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Short Communication

Effect of Casein Phosphopeptides on *In Vitro* Solubilization of Calcium in Fortified Cheese Pizza

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Abstract

Cheese pizzas fortified with casein phosphopeptide (CPP) and calcium were subjected to an *in vitro* digestion to assess whether CPP could prevent the precipitation of calcium. The total calcium content of the cheese pizzas was adjusted to 1,000 mg per pizza (~370 g) with the addition of calcium originating from eggshells. Two levels of trypsin-digested caseins (367 and 459 mg), with a CPP content of ~20%, were added to each pizza. The *in vitro* digested pizzas were then centrifuged and the supernatant was mixed with Na_2HPO_3 at 37℃ to estimate the possible soluble effect of CPP on calcium. After 24 h of reaction, the solution was centrifuged and the calcium content in the resultant supernatant was analyzed by inductively coupled plasma-atomic emission spectroscopy. One-way statistical analyses showed that CPP had a positive effect on the solubilization of calcium against phosphate ($p<0.05$). Cheese pizza supplemented with 459 mg of CPP powder was able to prevent precipitation of calcium by 98.8%, whereas no CPP-added cheese pizza solubilized 86.4% of the calcium. A sensory test was also carried out, revealing that panelists could not discern the bitter taste of the CPP added to the pizzas.

Keywords

in vitro calcium solubilization, casein phosphopeptide, pizza

Introduction

Calcium (Ca) is a crucial mineral for bone health through life span. A 99% of Ca in human is found in a bone, and as bone formation is increasing from baby to adult, more Ca is required. The Koreans should have, on average, 700–1,000 mg Ca daily [1]. Insufficient Ca intake may lead to osteoporosis [2], hypertension and stroke [3], or premenstrual syndrome [4]. According to the 2018 Korea National Health and Nutrition Examination Survey [5], 67.4% of them in 2018 failed to meet the daily requirement, and only 16.9% can take an optimum level of Ca. In light of it, Ministry of Health & Welfare [6] in Korea set its sights on 21% in 2020. Food industries in Korea have made much effort to relieve Ca deficiency in two ways, the addition of Ca, and the use of absorption enhancers for Ca (Ca-absorption enhancer), or both. The Ca-absorption enhancers include vitamin D_3 [7], casein phosphopeptide (CPP) [8], milk basic protein [9], and polycan [10]. According to a study by Kim et al. [11], the number of Ca fortified processed foods was 81 from 1998 to 1999, among which, 10 foods (7 from dairy products and 3 from fruit juice) had vitamin D. No foods were reported to contain CPP. Recent survey by authors, however, found that some dairy products with Ca fortified such as processed cheese and yoghurt contain not only Vitamin D but also CPP. We have not seen the application of CPP to other commercial processed foods, i.e., grain products, drinks, ramyuns, etc.

The active role of CPP as Ca-absorption enhancer can be deduced from the formation of bovine casein micelle. It has been well known that in the formation of casein micelle, calcium is bound to phosphorus of serine moiety of caseins. The attachment of Ca to caseins through serine phosphate would continue until available serine phosphate is depleted [12]. This observation could suggest that caseins that are phosphoprotein can be used as calcium sequestering agent. In fact, many studies [8] have demonstrated that tryptic digestion of caseins [13,14] or phosvitin [15] can help solubilize calcium in the presence of phosphorus. Among the trypsin digested peptides, the active components may be those peptides that comprise 1 or more serine phosphates in cluster, which are CPP. A number of research papers on effect of CPP on Ca are available. In as early as 1980, Mykkanen & Wasserman [16] implied that CPP could facilitate intestinal transport of Ca. Lee [17] reported the mechanism by which CPP helps absorb Ca. Lee [17] fed rats a CPP diet with a ratio of CPP/Ca being 0.35 and discovered that apparent Ca retention and absorption were 35 mg/day and 63.3%, which are higher than control of 23.1 mg/day and 43.0%, respectively. Perego et al. [18] observed that Ca-CPP complex enhanced the uptake of Ca ions in Caco 2 cells. In addition, there are *in vitro* studies. Kim et al. [19] found that CPP prevented the precipitation of Ca in the presence of phosphate. Several similar results were observed in Berrocal et al. [20], Jiang & Mine [15], and Lee et al. [14]. It should be noted that these *in vitro* studies did not evaluate CPP effect when food matrix was fortified with CPP. Instead, they looked at prevention of Ca precipitation when CPP alone was in test solution.

To the best of our knowledge, there is no published paper about effect of CPP on an *in vitro* solubilization of calcium added to food matrix. In our study, therefore, cheese pizza is chosen, for it is popular in especially teenager through the aged, and we decide to investigate how well CPP can solubilize Ca contained in cheese pizza.

Materials and Methods

1. Preparation of CPP powder

Food grade trypsin (Biocatalysts, Nantgarw, UK) was added to a 10% (w/v) sodium caseinate (Tatua, New Zealand) solution at final ratio of 0.1% (w/w) of substrate. Hydrolysis was performed with agitation at 100 rpm at 40℃ for 1 hr. The reaction was stopped by heating at 80℃ for 20 min. Tryptic-digested sodium caseinate were cooled down to 40℃, to which the second enzyme, flavorpro^{TM} (Biocatalysts, Wales, UK), was added at 0.005% of final solution to reduce bitter taste. After 1 hr, temperature was raised to 80℃ and held for 20 min. CPP powder was obtained by spray-drying the whole hydrolysate.

2. Measurement of CCP content

A 10% (w/v) solution of CPP powder was prepared. A pH of the solution was adjusted to 4.6 with 5 N HCl at 25℃, and centrifuged at 4,400 g for 15 min. To the recovered supernatant, the $CaCl_2$ [2% (w/v)] and equal volume of 95% ethanol were added, and stored at 4℃ for 8 hr. The precipitates were obtained by centrifugation at 4,400 g for 15 min, which were then oven dried at 102℃ overnight. Since the precipitates dried were known to have ~90% (w/w) CPP [21], the dried precipitate expressed as a percentage of the CPP powder was multiplied by 0.9. The CPP content of the CPP powder was ~20% (w/w).

3. Preparation of cheese pizzas fortified with Ca and CPP

Total Ca content per cheese pizza was targeted to be 1,000 mg. The Ca content per cheese pizza was predetermined to be ~849 mg, and the difference between the target and predetermined amounts was made up with 506 mg of eggshell powder (NewCal, Poonglim Foods, Korea) that has ~30% (w/w) Ca. The level of CPP addition was determined as follows. The maximum absorption rate of Ca in small intestine is ~50% when the amount of daily intake is 500 mg [22,23]. We, therefore, wanted to take care of the rest of 500 mg Ca. As in the results by Lee et al. [24], the amount of CPP required for 500 mg Ca was 30% of total Ca content. Analysis of cheese pizza showed that it already contained 58.3 mg CPP, and the gap between CCP required (150 mg) and 58.3 mg was filled with the CPP powder. In our study, two levels of CPP, 367 and 459 mg, were added to cheese pizza.

For the uniform distribution of Ca and CPP in the cheese pizzas, the determined amounts of Ca and CPP were thoroughly solubilized in water, to which other ingredients (flour, soybean oil, yeast, sugar, salt, etc.) were then added. Preparation for cheese pizza was made, as described in Choi et al. [25] with some modifications; the weights of dough, tomato sauce, and mozzarella cheese were 230, 30, and 110 g, respectively. The cheese pizzas were kept frozen until chemical analyses and sensory test were done.

4. *In vitro* digestion

As described in Oomen et al. [26], saliva, gastric juice, duodenal juice, and bile solutions were prepared. The thawed whole pizza (~370 g) was finely ground with home blender. An *in vitro* digestion was all carried out at 37℃ with an agitation of 100 rpm. A 30 g of sample was taken, to which 36 mL of saliva was added, and reacted for 15 min. A 72 mL of gastric juice was added and incubated for 2 h. A serial addition of 72 mL of duodenal juice, 36 mL of bile juice, and 24 mL of $NaHCO_3$ was done, and further incubated for 2 hr. The digested solution was finally centrifuged at 4,400 g for 20 min, and the supernatant was collected, and filtered with Whatman #2. To measure soluble Ca in the presence of Na_2HPO_4, 10 mL of filtrate was mixed with 20 mL of 0.24% (w/v) Na_2HPO_4 and 30 mL of distilled water. 10 mL of filtrate was mixed with 50 mL of distilled water, which serves as a control. They were incubated at 37℃ for 24 hr, and centrifuged at 10,000 g for 6 min. An amount of Ca in supernatant was measured with ICP-AES (Optima 8300, Perkin-Elmer, USA). The percent of Ca solubilization was calculated as follows. Total Ca of cheese pizza was also measured with ICP-AES.

$$\text{Ca solubilization (\%)} = \frac{\text{Ca content in supernatant reacted with } Na_2HPO_4}{\text{Ca content in supernatant reacted without } Na_2HPO_4} \times 100$$

5. Sensory analysis

Sixteen panelists were used to evaluate three fortified frozen pizzas that were cooked at 243℃ for 4.5 min in impinger gas conveyer oven (Gasro, Gastec Korea, Korea). Pizza samples were coded with randomly selected 4-digit numbers and served. Panelists were asked to evaluate two attributes, bitterness and graininess on a 1 to 4 scale, with 1 nothing, 2 difficult to detect, 3 slightly detectable, and 4 pronounced.

6. Statistical analysis

The data from total Ca, Ca solubilization, and sensory analysis were analyzed for their significance ($p<0.05$) with one-way ANOVA using the R software. Means were compared by Fisher's protected *t*-test ($p<0.05$).

Results and Discussion

Daily Ca recommendation, although it varies with age, body weight, pregnancy, and lactation, is 700–1,000 mg [1]. Currently, the amount of Ca consumption per person in Korea is about ~500 mg, and the gap of 500 mg should be supplied through either Ca fortified processed foods or Ca supplement. Therefore, eggshell powder (~30% Ca) was selected to fortify one whole cheese pizza to give a total of ~1,000 mg Ca. As one cheese pizza used in this study can serve 2 persons, it can provide 500 mg Ca per person. Having half piece of cheese pizza could satisfy the daily requirement only if one has 500 mg of Ca from other sources.

Calcium content of cheese pizza was predetermined to be ~849 mg per cheese pizza. Since 110 g of mozzarella was topped per pizza, and Ca content of the mozzarella used was determined to be 736±25 g/100 g, most Ca in cheese pizza originated from mozzarella. The second largest contributor of Ca to cheese pizza may be the flour. Khan et al. [27] reported that whole wheat flour had 47.53 mg Ca /100 g, and flour constituted ~60% of our dough. As in Table 1, the total Ca content for 0, 367, and 459 mg CPP powders after Ca fortification was found to be slightly higher than the target. There was no statistical significance in total Ca content ($p<0.05$). This could be attributed to higher Ca content in eggshell powder than ~30% Ca, and variable amounts of Ca contained in flour and mozzarella.

A Ca balance in human body is determined by the relationship among Ca intake,

Table 1. Total calcium content in cheese pizza fortified with Ca and CPP

Variable	CPP powder (mg/370 g)			SEM
	0	367	459	
Total calcium (mg/370 g)	1,226±72[a]	1,187±121[a]	1,165±68[a]	90.3

[a] Values (mean±SD) in the row without a common letter are significantly different ($p<0.05$).
CPP, casein phosphopeptide.

calcium absorption, and excretion. Ingested Ca is absorbed in the proximal small intestine. Loss of Ca absorbed through small intestine takes place in urine, skin, hair, and nail. When daily intake of Ca is 500 mg, the Ca absorption in small intestine is about 32%–50%, and it goes down to 28%–43%, when it is 1,000 mg/day [22]. The intended use of CPP powder is to increase the amount of soluble Ca, so that it enhances Ca absorption in small intestine. In our study, Ca absorption enhancer, CPP, was added to boost the possible Ca availability in small intestine. Table 2 revealed the CPP had an effect on *in vitro* solubilization of Ca in the presence of Na_2HPO_4 ($p<0.05$). When one whole cheese pizza with 1,165 mg Ca was fortified with 459 mg of a CPP powder, 98.5% of Ca present in the supernatant was solubilized. This finding is well in agreement with the results of Kim et al. [19] and Lee et al. [14]. In contrast, no CPP-added cheese pizza was able to solubilize 86.4% Ca. This value is much higher than expected. This is probably attributed to caseins in the mozzarella, which could produce CPP during *in vitro* digestion [8].

One of the prevailing problems limiting the application of CPP powder to the foods is its bitter taste [28]. The bitterness comes from hydrophobic amino acids, especially when hydrophobic residues are located centrally in the peptides rather than at either C- or N- terminal positions [29]. In our study, a tryptic digest of sodium caseinate was treated with flavorpro™ in an effort to debitter tryptic digests. The 16 panelists evaluated cheese pizzas with Ca and CPP powder, and could not detect any bitterness in cheese pizza, as shown in Table 3. Mouthfeel of eggshell powder was not grainy when 506 mg of eggshell powder was to one whole pizza (Table 3).

Conclusion

Cheese pizzas were developed as Ca-absorbing enhancer with the addition of a CPP powder and Ca from eggshell powder. The CPP content corresponding to 30% of total Ca content was found to enhance Ca availability by 12%. The bitter taste and the graininess in mouthfeel that are associated with the addition of Ca and CPP to the

Table 2. Effect of CPP on *in vitro* solubilization of Ca in the presence of Na_2HPO_4

Variable	CPP powder (mg/370 g)			SEM
	0	367	459	
Effect of CPP (%)	86.4±5.5[b]	91.5±4.6[ab]	98.5±1.4[a]	4.21

[ab] Values (mean±SD) in the row without a common letter are significantly different ($p<0.05$).
CPP, casein phosphopeptide.

Table 3. Sensory scores for cheese pizzas fortified with Ca and CPP

Attributes	CPP powder (mg/370 g)			SEM
	0	367	459	
Bitterness	1.75±0.86[a]	1.87±1.09[a]	1.87±1.02[a]	0.99
Graniness	1.56±0.81[a]	1.68±0.60[a]	1.25±0.45[a]	0.63

Values (mean±SD) are on a 1 to 4 scale, with 1 nothing, 2 difficult to detect, 3 slightly detectable, and 4 pronounced.
[a] Values (mean±SD) in the same row without a common letter are significantly different ($p<0.05$).
CPP, casein phosphopeptide.

cheese pizza were not detected.

Conflict of Interest

The authors declare no potential conflict of interest.

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