Note

Absorption of Zinc from Dietary Casein Phosphopeptide Complex with Zinc in Rats Given a Soybean Protein-Based Diet

Tohru MATSUI, Hiroaki OKUMURA and Hideo YANO

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawaoiwake-cho, Sakyo-ku, Kyoto, 606–8502, Japan

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Summary The absorption of zinc (Zn) in the form of casein phosphopeptide (CPP) complex was compared with that of its chloride form in rats given a soybean protein-based diet. We prepared $^{67}$Zn complex with CPP ($^{67}$Zn-CPP). Male rats were given a diet containing ZnCl$_2$ in the preliminary period. After overnight food deprivation, they were fed a test meal labeled $^{67}$Zn-CPP or $^{67}$ZnCl$_2$ (4 g Zn-free diet + 0.12 mg $^{67}$Zn) with 0.5 mg Dysprosium (Dy) as a fecal marker. Feces were collected for 5 d and analyzed for $^{67}$Zn isotopic enrichment and Dy concentration with an inductively coupled plasma mass spectrometer. Fecal Dy excretion closely paralleled that of $^{67}$Zn, and more than 89% of the administered Dy was recovered in the feces collected for 5 d. Moreover, the Zn source did not affect the excretion pattern of $^{67}$Zn. Since Dy and $^{67}$Zn were almost excreted within 3 d after the administration, the apparent absorption of $^{67}$Zn was calculated from the pooled data for 3 d. The source of Zn did not affect the apparent absorption of $^{67}$Zn. These results suggested that dietary Zn in the form of CPP complex did not show higher absorbability in rats.

Key Words zinc absorption, casein phosphopeptide, stable isotope, rat

The availability of zinc (Zn) in milk is well known to be high (1). Studies have shown that milk-derived ligands of Zn affect its absorption (2–4). Our previous study showed that Zn-binding peptide(s) was produced in the digestive tract of pigs given a skim milk powder-based diet and suggested that this peptide stimulated Zn absorption (5). Casein phosphopeptides (CPPs) are found in the digesta of rats given casein (6), and these peptides can bind to Zn (7). Therefore it is likely that CPPs produced in the digestive tract stimulate Zn absorption. Dietary CPP was reported to stimulate Zn absorption in human (8). Although soybean products contain a large amount of phytate that strongly interferes with zinc availability (9, 10), dietary CPP is also reported to eliminate the suppressive effect of phytate on Zn absorption in rats (11). Pérès et al. (12) report that the absorption of Zn in the form of a complex with CPP (Zn-CPP) is greater than in the form of sulfate using the perfused rat intestinal loop system. Therefore Zn-CPP is possibly used as a highly available Zn supplement in human and animals consuming soybean products.

However, no study has shown the absorption of Zn from dietary Zn-CPP. We compared the absorbability of Zn from dietary Zn-CPP with ZnCl$_2$ in rats given a phytate-rich diet based on soybean products using a stable isotope of Zn.

Materials and Methods

Preparation of $^{67}$Zn complex with casein phosphopeptide. Cation-free CPP was obtained from Meiji Milk Products Co. Ltd. (Higashimurayama, Japan). More than 90% CPP was the fragment of $\beta$-casein that constituted its first 28 amino acid residues from the N-terminal end (13). Enriched $^{67}$Zn (90.6%) as oxide was obtained from TV Isoflex (Moscow, Russia). We prepared $^{67}$Zn-CPP according to the method reported by Peres et al. (12). Briefly, enriched ZnO was dissolved in 6M HCl to transform the oxide into soluble ZnCl$_2$, and the solution’s pH was then adjusted to 5 by NaHCO$_3$. Casein phosphopeptide was dissolved in the solution and kept at room temperature for 1 h, at which time unbound Zn was removed by ultracentrifugation (3,000-Da cutoff; Centriprep-3, Amicon, Tokyo). The molar ratio of Zn/CPP was 3.1 in the final solution. This ratio was similar to that of Zn-CPP described by Peres et al. (12). The resulting complex was freeze-dried.

Animals and diets. Ten male Wistar rats weighing 200 g each were purchased from Japan SLC (Shizuoka, Japan) and were cared for according to the Guide for the Care and Use of Laboratory Animals (Animal Care Committee, Kyoto University). The rats were individually housed in metabolism cages in a room with controlled temperature (22°C), relative humidity (60%), and lighting (light, 7:00–19:00; dark, 19:00–7:00). They received a diet ad libitum during the dark period and allowed free access to demineralized water. The basal diet contained (g/kg) soybean protein isolate 200, starch 529.5, sucrose 100, corn oil 70, cellulose powder 50, Zn-free mineral mixture (based on AIN-93G) 35, vitamin mixture (AIN-93G) 10, L-cysteine 3, choline bitartrate 2.5. The concentration of Zn was 13.0 mg/kg in the basal diet. This diet contained 3.0 g/kg phytic acid. One hundred milliliters of aqueous
solution containing 3 mg Zn as ZnCl₂ was added to the basal diet (100 g) to form a semiliquid feed just before feeding (nonlabeled diet). The labeled meal was prepared by the addition of 100 mL aqueous solution containing 3 mg ⁶⁷Zn as ZnCl₂ or Zn-CPP and 12.5 mg Dysprosium (Dy) to 100 g basal diet.

**Experimental protocol.** The experimental protocol was similar to the report of Coudray et al. (14). In the 10 d preliminary period, the rats were fed the nonlabeled diet. The day before isotope administration, a 24 h fecal sample was collected from each animal. At the initiation of a dark period (19:00), 4 g labeled meal was offered to 12 h starved rats for 2 h. Eight rats completely consumed the meal. The amount of unconsumed meal was less than 0.3 g in 2 rats given the meal supplied with Zn-CPP. The rats were then given the nonlabeled diet for 10 h. In the following 4 d, the rats were given the nonlabeled diet during the dark period. Feces were collected at 1 d intervals for 5 d after the isotope administration.

**Analyzes.** Diets and feces were dried by an airflow oven and powdered. Subsamples (1 g) were digested by concentrated HNO₃ and 30% H₂O₂. The acid-digested samples were diluted by 0.1 M HNO₃ to an appropriate concentration to measure Zn concentration with an atomic absorption spectrophotometer (AA-6600E, Shimadzu, Kyoto, Japan). After Zn concentration was diluted to 100 pg/L by 0.1 M HNO₃, the isotope ratio of Zn was determined by an inductively coupled plasma mass spectrometer (Elan 6000, Perkin Elmer, USA). The concentration of Dy was determined as ¹⁶⁶Dy by the inductively coupled plasma mass spectrometer.

**Calculation and statistical analyses.** Fecal ⁶⁷Zn that had been derived only from the administered isotope was calculated by the equation reported by Coudray et al. (14). The apparent absorption of ⁶⁷Zn was calculated from the following formula:

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\text{Apparent absorption} = \frac{\text{administered } ⁶⁷\text{Zn} - (\text{fecal } ⁶⁷\text{Zn derived only from the administered isotope}) \times \text{ingested } \text{Dy}}{\text{administered } ⁶⁷\text{Zn}}.
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The data on the daily excretion of Dy or ⁶⁷Zn were analyzed by the MIXED PROCEDURE of SAS (15) in which the effects of days after administration as repeated measurements, Zn source, and their interaction were tested. To compare the excretion pattern of ⁶⁷Zn with that of Dy, the cumulative excretion of ⁶⁷Zn or Dy were expressed as a percentage of total excretion for 5 d. First the pattern of ⁶⁷Zn excretion was analyzed by the MIXED PROCEDURE of SAS (15). The model included days after administration as repeated measurements and Zn source and their interaction. Because the effects other than days after administration were not significant, these data were pooled and the excretion patterns of ⁶⁷Zn and Dy were reanalyzed by excluding the effect of Zn source in the second analysis. The data on total recovery of fecal Dy or ⁶⁷Zn absorption were tested by one-way ANOVA using the GLM procedure of SAS (16).

The data expressed as percentages were transformed by arcsine calculation before analyses to normalize their distributions (17).

**Results and Discussion**

A large amount of administered Dy and ⁶⁷Zn were excreted within a day (Fig. 1). The excretion of Dy and ⁶⁷Zn were then rapidly decreased, and almost all of Dy and ⁶⁷Zn were excreted for 3 d. Fecal Dy excretion closely paralleled that of ⁶⁷Zn, and more than 89% of the administered Dy was recovered in the feces collected for 5 d (Table 1). Furthermore, the Zn source did not affect the excretion pattern of ⁶⁷Zn. Coudray et al. (14) suggested that Dy could be used as a quantitative fecal marker in the ⁶⁷Zn absorption study of rats because fecal Dy excretion was nearly complete (<95%) in most of the rats, and ⁶⁷Zn and Dy exhibited almost identical fecal excretion patterns. Although the recovery of Dy was slightly less in the present experiment than in their report, we considered that the recovery of Dy was sufficient in the present experiment. Since Dy and ⁶⁷Zn were almost excreted within 3 d after the administration, the pooled data on fecal Dy and ⁶⁷Zn during this period were used to calculate the apparent absorption of ⁶⁷Zn.

The form of Zn did not affect the apparent absorption of ⁶⁷Zn in the rats given the soybean protein-based diet containing phytate (Fig. 2). The solubility of ZnCl₂ is reported to be very high in aqueous solution, which suggests that this zinc salt is highly absorbable (18). Seal and Heaton (19) reported that Zn absorption did not differ between rats given diets containing ZnSO₄ and ZnCl₂. Because these Zn salts are frequently used as Zn sources, we presume that Zn-CPP does not improve Zn absorption in rats given a soybean protein-based diet. An intestinal perfusion study showed that Zn in the form of Zn-CPP was more efficiently absorbed than in the form of sulfate (12).
Table 1. Fecal excretion patterns of administered Dysprosium (Dy) and $^{67}$Zn as the complex with casein phosphopeptide (Zn-CPP) or ZnCl$_2$.

| Day | ZnCl$_2$ | Zn-CPP | Zn source | ZnCl$_2$ | Zn-CPP |
|-----|----------|---------|-----------|----------|---------|
|     | Dy excretion$^{ac}$ | $^{67}$Zn excretion$^{ac}$ |           |          |         |
| 1   | 65.58±2.42 | 61.06±6.12 | 63.28±3.25 | 58.30±6.61 |
| 2   | 97.28±0.47 | 95.45±1.38 | 95.86±0.39 | 93.6±1.06 |
| 3   | 99.85±0.04 | 99.51±0.10 | 99.06±0.07 | 98.30±0.08 |
| 4   | 99.99±0.02 | 99.82±0.08 | 99.68±0.02 | 99.22±0.14 |
| 5   | 100.00    | 100.00   | 100.00    | 100.00   |
| Recovery (%)$^{b}$ | 92.37±2.39 | 89.69±1.23 |           |          |

Values are means±SE for 5 rats.

$^a$ The excretion pattern of Dy and $^{67}$Zn were expressed as a cumulative percentage of excreted elements for 5 d (total excretion).

$^b$ The ratio of excreted Dy for 5 d to administered Dy.

$^c$ Effect of day ($p<0.001$), effect of Zn source ($p=0.38$), effect of parameter ($p=0.38$), interactions ($p>0.40$).

$^d$ Effect of Zn source ($p=0.28$).

Zn-CPP and ZnCl$_2$ in the present experiment. Since ZnSO$_4$ and ZnCl$_2$ are similarly absorbed, the difference of inorganic Zn source does not explain the inconsistency between the intestinal perfusion study and the present experiment.

Hansen et al. (11) reported that dietary CPP stimulated Zn absorption in rats given a soybean protein-based diet. They also indicated that the addition of CPP increased Zn solubility in aqueous solution containing phytate. These results suggested that ingested CPP interacted with Zn in the digestive tract and increased Zn solubility, which enhanced Zn absorption. They gave CPP as a sodium or calcium complex and the estimated molar ratios of CPP/Zn were approximately 59 and 32, respectively. On the other hand, we supplied CPP as Zn complex to the labeled meal in which the molar ratio was approximately 0.3. If intact Zn-CPP reaches the small intestine, the major site of Zn absorption (20), Zn absorption is probably stimulated. Therefore we believe that Zn dissociates from CPP under the acidic condition in the stomach in rats administered Zn-CPP. When CPP concentration is high in small-intestinal digesta, CPP can make complex with Zn because pH increases in the small intestine. Therefore a large amount of dietary CPP enhances Zn absorption, as reported by Hansen et al. (11). On the other hand, the amount of administered CPP was very small in the present experiment. It is known that CPP can also bind to other cations such as calcium in the small intestine (21). Zinc and calcium may competitively bind to CPP in the small intestine. We suggest that the formation of Zn-CPP in the small intestine is not sufficient to stimulate Zn absorption in the present experiment. In conclusion, Zn-CPP is not superior to ZnCl$_2$, as a Zn supplement in rats fed a soybean protein-based diet.

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