymolysis technology. Immobilised trypsin was prepared using a covalent binding method, and then was added to degrade egg yolk phosvitin for the production of phosphopeptides. In our results, the prepared immobilised trypsin demonstrated a higher hydrolysing activity toward phosvitin than free trypsin, and the hydrolysing activity was retained well even after trypsin was repeatedly used up to five times. Interestingly, the phosvitin phosphopeptides prepared with immobilised trypsin demonstrated a lower N/P ratio and a higher calcium-binding efficiency than those prepared with free trypsin. Furthermore, phosphopeptides significantly increased the rate of calcium absorption and serum calcium content *in vivo*. Based on these results, we conclude that trypsin immobilised onto chitosan has a greater phosvitin hydrolysing activity than free trypsin, and the prepared phosphopeptides can be used as a new calcium supplement to significantly increase calcium absorption in growing rats.

Keywords: phosvitin; phosphopeptide; immobilisation; calcium-binding efficiency; calcium; absorption

Phosvitin from chicken egg yolk is a highly phosphorylated protein with a molecular weight of 35 kDa containing 10% phosphorus (Abe *et al*. 1982); this protein has been considered as a superior source for producing phosphopeptides (Castellani *et al*. 2004). Due to the high phosphoric acid content bound to serine residues, phosvitin phosphopeptides can behave as polyelectrolytes (polyanions) in liquid state (Grizzuti & Perlmann 1970) and show characteristics such as metal chelation, antioxidative properties, emulsifying capacities, and so on (Samaraweera *et al*. 2011). One interesting feature of phosvitin phosphopeptides is their ability to effectively bind calcium and inhibit the formation of insoluble calcium phosphates or complexes and to improve the absorption of calcium and bioavailability in vivo (Jiang & Mine 2000, 2001; Choi *et al*. 2005). Currently, phosvitin phosphopeptides are being considered as a new food additive in the food industry to promote the absorption of calcium.

Given that calcium deficiency can lead to metabolic bone disease, the lack of calcium has become a worldwide problem, causing effects such as osteoporosis. Because the absorption of calcium is influenced by numerous factors, such as the nature of the chemical matrix of food sources and the nutritional, metabolic, and physiological status of individuals, treatments for calcium deficiency consist of more than just adding calcium to diets. However, although phosvitin phosphopeptides are an attractive candidate for calcium supplements, the high price of phosvitin phosphopeptide production limits their potential for

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application (Samaraweera *et al*. 2011). The native structure of phosvitin is more stable and resistant to the proteolytic cleavage by trypsin because of containing a high amount of serine and phosphoric amino acids, and chelating of Fe, which results in low *in vivo* bioavailability (Ishikawa *et al*. 2007). Although it could be digested with trypsin after dephosphorisation, it is always time-consuming and difficult to separate free enzymes and products in the production of phosvitin phosphopeptides. Therefore, the development of improved and cost-effective preparation methods for phosvitin phosphopeptides is crucial to exploit their application in the food industry.

Based on many advantages such as easy recovery of both enzymes and products, multiple reuse of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and a greater variety of bioreactor designs, immobilised enzymolysis technology has been developed and is widely used in the food industry (Amjad & Mohammad 2010; Homaei *et al*. 2013). It has been reported that immobilised enzymes including immobilised trypsin and immobilised glutamic acid-specific endopeptidase have been successfully employed to hydrolyse casein for the preparation of casein phosphopeptides (CPP) (Park & Allen 1998; Park *et al*. 1998); this application of immobilised enzymolysis demonstrated a good mineral-binding ability and enhanced the absorption of Ca and iron in the gastrointestinal system even in the absence of vitamin D. To date, casein phosphopeptides have already been approved as nutraceuticals in Japan (Jiang & Mine 2000), and a product called "Capolac" containing CPP is available in Sweden as a mineral absorption facilitator (Korhonen & Pihlanto 2006). However, few studies have reported the production of bioactive phosphopeptides from egg yolk phosvitin using immobilised enzymolysis technology in the food industry.

Here, to investigate the effectiveness of immobilised enzyme technology on the preparation of phosvitin phosphopeptides from egg, immobilised trypsin was prepared and employed to hydrolyse purified egg yolk phosvitin; furthermore, the bioactivity of phosvitin phosphopeptides in terms of enhancing calcium absorption and accumulation in serum was evaluated *in vivo*.

MATERIAL AND METHODS

Preparation of immobilised trypsin. Immobilised trypsin was prepared as described previously with some modifications (Ling *et al*. 2007). In brief, 0.15 g of chitosan was dissolved with 15 ml of 1% CH_3COOH solution prior to the addition of 720 µl of 5 M NaOH to generate a white flocculent precipitate. The precipitate was filtered and washed with PBS until reaching neutral pH. To prepare the activated carrier, the white flocculent precipitate was added to 15 ml of 2% glutaraldehyde and placed in a shaker at 120 rpm for 5 h at 25°C. After washing with PBS repeatedly, the carrier was incubated with 10 ml of 3~14 mg/ml trypsin (Sigma Chemicals, St. Louis, USA) and shaken at 60 rpm for 8 h at 4°C to obtain immobilised trypsin. Finally, the immobilised trypsin was washed with PBS and stored at –20°C. Before and after immobilisation, the concentration of free trypsin was determined by the Lowry method. The enzyme immobilisation rate was calculated using the following equation:

$$R = [(U2 - U1)/U2] \times 100\%$$

where: U1 – concentration of free trypsin after immobilisation; U2 – original concentration of free trypsin before immobilisation

Determination of immobilised trypsin activity. The activity of immobilised trypsin and free trypsin was measured using the method of Klomklao *et al*. (2010), with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate. To determine the optimum pH and temperature of trypsin, the enzyme hydrolysing reaction system was operated at several pH values (pH 2~10) and temperatures (10~80°C).

Preparation of phosvitin and phosvitin phosphopeptides. The preparation of phosvitin was performed as described previously with some modifications (Ko *et al*. 2011). In brief, chicken egg yolk was lightly washed and rolled on filter paper to move any adhering albumen. Then, the yolk membrane was punctured with a needle, and the contents were collected and diluted with 8 volumes of 0.05 M sodium acetate at pH 5.0, stirred at 4°C for 30 min and stored at 4°C for 4 hours. After centrifugation at 10 000 rpm for 15 min at 4°C, the precipitate was collected and extracted with 8 volumes of hexane/ethanol mixture (3 : 1, v/v). Next, the pellet was centrifuged and dried; the pellet was then extracted with 4 volumes of 1.75 M NaCl overnight at 4°C. The suspension was centrifuged at 10 000 g for 15 min at 4°C, and the resulting supernatant was collected and dephosphorised for 40 min at room temperature with 0.1 M NaOH. After reaction, the solution was adjusted to pH 8.0 using 0.1 M HCl, dialysed against distilled water for 24 h at 4°C and freeze dried. A total of 5 of

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purified phosvitin from egg yolk was analysed using 12% SDS-PAGE.

The isolated phosvitin was dissolved in distilled water to a final concentration of 10 mg/ml, and the solution pH was adjusted to 8.0 with NaOH. Next, free trypsin and immobilised trypsin were added at an enzyme-to-substrate ratio of 1 : 20 (w/w), respectively. For the free trypsin group, the mixture was incubated at 40°C for 3 h, which was enough to yield better phosvitin phosphopeptide activity; the resulting phosvitin phosphopeptides were named PPP1. For the immobilised trypsin group, the mixture was incubated at 50°C for 2 h, and the immobilised trypsin was recovered by centrifugation and reused repeatedly for a total of 5 times; the resulting phosvitin phosphopeptides were named PPP2. The tryptic reaction was stopped by adjusting the pH to 5.0 with HCl. Then, the mixture was centrifuged at 3000 rpm for 5 min at 4°C and the supernatant was lyophilised.

Determination of the molar ratio of N/P. The nitrogen content (%) was determined using the Micro-Kjeldahl method with 6.25 as the N-to-protein ratio (Allen 1940), and the phosphorus content (%) was determined using the molybdenum blue colorimetric method (GB/T 5537-2008 standard protocol; China). Each experiment was performed in triplicate for quality control and statistical purposes.

The N/P ratio was calculated using the following equation:

$$N/P = (N\%/P\%) \times (31/14)$$

where: N% – protein nitrogen content; P% – protein phosphorus content

Effects of phosvitin phosphopeptides (PPPs) on Ca-solubilising ability. A total of 0.1 g of PPP1 was dissolved in distilled water by serial dilution to concentrations of 100, 200, 400, 800, and 1000 ppm in the reaction system, and the effect of phosvitin peptides on Ca-solubilising ability was determined by the pH-stat method as described previously (Berrocal *et al*. 1989). To compare the effects on Ca-solubilising ability, 0.1 g of PPP1 or PPP2 was dissolved in distilled water to a final concentration of 400 ppm in the reaction system, and the Ca-solubilising ability was determined as above.

Animal and experimental design. Thirty four-week-old male SD rats were supplied by the Sichuan University Experimental Animal Center. All animals were housed in mesh-bottom plastic cages in a controlled environment and acclimatised for 1 week. A low calcium diet (containing 1.0 mg Ca/g) and water were provided ad libitum. All rats were randomly allocated into five groups and were given 0.5 ml vehicle (1.5% carboxymethyl cellulose), 0.5 ml $CaCl_2$ (0.02 g Ca/ml), 0.5 ml PPP1-Ca, and 0.5 ml PPP2-Ca by gavages once daily for 3 weeks, respectively. For the PPP1-Ca and PPP2-Ca groups, 5.0 mg PPP1 or PPP2 was dissolved in 0.5 ml of 1.5% carboxymethyl cellulose containing 10 mg calcium. All studies were approved by the Institutional Animal Care and Use Committee of Sichuan University. Body weights and food intake quantities were recorded weekly.

Apparent absorption rate of Ca. To determine the apparent absorption rate of calcium, the amount of food consumption was recorded and the faeces were collected every day for 3 weeks. For determination of calcium content, the faeces were dried at 70°C and weighed, then ashed. The ashed sample was then dissolved in 1.0 M HNO_3 for calcium determination with an atomic absorption spectrophotometer (AA-6500F; Shimadzu, Kyoto, Japan) as described previously (Maqueda & Morillo 1990).

The apparent absorption rate of calcium was calculated as follows:

$$\text{Apparent absorption rate of calcium} = [(\text{Intake of calcium} - \text{Faeces calcium})/\text{Intake of calcium}] \times 100\%$$

Determination of serum Ca. To determine the serum calcium content, after fasting for 12 h, the rats were anaesthetised by inhalation of isoflurane and the blood was collected from the eye at 3 weeks. After stayed for 8 h or overnight at 4°C, the serum was collected by centrifuging at 3000 *g* for 10 minutes. The content of serum calcium was determined by atomic absorption spectrometry as above.

Statistical analysis. Results are shown as the means ± SD from the indicated set of experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) by the Student-Newman-Keuls multiple comparison tests with the SPSS 17.0 software. The P values ≤ 0.05 and ≤ 0.01 mean significant and very significant, respectively. Each experiment was performed three times.

RESULTS AND DISCUSSION

Preparation and characterisation of immobilised trypsin and its activity. Chitosan, a natural polymer, has been used previously as a support for enzyme immobilisation (Kapoor & Kuhad 2007). Here, chitosan was employed as a support to prepare the

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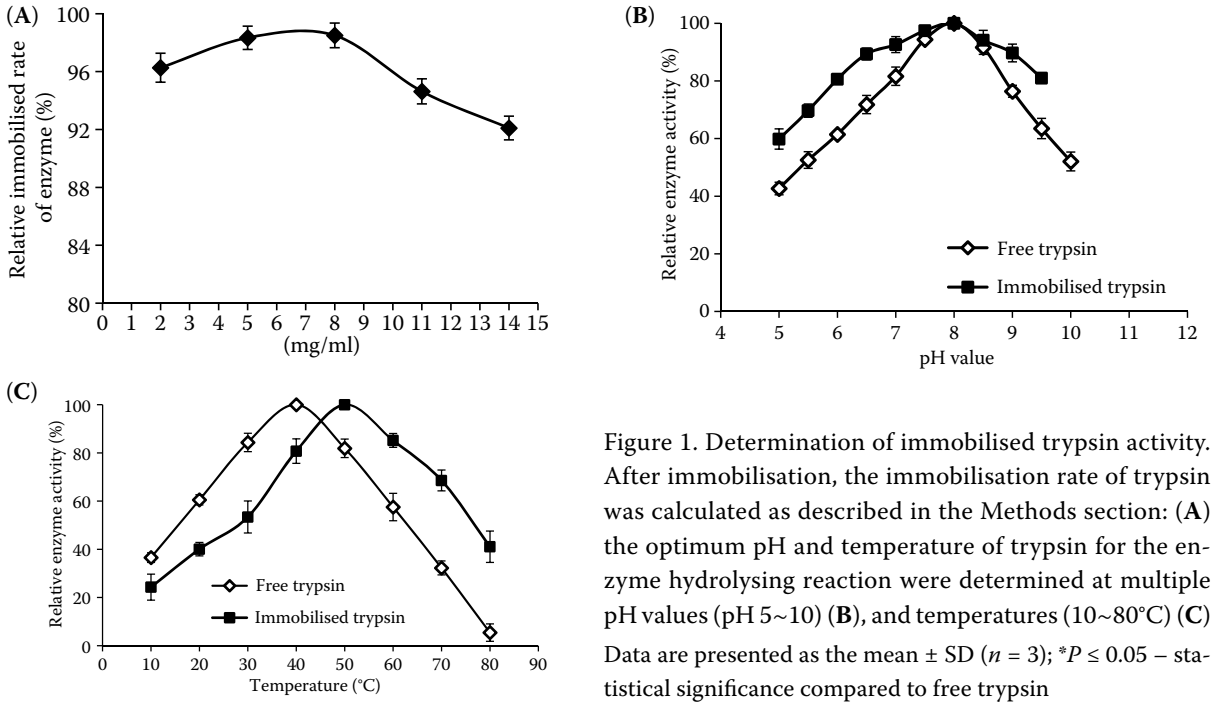

Figure 1. Determination of immobilised trypsin activity. After immobilisation, the immobilisation rate of trypsin was calculated as described in the Methods section: (**A**) the optimum pH and temperature of trypsin for the enzyme hydrolysing reaction were determined at multiple pH values (pH 5~10) (**B**), and temperatures (10~80°C) (**C**)

Data are presented as the mean ± SD (n = 3); *$P \leq 0.05$ – statistical significance compared to free trypsin

immobilised trypsin using a covalent binding method, and the prepared immobilised trypsin demonstrated some characteristics which were similar to those in the previous report (Ling *et al.* 2007). Figure 1A presents the efficiency of trypsin immobilisation. The rate of trypsin immobilisation gradually increased, exceeding 98% at an original enzyme concentration of over 5 mg/ml, and markedly decreased at an original enzyme concentration of over 8 mg/ml, suggesting that the optimal original enzyme concentration for trypsin immobilisation was between 5 mg/ml and 8 mg/ml. Furthermore, the optimum pH and temperature of enzyme activity were evaluated. As shown in Figure 1B, although the optimum pH of immobilised trypsin is similar to that of free trypsin, pH 8.0, the immobilised trypsin has an obvious advantage with its greater adaptability to changes in pH than free trypsin. Figure 1C shows the changes in the optimum temperature of trypsin: the optimum value changed from ~40°C to ~50°C after immobilisation. These results suggest that immobilised trypsin can be well prepared in our reaction system. In addition, the stability of immobilised trypsin was also evaluated. The immobilised trypsin still retains over 90% activity compared to its original activity in 0.01 M Tris-HCl buffer (pH 8.0) for 30 days at –20°C (data not shown).

Preparation of phosvitin phosphopeptides with immobilised trypsin. Next, the immobilised-trypsin enzymolysis technology was employed to degrade phosvitin for the preparation of phosvitin phosphopeptides. Figure 2A shows the SDS-PAGE results for purified egg yolk phosvitin, displaying a major band at ≈ 35 kDa with purity of approximately 90%, which is similar to previous reports (Ko *et al.* 2011; Lei & Wu 2012). In our system, the yield of phosvitin was approximately 150 mg per egg. After dephosphorisation, the purified phosvitin was digested with free trypsin and immobilised trypsin to produce phosvitin phosphopeptides, PPP1 and PPP2, respectively. Our

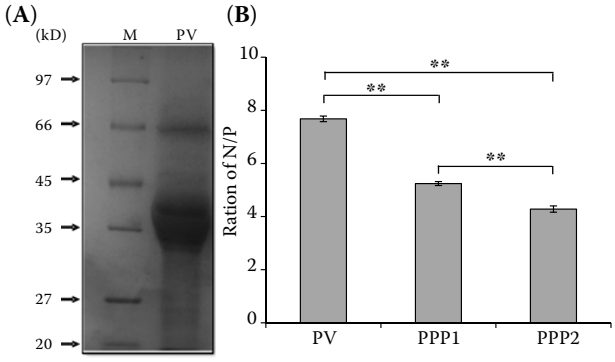

Figure 2. Preparation of chicken egg yolk phosvitin and phosvitin phosphopeptides: (**A**) purified phosvitin from chicken egg yolk analysed by SDS-PAGE and (**B**) the purified phosvitin was digested with free trypsin and immobilised trypsin to produce phosvitin phosphopeptides, named PPP1 and PPP2, respectively

PPP1 and PPP2 were subjected to analysis to determine the N and P contents; N/P ratio was calculated as described in the Methods section; data are presented as the mean ± SD (n = 3); **$P \leq 0.01$ – statistically very significant values

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results show that the N/P ratio of phosvitin phosphopeptides is significantly decreased compared to original phosvitin (5.0 ± 0.08 vs. 7.7 ± 0.12, $P \leq 0.05$) (Figure 2B). Furthermore, PPP2 prepared by immobilised trypsin demonstrate a lower N/P ratio than PPP1 (4.2 ± 0.12 vs 5.4 ± 0.08, $P \leq 0.05$) (Figure 2B), which indirectly provides information regarding the Ca-binding abilities of these compounds.

Ca-solubilising abilities of phosvitin phosphopeptides. To evaluate the Ca-solubilising abilities of phosvitin phosphopeptides, their ability to block calcium phosphate precipitation was investigated by the pH-stat method. Figure 3 presents the time required for the consumption of NaOH in PPP1 and PPP2 reaction systems. The NaOH consumption time was significantly delayed following an increase in PPP1 concentration, with a delay of more than 60 min at a PPP1 concentration of 1000 ppm (Figure 3A), whereas PPP2 delayed the consumption of NaOH more than PPP1 (Figure 3B). Interestingly, even though the immobilised trypsin was used repeatedly for a total of 5 times, PPP2 still delayed the consumption of NaOH more than PPP1. These results suggest that the catalytic activity and stability of immobilised trypsin are retained well even after the material is repeatedly used and that PPP2 have a better Ca-solubilising ability than PPP1. In our system, PPP2 had a better calcium binding ability (≈ 11.5%) than PPP1 (≈ 9.1%) (m/m). In addition, no significant difference in the yield of PPPs was observed between PPP1 and PPP2.

The difference in N/P ratio and Ca-solubilising ability between PPP1 and PPP2 can be explained by the different amino acid composition in phosvitin phosphopeptides, such as the phosphorylated serine moiety, which plays a major role in binding divalent metal ions such as Ca (Li *et al.* 1989; Hansen *et al.* 1996; Kitts 2005). Importantly, the effects of the molecular size of different phosvitin phosphopeptide (PPP) fragments prepared by tryptic digestion on Ca^{2+} binding property are obviously different (Jiang & Mine 2001). The smaller fragment of less than 1 kDa and *O*-phospho-1-serine did not bind Ca^{2+} to any significant extent, while PPP of 1–3 kDa showed a higher ability than commercial casein phosphopeptides (CPP) to render soluble calcium, suggesting that not only the phosphorylated serine moiety or residues are critical for Ca^{2+} binding, but also the molecular size of the phosvitin phosphopeptide fragments (Jiang & Mine 2001). So, it is worthwhile to further investigate whether the amino acid composition and PPP size result in this function difference between PPP1 and PPP2.

Enhancement of the apparent absorption rate of calcium and serum calcium content in vivo by phosvitin phosphopeptides. Furthermore, the effects of phosvitin phosphopeptides on the absorption rate of calcium were evaluated *in vivo*. Figure 4A presents the apparent absorption rate of calcium after treatment with PPP1-Ca and PPP2-Ca, respectively. Both the PPP1-Ca and PPP2-Ca treatments display a higher absorption rate than the $CaCl_2$ and vehicle groups, suggesting that phosvitin phosphopeptides have a beneficial effect on enhancing calcium absorption *in vivo*. Interestingly, compared to PPP1-Ca, the PPP2-Ca group always shows a higher apparent absorption rate of calcium ($P \leq 0.05$). Additionally, PPP2-Ca displayed a consistently greater increase of serum calcium content than PPP1-Ca (Figure 4B), suggesting that PPP2-Ca have a higher efficiency on

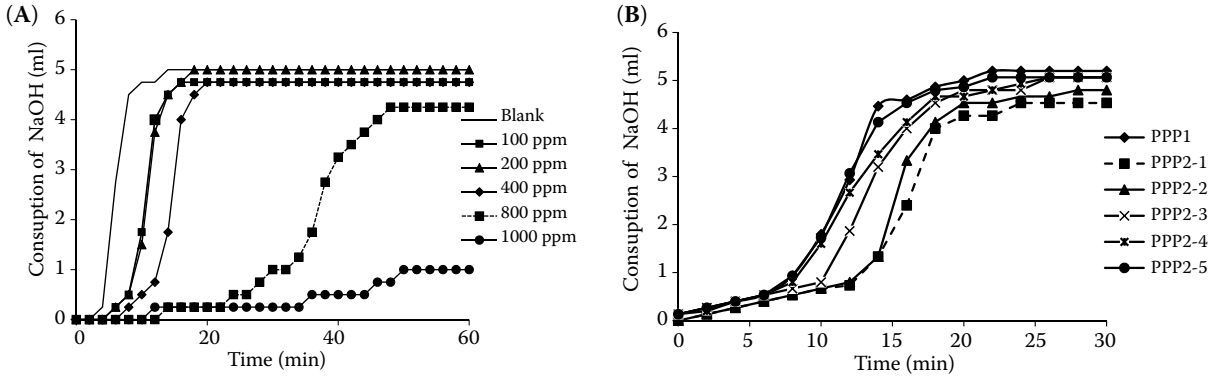

Figure 3. Ca-solubilising abilities of phosvitin phosphopeptides. Ca-solubilising ability was determined by the pH-stat method: (**A**) comparison of effectiveness of blocking calcium phosphate precipitation with different concentrations of PPP1 and (**B**) blocking activity of PPP2 prepared by repeatedly using immobilised trypsin on calcium phosphate precipitation compared to PPP1

PPP2-1~5 represent the products prepared by repeatedly using immobilised trypsin for up to 5 times

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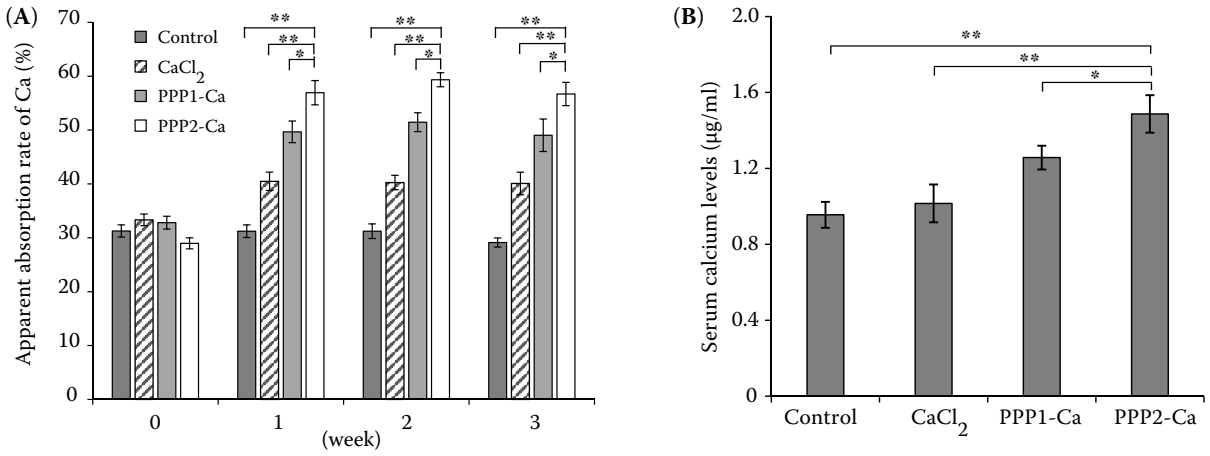

Figure 4. Effects of phosvitin phosphopeptides on the apparent absorption rate of Ca in rats. Twenty-four four-week-old male SD rats were randomly allocated into four groups of 6 rats each: Control (Vehicle), $CaCl_2$, PPP1-Ca, and PPP2-Ca: (**A**) the feces were collected to determine feces calcium content every day for 3 weeks. The apparent absorption rate of calcium was calculated as described in the Methods section and (**B**) at 3 weeks of treatment, the blood was collected from eye after fasting 12 hours

After stayed for 8 h or overnight at 4°C, the serum was collected by centrifuging at 3000 *g* for 10 min; serum calcium was described in the Methods section; data are presented as the mean ± SD ($n = 6$); $^*P \leq 0.05$ and $^{**}P \leq 0.01$ – statistically significant and very significant values, respectively

promoting calcium absorption than PPP1-Ca and $CaCl_2$ supplements *in vivo*.

It has been reported that phosvitin peptides prepared with free trypsin, like PPP1 in our study, significantly enhance the absorption and incorporation of calcium in bones and promote the bone growth (Choi *et al.* 2005). After phosvitin peptide treatment, the average wet weight of tibia and femur bones was higher than that of the control, and the contents of both ash and calcium in bones of phosvitin peptide groups were significantly higher than those of the control group. Additionally, the ratios of Ca/bone weight and Ca/bone ash, which directly provide information on the efficiency of calcium incorporation into bones, are significantly increased by phosvitin peptides. In the present study, we compared the Ca-solubilising ability and Ca absorption rate of PPP1 and PPP2, respectively. PPP2 demonstrated a significantly better calcium-binding efficiency, and obviously enhanced the absorption of Ca and increased the serum Ca content more than PPP1, suggesting that a lower dose of PPP2 can achieve the same effect on enhancing Ca absorption and accumulation *in vivo* as PPP1.

CONCLUSIONS

The effectiveness of immobilised enzymolysis technology for the preparation of PPP2 and the ability of PPP2 to enhance calcium absorption *in vivo* were systematically analysed and discussed. The prepared immobilised trypsin demonstrates many advantages, such as stability, allowing multiple reuses for hydrolysis of phosvitin. Furthermore, PPP2, the phosvitin phosphopeptides prepared with immobilised trypsin, displayed a higher calcium-binding efficiency than those prepared with free trypsin, and greatly increased the rate of calcium absorption and serum calcium content in growing rats. Taken together, our results indicate that the immobilised-trypsin enzymolysis technology can be used as a promising and efficient method to prepare phosvitin phosphopeptides for improved utilisation of PPPs in future industrial applications.

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