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Effects of Copper Source and Concentration on in Vitro Phytate Phosphorus Hydrolysis by Phytase

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Five copper (Cu) sources were studied at pH 2.5, 5.5, and 6.5 to determine how Cu affects phytate phosphorus (PP) hydrolysis by phytase at concentrations up to 500 mg/kg diet (60 min, 40−41 °C). Subsequently, Cu solubility with and without sodium phytate was measured. Adding Cu inhibited PP hydrolysis at pH 5.5 and pH 6.5 ($P < 0.05$). This inhibition was greater with higher concentrations of Cu. Tri-basic copper chloride and copper lysinate inhibited PP hydrolysis much less than copper sulfate pentahydrate, copper chloride, and copper citrate ($P < 0.05$). A strong negative relationship was observed between PP hydrolysis and soluble Cu at pH 5.5 ($r = -0.76$, $P < 0.0001$) and 6.5 ($r = -0.54$, $P < 0.0001$). In conclusion, pH, Cu concentration, and source influenced PP hydrolysis by phytase in vitro and were related to the amount of soluble Cu and the formation of insoluble copper−phytin complexes.

KEYWORDS: Copper source; phytate phosphorus; phytase; solubility

INTRODUCTION

Poultry diets are comprised mainly of plant-based materials. The major storage form of phosphorus in plant seeds is phytate (*myo*-inositol hexakisphosphate). In the phytic acid molecule, six of the twelve replaceable protons are strongly acidic ($pK_a < 3.5$), three are weakly acidic with pK_a values between 5.7 and 7.6, and the remaining three are very weakly acidic with pK_a values greater than 10. Therefore, phytic acid is strongly negatively charged over a wide pH range (*1*). Phytic acid can easily chelate divalent and trivalent metal ions, such as Ca^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+} and form mineral−phytin complexes existing in soluble and/or insoluble forms (*2*−*4*). At pH 6.5, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, and Zn^{2+} inhibit phytate phosphorus (PP) hydrolysis by microbial phytase and reduce P release from PP. The concentrations that cause 50% inhibition of PP hydrolysis for Zn^{2+} and Ca^{2+} at pH 6.5 are 0.47 and 5.1 mM, respectively (*4*).

The normal pH of gastric and intestinal digesta of chickens is approximately 2.5 and 6.5, respectively (*5*). Researchers have also studied the impact of this range of pHs in the gastrointestinal tract (GIT) on chelation of macro-minerals to PP and therefore PP hydrolysis by phytase. For example, the addition of 1.0, 4.0, or 9.0 g of Ca/(kg of poultry diet) reduced ($P < 0.05$) PP hydrolysis at pH 2.5 and pH 6.5 in vitro, and the inhibitory effect was greater at pH 6.5 than at pH 2.5 (*6*). Although Ca causes significant suppression of PP hydrolysis due to its high concentration in the diet, it has a lower affinity for phytic acid than Cu or Zn, which have the highest affinity for phytic acid (*7*). The effect of Cu on PP hydrolysis is not known yet.

A 125−250 mg Cu/kg diet (from copper sulfate pentahydrate) is usually added to poultry diets by the poultry industry due to its positive effects on bird growth and purported antimicrobial properties (*8*). However, the high amount of Cu in diets may reduce the capacity of PP to be hydrolyzed by phytase because of its high affinity for phytate. Up to 250 mg/kg diet supplementation of Cu from $CuSO_4 \cdot 5H_2O$ (Cu Sul) did not affect the efficacy of phytase in broilers but lowered apparent P retention regardless of phytase supplementation by 11−15 percentage units (*9*). Supplementation with 250 mg Cu/kg diet from copper citrate (Cu CIT) also decreased apparent P retention ($P < 0.05$) but copper chloride (Cu CL) and copper lysinate (Cu Lys) did not (*10*). The most commonly used source of Cu as a dietary supplement for poultry is Cu Sul. Other dietary Cu sources may include Cu CL, Cu Lys, Cu CIT, and tri-basic copper chloride ($Cu_2(OH)_3Cl$; TBCC). Because different chemical sources of Cu have different chemical properties, the solubility and chelation to PP within the GIT may be different with different Cu sources.

Phytases catalyze PP hydrolysis and are available commercially from a number of different vendors. Natuphos (BASF, Mt. Olive, NJ) is a phyA gene product produced by *Aspergillus ficuum* (niger), with two pH optima at 2.5−3 and 5−5.5. This 3-phytase has a $K_m = 27$ μM and a $K_{cat.} = 348$ S^{1-} using inositol hexaphosphate (IP_6) as the only substrate (*11*). Ronozyme (DSM Inc, Parsippany, NJ) is produced by *Aspergillus oryzae* carrying the *Peniophora lycii* phytase gene and is a 6-phytase. Its optimal pH is 5.0−5.5 and K_m and $K_{cat.}$ are 33 μM and 2200 S^{-1}, respectively, using IP_6 as the only substrate (*11*). A third phytase, *Escherichia coli* (*E. coli*) appA phytase, has been expressed in different yeasts including *Saccharomyces cerevisiae* (*S. cerevisiae*). It hydrolyzes PP over a broad pH range (2.5−5.5) and is a 6-phytase with $K_m = 130$ μM and $K_{cat.} = 6209$ S^{-1} using

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IP_6 as the only substrate (*12*). Because different phytases have different kinetic properties, the in vivo efficacy may vary considerably due to retention time in the GIT, differences in pH, and solubility properties of PP on exposure to macro- and micro-minerals and other factors.

Therefore, the present study was designed to determine the effects of Cu concentration and source on in vitro PP hydrolysis at different pH conditions by three phytases. Subsequently, the effect of Cu source on the solubility of Cu and copper−phytate complexes was determined in vitro.

MATERIALS AND METHODS

Materials. The phytate used was the dodecasodium salt from rice (Sigma, St. Louis, MO; analytical reagent). Copper sulfate pentahydrate, tri-basic copper chloride (TBCC, Micronutrients, LLC, Indianapolis, IN), and Cu Lys and Cu CIT (Monarch Nutrition Laboratories, Inc., Park City, UT) were feed grade. Copper chloride dihydrate was analytical reagent grade (Sigma-Aldrich Co., St. Louis, MO; analytical reagent). A proprietary experimental *E. coli* appA2 phytase was expressed in *S. cerevisiae*. The other two phytases: Ronozyme PL (DSM Inc., Parsippany, NJ) and BASF Natuphos 5000 L (BASF, Mt. Olive, NJ) were purchased. Phosphorus standard (1027 ug/mL P in H_2O) was purchased from Sigma. Standard Cu solution (1000 mmol/L in 2% HCl for atomic absorption spectrophotometry) was produced from Spex Certi Prep (Metuchen, NJ), and HCl was trace-metal grade (Fisher Scientific, Pittsburgh, PA). All glassware was acid-washed in 3% H_2SO_4 solution and rinsed with distilled, deionized water.

In Vitro Phytate Phosphorus Hydrolysis by Phytase. The in vitro PP hydrolysis assay was modified from that of Tamim and Angel (*6*). One phytase unit (U) is defined as the amount of enzyme that releases 1 μmol of inorganic phosphate from sodium phytate/min from 5.0 mM phytate at pH 5.5 and at 40−41 °C. Assuming that the typical PP content in corn−soybean meal-based broiler diets is 2.7 g PP/kg, the phytase concentration is 500 units/kg, and feed to water consumption is 2:1, the concentrations of phytic acid and phytase in the GIT are 2.8 mM and 5.4 units/L, respectively. Considering the dilution effect when mixing phytate and phytase solution, 2.9 mM phytate and 166.7 units/L phytase solutions were made. Subsequently 3 mL of phytic acid and 0.1 mL of phytase were mixed and incubated at pH 2.5, 5.5, and 6.5 (simulating pHs in birds' GIT) at 41 °C (simulating birds' body temperature) for 60 min. The reaction was stopped by addition of 2.07 mL of ammonium molybdate−metavanadate reagent (*13*). The amount of P hydrolyzed was then measured colorimetrically at 410 nm, using inorganic P as a standard. All incubations were done at least in triplicate. The buffers used were 0.2 M glycine−HCl (pH 2.5) and 0.2 M sodium acetate (pH 5.5 and pH 6.5).

Effect of Cu on PP Hydrolysis by Phytase. Five Cu solutions with concentrations of 0, 22.0, 43.0, 86.0, and 172.0 mmol/L were prepared to represent dietary Cu concentrations (0, 62.5, 125, 250, and 500 mg/kg diet) and 2:1 feed to water consumption assumed. In the poultry industry, 125−250 mg Cu/kg diet is normally added to diets (*8*). When fed at up to 250 mg Cu/kg diet, the efficacy of phytase in broiler chicks was not affected but apparent P retention was greatly reduced regardless of phytase supplementation by 11−15 percentage units (*9*). Therefore, up to 500 mg Cu/kg diet was chosen in this study. Substrate solutions were prepared by mixing Cu with phytate and adjusting the pH to 2.5, 5.5, and 6.5 to represent gastric, duodenal, and ileal pHs in chickens, respectively (*5*). Substrate solution (3.0 mL) and phytase solution (0.1 mL, the same pH as the substrate solution) were added into test tubes in triplicate and vortexed for 5 s and incubated at 41 °C for 1 h. The reaction was stopped by addition of 2.07 mL of ammonium molybdate−metavanadate reagent (*13*). The P released was expressed as a percentage relative to the P released when no copper was added to the reaction mixtures. Solutions were incubated at room temperature for 20 min. The amount of P hydrolyzed was then measured colorimetrically at 410 nm, using inorganic P as a standard. The experiment was repeated three times.

Solubility of Cu Sources with and without Phytate. The solubility of five sources of Cu (Cu CIT, Cu CL, Cu Lys, Cu Sul, and TBCC) was measured at concentrations of 22.0, 43.0, 86.0, and 172.0 mmol/L in 0.2 mM glycine−HCl (pH 2.5) and 0.2 mM sodium acetate buffers (pH 5.5 and pH 6.5). Each Cu source was mixed with 40 mL of buffer with and without 4.6 M phytate in triplicate and incubated at 41 °C in a shaking water bath for 1 h and filtered through 42 μm Whatman filter paper for Cu analysis (*14*) by atomic absorption spectroscopy. Soluble and insoluble copper phytate was calculated as follows:

$$\text{solubility (\%)} = (\text{soluble Cu/total Cu}) \times 100$$

$$\text{insoluble copper phytate (\%)} = 100 \times (\text{soluble Cu A1} - \text{soluble Cu A2})/(\text{total Cu})$$

where A1 = soluble Cu in the solution without phytate addition and A2 = soluble Cu in the solution with phytate addition.

Statistical Analysis. Statistical analysis was completed by ANOVA, using the general linear model procedures of SAS (SAS Institute, Inc., Cary, NC). Differences between means were determined by Student−Newman−Keuls (SNK) multiple comparison test. The significance level (α) was 0.05. Correlation coefficients between PP hydrolysis and soluble Cu without phytate addition were also determined.

RESULTS

Effect of Cu Addition on PP Hydrolysis by Different Sources of Phytase. No substantial differences were observed among phytase sources within any experiment; therefore, the results of only one phytase source (Ronozyme phytase) are presented.

Effect of Cu Addition on PP Hydrolysis by Phytase at Different pH Values. Copper influenced PP hydrolysis differently at different pHs ($P < 0.0001$). At pH 2.5, no inhibition was noted among the treatments except 250 and 500 mg Cu/kg diet from Cu CL and 500 mg Cu/kg diet from Cu Sul. At pH 5.5 and pH 6.5, Cu addition greatly inhibited PP hydrolysis ($P < 0.0001$). The inhibition was greater at pH 6.5 than at pH 5.5, which was greater than at pH 2.5 ($P < 0.0001$). At pH 2.5, over 80.0% of phytase activity was retained for all Cu sources and concentrations (**Figure 1**). Addition of 500 mg Cu/kg diet from Cu Sul inhibited PP hydrolysis by 12.6% ($P \leq 0.05$), whereas, both 250 and 500 mg Cu/kg diet from Cu CL inhibited PP hydrolysis by 10.4 and 19.4%, respectively ($P \leq 0.05$). Concentrations up to 500 mg Cu/kg diet from TBCC, Cu Lys, or Cu CIT did not inhibit PP hydrolysis ($P \leq 0.05$).

At pH 5.5, addition of Cu Sul, Cu CL, and Cu CIT inhibited PP hydrolysis ($P \leq 0.05$) (**Figure 2**). Phytate P hydrolysis was inhibited more by Cu Sul and Cu CL than other Cu sources and was 22.0 and 26.4% of PP hydrolysis in control groups, respectively, when 500 mg Cu/kg diet was added. Addition up to 500 mg Cu/kg diet from Cu Lys did not affect PP hydrolysis. Phytate P hydrolysis was decreased 13.4% ($P \leq 0.05$) by 500 mg Cu/kg diet from TBCC but was not affected by lower Cu concentrations.

Increasing pH to 6.5 greatly increased the extent of inhibition of PP hydrolysis by Cu Sul and Cu CL (**Figure 3**). The addition of 62.5 mg Cu/kg diet from Cu Sul and Cu CL inhibited P release by 89.8 and 92.0%, respectively. Tri-basic copper chloride and Cu Lys caused less inhibition of PP hydrolysis than the other three Cu sources. The extent of inhibition ranged from 10.9 to 51.4% ($P \leq 0.05$). Tri-basic copper chloride inhibited PP hydrolysis in a concentration-dependent manner, but Cu Lys did not. The addition of Cu from Cu CIT also decreased PP hydrolysis. However, the extent of inhibition was moderate compared to Cu CL and Cu Sul, not Cu Lys and Cu Sul ($P \leq 0.05$).

Solubility of Different Sources of Cu at Different pH Values. At pH 2.5, no significant solubility differences were observed among the five Cu sources (**Table 1**). At pH 5.5, the order of solubility for five sources was TBCC < Cu Lys or Cu CIT < Cu Sul or Cu CL. At pH 6.5, the order of solubility was TBCC < Cu Lys < Cu CIT < Cu Sul < Cu CL (**Table 1**).

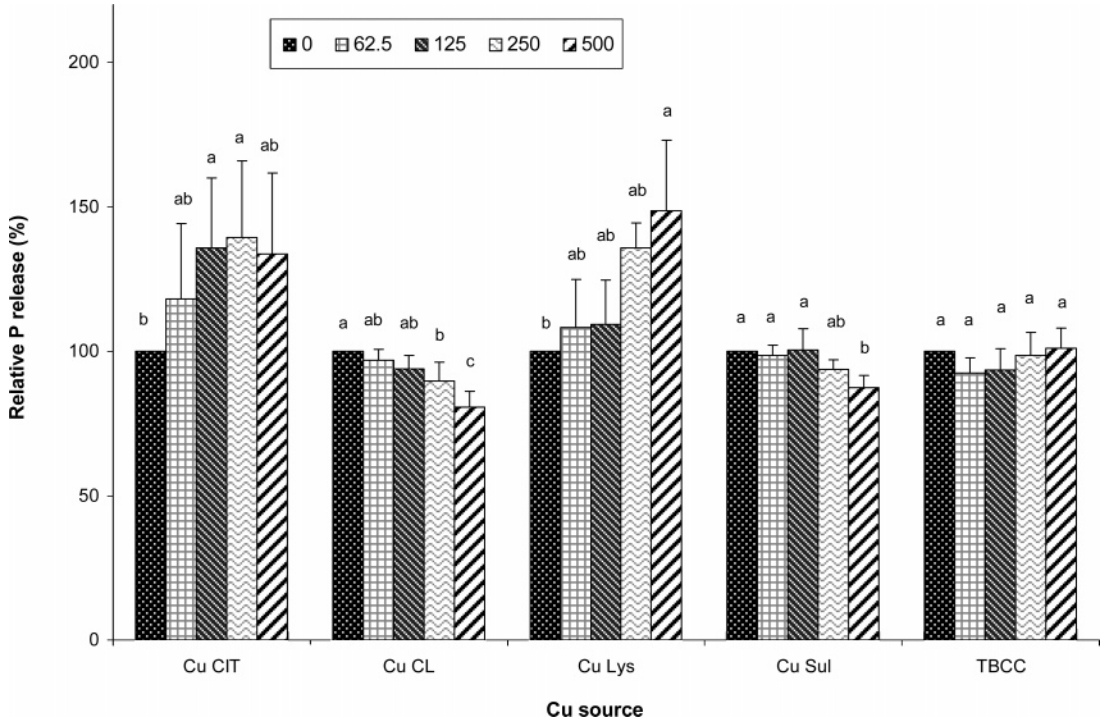

Figure 1. Effect of Cu source and concentration on phytase efficacy at pH 2.5 (a–d) Means within each Cu source with no common superscript differ significantly ($P \leq 0.05$). Error bars represent standard deviation ($n = 3$).

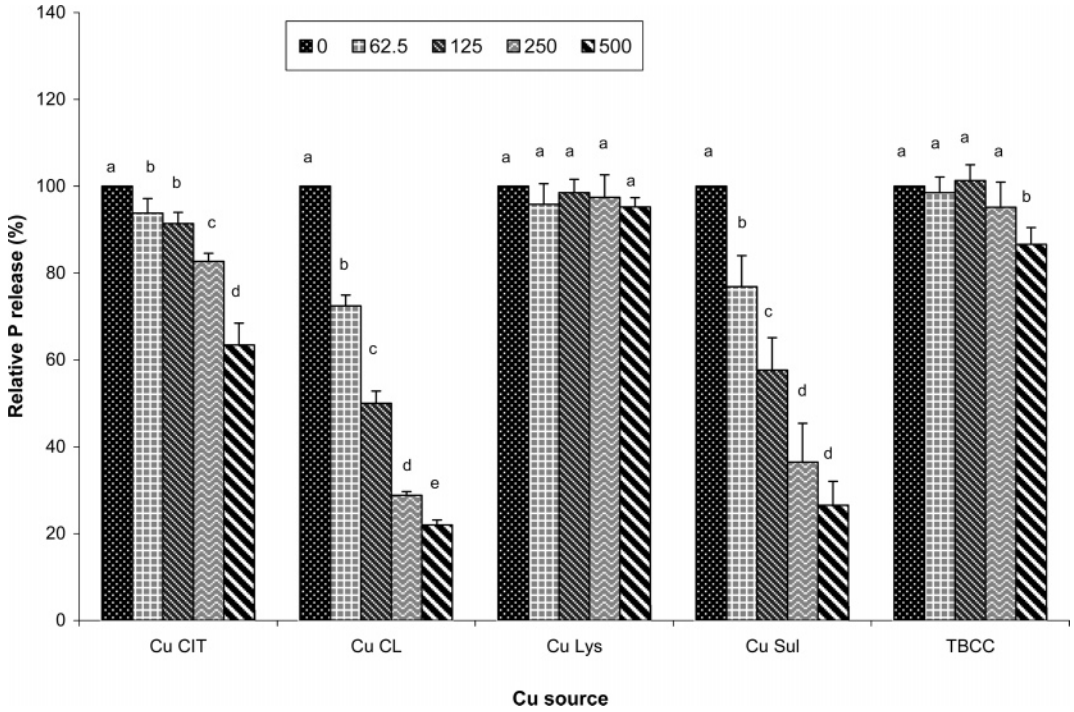

Figure 2. Effect of Cu source and concentration on phytase efficacy at pH 5.5 (a–d) Means within each Cu source with no common superscript differ significantly ($P \leq 0.05$). Error bars represent standard deviation ($n = 3$).

The amount of insoluble copper−phytate complex was also measured at each pH. At pH 2.5, among all the five sources, no insoluble copper−phytate was measured. At pH 5.5, Cu Sul formed the most (41.3%) insoluble copper−phytate among the five Cu sources and Cu CIT formed the least (9.6%) (**Table 2**). At pH 6.5, Cu Sul still formed the greatest amount of insoluble copper−phytate (48.2%). The TBCC, Cu CIT, and Cu Lys formed the least, and there was no significant difference among the three sources. Copper chloride formed 39.1% insoluble copper−phytate.

Correlation between PP Hydrolysis and Soluble Cu without Phytate. At pH 5.5 and pH 6.5, a strong negative relationship was observed between PP hydrolysis and soluble Cu (**Table 3**). The more dissolved Cu resulted in less PP hydrolysis.

DISCUSSION

Copper addition inhibited PP hydrolysis by phytase at pH 5.5 and pH 6.5 in vitro. These results do not agree with the in

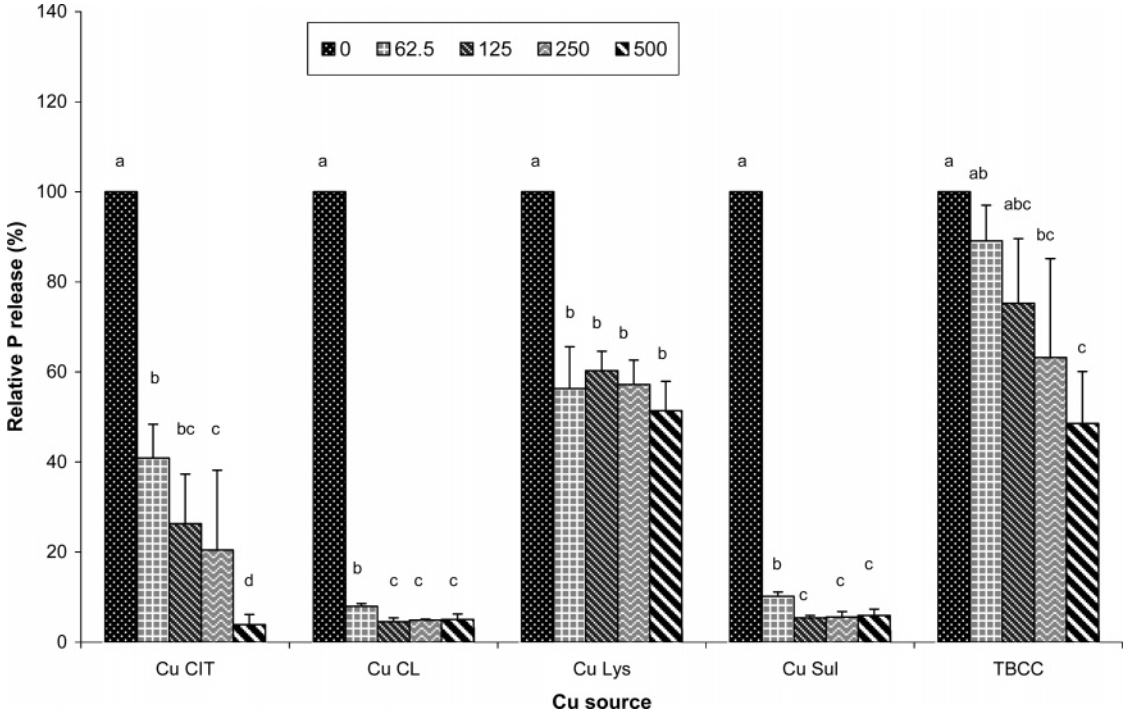

Figure 3. Effect of Cu source and concentration on phytase efficacy at pH 6.5 (a–d) Means within each Cu source with no common superscript letters differ ($P \leq 0.05$), $n = 3$. Error bars represent standard deviation ($n = 3$).

Table 1. Solubility of Different Sources of Copper without Phytate at Different pH Values[a]

	solubility[a] (%)		
Cu source	pH 2.5	pH 5.5	pH 6.5
Cu CIT	99.2	61.6b	59.4c
Cu CL	100.0	97.7a	91.8a
Cu Lys	99.4	59.4b	47.1d
Cu Sul	99.1	97.0a	87.9b
TBCC	100.0	42.1c	9.4e
SEM[b]	0.4	1.3	0.9

[a] Solubility means within each column with no common letter (a–e) differ significantly ($P \leq 0.05$). Means are averages across 62.5, 125, 250, and 500 mmol/L Cu concentrations for each Cu source from one experiment, each treatment conducted in triplicate. [b] Standard error of the means.

Table 2. Insoluble Copper Phytate[a] of Different Sources of Copper at Different pH Values

	insoluble copper phytate (%)	
Cu source	pH 5.5	pH 6.5
Cu CIT	9.6e	4.2c
Cu CL	25.4c	39.1b
Cu Lys	17.6d	5.7c
Cu Sul	41.4a	48.2a
TBCC	33.0b	6.1c
SEM	0.69	0.62

[a] Solubility means within each column with no common letter (a–e) differ significantly ($P \leq 0.05$). Insoluble copper phytate (%) = (soluble Cu A1 − soluble Cu A2)/(total Cu) × 100, where A1 = without phytate addition and A2 = with phytate addition. Means are averages across 62.5, 125, 250, and 500 mmol/L Cu concentrations for each Cu source from one experiment, each treatment conducted in triplicate.

Table 3. Correlation between PP Hydrolysis and Soluble Copper without Phytate Addition

	soluble Cu		
	pH 2.5	pH 5.5	pH 6.5
PP hydrolysis[a]	0.04	–0.76	–0.54
P value	0.75	<0.0001	<0.0001

[a] Correlation coefficients between PP hydrolysis and soluble Cu, representing the averages across 62.5, 125, 250, and 500 mmol/L Cu concentrations and Cu sources from one experiment, each treatment conducted in triplicate.

vivo results of Banks et al. (*9*). They reported that the addition of up to 250 mg Cu/kg diet from Cu Sul did not affect the efficacy of phytase but greatly reduced P retention whether dietary phytase was supplemented or not. The reasons are perhaps the differences in the substrates and phytases between the in vitro and in vivo experiments as was described for experiments with Ca by Tamim Angel (*6*). In the in vitro experiment of Tamim and Angel, sodium phytate was the only phytate source. In the in vivo trial, phytate was derived from corn and soybean meal based diet, where it existed as mixed salts of K, Mg, and Ca with the cations already bound to phytic acid. Therefore, dietary Cu may not have bound to phytic acid as readily as it did in vitro because of binding affinity competition with other cations. In addition, IP_6 was the only form of phytate in the in vitro experiment, whereas in the in vivo trial, there might have been other forms of inositol phosphates (IP_5, IP_4, IP_3, or IP_2). Compared with IP_6, the lower inositol phosphates are much easier for birds to utilize because of a higher solubility of the complexes formed (*15*) and are more easily hydrolyzed by nonspecific acid phosphatases.

Results demonstrate that the effect of Cu on the ability of phytase to hydrolyze PP is dependent on pH. At pH 2.5 (gastric pH), no inhibition was noted among the treatments except that the addition of 250 and 500 mg Cu/kg diet from Cu CL and 500 mg Cu/kg diet from Cu Sul slightly inhibited PP hydrolysis. When pH was increased to the pHs of small intestinal digesta (5.5 and 6.5), PP hydrolysis was greatly inhibited. These results agree with Tamim and Angel (*6*), where adding Ca reduced PP hydrolysis at pH 6.5, with the more acidic pH causing a less pronounced effect than at a more neutral pH. Hydrolysis of PP by phytases occurs when phytate is soluble (*16*). Most phytate−

mineral complexes are soluble at low pHs (less than 3.5) (*17*). Phytate chelates with Ca and/or other cations and forms insoluble complexes (*18*) at higher pHs (5.50, 6.06, and 6.62) (*5*). These insoluble complexes are less available for phytase to catalyze hydrolysis, thus decreasing utilization of PP by animals. There are 12 proton dissociation sites in the phytic acid molecule. Six sites are strongly acidic ($pK_a < 3.5$), three are weakly acidic with pK_a values between 5.7 and 7.6, and the remaining three are very weakly acidic with pK_a values greater than 10 (*1*). Dissociation of the protons leaves the phytic acid molecule negatively charged, which makes phytic acid able to easily bind cations. At pH 2.5, phytic acid has fewer protons dissociated than at pH 5.5 and pH 6.5, resulting in less Cu chelated to phytic acid. In addition, at pH 2.5, Cu was bound either as a soluble complex with phytate or did not bind with the phytate molecule. However, at pH 5.5 and pH 6.5, insoluble copper phytate was formed (**Table 3**). Therefore, one of the possible reasons causing the pH dependence of Cu on phytase efficacy is that the copper−phytate complex may form more insoluble complexes at pH 5.5 and pH 6.5, thereby reducing the efficacy of phytase.

The results demonstrate that inhibition of Cu on PP hydrolysis by phytase was dependent on Cu concentration at pH 5.5 (except TBCC and Cu Lys) and pH 6.5. In solution, Cu exists in Cu^{2+} and or bound forms. When phytate is added, it chelates Cu^{2+}. The copper−phytate complex may exist in soluble and/or insoluble forms. The insoluble complex is less available to be hydrolyzed by phytase, thus decreasing utilization of PP in animals. In this study, we observed that the higher the Cu concentration, the more Cu was dissolved with all Cu sources and pHs (data not shown). Therefore, this influence of Cu concentration on PP hydrolysis may result from the more soluble Cu and more copper−phytate complex at high Cu concentration treatment. In addition, the size of the soluble mineral complexes also influences the availability of PP for absorption. The smaller the size of the phytate−mineral complex, the greater the surface area available for the phytase to bind and hydrolyze PP. Shafey et al. (*5*) observed that as dietary Ca increased, the proportion of complexes having a molecular size greater than 100 000 also increased. Therefore, differences between sizes of soluble phytate−copper complexes formed at different Cu concentrations may also be a factor resulting in the Cu concentration effect. However, the size of copper−phytate complexes was not measured in this study.

The results demonstrate the effect of Cu on phytase's ability to hydrolyze PP was dependent on the Cu source, which agrees with in vivo experiment results. Banks et al. (*10*) observed that supplementation with 250 mg Cu/kg diet from Cu CIT or Cu Sul decreased apparent P retention; however, supplementation with 250 mg Cu/ kg diet from Cu Lys or Cu CL did not affect apparent P retention. In this study, TBCC and Cu Lys sources had less inhibition on PP hydrolysis than Cu Sul, Cu CL, and Cu CIT at pH 5.5 and pH 6.5. This difference may result from the different solubilities of Cu sources. Copper Lys and TBCC had lower solubilities than Cu Sul, Cu CIT, and Cu CL. Lower solubility resulted in less Cu^{2+} in solution and less Cu^{2+} bound to phytate and higher PP hydrolysis. Moreover, Cu Lys formed less insoluble copper−phytate than Cu Sul and Cu CL at both pH 5.5 and pH 6.5. Tri-basic copper chloride formed less insoluble copper−phytate than Cu Sul and Cu CL at pH 6.5 but not at pH 5.5. The less insoluble copper−phytate present, the more soluble phytate was available for hydrolysis. This may contribute to the lower inhibition of Cu Lys on PP hydrolysis at pH 5.5 and 6.5 and TBCC at pH 6.5. Moreover, difference between sizes of soluble phytate−copper complexes formed by different Cu sources may also contribute to this phenomenon.

In conclusion, pH, Cu concentration, and Cu source all influence PP hydrolysis by phytase. Copper greatly inhibited the efficacy of phytase as pH increased toward neutrality, with higher concentrations of Cu causing more inhibition. Cu Lys and TBCC inhibited P release less than Cu CIT, Cu Sul, and Cu CL, which may be a result of lower solubility and less insoluble copper−phytate being formed.

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