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Zinc proteinate with moderate chelation strength enhances zinc absorption by upregulating the expression of zinc and amino acid transporters in primary cultured duodenal epithelial cells of broiler embryos

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Abstract

Recent study showed that zinc (**Zn**) and amino acid transporters may be involved in enhancing Zn absorption from Zn proteinate with moderate chelation strength (**Zn-Prot M**) in the duodenum of broilers. However, the specific mechanisms by which Zn-Prot M promotes the above Zn absorption are unknown. Therefore, in this study, 3 experiments were conducted to investigate specific and direct effects of Zn-Prot M and Zn sulfate (**ZnS**) on Zn absorption and expression of related transporters in primary duodenal epithelial cells of broiler embryos so as to prelim-inarily address possible mechanisms. In experiment 1, cells were treated with 100 μmol Zn/L as ZnS or Zn-Prot M for 20, 40, 60, 80, 100, or 120 min. Experiment 2 consisted of 3 sub-experiments. In experiment 2A, cells were treated with a Zn-unsupplemented basal medium (Control) or the basal medium supplemented with 100 or 200 μmol Zn/L as ZnS or Zn-Prot M for 60 min; in experiment 2B, cells were treated with a Zn-unsupplemented basal medium (Control) or the basal medium supplemented with 200 μmol Zn/L of as the ZnS or Zn-Prot M for 120 min; in experiment 2C, cells were treated with a Zn-unsupplemented basal medium (Control) or the basal medium supplemented with 400 or 800 μmol Zn/L as ZnS or Zn-Prot M for 120 min. In experiment 3, cells were treated with a Zn-unsupplemented basal medium (Control) or the basal medium supplemented with 400 μmol Zn/L as ZnS or Zn-Prot M for 120 min. The results of experiment 1 indicated that the minimum incubation time for saturable Zn absorption was determined to be 50.83 min using the best fit line. The results in experiment 2 demonstrated that a Zn concentration of 400 μmol/L and an incubation time of 120 min were suitable to increase the absorption of Zn from Zn-Prot M compared to ZnS. In experiment 3, Zn absorption across cell monolayers was significantly increased by Zn addition ($P < 0.05$), and was significantly greater with Zn-Prot M than with ZnS ($P < 0.05$). Compared to the control, Zn addition significantly decreased Zn transporter 10 and peptide-transporter 1 mRNA expression levels and increased y + L-type amino transporter 2 (**y + LAT2**) protein abundance ($P < 0.05$). Moreover, protein expression levels of zrt/irt-like protein 3 (**ZIP3**), zrt–irt-like protein 5 (**ZIP5**), and y + LAT2 were significantly greater for Zn-Prot M than for ZnS ($P < 0.05$). These findings suggest that Zn-Prot M promote Zn absorption by increasing ZIP3, ZIP5 and y + LAT2 protein expression levels in primary duo-denal epithelial cells.

Lay Summary

Our previous studies demonstrated that zinc (**Zn**) proteinate with moderate chelation strength (**Zn-Prot M**) exhibited the greatest bioavailability compared to the inorganic Zn sulfate (**ZnS**) and other organic Zn sources with either weak or strong chelation strength in broilers. Our recent study further showed that Zn and amino acid transporters could be potentially involved in promoting the absorption of Zn as Zn-Prot M in the duodenum of broilers. Nevertheless, further in vitro experiments are required to reveal the specific mechanisms by which Zn-Prot M promotes Zn absorption, where it is necessary first to investigate the specific and direct effect of Zn-Prot M on Zn absorption and the expression of Zn and amino acid transporters compared to that of ZnS. Therefore, we performed an in vitro study and found that Zn-Prot M increased Zn absorp-tion and protein expression levels of the zrt–irt-like protein 3 (**ZIP3**), zrt–irt-like protein 5 (**ZIP5**), and y + L-type amino transporter 2 (**y + LAT2**) compared to ZnS, suggesting that ZIP3, ZIP5, and y + LAT2 might be involved in promoting the absorption of Zn from Zn-Prot M in the primary cultured duodenal epithelial cells of broiler embryos.

Key words: amino acid transporters, broilers, duodenal epithelial cells, zinc absorption, zinc proteinate, zinc transporters

Introduction

Zinc (**Zn**) is a trace element that is essential for animal and human nutrition and biochemical functions (Saper and Rash, 2009; Muhamed and Vadstrup, 2014). This element is required for the catalytic activity of more than 300 enzymes (Vallee and Auld, 1990; Li et al., 2021). Moreover, dietary Zn deficiency has been found to cause growth retardation, oxidative stress, and impaired immune responses in chickens (Ogbuewu and Mbajiorgu, 2023). Zinc content in commonly used feedstuffs is low, and chickens poorly utilize Zn because of the presence of phytic acid and other anti-nutritional factors in plant feedstuffs (Yu et al., 2008). Therefore, Zn supplementation in poultry diets is generally used to meet their Zn demands and prevent Zn deficiency.

Inorganic Zn sulfate (**ZnS**) is the most commonly used Zn source in poultry diets, however, some unique organic Zn sources have received increased attention and have been incorporated into poultry diets due to their higher bioavailability (Chen et al., 2022; Khan et al., 2024). Min et al. (2019) reported that the methionine hydroxy analog chelated Zn was more readily available in laying hens than ZnS, using tibial Zn content and tibial strength as the responsive criteria. Chen et al. (2022) discovered that Zn-methionine had a higher bioavailability than ZnS, as reflected by pancreatic metallothionein content and mRNA expression in broilers. Yu et al. (2017) demonstrated that the organic Zn sources exhibited greater absorptivity than ZnS in the ligated duodenal segment, and that the relative absorptivity of organic Zn sources in broilers was strongly associated with the chelation strengths (measured by quotient of formation [Q_f] values) between Zn and its ligands. Moreover, Huang et al. (2009) observed the highest bioavailability of Zn proteinate with a moderate Q_f value (**Zn-Prot M**) in broilers, compared to ZnS and other organic Zn sources with either weak or strong Q_f values. However, the reasons for the highest Zn bioavailability of Zn-Prot M in broilers remain unclear. Although in vivo studies on the absorption of Zn as Zn-Prot M have been performed in the small intestine of broilers (Yu et al., 2017; Hu et al., 2022, 2023), further in vitro investigations are required to elucidate the precise molecular mechanisms of the above Zn absorption.

Zn transporters include the Zn transporter families (**ZnTs**) and zrt/irt-like protein families (**ZIPs**), which are required to maintain cellular Zn homeostasis (Yin et al., 2023). About 14 ZIPs and 10 ZnTs have been identified and named in human cells (Kambe, 2013). Furthermore, ZIPs transport Zn into the cytoplasm from internal organelles and vesicles. In contrast, ZnTs mediate Zn efflux into the organelles and vesicles (Sekler et al., 2007; Kimura and Kambe, 2016). Studies on broilers have suggested that ZnTs and ZIPs may play a role in the transport of organic Zn in the small intestine (He et al., 2019; Hu et al., 2022, 2023). He et al. (2019) identified that broilers fed a Zn-amino acid complex exhibited an increased mRNA expression of jejunal ZnT1. Our recent in vivo studies demonstrated that Zn-Prot M promoted the protein abundances of small intestinal ZIP3, ZnT4, ZIP5, ZnT7, ZnT9, or ZnT10 in broilers compared to ZnS (Hu et al., 2022, 2023). Furthermore, Zn-Prot M enhanced the protein abundances of the small intestinal y + L-type amino transporter 2 (**y + LAT2**), b0,+-type amino acid transporter (**rBAT**), and peptide-transporter 1 (**PepT1**) in broilers (Hu et al., 2022, 2023).

These studies indicated that ZnT, ZIP, peptide, and amino acid transporters could potentially be involved in the transport of Zn as organic Zn sources in the small intestine of broilers. Nevertheless, it is recognized that the aforementioned in vivo studies in broilers might be influenced by dietary, environmental, and physiological factors other than Zn. Therefore, additional in vitro investigations are required to determine the specific and direct impacts of ZnS and Zn-Prot M on the expression of the carriers mentioned above using a primary cultured model of duodenal epithelial cells from broiler embryos, which was previously established in our laboratory (Zhang et al., 2018).

Based on the above in vivo studies performed in our laboratory and by other researchers, we hypothesized that organic Zn-Prot M would increase Zn absorption potentially by upregulating the expression of Zn, amino acid and peptide transporters in the primary duodenal epithelial cells. Therefore, the objective of the present study was to determine the effect of supplemental Zn-Prot M and ZnS on Zn absorption and gene expression of ZnT, ZIP, peptide, and amino acid transporters in the primary cultured duodenal epithelial cells of chick embryos.

Materials and Methods

The experiment protocols were reviewed and approved by the Animal Ethics Committee of Yangzhou University (approval number: SYXK (Su) 2021-0027).

Isolation and cultivation of duodenal epithelial cells

Duodenal epithelial cells were isolated from 18-d-old chick embryos (Zhang et al., 2018), and a total of 2,000 chicken embryos were used to isolate the cells in this study. The isolated epithelial cells were seeded into 6-well Transwell plates (Cat. No. 3450, Corning Incorporated, Corning, NY, USA) at an initial density of 4.5×10^6 cells/well, and then cultured in a growth medium containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (**DMEM/F12**) basal medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), 100 μg/mL of heparin sodium, 20 ng/mL of epidermal growth factor, 1% penicillin, 1% insulin–transferrin–selenium, and 3% fetal bovine serum, and kept in a humidified incubator for 48 h of cultivation at 37 °C with 5% CO_2. To assess the integrity of the duodenal epithelial cell monolayers at 48 h of cultivation, the trans-epithelial electrical resistance (**TEER**) and phenol red transmittance were measured using the techniques detailed by Cao et al. (2020). A commercial lactate dehydrogenase (**LDH**) kit (Nanjing Jiancheng Bioengineering Institute, China) was used to measure the LDH activity in the culture medium to assess the cells' integrity at 48 h after seeding. Only when the duodenal epithelial cell monolayers reached a TEER > 300 Ω cm², phenol red transmittance < 5%, and LDH activity < 400 U/L, such an absorption model is successful (Zhang et al., 2018). The results indicated that the TEER value and phenol red transmittance of the cell monolayers were 434 ± 10 Ω·cm² and 2.42 ± 0.35 % (*n* = 6) at 48 h, respectively. The LDH activity in the culture medium was 315 ± 16 U/L (*n* = 6) at 48 h. Therefore, the established primary cultured duodenal epithelial cell monolayer model of chick embryos at 48 h in the present study was acceptable to be used in the subsequent Zn absorption experiments.

Preparation of Zn solutions

A Zn stock medium containing 1,000 μmol Zn/L in the form of ZnS (ZnSO$_4$.7H$_2$O) or Zn-Prot M (feed grade, Q_f = 51.6, comprising 17.09% of Zn by analysis) was freshly prepared. The ZnS and Zn-Prot M used in this study were identical to those used in our previous study (Hu et al., 2022). The Zn media were prepared by mixing the DMEM/F12 basal medium buffer with the Zn stock medium to obtain Zn concentrations of 100, 200, 400, and 800 μmol/L, respectively.

A completely randomized design was adopted, and there were 3 to 8 replicate wells depending on the different indices for each treatment in the following experiments. According to power and sample size analysis performed using an online Power and Sample Size Calculator (https://www.stat.ubc.ca/~rollin/stats/ssize/b2.html), as well as a previous publication (Huang et al., 2024), 3 replicate wells were sufficient to obtain reliable TEER value, phenol red transmittance, and LDH enzyme activity data for statistical analyses, and 6 replicate wells were sufficient to obtain reliable data of Zn absorption and molecular indices for statistical analyses.

Time responses of Zn absorption across the duodenal epithelial cell monolayers (experiment 1)

Experiment 1 was conducted to determine the appropriate cell incubation time. A 2 (Zn treatment) × 6 (incubation time) factorial arrangement of treatments was used, with ZnS and Zn-Prot M as the 2 Zn treatments as well as 6 incubation timepoints of 20, 40, 60, 80, 100, and 120 min. Yu et al. (2008) reported that the time points for maximum Zn absorption were 49, 46, and 60 min in the ligated duodenum, jejunum, and ileum of broilers, respectively. Therefore, in order to ensure the adequate Zn absorption in the primary cultured duodenal epithelial cells, the aforementioned 6 incubation time points were selected. In addition, the supplemental Zn level of 100 μmol/L was adopted according to a previous study (Shao et al., 2017). Moreover, to assess the integrity of cell monolayers after Zn treatment at 120 min of incubation, a basal medium control with no Zn was included. At 48 h of cultivation after seeding, the cells were gently washed thrice with DMEM/F12 basal medium at 37 °C. Subsequently, 1.5 mL of growth media supplemented with 100 μmol Zn/L in the form of either ZnS (containing 98 μmol/L of Zn by analysis) or Zn-Prot M (containing 101 μmol/L of Zn by analysis) were supplemented into the apical chamber (donor chamber) of one well in each 6-well Transwell plate. Moreover, 2.6 mL of DMEM/F12 basal media (containing 1.82 μmol/L of Zn by analysis) were added to its basolateral chamber (the receiver chamber). After Zn treatments, all cells were incubated in a humidified culture incubator for 20, 40, 60, 80, 100, or 120 min according to the above-mentioned incubation timepoint design. At each incubation time point, 2.2 mL of incubation media were sampled from the basolateral chamber of each well to analyze Zn concentration (except for the control). At 120 min, the integrity of the cell monolayers was assessed as described above.

Effect of Zn source and added Zn level on Zn absorption in duodenal epithelial cells (experiment 2)

Experiment 2 was a serial trial consisting of 3 sub-experiments 2A to C to determine the appropriate added Zn level by investigating the effects of supplemental Zn-Prot M and ZnS on Zn absorption under different levels of added Zn in the primary cultured duodenal epithelial cells based on the appropriate cell incubation time (50.82 min), which was determined in experiment 1. In experiment 2A, a 1 (control) + 2 (Zn source) × 2 (added Zn level) factorial arrangement of treatments was used. The 2 Zn sources were ZnS and Zn-Prot M, and the 2 added Zn levels were 100 and 200 μmol/L based on the previous study (Shao et al., 2017), while the Zn-unsupplemented cells were set as the control. The Zn incubation time was set to 60 min, according to the results of experiment 1.

According to the results of experiment 2A, there was no difference in Zn absorption between the 2 Zn sources at either 100 or 200 μmol Zn/L, possibly due to the shorter incubation time (60 min) in this trial. Therefore, experiment 2B was conducted to evaluate the effects of supplemental Zn-Prot M and ZnS on Zn absorption across the primary cultured duodenal epithelial cells, when the incubation time was extended to 120 min at 200 μmol Zn/L. Therefore, 3 treatments were designed as the control with no Zn addition, ZnS and Zn-Prot M treatments, respectively.

According to the results of experiment 2B, there was still no difference in Zn absorption between the 2 Zn sources, possibly due to the lower added Zn level (200 μmol/L). Therefore, experiment 2C was conducted to further investigate the effects of supplemental Zn-Prot M and ZnS on Zn absorption across the duodenal epithelial cells at increased supplemental Zn levels of 400 and 800 μmol/L and the incubation time of 120 min. A 1 (control) + 2 (Zn source) × 2 (added Zn level) factorial arrangement of treatments was used. The 2 Zn sources were ZnS and Zn-Prot M, and the 2 added Zn levels were 400 and 800 μmol/L, while the Zn-unsupplemented cells were set as the control.

At 48 h of cultivation after seeding, the cells were gently washed thrice with DMEM/F12 basal medium at 37 °C. Then 1.5 mL of growth basal media with no Zn addition (control, containing 2.97 μmol/L of Zn by analysis) or the growth basal media supplemented with 100, 200, 400, or 800 μmol/L of Zn in the form of ZnS (containing 98, 195, 395, or 778 μmol/L of Zn by analysis) or Zn-Prot M (containing 101, 195, 397, or 783 μmol/L of Zn by analysis) were added to the apical chamber of one well in each 6-well Transwell plate. Furthermore, 2.6 mL of DMEM/F12 basal media (containing 1.82 μmol/L of Zn by analysis) were added to its basolateral chamber according to the experimental designs described. After Zn treatment, all cells were incubated for 60 or 120 min. At 60 or 120 min, 2.2 mL of the incubation media were sampled from the basolateral chamber of each well for the analysis of Zn concentration, and also the integrity of the cell monolayers was assessed as described above.

Effect of supplemental Zn source on Zn absorption and gene expression of related transporters in duodenal epithelial cells (experiment 3)

According to the results of experiment 2C, Zn-Prot M significantly enhanced Zn absorption compared with ZnS at either 400 or 800 μmol Zn/L. However, 800 μmol Zn/L was excessive for the duodenal epithelial cells at 120 min of incubation. Therefore, in experiment 3, we used the added Zn level of 400 μmol/L and the cell incubation time of 120 min. Three treatments were designed, including the control with no Zn addition, ZnS, and the Zn-Prot M treatments with 6 replicate wells per treatment.

At 48 h of cultivation after seeding, the cells were gently washed thrice with DMEM/F12 basal medium at 37 °C. Then 1.5 mL of growth basal media with no Zn addition (control, containing 2.97 µmol/L of Zn by analysis) or the growth basal media supplemented with 400 µmol Znl/L from ZnS (containing 397 µmol/L of Zn by analysis) or Zn-Prot M (containing 397 µmol/L of Zn by analysis) were added to the apical chamber of one well in each 6-well Transwell plate, along with the addition of 2.6 mL DMEM/F12 basal media (containing 1.82 µmol/L of Zn by analysis) to its basolateral chamber. After Zn treatment, all cells were incubated for 120 min. After incubation for 120 min, 2.2 mL of the incubation media were sampled from the basolateral chamber of each well for analysis of Zn concentration, and the duodenal epithelial cells were collected to examine the mRNA and protein expression levels of ZnT, ZIP, peptide, and amino acid transporters. The integrity of the cell monolayers was assessed as described above.

Measurement of Zn contents and calculation of Zn absorption amount

An Agilent Technologies 5110 plasma emission spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used to analyze the Zn content in the culture media before and after incubation, according to the method described by Li et al. (2013). The amount of Zn absorption was calculated as follows:

$$\text{The amount of Zn Absorption } (nmol/cm^2)$$
$$= \frac{\begin{array}{c}\text{Zn Concentration in}\\ \text{the receiver chamber of}\\ \text{Zn treatment } (\mu mol/L) \times 2.6 \ (mL)\end{array}}{4.67 (cm^2)}$$

In the equation, the 2.6 mL is the liquid volume in the receiver chamber of the Zn treatment, and the 4.67 cm^2 is the area of the bottom of the receiver chamber in each well of a 6-well Transwell plate.

Real-time qPCR (RT-qPCR)

Total RNA was extracted from the duodenal epithelial cells using TRIzol reagent (Vazyme Biotech Co., Ltd, Nanjing, China) (Cao et al., 2020). The synthesis of cDNA was performed using 1,000 ng of total RNA with a commercially available kit obtained from Vazyme Biotech Co., Ltd. AceQ SYBR Green Master Mix (Vazyme Biotech Co., Ltd.) was used for the RT-qPCR procedure. The relative mRNA expression level was determined using the $2^{-\Delta\Delta CT}$ method, with glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and 18S ribosomal RNA (**18S rRNA**) serving as the internal controls (Livak and Schmittgen, 2001). All primers and their respective PCR fragment lengths are listed in Table 1.

Total protein extraction and western blotting

Epithelial cells were homogenized in radioimmunoprecipitation assay buffer supplemented with 1% (v/v) phenylmethylsulfonyl fluoride using a homogenizer (Beijing Hede Technology Co., Ltd, China). The cell lysate was centrifuged at 10,000 × *g* for 10 min. The supernatant was collected, and the protein concentrations were determined. The cell lysates with a protein content of 40 µg were separated using either 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The primary antibodies for ZnT1 (ab214356), ZnT10 (ab229954), ZIP3 (ab254868), ZIP5 (ab105194), and B^{0}AT1 (ab180516) were purchased from Abcam (Cambridge, MA, USA). Primary antibodies against ZnT5 (25604-1-AP), ZnT7 (13966-1-AP), and y + LAT2 (13823-1-AP) were purchased from Proteintech Group, Inc. (Chicago, IL, USA). The primary antibodies for ZnT9 (BS62531), LAT1 (DF8065), and rBAT (DF7379) were purchased from Bioworld Technology, Inc. (Louis Park, MN, USA) or Affinity Biosciences (Cincinnati, OH, USA). In addition, the primary antibodies for ZnT4 (BA3484) and PepT1 (A03672-1) were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). All antibodies used in the present study were either mouse- or human-derived antibodies. The protein bands were scanned using an ECL system (Tanon Technology Co. Ltd., Shanghai, China), and the intensity of the protein bands was quantified using Tanon Gis 1D software.

Statistical analyses

TEER value, phenol red transmittance, and LDH enzyme activity in experiment 1, and all data in experiments 2B and 3 were analyzed by one-way ANOVA using the generalized linear model (**GLM**) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data on the Zn absorption amount in experiment 1 were analyzed by 2-way ANOVA using the GLM procedure, and the statistical model included Zn treatment, incubation time, and their interactions. Orthogonal comparisons were applied for linear and quadratic responses of the dependent variables to independent variables. Regression analyses of quadratic, asymptotic, and broken-line models were performed, and the best-fitted model was selected to estimate the suitable incubation time (the break point from the broken-line model). Data in experiments 2A and C were analyzed using a single degree of freedom contrast to compare all supplemental Zn treatments with the control. Data excluding the control were analyzed by 2-way ANOVA using the GLM procedure, and the statistical model included Zn source, added Zn level and their interaction. Differences among the means were tested using the least significant difference method. The replicate well served as the experimental unit, and the statistical significance was set at $P < 0.05$.

Results

Effects of Zn treatment and incubation time on Zn absorption in duodenal epithelial cells (experiment 1)

Zinc treatment did not affect the TEER value (>300 Ω·cm²), phenol red transmittance (<5%) of the cells, and LDH activity (<400 U/L) in the culture medium after incubation for 120 min ($P > 0.34$) (Table 2), indicating that the integrity of the duodenal epithelial cells was not affected by Zn treatment, and thus the following results of Zn absorption in this experiment are reliable.

As shown in Table 3, Zn treatment and its interaction with the incubation time did not affect the amount of Zn absorption in the primary cultured duodenal epithelial cells ($P > 0.27$), but the incubation time significantly affected it ($P < 0.001$). Cells at either 40, 60, 80, 100, or 120 min incubation had a higher amount of Zn absorption than those at 20 min ($P < 0.05$), but no differences were observed among the 40, 60, 80, 100, and 120 min of incubation time points

Table 1. Primer sequences used for real-time qPCR

Genes	GenBank ID	Primer sequences (5′ to 3′)	Product length (bp)
ZnT1	XM_421021.5	F: AGAGCCTGGGTTTGGATTCG	229
		R: AGCCCATGCATGAACACTGA	
ZnT4	XM_423325	F: GCCATCTTGACGGACGTAGT	190
		R: CAAGGTACACCAGGACACCC	
ZnT5	NM_001031419.2	F: ATGGAGGAAAAGTACAGCAGCC	118
		R: TCAGAAACTTGGCGAAGCAC	
ZnT6	XM_045691225.1	F: CTACGGTTCTGGCACAGCTT	126
		R: GTCGCCCCGTGTGTATCTC	
ZnT7	NM_001008788.1	F: TGCTGCCCCTCTCCATTAAG	114
		R: AGAGGTTGCGGGATGTCTTG	
ZnT9	XM_015285471.1	F: ACATGTTTTTCCGTGCAGCC	265
		R: CGGAACACAACCTTTACCAGC	
ZnT10	XM_015283897.1	F: GAGCTGTAGAGATGGGTCGC	184
		R: ACACCGAGCAAACCGATGAT	
ZIP3	XM_015299966.2	F: CATACATCCAGGAGGCAGAGG	246
		R: CCTGGATGATCTTGACGGGG	
ZIP5	XM_025145573.1	F: CCAAGATGAAACGCACGCAA	284
		R: TAAGCTGCGACCAAGTCCTG	
B^{0}AT1	XM_419056.6	F: CTTGGGTGAGGTAGGTGGGA	167
		R: GATGCGGGTGCTCTCATGTA	
LAT1	NM_001030579.2	F: GGAAAGGCCCATCAAGGTGA	248
		R: ACGATTCTTGGGGAACCAC	
rBAT	XM_004935370.3	F: CCTAGGAGGAGAGGCACGAA	223
		R: TCCTGCATAGGGCTGCAATG	
EAAT3	XM_424930.6	F: CGTCCAGGCCTGTTTTCAAC	171
		R: TCGGAATACATGCCCACGAT	
PepT1	NM_204365.1	F: AAAACAGGTTTCGGCATCGC	167
		R: CTGCTGGTCAAAAAGTGCCC	
GAPDH	NM_204305.1	F: CTTTGGCATTGTGGAGGGTC	128
		R: ACGCTGGGATGATGTTCTGG	
18S rRNA	NM_001182073.1	F: ATAACGAACGAGACTCTGGCA	147
		R: CGGACATCTAAGGGCATCACA	

ZnT1, zinc transporter 1; ZnT4, zinc transporter 4; ZnT5, zinc transporter 5; ZnT6, zinc transporter 6; ZnT7, zinc transporter 7; ZnT9, zinc transporter 9; ZnT10, zinc transporter 10; ZIP3, zrt–irt-like protein 3; ZIP5, zrt–irt-like protein 5; B^{0}AT1, b-0-system neutral amino acid co-transporter; LAT1, L-type amino acid transporter 1; rBAT, b0,+-type amino acid transporter; EAAT3, excitatory amino acid transporter 3; PepT1, peptide-transporter 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 18S rRNA, 18S ribosome RNA; F, forward; R, reverse.

Table 2. Effect of supplemental Zn on TEER value and phenol red transmittance of primary cultured duodenal epithelial cells and LDH activity in the culture medium at 120 min of incubation[1] (experiment 1)

Zn treatment	TEER value, $\Omega.cm^2$	Phenol red transmittance, %	LDH enzyme activity, U/L
Control	424	2.83	141
ZnS	420	3.04	149
Zn-Prot M	418	3.05	139
Pooled SE	3	0.11	8
P value	0.3869	0.3438	0.6461

[1]Data represent the means of 5 to 8 replicates (n = 5 to 8).
LDH, lactate dehydrogenase; TEER, the trans-epithelial electrical resistance; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6).

($P > 0.05$). Moreover, the amount of Zn absorption increased linearly and quadratically with the increase in incubation time ($P < 0.03$).

The appropriate incubation time for Zn absorption by the primary cultured duodenal epithelial cells was estimated based on the best-fitted broken-line model [$Y = 0.6296 + 0.039X$

Table 3. Effects of Zn treatment (added Zn level of 100 µmol/L) and incubation time on Zn absorption in primary cultured duodenal epithelial cells (experiment 1)

Zn treatment	Incubation time, min	Amount of Zn absorption, nmol/cm²
ZnS[1]	20	1.35
	40	2.27
	60	2.18
	80	2.47
	100	2.37
	120	2.75
Zn-Prot M[1]	20	1.47
	40	2.10
	60	3.29
	80	2.46
	100	2.73
	120	2.63
Pooled SE		0.31
Zn treatment[2]	ZnS	2.23
	Zn-Prot M	2.45
Pooled SE		0.13
Incubation time[3]	20	1.41[b]
	40	2.19[a]
	60	2.73[a]
	80	2.46[a]
	100	2.55[a]
	120	2.69[a]
Pooled SE		0.22
P value	Zn treatment	0.2758
	Incubation time	0.0009
	Interaction	0.4775
	Linear	0.0004
	Quadratic	0.0206

[1]Data represent the means of 6 to 8 replicates (n = 6 to 8).
[2]Data represent the means of 40 to 44 replicates (n = 40 to 44).
[3]Data represent the means of 14 to 16 replicates (n = 14 to 16).
[a,b]Means with different superscripts within the same column differ significantly ($P < 0.05$).
ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6).

$(0 < X \leq 50.82)$; $Y = 0.6347 + 0.0389X$ $(50.82 < X \leq 120)$]. Therefore, to maintain adequate Zn absorption across the cell monolayers, the minimum incubation time for the duodenal epithelial cells was 50.82 min.

Effect of Zn source and added Zn level on Zn absorption in the duodenal epithelial cells at 60 min of incubation (experiment 2A)

The TEER value and phenol red transmittance of the cells, as well as LDH activity in the culture media were unaffected by Zn treatment at 60 min of incubation compared to the control ($P > 0.05$) (Table 4). The above-mentioned indices were not affected by the Zn source, added Zn level, and their interaction ($P > 0.16$), indicating that the integrity of the duodenal epithelial cells was not affected by the Zn source and added Zn level, and thus the following results of Zn absorption in this experiment are reliable.

Compared to the control, the addition of Zn significantly increased the absorption of Zn in the duodenal epithelial cells ($P < 0.05$) (Table 4). The amount of Zn absorption was significantly influenced by the added Zn level ($P < 0.02$), but not by Zn source or its interaction with the added Zn level ($P > 0.75$). The Zn absorption was significantly greater when the cells were exposed to a concentration of 200 µmol Zn/L than 100 µmol Zn/L ($P < 0.05$).

Effects of Zn source (added Zn level of 200 µmol/L) on Zn absorption in duodenal epithelial cells at 120 min of incubation (experiment 2B)

The integrity of duodenal epithelial cells was not affected by Zn treatment, as evidenced by the unaffected TEER value, phenol red transmittance, and LDH activity at 120 min of incubation ($P > 0.30$) (Table 5), and thus the following results of Zn absorption in this experiment are reliable.

Zinc source significantly influenced the Zn absorption amount in the duodenal epithelial cells ($P < 0.0001$) (Table 5). Compared to the control, 2 Zn sources significantly increased the amount of Zn absorption by 1,256% (ZnS) and 1,108% (Zn-Prot M) ($P < 0.05$), respectively with no significant difference between the 2 Zn sources ($P > 0.05$). However, it was expected that Zn-Prot M should promote Zn absorption compared to ZnS based on Zn biologically relevant (Hu et al., 2022).

Effect of Zn source and added Zn level on Zn absorption in the duodenal epithelial cells of broiler embryos at 120 min of incubation (experiment 2C)

The TEER value and phenol red transmittance of the cells, as well as LDH activity in the culture media at 120 min of incubation were unaffected by Zn treatment when compared to the control ($P > 0.05$) (Table 6). The TEER value and LDH activity were not affected by Zn source, added Zn level, or their interaction ($P > 0.34$). The added Zn level significantly affected the phenol red transmittance ($P < 0.006$), whereas the Zn source and its interaction with the added Zn level did not affect it ($P > 0.11$). The phenol red transmittance was significantly greater at a concentration of 800 µmol Zn/L compared to that at 400 µmol Zn/L ($P < 0.05$), indicating that the supplementation of 800 µmol Zn/L decreased the tight junctions between the epithelial cells, and induced a certain degree of damage on the barrier function of the epithelial cell monolayers. Therefore, the following results of Zn absorption at 400 µmol Zn/L, regardless of Zn source, are reliable in this experiment.

Zinc supplementation resulted in a significant increase in the Zn absorption amount in the duodenal epithelial cells compared to the control ($P < 0.05$) (Table 6). The Zn source and added Zn level significantly affected the Zn absorption amount ($P < 0.04$), whereas their interaction did not affect it ($P > 0.30$). Zn-Prot M significantly enhanced the amount of Zn absorption compared with ZnS ($P < 0.05$). The absorption of Zn was significantly greater when cells were exposed to a concentration of 800 µmol Zn/L than 400 µmol Zn/L ($P < 0.05$).

Effect of Zn source (added Zn level of 400 µmol/L) on Zn absorption in duodenal epithelial cells at 120 min of incubation (experiment 3)

Zinc source did not affect the TEER value and phenol red transmittance of the cells, and LDH activity in the culture medium at 120 min of incubation ($P > 0.55$) (Table 7), indicating that the integrity of duodenal epithelial cells was not affected by the

Table 4. Effects of Zn source and added Zn level on TEER value, phenol red transmittance and Zn absorption of primary cultured duodenal epithelial cells and LDH activity in the culture medium at 60 min of incubation (experiment 2A)

Zn source	Added Zn level, μmol/L	TEER value, Ω.cm²	Phenol red transmittance, %	LDH enzyme activity, U/L	Amount of Zn absorption, nmol/cm²
Control[1]	0	416	2.44	134	0.77*
ZnS[1]	100	427	2.53	139	2.87
	200	409	2.62	156	4.00
Zn-Prot M[1]	100	442	2.42	162	2.58
	200	431	2.55	133	3.98
Pooled SE		12.16	0.09	28	0.50
Zn source[2]	ZnS	418	2.58	148	3.44
	Zn-Prot M	437	2.49	147	3.28
Pooled SE		8.60	0.07	20	0.35
Added Zn level[3]	100	434	2.47	150	2.73b
	200	420	2.59	145	3.99a
Pooled SE		8.60	0.07	20	0.35
P value	Zn source	0.2825	0.4576	0.9919	0.7528
	Added Zn level	0.1630	0.3662	0.8398	0.0194
	Interaction	0.8044	0.9726	0.4471	0.7856

[1]Data represent the means of 3 to 6 replicates (n = 3 to 6).
[2]Data represent the means of 6 to 12 replicates (n = 6 to 12).
[3]Data represent the means of 6 to 12 replicates (n = 6 to 12).
*Different from all Zn supplemental groups ($P < 0.05$).
a,bMeans with different superscripts within the same column differ significantly ($P < 0.05$).
LDH, lactate dehydrogenase; TEER, the trans-epithelial electrical resistance; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6).

Table 5. Effect of Zn source (added Zn level of 200 μmol/L) on TEER value, phenol red transmittance and Zn absorption of primary cultured duodenal epithelial cells and LDH activity in the culture medium at 120 min of incubation[1] (experiment 2B)

Zn source	TEER value, Ω.cm²	Phenol red transmittance, %	LDH enzyme activity, U/L	Amount of Zn absorption, nmol/cm²
Control	422	2.26	100	0.36b
ZnS	417	2.41	144	4.88a
Zn-Prot M	430	2.25	115	4.35a
Pooled SE	10	0.07	21	0.28
P value	0.7082	0.3034	0.3681	<0.0001

[1]Data represent the means of 3 to 6 replicates (n = 3 to 6).
a,bMeans with different superscripts within the same column differ significantly ($P < 0.05$).
LDH, lactate dehydrogenase; TEER, the trans-epithelial electrical resistance; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6).

Zn source, and thus the following results of Zn absorption and other indices in the present experiment are reliable.

Zinc source significantly influenced the Zn absorption amount in the duodenal epithelial cells ($P < 0.0001$) (Table 7). Compared with the control group, the 2 Zn sources exhibited a significant increase in the amount of Zn absorption ($P < 0.05$). Furthermore, when compared with ZnS, Zn-Prot M demonstrated a significant increase in Zn absorption by 91% ($P < 0.05$).

Effect of Zn source (added Zn level of 400 μmol/L) on mRNA expression levels of ZnT, ZIP, small peptide, and amino acid transporters in the duodenal epithelial cells at 120 min of incubation (experiment 3)

Zinc source did not affect the mRNA expression levels of *ZnT1, ZnT4, ZnT5, ZnT6, ZnT7, ZnT9, ZIP3, ZIP5, rBAT, and EAAT3* in the duodenal epithelial cells ($P > 0.11$) (Figure 1).

However, the Zn source significantly influenced the expression levels of *ZnT10, PepT1, LAT1, and B⁰AT1* ($P < 0.04$) (Figure 1). Compared with the control, the mRNA expression levels of *ZnT10* and *PepT1* were significantly reduced by 2 Zn sources ($P < 0.05$). Zn-Prot M significantly increased *LAT1* mRNA expression compared to the control ($P < 0.05$), while Zn-Prot M significantly decreased the mRNA expression of *B⁰AT1* compared to the control and ZnS ($P < 0.05$).

Effect of Zn source (added Zn level of 400 μmol/L) on the protein expression levels of ZnT, ZIP, small peptide, and amino acid transporters in the duodenal epithelial cells at 120 min of incubation (experiment 3)

Zinc source did not affect the protein abundances of ZnT1, ZnT4, ZnT5, ZnT7, ZnT9, ZnT10, rBAT, LAT1, B⁰AT1, EAAT3, and PepT1 ($P > 0.32$) (Figure 2). However, the protein abundances of ZIP3, ZIP5, and y + LAT2 in the

Table 6. Effects of Zn source and added Zn level on TEER value, phenol red transmittance and Zn absorption of primary cultured duodenal epithelial cells and LDH activity in the culture medium at 120 min of incubation (experiment 2C)

Zn source	Added Zn level, μmol/L	TEER value, Ω.cm²	Phenol red transmittance, %	LDH enzyme activity, U/L	Amount of Zn absorption, nmol/cm²
Control[1]	0	413	2.11	142	0.26[*]
ZnS[1]	400	406	2.18	143	6.95
	800	405	2.49	157	11.99
Zn-Prot M[1]	400	413	2.13	161	9.19
	800	400	2.31	125	12.82
Pooled SE		12	0.06	25	0.64
Zn source[2]	ZnS	406	2.33	150	9.47[b]
	Zn-Prot M	406	2.22	143	11.01[a]
Pooled SE		8	0.05	18	0.45
Added Zn level[3]	400	409	2.16[b]	152	8.17[b]
	800	402	2.40[a]	141	12.44[a]
Pooled SE		8	0.05	18	0.48
P value	Zn source	0.5885	0.1147	0.7852	0.0345
	Added Zn level	0.9516	0.0052	0.6833	<0.0001
	Interaction	0.6727	0.3061	0.3475	0.3063

[1]Data represent the means of 3 to 6 replicates (n = 3 to 6).
[2]Data represent the means of 6 to 12 replicates (n = 6 to 12).
[3]Data represent the means of 6 to 12 replicates (n = 6 to 12).
[*]Different from all Zn supplemental groups ($P < 0.05$).
[a,b]Means with different superscripts within the same category of the same column differ significantly ($P < 0.05$).
LDH, lactate dehydrogenase; TEER, the trans-epithelial electrical resistance; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6).

Table 7. Effect of Zn source (added Zn level of 400 μmol/L) on TEER value, phenol red transmittance and Zn absorption of primary cultured duodenal epithelial cells and LDH activity in the culture medium at 120 min of incubation[1] (experiment 3)

Zn source	TEER value, Ω.cm²	Phenol red transmittance, %	LDH enzyme activity, U/L	Amount of Zn absorption, nmol/cm²
Control	430	1.63	153	0.27[c]
ZnS	416	1.74	141	2.62[b]
Zn-Prot M	423	1.67	153	5.01[a]
Pooled SE	8	0.08	33	0.34
P value	0.5557	0.6299	0.9327	<0.0001

[1]Data represent the means of 3 to 6 replicates (n = 3 to 6).
[a,b,c]Means with different superscripts within the same column differ significantly ($P < 0.05$).
LDH, lactate dehydrogenase; TEER, the trans-epithelial electrical resistance; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6).

duodenal epithelial cells were significantly affected by Zn source ($P < 0.04$) (Figure 2). Compared to the control, 2 Zn sources demonstrated a significant increase in protein abundance of y + LAT2 ($P < 0.05$). Furthermore, when compared to ZnS, Zn-Prot M exhibited a significant increase ($P < 0.05$). The protein abundances of ZIP3 and ZIP5 were significantly greater for Zn-Prot M compared to the control and ZnS ($P < 0.05$), with no significant difference between the control and ZnS ($P > 0.05$).

Discussion

In this study, Zn-Prot M notably enhanced the absorption of Zn and the protein abundances of ZIP3, ZIP5, and y + LAT2 in the primary cultured duodenal epithelial cells compared to the control and ZnS, suggesting that Zn-Prot M enhanced the Zn transport possibly by upregulating the protein expression levels of ZIP3, ZIP5, and y + LAT2, which has supported our

hypothesis. These findings have not been previously reported, and are presented as a new perspective on the molecular modes of Zn absorption as the organic Zn-Prot M in the duodenum of broilers.

In the current study, Zn-Prot M increased Zn absorption in cells compared to ZnS, which is in accordance with our previous findings in broilers where Zn-Prot M displayed greater Zn absorption than ZnS in both ligated duodenal loops and the small intestine (Yu et al., 2017; Hu et al., 2022). The absorption of Zn in the in situ ligated duodenum of broilers is a saturable carrier-mediated process (Yu et al., 2017), and is primarily regulated by ZnTs and ZIPs (Tang et al., 2015; Wu et al., 2020; Hu et al., 2022, 2023; Rao et al., 2023). A previous study demonstrated that broilers fed a Zn-amino acid complex had higher mRNA expression level of jejunal ZnT1 (Huang and Gitschier, 1997). According to our recent researches (Hu et al., 2022, 2023), the increased transport of Zn as Zn-Prot M may be linked to
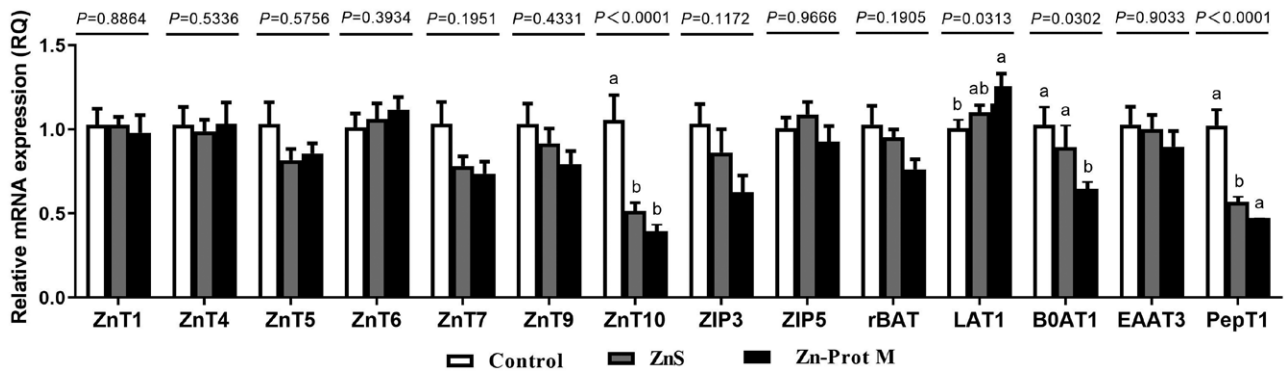
Figure 1. Effect of Zn source (added Zn level of 400 µmol/L) on mRNA expression levels of ZnT, ZIP, amino acid, and small peptide transporters in primary cultured duodenal epithelial cells at 120 min of incubation (experiment 3). Values are means ± SE (*n* = 4 to 6). Lacking the same letters (a, b) means significant differences (*P* < 0.05). ZnT1, zinc transporter 1; ZnT4, zinc transporter 4; ZnT5, zinc transporter 5; ZnT6, zinc transporter 6; ZnT7, zinc transporter 7; ZnT9, zinc transporter 9; ZnT10, zinc transporter 10; ZIP3, zrt–irt-like protein 3; ZIP5, zrt–irt-like protein 5; B⁰AT1, b-0-system neutral amino acid co-transporter; LAT1, L-type amino acid transporter 1; rBAT, b0,+-type amino acid transporter; EAAT3, excitatory amino acid transporter 3; PepT1, peptide-transporter 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6). The mRNA expression levels were calculated as the relative quantities of the target gene mRNA to the geometric mean of 18S rRNA and GAPDH mRNA using the $2^{-\Delta\Delta CT}$ method.

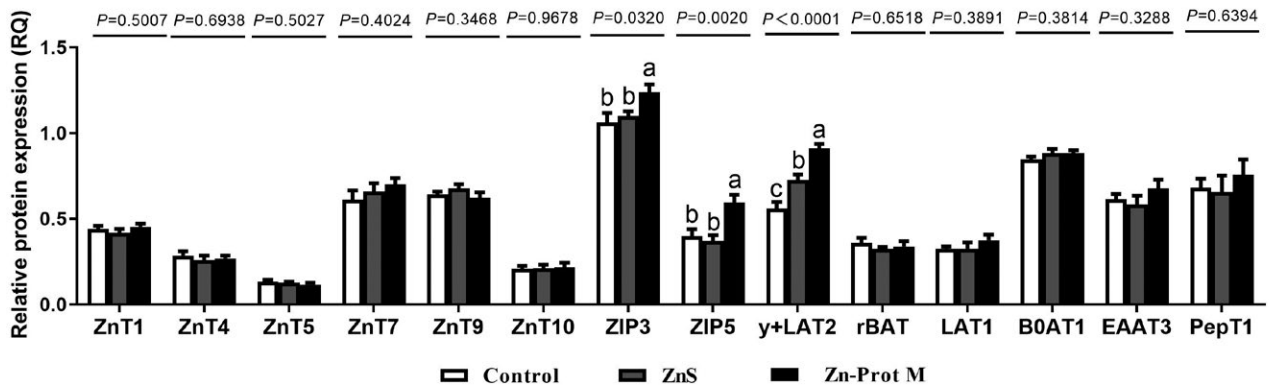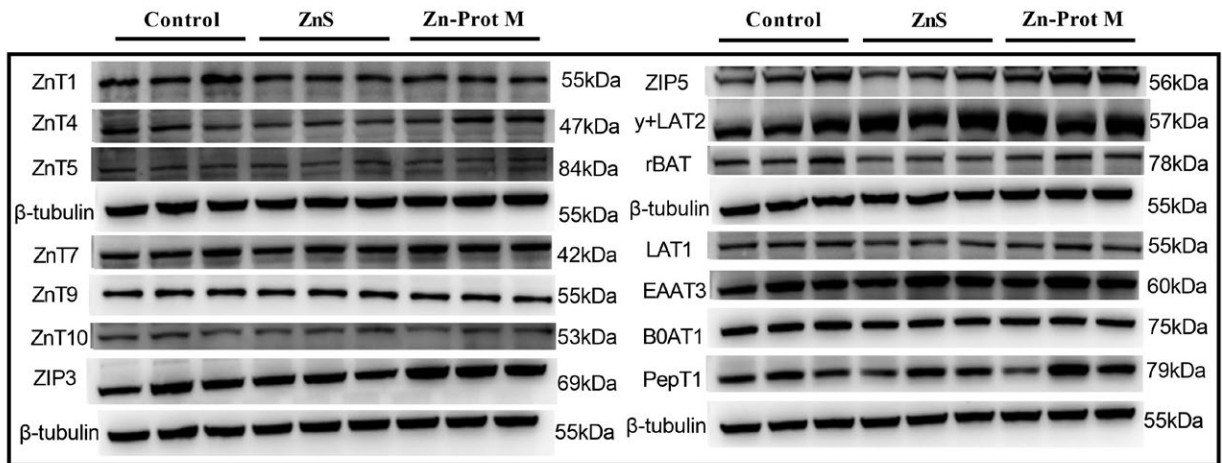**Figure 2.** Effect of Zn source (added Zn level of 400 µmol/L) on protein expression levels of ZnT, ZIP, amino acid, and small peptide transporters in primary cultured duodenal epithelial cells at 120 min of incubation (experiment 3). Values are means ± SE (*n* = 5 to 6). Lacking the same letters (a, b, c) means significant differences (*P* < 0.05). ZnT1, zinc transporter 1; ZnT4, zinc transporter 4; ZnT5, zinc transporter 5; ZnT6, zinc transporter 6; ZnT7, zinc transporter 7; ZnT9, zinc transporter 9; ZnT10, zinc transporter 10; ZIP3, zrt–irt-like protein 3; ZIP5, zrt–irt-like protein 5; B0AT1, b-0-system neutral amino acid co-transporter; LAT1, L-type amino acid transporter 1; rBAT, b0,+-type amino acid transporter; EAAT3, excitatory amino acid transporter 3; PepT1, peptide-transporter 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ZnS, zinc sulfate; Zn-Prot M, zinc proteinate with moderate chelation strength (Q_f = 51.6). The protein expression levels were calculated as the relative quantities of the target gene protein band intensity to the β-tubulin protein band intensity.

elevated protein expression levels of ZnT1, ZnT4, ZnT7, ZnT10, ZIP3, and ZIP5 in the small intestine of broilers. Similarly, in the current study, it was found that Zn-Prot M upregulated the protein abundances of ZIP3 and ZIP5 in the primary cultured duodenal epithelial cells compared to the control and ZnS. However, no significant changes were observed in the protein abundances of ZnTs. Factors other than Zn source, such as feed consumption, environmental, and physiological factors, might be responsible for the disparities mentioned above in findings between in vivo and in vitro studies. For example, feed consumption change as affected by Zn may regulate the protein expression levels of ZnTs in the small intestine of broilers possibly by affecting the intake of Zn and other nutrients. Dufner-Beattie et al. (2006) reported that mice become more vulnerable to Zn deficiency in the absence of the ZIP3 gene, indicating that ZIP3 participated in the absorption of Zn. Geiser et al. (2013) demonstrated that ZIP5 was involved in the control of Zn excretion in mice. Therefore, the results of this study suggest that ZIP3 and ZIP5 could potentially be involved in the transport of Zn as the organic Zn-Prot M in the primary duodenal epithelial cells of boilers.

According to previous studies, the absorption of organic trace elements might be influenced by amino acid and peptide transporters (Gao et al., 2014; Hu et al., 2022, 2023). Gao et al. (2014) reported that Cu could be transported as a Cu-methionine complex in the Caco-2 cells, which was facilitated by amino acids transporters. Our recent studies revealed that broilers fed a Zn-Prot M diet exhibited increased protein expression levels of y + LAT2 and PepT1 in the duodenum, jejunum, or ileum of broilers compared to those fed a ZnS diet, suggesting that these amino acid and peptide transporters might play an important role in the absorption of Zn from the organic Zn-Prot M (Hu et al., 2022, 2023). Similar results were observed in our current study regarding y + LAT2 protein expression level in the cells, suggesting that the absorption of Zn from the organic Zn-Prot M in primary duodenal epithelial cells might also be enhanced by the neutral amino acid transporter y + LAT2.

In the present study, we also observed the discordant mRNA and protein expression levels of certain Zn and amino acid transporters in the primary duodenal epithelial cells. For example, the mRNA expression of B^0AT1 was significantly decreased by Zn-Prot M compared to the control and ZnS, whereas no significant change was detected in its corresponding protein expression. The protein expression levels of ZIP3 and ZIP5 were significantly greater in the Zn-Prot M than in the control and ZnS. However, no changes were observed in the mRNA expression levels of these proteins. This might reflect negative feedbacks for the expression of their mRNA or protein, or the presence of other regulatory factors that are not currently understood (Chen et al., 2002).

The findings from the present study suggest that ZIP3, ZIP5, and y + LAT2 might play an important role in the transport of Zn from the organic Zn-Prot M in the primary duodenal epithelial cells of chick embryos. Nevertheless, further studies are needed to be conducted to validate the functions of the aforementioned carriers in promoting the transport of Zn as Zn-Prot M in primary epithelial cells using gene silencing and overexpression techniques.

Conclusions

Zn-Prot M increased Zn absorption in the primary duodenal epithelial cells of broiler embryos compared to ZnS possibly by elevating the protein expression levels of ZIP3, ZIP5, and y + LAT2 at a Zn concentration of 400 µmol/L.

Acknowledgments

Conflict of interest statement

None of the authors have any conflicts of interest to declare.

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