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Chloroform Fraction of *Panax Ginseng* Extract Enhances Zip4-Mediated Zinc Influx into the Cytosol

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Zinc is an essential nutrient with important biological functions, and its deficiency can lead to several diseases. The zinc transporter families, ZIP and ZNT, play essential roles in regulating zinc homeostasis and dynamics in the body and cells. Specifically, ZIP4 is the primary zinc transporter responsible for zinc absorption in the small intestine. Previous studies have shown that *Panax ginseng* (*P. ginseng*) extract can promote mouse Zip4 expression, and ginsenosides, including Rc and Re, enhance zinc uptake. However, the effects of other metabolites present in *P. ginseng* extract remain unclear. Therefore, we fractionated *P. ginseng* extract using chloroform, ethyl acetate, and n-butyl alcohol, and evaluated the effect of each fraction on zinc uptake using mouse Hepa and Hepa/MRE-Luc cells that stably expressed luciferase under the promoter of metal-responsive elements. Luciferase activity assays demonstrated that the chloroform (F1), ethyl acetate (F2), and n-butyl alcohol (F3) fractions increased cellular zinc uptake. In particular, F1 fraction was found to induce *Zip4* mRNA and protein expressions, which significantly enhanced zinc uptake. Ginsenosides were mainly present in the F2 and F3 fractions, indicating that metabolites other than ginsenosides in the F1 fraction would enhance zinc uptake by inducing *Zip4* mRNA and protein expressions. Our study offers novel insights into the molecular mechanisms underlying zinc uptake by *P. ginseng*.

Key words *panax ginseng* C. A. Meyer, chloroform fraction, transporter, zinc, Zip4

INTRODUCTION

Zinc is an essential nutrient, and its deficiency leads to several physical disorders including dysgeusia, anorexia, dermatitis, diarrhea, alopecia, hypogonadism, and immune system dysfunction.[1-4] Owing to this, zinc homeostasis is important and is regulated mainly by two zinc transporter families, zinc transporters (ZNT) and Zrt-, Irt-like protein (ZIP) transporters.[5] These transporters belong to the solute carrier family and do not require ATP hydrolysis for zinc transfer. The ZNT family accelerates zinc mobilization from the cytosol into the extracellular space or lumen of intracellular compartments. In contrast, the ZIP family promotes zinc influx into the cytosol from the extracellular space or lumen of intracellular compartments. Among these, ZIP4 (SLC39A4), which is present in the small intestine, plays an essential role in dietary zinc absorption.[6] Therefore, human *ZIP4* mutations cause severe zinc deficiency within a few days to weeks after birth, resulting in acrodermatitis enteropathica.[7] Several studies on ZIP4 expression have revealed its regulatory mechanisms. During zinc deficiency, *ZIP4* mRNA is stabilized and ZIP4 degradation is attenuated, which results in ZIP4 accumulation on the apical membranes of enterocytes in the small intestine.[8-10] Therefore, chemicals that enhance ZIP4 expression may be used as avenues to develop new strategies for preventing zinc deficiency. For screening such chemicals, mouse Hepa cells have been used, since this cell line, among several mouse cell lines examined, mimics the regulation of Zip4 expression in the small intestine, in a zinc concentration-dependent manner with multiple post-transcriptional mechanisms.[10-12] Screening of foods and food chemicals using Hepa cells showed that soybean extracts and soyasaponin Bb increase Zip4 expression.[11]

We screened herbal medicines for Zip4 inducing activity in our previous study; the study that used mouse Hepa cells revealed that the methanol extract from *Panax ginseng* (*P. ginseng*) induced *Zip4* mRNA and protein expressions, which resulted in higher intracellular zinc levels.[13] *P. ginseng* contains various triterpene saponins, that is, ginsenosides, which are the major bioactive compounds that contribute to several pharmacological actions, such as anti-inflammatory,[14,15] anti-cardiovascular,[16] anti-obesity[17], and anticancer properties.[18] More than 70 ginsenosides have been identified in *P. ginseng*. Among them, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2 were the major constituents.[19] Our previous study

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showed that Rc and Re are potential metabolites that increase intracellular zinc levels.[13] However, it is unclear which metabolites in *P. ginseng* extract induce Zip4 expression or whether metabolites other than ginsenosides are involved in zinc uptake. In addition to ginsenosides, *P. ginseng* contains various biological metabolites, including polyacetylenes, polysaccharides, alkaloids, and phenolic acids.[19] Therefore, in this study, *P. ginseng* extracts were fractionated with chloroform, ethyl acetate, and n-butyl alcohol. Following this, the effect of these fractions on zinc uptake and Zip4 expression were determined using mouse Hepa and Hepa/MRE-Luc cells.

MATERIALS AND METHODS

Materials Dried root of *P. ginseng* C. A. Meyer (*Panax schinseng* Nees) (*Araliaceae*) (China, lot. 008617007) was obtained from Tochimoto Tenkaido (Osaka, Japan). Falcarinol was purchased from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals used were of the highest available grade.

Preparation of *P. Ginseng* Extracts *P. ginseng* methanol extract (F0) was prepared as described in our previous study.[13] Herein, 51.4 g of dried *P. ginseng* powder was extracted three times with 100 mL of 100% methanol under stirring at 25-27°C for 50 min and sonication for 10 min. The filtrates were then pooled and evaporated to dryness using a rotary vacuum evaporator at 40°C (F0, 4.80 g, 9.34% w/w). The residue was dissolved in equal volumes of distilled water and chloroform. The extract was then fractionated using equal volumes of ethyl acetate and n-butyl alcohol (Fig. 1). All fractions were concentrated using a rotary vacuum evaporator at 40°C or freeze-dryer at -80ºC. Residues of the chloroform (F1, 383.25 mg, 0.75% w/w), ethyl acetate (F2, 73.80 mg, 0.14% w/w), n-butyl alcohol (F3, 1129.10 mg, 2.20% w/w), and water (F4, 3063.59 mg, 5.96% w/w) fractions were dissolved in dimethyl sulfoxide or distilled water, sterilized through filtration with a 0.22 μm filter, and stored at -20°C until use. F0 fraction used for bioassay have been prepared in our previous study.[13]

Cell Culture and Stable Transfection Mouse Hepa cells, kindly provided by Dr. Glen K. Andrews (University of Kansas Medical Center), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in an incubator humidified with 5% CO_2.[13] To generate a zinc-deficient conditioned medium (CX), heat-inactivated FBS was treated with Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA, USA) as described previously.[13]

In our previous study, to monitor changes in intracellular zinc levels, a luciferase reporter plasmid, pGL4.26-MRE, containing five tandem repeats of a metal-responsive element (MRE) was constructed.[13] The hygromycin resistance coding region in pGL4.26-MRE was replaced with the neomycin resistance coding region from pGL4.18 (Promega, Madison, WI, USA) using *Bam*HI/*Sal*I restriction enzymes to generate the plasmid pGL-MRE-Neo. Hepa cells were transfected with the luciferase reporter plasmid pGL-MRE-Neo using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After transfection, cells stably harboring the plasmid were selected using 5.0 mg/mL G418 (Nacalai Tesque, Kyoto, Japan), and a large number of G418-resistant clones (Hepa/MRE-Luc cells) were pooled into one dish.

Cytotoxicity Assay The conversion of WST-8 to formazan by viable cells was quantified using a Cell Counting kit-8 (Dojindo, Kumamoto, Japan) as previously described.[13] Briefly, Hepa and Hepa/MRE-Luc cells in 96-well plates were incubated for 24 h with DMEM supplemented with 10% FBS in the absence or presence of various concentrations of each fraction. The precipitation of components was not observed in F0 at the concentration of 1000 μg/mL under microscopic observation. After addition of Cell Counting kit-8 solution and incubation for 1 h, viable cells were evaluated by measuring the absorbance at 450 nm (reference at 630 nm) using a Varioskan LUX (Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Assay Hepa/MRE-Luc cells were subcultured in 24-well plates at a density of 3.0×10^5 cells for 24 h and incubated for 24 h with DMEM supplemented with 10% FBS in the absence or presence of various concentrations of each fraction. Cells were dissolved in Glo Lysis buffer (Promega), and the protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific) to normalize luciferase activity. Luciferase activity was measured with a GloMax 20/20 Luminometer (Promega) using the ONE-Glo Luciferase assay system (Promega) according to the manufacturer's instructions.

Zinc Uptake Zinc uptake was quantified using $^{65}ZnCl_2$ as previously described.[13] Briefly, Hepa cells were seeded in 24-well plates at a density of 3.0×10^5 cells, incubated for 18 h, and treated for an additional 24 h with DMEM supplemented with 10% FBS in the absence or presence of various fraction concentrations. The cells were then washed and incubated with 0.5 μM $ZnCl_2$ containing 1.13 nM $^{65}ZnCl_2$ (11.1 kBq/well) in zinc-deficient conditioned medium at 37°C for one hour. Cell-associated radioactivity was measured using a 2480 Wizard^2 automatic gamma counter (Perkin Elmer, Waltham, MA, USA). Zinc uptake rates were calculated and normalized to the protein content determined using a BCA Protein Assay Kit.

RNA Extraction and Quantitative RT-PCR Total RNA extraction and reverse transcription for real-time PCR were performed as previously described.[13] Real-time PCR was performed using the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) and a LightCycler 96 (Roche, Basel, Switzerland). The primer sequences used for this study were as follows: Zip4 forward, 5'-TGGCACCAAGCAATCTCCGAC-3'; Zip4 reverse, 5'-CAGCCCAGTCTTCCACGAG-3'; β-actin forward, 5'-GACGGCCAGGTCATCACTATTG-3'; β-actin reverse, 5'-CCACAGGATTCCATACCCAAGA-3'; Mt1 forward, 5'-GCTGTGCCTGATGTGACGAA-3'; Mt1 reverse, 5'-AGGAAGACGCTGGGTTGGT-3'. Relative *Zip4* mRNA expression levels between the samples were calculated using the standard curve method, with the expression level of *β-actin* mRNA as a reference gene.

Immunoblot Analysis Immunoblot analysis was performed as previously described.[13] Briefly, membrane proteins were isolated, using a tight-fitting Dounce homogenizer, from Hepa cells which were incubated for 24 h with normal medium or zinc-deficient conditioned medium in the absence or presence of each fraction at 37°C. After determining the protein concentration using the BCA Protein Assay Kit, proteins were separated on an SDS-PAGE gel. The primary antibodies used for this study were as follows: monoclonal anti-Zip4 antibody (1:1000 dilution), which was generated in our previous study and detects the extracellular amino-terminal portion[11],

and monoclonal anti-Na,K-ATPase antibody (Cell Signaling Technology, Danvers, MA, USA) (1:1000 dilution).

UPLC-UV-MS Analysis Each fraction of *P. ginseng* extract was analyzed using an ACQUITY ultra-performance liquid chromatography (UPLC) system with a QDa mass detector (Waters Corp., Milford, MA, USA) under the following conditions: column, ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm); H_2O (solvent A)/acetonitrile (solvent B) gradient containing 0.1% formic acid, 0–2 min, 5–33% B; 2–9 min, 33–38% B; 9–16 min, 38–100% B; 16–16.5 min, 100–5% B; and 16.5–20 min, 5% B; UV detection, absorbance measurements from 210 nm to 700 nm with a PDA eλ Detector (Waters Corp.); flow rate, 0.3 mL/min; column temperature, 40°C; injection volume, 2 μL. The QDa conditions were set as follows: positive or negative scan mode = 100–1000 Da; cone voltage, 30 V; capillary voltage = 0.8 kV, and source temperature, 600°C. The detection of ginsenosides (Rd, Re, Rf, Rg1, and Rg2) in each fraction was performed as previously described.[13]

Statistical Analysis ANOVA with Dunnett's test was used to perform multiple comparisons. Differences were considered statistically significant at $P < 0.05$ (two-tailed).

RESULTS AND DISCUSSION

Cytotoxicity of *P. Ginseng* Extraction Fraction In this study, we first fractionated the crude extract of *P. ginseng* to investigate the various effects of metabolites other than ginsenosides. The crude extract (F0) was fractionated using equal volumes of chloroform (F1), ethyl acetate (F2), n-butyl alcohol (F3), and distilled water (F4) (Fig. 1). Additionally, we established a Hepa cell line stably harboring the luciferase reporter plasmid pGL-MRE-Neo (Hepa/MRE-Luc cells) for more stable analysis of intracellular zinc levels. Using these cells, we evaluated the cytotoxicity of each fraction (F1–F4). Hepa and Hepa/MRE-Luc cells were incubated with various concentrations of each fraction for 24 h and then the relative absorbance of formazan, an index of viable cells, was measured. Although F1 and F2 were cytotoxic at high concentrations, no cytotoxicity was observed at low concentrations. F3 and F4 showed almost no cytotoxicity to Hepa and Hepa/MRE-Luc cells at concentrations below 400 μg/mL (Fig. 2).

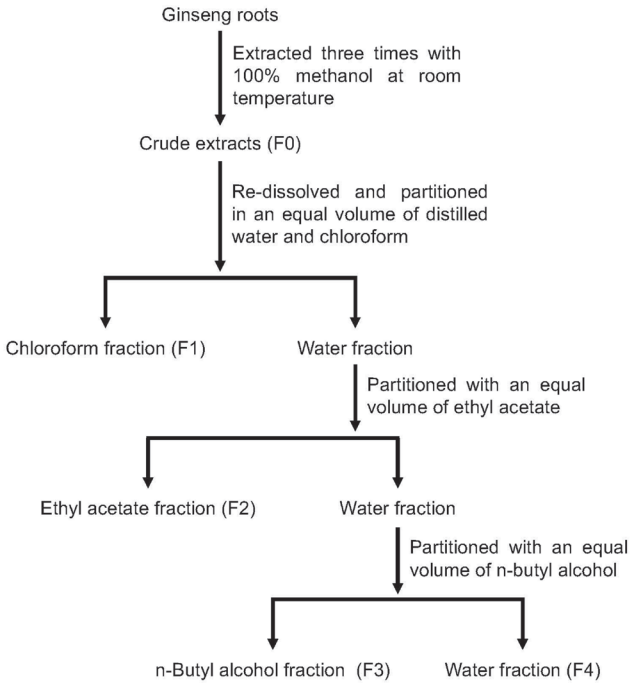

Fig. 1. Preparation of Different Polar Fractions from *P. Ginseng*

Effects of Each Fraction on Intracellular Zinc Levels To evaluate the effect of each fraction on luciferase activity driven by intracellular zinc levels, Hepa/MRE-Luc cells were incubated for 24 h in the presence of each fraction at a concentration that showed no cytotoxicity (Fig. 3). Luciferase activity in cells incubated in zinc-deficient medium (CX) was significantly lower than that in control cells (normal medium). Consistent with our previous study using Hepa cells, the crude extract of *P. ginseng* (F0) (1000 μg/mL) increased the luciferase activity.[13] Treatment with 100 μg/mL F1, 100 μg/mL F2, and 400 μg/mL F3 showed a significant increase in luciferase activity. The F3 fraction did not show a concentration-dependent increase. In our previous study,[13] ginsenoside Rc and Re increased intracellular zinc levels only at certain concentrations (1 μM and 2.5 μM, respectively) but not at other concentrations. Similarly, the F3 fraction might increase intracellular zinc concentration only at specific concentrations. Treatment with F4 also showed the tendency to increase the luciferase activity at some concentrations. These results suggest that the intracellular zinc levels in Hepa/MRE-Luc cells were increased by treatment with these fractions, especially F1, F2, and F3. Therefore, we examined whether these fractions affected zinc uptake into the cytosol from the medium using $^{65}ZnCl_2$ and Hepa cells. After cells were treated with each fraction for 24 h, they were incubated with a medium containing $^{65}ZnCl_2$ for one hour. As depicted in Fig. 4, the zinc uptake was significantly increased by the addition of each fraction. At these concentrations, F1 potently enhanced zinc uptake into Hepa cells. The results of zinc uptake after F4 treatment were not consistent with luciferase activity. This inconsistency may be due to the differences detected between these two tests. The luciferase activity experiment reflected the cytosolic zinc levels during 24 h of treatment, whereas the zinc uptake test reflected the zinc imported from the medium for only one hour after 24 h of treatment. Therefore, the mechanism of F4 in zinc uptake by Hepa cells might be different from those of F1–F3. We performed subsequent experiments with F1–F3 since we observed significant differences in these fractions in both experiments.

Effects of *P. Ginseng* Fractions on Expression of Metallothionein 1 and Zip4 We examined the effects of F1–F3 fractions on the expression of *metallothionein 1* (*Mt1*) mRNA. Because the MT1 promoter has an MRE, the assessment of *Mt1* mRNA expression reflects the change in intracellular zinc levels.[11] We previously reported that F0 (*P. ginseng* extract) increases *Mt1* mRNA expression.[13] In this study, we treated Hepa cells for 24 h with F0–F3 fractions at the indicated concentrations and examined the effects on *Mt1* mRNA expression (Fig. 5A). *Mt1* mRNA expression was significantly increased following the addition of 100 μg/mL of F1 and F2. Treatment with F3 at 400 μg/mL also tended to increase *Mt1* mRNA expression ($P = 0.09$). Zip4 is a well-studied zinc transporter that contributes to zinc uptake in Hepa cells. Therefore, we previously examined the F0 effect on Zip4 expression and

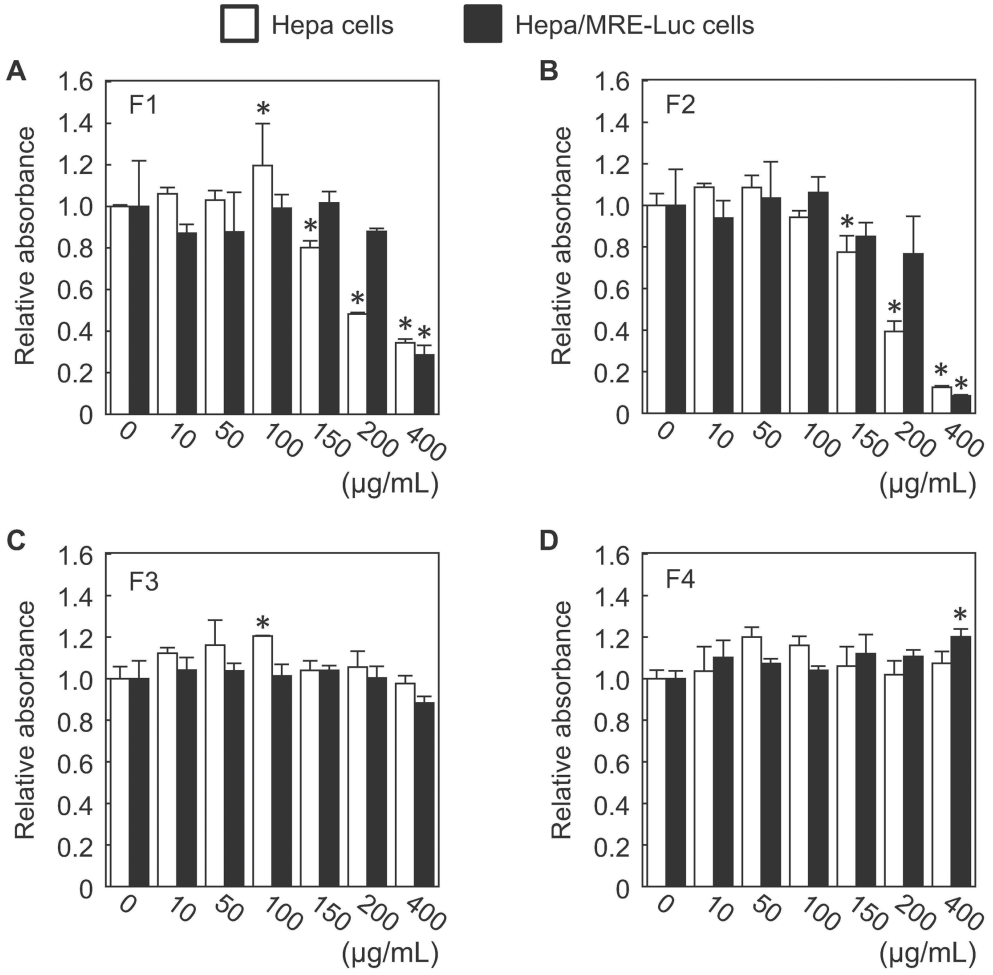

Fig. 2. Cytotoxicity of *P. Ginseng* Fractions

The cytotoxicity of each fraction against Hepa and Hepa/MRE-Luc cells was evaluated using a Cell Counting kit-8. Statistical significance was determined using ANOVA with Dunnett's test. **P* < 0.05, significantly different from control (0 μg/mL). Each bar represents the mean ± S.D. (n = 3).

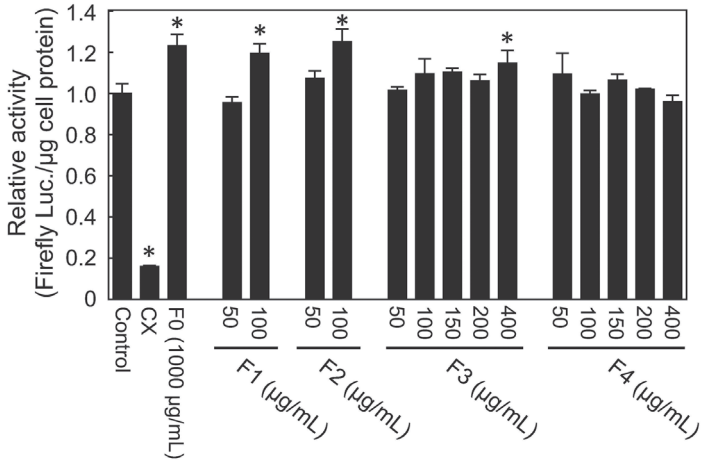

Fig. 3. Effects of *P. Ginseng* Fractions on Luciferase Activity Driven by Intracellular Zinc Levels

Hepa/MRE-Luc cells were incubated for 24 h in normal medium (control), zinc-deficient medium (CX), and in the presence of the indicated concentrations of *P. ginseng* extract fraction in normal medium. Statistical significance was determined using ANOVA with Dunnett's test. **P* < 0.05, significantly different from the control group. Each bar represents the mean ± S.D. (n = 3).

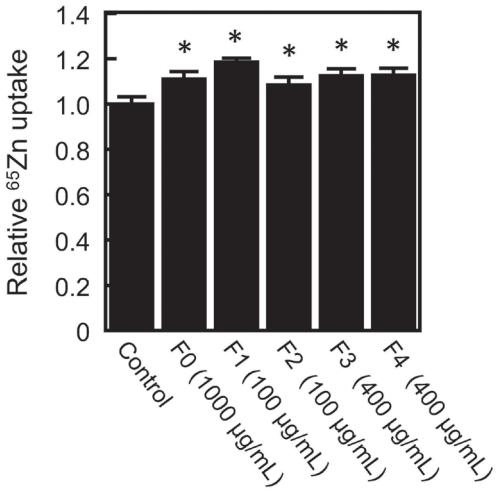

Fig. 4. Effects of *P. Ginseng* Fractions on Zinc Uptake in Hepa Cells

Hepa cells were incubated for 24 h in normal medium in the presence of the indicated concentrations of each fraction. Zinc uptake rates for 60 min were calculated and normalized to the protein concentrations. Statistical significance was determined using ANOVA with Dunnett's test. **P* < 0.05, significantly different from the control group. Each bar represents the mean ± S.D. (n = 3).

reported enhancements in *Zip4* mRNA and protein expressions following treatment with F0.[13] We also investigated the effect of F1–F3 fractions in this study (Fig. 5B and C). Consistent with our previous reports, *Zip4* mRNA and protein expressions were increased following treatment with zinc-deficient conditions and F0. Although treatment with F2 and F3 did not increase Zip4 expression, treatment with 100 μg/mL F1 clearly increased Zip4 expression at both the mRNA and protein levels. These results suggest that F1 enhances zinc uptake via the upregulation of Zip4, resulting in intracellular zinc accumulation. Since treatment with the F1 fraction at a lower concentration (100 μg/mL) compared to the F0 fraction (1000 μg/mL) showed a similar induction level of Zip4, the main metabolites of *P. ginseng*, which induced *Zip4* mRNA and protein expressions in our previous study[13] and this study, would likely be concentrated in the F1 fraction.

UPLC Analysis of F1-F3 Fractions We analyzed the F1–F3 fractions using UPLC-UV-MS to investigate the metabolites responsible for Zip4 induction (Supplementary Fig. 1). Characteristic ions (m/z), which correspond to ginsenosides, were detected in the F2 and F3 fractions (Supplementary Fig. 2). These ions were not detected in the F1 fraction. These

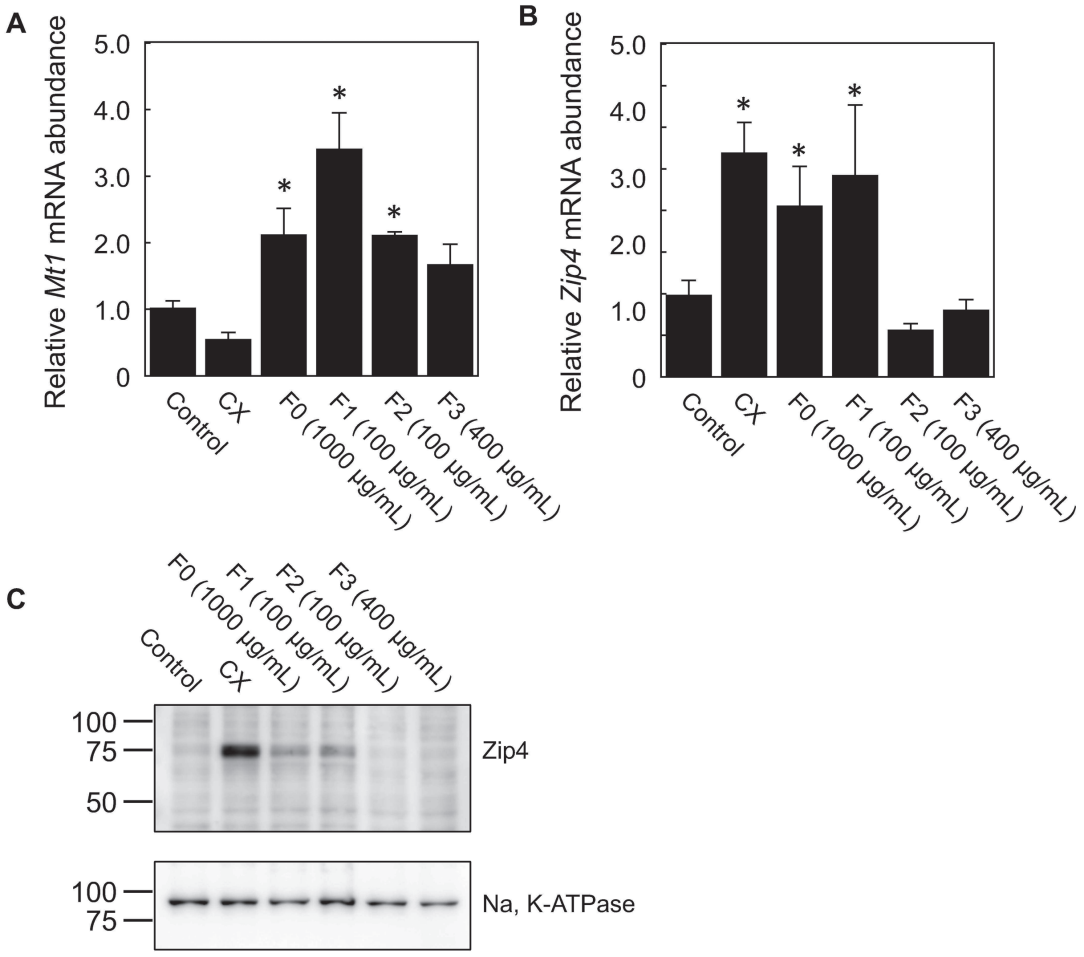

Fig. 5. Effects of *P. Ginseng* Fractions on Expressions of Mt1 and Zip4

(A) *Mt1* mRNA and (B) *Zip4* mRNA expression levels in Hepa cells cultured for 24 h in normal medium (control), zinc-deficient medium (CX), and in the presence of the indicated concentrations of *P. ginseng* extraction fraction in normal medium, were determined using qRT-PCR. Statistical significance was determined using ANOVA with Dunnett's test. **P* < 0.05, significantly different from control. Each bar represents the mean ± S.D. (n = 3). (C) Membrane proteins (9.0 μg) from Hepa cells treated with the indicated conditions were separated using 10% SDS-PAGE.

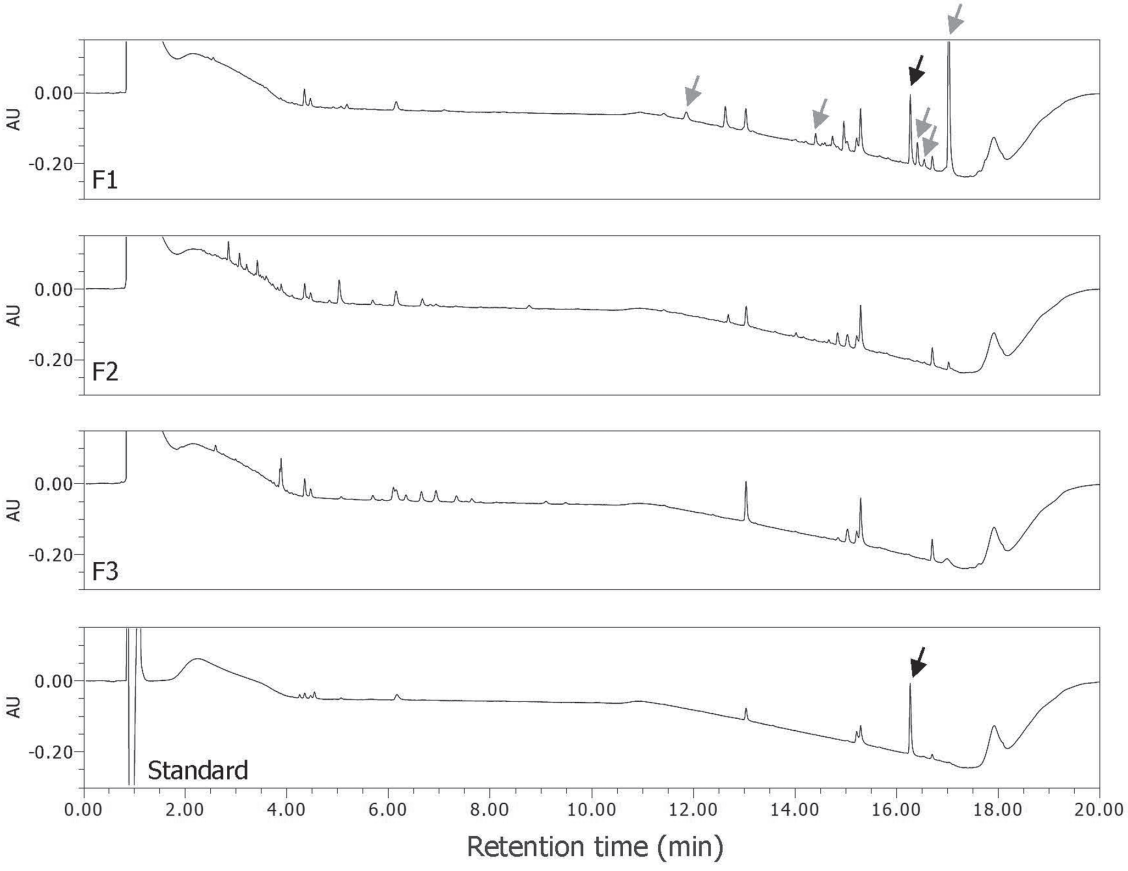

Fig. 6. UPLC Analysis of *P. Ginseng* Fractions

The UPLC chromatogram was generated at 200 nm for each *P. ginseng* fraction (F1–F3) and 20 μM falcarinol standard. AU, arbitrary unit. Arrows indicate the peaks specifically observed in the F1 fraction. Black arrow indicates the peak of falcarinol.

results suggest that the enhanced zinc uptake by the F2 and F3 fractions might be due to ginsenosides. In contrast, some peaks characteristic of the F1 fraction were observed using UPLC-UV at 200 nm (Fig. 6 and Supplementary Fig. 1). We attempted to detect specific ions at these peaks using MS analysis. However, after several attempts in both positive and negative ion modes and various cone voltage values, most ions corresponding to the apparent F1-specific peaks were not observed except for one peak found at about 16.4 min (Supplementary Fig. 2). Most of the metabolites associated to the characteristic peaks may be compounds that are difficult to ionize with the MS instrument that was used in this study. Among those, we could identify one F1-specific peak at 16.4 min as falcarinol in comparison to the authentic standard (Fig. 6 and Supplementary Fig. 3). The presence of this metabolite is consistent with the previous studies that reported several bioactive metabolites, including sitosterol and 10,12-octadecadiynoic acid, in the chloroform fraction.[20-22] Falcarinol and other polyacetylenes enhance glucose uptake by functioning as peroxisome proliferator-activated receptor gamma agonists.[23] Therefore, these metabolites might be responsible for inducing Zip4 expression and enhancing zinc uptake via a single or mixture of these metabolites.

Conclusions

Herein, we analyzed the *P. ginseng* extract, which enhances zinc uptake via Zip4 induction. We fractionated it with chloroform, ethyl acetate, and n-butyl alcohol, and examined the zinc uptake activity of the resultant fractions. Analyses using luciferase activity and radiolabeled zinc uptake showed that the F1–F3 fractions increased zinc uptake. Furthermore, the expression of Zip4, which is responsible for zinc uptake into Hepa cells, was examined. The F1 fraction induced *Zip4* mRNA and protein expressions. UPLC-UV-MS analysis of the F1 fraction detected several specific peaks, including one corresponding to falcarinol. These results indicate that metabolites other than ginsenosides promote zinc uptake by inducing Zip4 expression. Although it has been reported that the stabilization of *Zip4* mRNA depends on zinc concentration and that KLF4 (Krüppel-like factor 4), a transcription factor, regulates its transcription,[24] the detailed mechanism remains largely unknown. Such *Zip4* mRNA transcription and stabilization mechanism might be elucidated by further analysis of the F1 fraction and metabolites included.

These findings provide new insights into the mechanisms of *P. ginseng*-mediated zinc uptake and suggest that further analysis might identify additional metabolites and mechanisms involved in Zip4 induction. Such analyses will lead to the pre-

vention of zinc deficiency when using Kampo medicines containing *P. ginseng*, thus promoting human health.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Prasad AS. Discovery of human zinc deficiency and studies in an experimental human model. *Am. J. Clin. Nutr.*, **53**, 403–412 (1991).
2) Van Wouwe JP. Clinical and laboratory assessment of zinc deficiency in Dutch children. A review. *Biol. Trace Elem. Res.*, **49**, 211–225 (1995).
3) Goto T, Komai M, Suzuki H, Furukawa Y. Long-term zinc deficiency decreases taste sensitivity in rats. *J. Nutr.*, **131**, 305–310 (2001).
4) Hojyo S, Fukada T. Roles of zinc signaling in the immune system. *J. Immunol. Res.*, **2016**, 6762343 (2016).
5) Kambe T, Tsuji T, Hashimoto A, Itsumura N. The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. *Physiol. Rev.*, **95**, 749–784 (2015).
6) Dufner-Beattie J, Wang F, Kuo YM, Gitschier J, Eide D, Andrews GK. The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. *J. Biol. Chem.*, **278**, 33474–33481 (2003).
7) Maverakis E, Fung MA, Lynch PJ, Draznin M, Michael DJ, Ruben B, Fazel N. Acrodermatitis enteropathica and an overview of zinc metabolism. *J. Am. Acad. Dermatol.*, **56**, 116–124 (2007).
8) Dufner-Beattie J, Kuo YM, Gitschier J, Andrews GK. The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5. *J. Biol. Chem.*, **279**, 49082–49090 (2004).
9) Liuzzi JP, Bobo JA, Lichten LA, Samuelson DA, Cousins RJ. Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis. *Proc. Natl. Acad. Sci. USA*, **101**, 14355–14360 (2004).
10) Weaver BP, Dufner-Beattie J, Kambe T, Andrews GK. Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zip5). *Biol. Chem.*, **388**, 1301–1312 (2007).
11) Hashimoto A, Ohkura K, Takahashi M, Kizu K, Narita H, Enomoto S, Miyamae Y, Masuda S, Nagao M, Irie K, Ohigashi H, Andrews GK, Kambe T. Soybean extracts increase cell surface ZIP4 abundance and cellular zinc levels: a potential novel strategy to enhance zinc absorption by ZIP4 targeting. *Biochem. J.*, **472**, 183–193 (2015).
12) Kambe T, Andrews GK. Novel proteolytic processing of the ectodomain of the zinc transporter ZIP4 (SLC39A4) during zinc deficiency is inhibited by acrodermatitis enteropathica mutations. *Mol. Cell. Biol.*, **29**, 129–139 (2009).
13) Ikeda Y, Munekane M, Yamada Y, Kawakami M, Amano I, Sano K, Mukai T, Kambe T, Shitan N. Enhancing effect of *panax ginseng* on Zip4-mediated zinc influx into the cytosol. *J. Ginseng Res.*, **46**, 248–254 (2022).
14) Huang Q, Wang T, Wang HY. Ginsenoside Rb2 enhances the anti-inflammatory effect of omega-3 fatty acid in LPS-stimulated RAW264.7 macrophages by upregulating GPR120 expression. *Acta Pharmacol. Sin.*, **38**, 192–200 (2017).
15) Yu T, Yang Y, Kwak YS, Song GG, Kim MY, Rhee MH, Cho JY. Ginsenoside Rc from *panax ginseng* exerts anti-inflammatory activity by targeting TANK-binding kinase 1/interferon regulatory factor-3 and p38/ATF-2. *J. Ginseng Res.*, **41**, 127–133 (2017).
16) Kim JH. Cardiovascular Diseases and *panax ginseng*: a review on Molecular Mechanisms and Medical Applications. *J. Ginseng Res.*, **36**, 16–26 (2012).
17) Yang JW, Kim SS. Ginsenoside Rc promotes anti-adipogenic activity on 3T3-L1 adipocytes by down-regulating C/EBPα and PPARγ. *Molecules*, **20**, 1293–1303 (2015).
18) Sun M, Ye Y, Xiao L, Duan X, Zhang Y, Zhang H. Anticancer effects of ginsenoside Rg3. *Int. J. Mol. Med.*, **39**, 507–518 (2017). (Review)
19) Ru W, Wang D, Xu Y, He X, Sun YE, Qian L, Zhou X, Qin Y. Chemical constituents and bioactivities of *panax ginseng* (C.A. Mey.). *Drug Discov. Ther.*, **9**, 23–32 (2015).
20) Zhang Y, Lyu X, Liu T, Luo J, Zhang W, Mu Q. Analysis of nonpolar components from ginseng of different ages. *Am. J. Plant Sci.*, **04**, 92–97 (2013).
21) Stefanson AL, Bakovic M. Falcarinol is a potent inducer of heme Oxygenase-1 and was more effective than sulforaphane in attenuating intestinal inflammation at diet-achievable doses. *Oxid. Med. Cell. Longev.*, **2018**, 3153527 (2018).
22) Babu S, Jayaraman S. An update on β-sitosterol: A potential herbal nutraceutical for diabetic management. *Biomed. Pharmacother.*, **131**, 110702 (2020).
23) El-Houri RB, Kotowska D, Christensen KB, Bhattacharya S, Oksbjerg N, Wolber G, Kristiansen K, Christensen LP. Polyacetylenes from carrots (Daucus carota) improve glucose uptake *in vitro* in adipocytes and myotubes. *Food Funct.*, **6**, 2135–2144 (2015).
24) Liuzzi JP, Guo L, Chang SM, Cousins RJ. Krüppel-like factor 4 regulates adaptive expression of the zinc transporter *Zip4* in mouse small intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **296**, G517–G523 (2009).