Effect of phytate on laying hen performance, carbonic anhydrase activity and total cholesterol level

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Phosphorus is an indispensable mineral, crucial to growth and development both structurally and metabolically but phytate (a major storage form of phosphorus in seeds of plants) decreases mineral bioavailability and nutrient digestibility. Subsequently, in determining the influence of phytate with/without phytase or citrate on laying performance, 5 soybean-maize based diets of similar nutrient density were formulated: a control diet with 0.21% phytate-P and 0.36 % non-phytate-P but no added phytate, and a second similar diet except that the inorganic phosphorus source was replaced by added phytate-P. The third, fourth and fifth diets were similar to the second diet but they contained 0.3% phytase, 1.5% citrate, 0.3% phytase plus 1.5% citrate, respectively. Each treatment was randomly assigned to 35-week-old laying hens. Hens fed the phytase diet showed a pronounced increase in egg production ($P<0.05$), followed by the citrate treatment, while the phytate-P based diet depressed egg production compared to the rest of the treatments. Though the differences in eggshell thickness were insignificant ($P>0.05$), egg weights were affected ($P<0.05$) by the various dietary treatments; the phytase plus citrate diet yielded the highest egg weight improvement. Carbonic anhydrase in the shell gland mucosal extracts depicted significant variation in its specific activity ($P<0.05$), with no observed differences between the control and the phytate-P, phytase or citrate treatments, but phytase combined with citrate had significantly the highest activity of carbonic anhydrase. Serum zinc and yolk iron were significantly improved ($P<0.05$) by phytase and/or citrate treatments, but serum copper or yolk total lipid and mineral concentrations were not affected by the dietary treatments ($P>0.05$). Both phytase and phytase plus citrate treatments yielded high serum and yolk total cholesterol, followed by the citrate and phytate-P, and the least for the control diet. Supplementary phytase and/or citrate seems to increase nutrients availability. Whether citrate increases susceptibility of phytate to phytase by removing cations bound to phytate or creating digesta pH conducive for phytate hydrolysis is yet to be determined.

Keywords: Phytate; phytase; citrate; hen performance; carbonic anhydrase; cholesterol

Introduction

Phosphorus is an indispensable mineral, crucial to growth and development both structurally and metabolically (Boiling *et al.,* 2000a,b). Laying hen requires phosphorus for production of eggs, replacement of tissue metabolites such as nucleotides and phospholipids and to maintain skeletal integrity, but its excess in faeces poses hazard to the environment. However, about two-thirds of the

organic phosphorus of cereals and oil seed meals is present with a phytin-linkage, its phosphate groups can form either simple or mixed salts *i.e. chelates.* Phytate was observed to decrease mineral bioavailability as well as nutrient digestibility, it complexes with protein and inhibits ∝-amylases, trypsin, tyrosinase and pepsin (Singh and Krikorian, 1982; Deshpande and Cheryan, 1984; Caldwell, 1992).

Furthermore, there is a proposition that negatively charged phytate can form insoluble complexes of less digestibility with positively charged protein at low pH (Cheryan, 1980; Thomson and Serraino, 1986), this phytate–protein binding might occur at high pH also, mediated by polyvalent cations, such as calcium, magnesium, or zinc (Anderson, 1985). Subsequently the current study was undertaken to examine the hypothesis that; phytate as a dietary phosphorus resource in an entire plant-based diet negatively influences hens laying performance, activity of carbonic anhydrase in the eggshell gland, as well as reduces both egg and serum cholesterol levels which could be reversed by phytate hydrolysis, either through phytase supplementation and/or its acidification in a plant-diet.

Materials and methods

Animals and diets

Fifty laying hens were selected from a stock of 250 commercial strain of layers which had been monitored for a fortnight, to exclude any non-laying hen and hen that laid deformed eggs or eggshells, and were randomly allocated into individual experimental cages, and allowed two weeks to adapt to the cages, with *ad libitum* access to a proprietary layer mash and water.

Five soybean-maize based mash diets (Table 1) of similar nutrient density were formulated to be isonitrogenous, isocaloric, equal in sulphur amino acids and lysine and to meet the requirements for the essential nutrients as outlined by the Ministry of Agriculture Fisheries and Food (1974). The control treatment was a conventional diet with 0.21 % phytate-P and 0.35 % non-phytate-P, the non-phytate-P was mainly provided by addition of dicalcium phosphate with no added phytate. But in the second treatment, the source of inorganic phosphorus as in the control diet was replaced by added phytate in order to raise the level of total phytate-P to 0.36% and to reduce the level of non-phyate-P to 0.04%. The third, fourth and fifth dietary treatments were similar to the second except that they contained 0.3% phytase, 1.5% citrate and the combination of 0.3% phytase and 1.5% citrate, respectively.

Husbandry and procedure

Each dietary treatment was assigned to group of 10 individually caged layers (10 replicates/dietary treatment) in a completely randomised design. The birds were daily observed for the form of droppings, incidences of egg deformation. Both the photoperiod (16hrs/day) and the thermostatically controlled temperature (22°C) were maintained throughout the eight weeks experimental period. Live weights were determined on the first and the last days of the experiment. Feed intake was measured weekly, egg production and egg weight were recorded daily, and eggshell thickness was measured twice a week by micrometer at the average of two points at the equatorial region excluding the shell membrane.

Sample collection and tissue preparation

At day 56, the birds were sacrificed by cervical dislocation. Blood samples were obtained through a cardiac heart puncture (using a 20-gauge needle syringe), each sample was then divided between a heparinised tubes (50 units/ml) placed on ice, and a non-heparinised blood which were allowed to clot and the serum was separated by centrifugation at 600g for 10 minutes, then preserved at 4ºC pending determinations of the analytes: alkaline phosphatase, inorganic phosphorus, iron, zinc, copper and total cholesterol.

Tubular oviduct was removed from each experimental bird. The shell gland was rapidly excised and placed on ice, cut open and the mucosa was scraped gently with the edge of a microscope slide to minimise contamination of the enzyme extract with blood.

Table 1 Composition of the Experimental Diets (g/kg as fed)

Ingredient	Dietary Treatments				
	Control	Phytate-P	Phytase	Citrate	Phytase + Citrate
Soybean meal	245.0	245.0	245.0	245.0	245.0
Maize	554.0	554.0	554.0	554.0	554.0
Maize starch	65.5	63.1	60.1	40.6	37.6
Maize oil	27.5	27.5	27.5	35.0	35.0
Dl-methionine	2.0	2.0	2.0	2.0	2.0
Dicalcium Phosphate	14.4	0.0	0.0	0.0	0.0
Limestone	40.3	40.3	40.3	40.3	40.3
Oyster shell	27.4	27.4	27.4	27.4	27.4
Feed supplement[1]	12.5	12.5	12.5	12.5	12.5
Calcium carbonate	9.0	16.7	16.7	16.7	16.7
Salt	2.4	2.4	2.4	2.4	2.4
Sodium phytate	0.0	9.1	9.1	9.1	9.1
Phytase	0.0	0.0	3.0	0.0	3.0
Citric acid	0.0	0.0	0.0	15.0	15.0
	1000	1000	1000	1000	1000
Calculated Composition					
CP	165.0	165.0	165.0	165.0	165.0
ME, Kcal/kg	2869.6	2832.5	2820.3	2817.5	2805.4
Lysine	8.8	8.8	8.8	8.8	8.8
Methionine	5.0	5.0	5.0	5.0	5.0
Calcium	36.1	36.1	36.1	36.1	36.1
Non-phytate-P	3.5	0.4	0.4	0.4	0.4
Phytate-P	2.1	3.6	3.6	3.6	3.6
Determined Composition					
Non-Phytate-P	4.30	0.61	0.58	0.53	0.56
Phytate-P	3.30	4.23	4.40	4.21	4.33

[1]Feed supplements provides per kilogram diet: vitamin A 10 IU, vitamin D3 3 IU, vitamin E 0.008 IU, copper (cupric sulphate) 0.005mg, vitamin B1 0.01g, vitamin B2 0.005g, vitamin B6 0.001g, vitamin B12 0.008mg, vitamin K 0.002g, nicotinic acid 0.02g, pantothenic acid 0.01g, folic acid 0.001g, biotin 0.05mg, choline 0.075, iron 0.01g, cobalt 0.025g, manganese 0.08g, zinc 0.06g, iodine 0.001g, selenium 0.00015g.

The tissue was weighed, suspended in deionised water and ultrasonicated in an ice-bath to maintain the suspension temperature below 4°C (Yoselewitz and Balnave, 1989). The homogenate was then centrifuged at 4500 rpm for 35 minutes at 4°C, and the clear supernatants obtained were transferred individually into 20 ml plastic syringes, immediately sealed and stored at 4°C pending measurement of carbonic anhydrase activity.

Determinations and statistical analyses

Dietary nutrient concentration; Ca, Zn, Fe, Mn, Mg, Cu and Zn, and Cu in plasma and egg yolk were measured using atomic absorption photometry after wet ashing (AOAC, 1984), while dietary nitrogen content and P were determined using auto-analyser. Serum alkaline phosphatase and inorganic phosphorus were determined according to Sigma Diagnostics ALP (Sigma ALP optimised Alkaline Phosphatase EC 3.1.3.1 Colorimetric Test) and Fiske and SubbaRow (1925), respectively. The influence of dietary treatments on the activity of carbonic anhydrase in the shell gland mucosa was determined following the electrometric method of Wilbur and Anderson (1948) as amended by Yoselewitz and Balnave (1989). Plasma samples from the respective treatment replicates were assayed for total cholesterol according to Menagent Cholesterol Tinder Method, which is based on the concentration proportionality of the total cholesterol to the absorption of the quinoneimine when read at 500 nm. Yolks from randomly collected eggs

representing each of the dietary treatment were assayed for total cholesterol content according to Bair and Marion (1978) modified colorimetric procedure of Pearson *et al.,* (1953).

All the generated data were subjected to analysis of variance, and the treatment means were compared using the least significant difference effect as outlined by Gomez and Gomez (1984).

Results and discussion

Performance of the laying hens

The effect of phytate with or without phytase or citrate on laying hens performance are summarised in Table 2. Both body weight change and feed intake showed no significant differences (P>0.05) among the dietary treatments, however feed conversion ratio showed significant differences (P<0.05) with phytate diet showed the highest grams of feed required to produce one gram of the egg, while the lowest was depicted by the phytase diet. Egg mass showed significant differences amongst the dietary treatments, with phytase diet had the highest egg mass followed by phytase combined with citrate, whilst, phytate diet gave the lowest egg mass compared to the rest of the dietary treatments. Egg production expressed as percent day production varies significantly with hens fed phytase showed a pronounced increase in egg production (P<0.05), followed by the citrate treatment, however, hens fed on the combination of phytase plus citrate gave lower value lower than that of the control diet, but phytate-P based diet had the lowest percent egg production compared to the rest of the treatments. Though the egg weights were significantly affected (P<0.05) by the various dietary treatments, they seemed to respond in the same pattern as percent egg production; with the exception that supplementary phytase in combination with citrate had the highest improvement in egg weight.

Feeding supplementary phytase and or citrate did not significantly (P>0.05) affect eggshell thickness although shell thickness values for the respective treatments had a similar response pattern compared to that of the production. In comparison to the rest of the dietary treatments, phytate depresses egg production and reduces egg weight and shell thickness, these deteriorations in egg quality attributes were alleviated by incorporation of phytase with or without citrate.

Serum and yolk mineral concentrations

Supplementary phytase and/or citrate resulted in insignificant differences (P>0.05) among serum copper concentrations (Table 3). However, serum zinc concentrations vary significantly (P<0.05), with observed enhancement in serum zinc concentration when phytase combined with citrate was fed than either citrate or phytase, but phytate treatment had the lowest concentration of serum zinc.

Table 2 Effect of phytase and/or citrate on laying hen performances

Parameter	Dietary treatments					
	Control	Phytate	Phytase	Citrate	Phytase + Citrate	±SEM
Body change (g/hen)	32.8	24.7	33.0	27.4	32.3	4.31^{ns}
Feed intake (g/day)	111.6	124.0	124.1	113.8	112.9	8.34^{ns}
FCR (g feed/g egg)	2.39^{a}	3.02^{b}	2.27^{a}	2.35^{a}	2.34^{a}	0.18*
Egg production (%)	75.7^{ab}	67.9^{b}	82.9^{a}	76.4^{ab}	72.1^{b}	1.277*
Egg weight (g)	61.6^{b}	60.4^{b}	62.7^{ab}	62.4^{ab}	66.2^{a}	1.85*
Shell thickness (mm)	0.41	0.38	0.42	0.41	0.40	0.013^{ns}
Egg mass (g/hen/day)	46.63^{ad}	41.01^{b}	51.98^{c}	47.68^{d}	47.78^{d}	0.70*

Values are means of 10 replicates per treatment ±SEM.
[a-d] Means within a row lacking common superscripts differ significantly.
* (P<0.05). [ns] insignificant (P>0.05).

The effects of phytate with or without phytase or citrate on yolk mineral concentrations are presented in Table 3, with the exception of iron which showed significant difference (P<0.05) among the treatments, dietary treatments did not result in any significant differences (P>0.05) in yolk dry matter, phosphorus, magnesium and zinc concentrations.

Table 3 Effect of phytase and/or citrate on serum and yolk mineral concentration

Parameter	Dietary treatments				Phytase +	
	Control	Phytate	Phytase	Citrate	Citrate	±SEM
Serum Cu, mg/ml	0.3	0.4	0.3	0.4	0.4	0.08^{ns}
Serum Zn, mg/ml	6.0^{c}	5.2^{cd}	6.9^{bc}	7.5^{b}	9.0^{a}	1.13*
Yolk DM, %	50.5	48.7	49.4	50.0	51.3	1.33^{ns}
Yolk P, %	1.0	1.1	1.2	1.1	1.1	0.05^{ns}
Yolk Mg, %	0.01	0.02	0.02	0.02	0.02	0.002^{ns}
Yolk Fe, ppm	137.3^{a}	70.5^{c}	105.3^{b}	100.0^{bc}	107.5^{ab}	12.33*
Yolk Zn, ppm	132.5	109.3	119.0	129.8	130.8	11.14^{ns}

Values are means of 5 replicates per treatment ±SEM.
[a-d] Means within a row lacking a common superscript differ significantly.
* (P<0.05). [ns] insignificant (P>0.05).

Though yolk iron concentrations were significantly different, the control diet that had a source of inorganic phosphorus without any added phytate yielded the highest iron concentration, followed by phytase plus citrate, but phytate diet was observed to yield the lowest yolk iron level.

Carbonic anhydrase activity

Table 4 shows the average mean times for the pH to change over the selected reference range for the five dietary treatments, as well as the effect of the dietary treatments on the activity of carbonic anhydrase. It is noted that the control treatment gave the longest carbon dioxide hydration time followed by the phytate-P, phytase and citrate (P<0.05), however phytase plus citrate diet had the shortest carbon dioxide hydration time.

The specific activity of carbonic anhydrase showed significant differences amongst the dietary treatments (P<0.05), however, there was no observed significant difference (P>0.05) in carbonic anhydrase activity between the control and the phytate-P dietary treatments. Though the differences between the phytase and citrate treatments were not significant (P>0.05) both treatments were significantly different (P<0.05) compared to both the control and phytate-P treatments. Supplementing phytate-P based diet with phytase combined with citrate at the level fed resulted in significantly higher specific carbonic anhydrase activity (P<0.05) compared to the rest of the dietary treatments.

Serum and yolk total cholesterol contents

Table 4 shows that feeding dietary treatments resulted in significant differences (P<0.05) among serum cholesterol levels, both phytate-P and citrate treatments showed no significant difference (P>0.05) in serum cholesterol level compared to the control, but they were significantly different (P<0.05) compared to phytase and phytase plus citrate. A high serum cholesterol level was depicted by both phytase and phytase combined with citrate compared to the control (P<0.05). Despite the highest yolk total lipids percent (69.4%) in the group that were fed on phytate-P diet supplemented with citrate and the lowest were in phytate-P diet group, dietary supplementations did not result in any significant differences among the dietary treatments (P>0.05). However, supplementing phytate-P diet with either phytase and/or citrate resulted in phytase or phytase plus citrate having the highest cholesterol level (P<0.05) compared to the control treatment, with exception of citrate treatment which

was insignificantly different compared to the control, the rest of dietary supplements showed higher cholesterol content ($P<0.05$).

The current observed reductions in egg production, egg weight and shell thickness associated with phytate diet are consistent with the findings that hens consuming phosphorus unsupplemented soybean meal diets had a depression in egg production (Carlos and Edwards, 1998) which were alleviated upon feeding phytase and/or citrate, which seems to agree with the proposition that at low pH a negatively charged phytate can form an insoluble complex with positively charged protein (Cheryan, 1980), and this protein-phytate complex has been indicated to have low digestibility (Thompson and Serraino 1986), but alternatively, at higher pH and in the absence of citric acid, phytate-protein complexes may form via polyvalent cations (mediators), such as calcium, magnesium or zinc (Anderson, 1985).

Though avian blood have been reported to contain appreciable amounts of carbonic anhydrase (Heald *et al*., 1968), this study has kept contamination of the enzyme extracts with blood to extremely minimal, however, both Yoselewitz and Balnave (1989) have found that the haemoglobin content of the enzyme extract was insignificant, and the poor shell quality may be associated with the lack of bicarbonate ions to the lumen of the shell gland, which raises the possibility that a reduced carbonic anhydrase in the shell gland may be an important factor in poor egg shell calcification. This seems to concur with the current findings, given that phytate-P dietary treatment had the lowest carbonic anhydrase activity, and the lowest of all dietary treatments in terms of egg production, egg weight and shell thickness, furthermore, the observed highest carbonic anhydrase activity shown when phytase was combined with citrate might be attributed to their associated effect, as in separate experiment Jongbloed *et al.* (1996) have reported a synergistic effect between microbial phytase and lactic acid or formic acid on the total phosphorus digestibility in growing pigs. However, in the current study with laying hens, supplementing citrate plus phytase seems to interact positively. But the problems associated with poor eggshell formation are normally considered to reflect an inadequate supply of calcium, that might be aggravated by the presence of phytate, since carbonic anhydrase plays an important role in eggshell calcification through the hydration of carbon dioxide produced in uterine tissue to carbonic acid, a weak acid which is the major source of carbonate ions for eggshell formation (Balnave *et al*., 1992).

Table 4 Effect of phytase and/or citrate on activity of carbonic anhydrase, yolk lipid, serum and yolk cholesterol levels

Parameter	Dietary treatments					
	Control	Phytate	Phytase	Citrate	Phytase + Citrate	±SEM
CO_2 hydration, sec[1]	56.53[a]	43.55[b]	39.14[c]	31.28[d]	20.07[e]	2.55
Carbonic anhydrase activity[2]	2.53[c]	2.23[c]	4.89[b]	5.44[b]	6.65[a]	0.53
Serum cholesterol, µg/assay	5.99[a]	13.24[a]	22.44[b]	13.69[a]	27.29[b]	5.83
Yolk Total Lipid, %	63.10	60.20	63.30	69.40	63.30	4.02 [ns]
Cholesterol, µg/yolk DM	2.79[b]	3.67[ab]	4.02[a]	3.26[b]	3.76[a]	0.38

Values are means of 5 replicates per treatment ±SEM.
[1] Time in seconds required for the hydration of CO_2 within the pH range from 7 to 5.
[2] Unit/gm tissues.
[a-d] Means within a row lacking a common superscript differ significantly.
* ($P<0.05$). [ns] insignificant ($P>0.05$).

The observed lowest total lipids level due to intakes of phytate diet are consistent with the findings of Sharma (1986), he associated lower serum lipids levels with higher intake of the phytic acid rich legumes, also an earlier findings have shown that addition of purified phytic acid to a rat diet have significantly reduced plasma lipids levels (Klevay, 1977). However, the hypolipidemic effect of phytic acid was thought by Klevay (1977) to be related to its higher affinity for binding zinc compared to copper, since higher plasma zinc:copper ratio predisposes human to cardiovascular disease, but separate results with rats have indicated that the rise in hepatic total lipids, hepatic and serum triacylglycerols, serum phospholipids and hepatic NADPH-generating enzymes by sucrose feeding,

were all significantly depressed by dietary sodium phytate at the 0.5% level (Katayama, 1995). However, in the current findings where the levels of copper fed were maintained the same in all the dietary treatments, any variability might be attributable to phytate hydrolysis, since phytate might induce copper deficiency.

A positive relationship had been noted between serum calcium and serum cholesterol (Sloan *et al.,* 1994) which was consistent with the earlier reports that hens having elevated serum cholesterol also had elevated concentrations of serum calcium (Harms and Simpson, 1979), however, in the current study serum calcium was not determined, but the fed diets were of similar levels of calcium.

In the current studies, both zinc and copper levels were maintained at equal levels in all the dietary treatments, but the addition of phytate-P as phosphorus source specially in the entire phytate-P diet might have resulted in a decrease in both zinc and copper levels which were rendered available by both phytase and or citrate dietary supplements. Supplementary phytase and citrate seems to improve nutrients availability, given the observed association of phytate diet with low level of carbonic anhydrase activity, and being the lowest of all dietary treatments in terms of egg production, egg weight, shell thickness and total lipids, however, and any emerged variability could be attributed to phytate hydrolysis. And with reference to recommendations that the chelator must be non-toxic and the minerals bound to it should be available to the monogastric animal either by absorption of mineral-chelate complex or by the dissociation and absorption of the minerals. Such carboxylic acid as "citric acid" has been reported to have higher affinity than phytic acid for mineral binding (Cheryan, 1980, Boiling *et al.,* 2000a,b). Therefore, dietary supplementation with phytase augmented with citrate seems to have the potential to improve the efficacy of dietary phytase supplement. Or alternatively, the citrate might have served in removing cations from binding to phytate and subsequently increases the susceptibility of the phytate to the enzyme or it might have created a digesta pH conducive for phytate hydrolysis.

Phytase and phytase plus citrate treatments apparently had high serum and yolk total cholesterol followed by that of citrate compared to either phytate-P or the control diet, where only one third of the required phosphorus was added to replenish the two third of phytate-P considered to be available. However, it is very difficult to compare egg cholesterol levels between research reports because, not only do analytical methods differ, but birds might also differ genotypically. The possibility of phytate reducing egg cholesterol levels might provide a new approach for production of low cholesterol table eggs, but more research is needed as far as lower inositols (which are hydrolysable) and the chelative effects of higher inositols are concerned.

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