Effects of dietary P and Ca on phytate degradation in broiler chickens – *in vitro* and *in vivo* investigations

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ABSTRACT

Broiler chickens show a great potential to degrade phytate, provided that the diet has a low phosphorus (P) and calcium (Ca) content.

Several studies reported that supplementing mineral P and Ca can diminish this potential and provoke the excretion of unused phytate-P.

In this article, two experiments investigating and separating effects of these minerals on phytate degradation are presented.

The first experiment was conducted in an *in vitro* assay simulating the digestive tract of poultry in three steps to study phytate degradation under standardized conditions.

The second experiment was an *in vivo* experiment, conducted to study single or interactive effects of P, Ca, and phytase supplementation on phytate degradation in broiler chickens.

It was concluded that the supplementation of either P or Ca can have detrimental effects on the degradation of phytate.

The supplementation of mineral P and Ca to poultry diets should be kept on a minimum to use the full potential of broiler chickens to degrade phytate and thus a maximum of plant-based P.

Keywords: Broiler chicken, calcium, *in vitro*, *in vivo*, phosphorus, phytate degradation.

THE ENDOGENOUS POTENTIAL OF BROILER CHICKENS TO DEGRADE PHYTATE

Phytate, the salt of myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate), or short $InsP_6$, is the main storage form of phosphorus (P) in plant seeds.

The six phosphate groups adhering to the inositol ring can theoretically be utilized by the animal after stepwise cleavage initiated by phytate degrading enzymes, so-called phytases.

The cleavage of P yields isomers of lower inositol phosphates (InsP) with different degrees of phosphorylation as intermediate products.

In contrast to ruminants, non-ruminant animals like poultry and swine have only a limited endogenous phytase equipment in their digestive tract and it has long been thought that they are not capable to use phytate-bound P.

That is why in practice either mineral P or microbial phytase products are supplemented –sometimes even both– to meet the P requirements of non-ruminants. During the last years, the view on the non-usable phytate-P has changed, and it is known now that even non-ruminants can utilize some of the phytate-P deriving from the plant components of their feed.

Among non-ruminants, especially broiler chickens show a high potential to degrade phytate and thus use plant-based P. As reviewed by *Rodehutscord and Rosenfelder (2016)*, several studies showed that broilers can degrade up to 62 – 89% phytate when no intrinsic plant phytase or microbial phytase products are included in the diets.

This high degradation is achieved by phytases and other phosphatases produced by the epithelial cells and the microbiota of the intestine. The quantitative contribution of each of the phytase sources is still unknown.

However, a study with gnotobiotic broiler chickens suggests a considerable contribution of epithelial-derived phytases to phytate degradation (42% $InsP_6$ disappearance up to the end of the ileum (prececal) (*Sommerfeld et al. 2019*).

The high potential of broiler chickens to use phytate-P is usually observed when the diets are low in P and calcium (Ca). It has been shown that phytate-P usage is reduced when feed with standard P and Ca formulations is fed.

EFFECTS OF P AND CA SUPPLEMENTATION ON PHYTATE DEGRADATION

Indeed, it was reported that supplementing mineral P sources like monocalcium phosphate (MCP) or Ca, for example in the form of limestone, can diminish phytate degradation (Table 1 and 2).

Table 1.
Results of selected studies regarding prececal $InsP_6$ disappearance in broiler chicken as affected by dietary P, Ca, and phytase level.

Reference	Diet based on	Mineral P source	P level	Ca level	Phytase	pc $InsP_6$ disappearance
			g/kg DM	g/kg DM	FTU/kg	%
Shastak et al. 2014[1,2]	corn	-	3.4	4.8	-	62
	corn	MCP[3]	4.2	5.6	-	50
	corn	MCP	4.8	6.6	-	21
	wheat	-	3.3	4.7	-	61
	wheat	MCP	4.1	5.5	-	47
	wheat	MCP	4.9	6.8	-	23
Zeller et al. 2015[1]	corn-soybean meal	-	4.4	6.0	-	67
	corn-soybean meal	-	4.4	6.5	500	78
	corn-soybean meal	-	4.3	6.3	12500	92
	corn-soybean meal	MCP	5.2	7.6	-	51
	corn-soybean meal	MCP	5.2	7.4	500	58
	corn-soybean meal	MCP	5.3	7.8	12500	92
Siegert et al. 2019[1]	corn-soybean meal	-	4.1	9.2	-	45
	corn-soybean meal	-	4.2	8.9	1500	75
	corn-soybean meal	-	4.1	9.6	3000	92
	corn-soybean meal	MCP	5.6	10.3	-	21
	corn-soybean meal	MCP	5.6	10.5	1500	74
	corn-soybean meal	MCP	5.7	10.2	3000	89

[1]Ca level adjusted with limestone
[2]Dietary Ca and P concentration (g/kg DM) calculated assuming 90% dry matter
[3]MCP, monocalcium phosphate

Table 2.
Results of selected studies regarding prececal $InsP_6$ disappearance in broiler chicken as affected by dietary Ca level and phytase.

Reference	Diet based on	Ca source	Ca level	P level	Phytase	pc $InsP_6$ disappearance
			g/kg DM	g/kg DM	FTU/kg	%
Tamim et al. 2003[1]	corn-soybean meal	-	2.0	4.4	-	67
	corn-soybean meal	$CaCO_3$	7.6	4.4	-	19
Tamim et al. 2004[1]	corn-soybean meal	-	1.9	4.4	-	69
	corn-soybean meal	-	1.9	4.4	500[2]	80
	corn-soybean meal	-	1.9	4.4	500[2]	76
	corn-soybean meal	$CaCO_3$	7.2	4.4	-	25
	corn-soybean meal	$CaCO_3$	7.6	4.4	500[2]	59
	corn-soybean meal	$CaCO_3$	7.2	4.4	500[2]	45
Plumstead et al 2008[1]	corn-soybean meal	CaCO3	5.2	6.0	-	20
	corn-soybean meal	$CaCO_3$	7.8	6.0	-	15
	corn-soybean meal	$CaCO_3$	10.3	6.0	-	9
	corn-soybean meal	$CaCO_3$	12.9	6.0	-	6

[1]Dietary Ca and P concentration (g/kg DM) calculated assuming 90% dry matter
[2]2 different phytases were used

Several studies showed a detrimental effect of supplementing Ca on phytate degradation in broiler chickens (*Tamim et al. 2003, 2004; Plumstead et al. 2008; Amerah et al. 2014; Li et al. 2017*).

Those effects were usually more pronounced when Ca was supplemented together with P (*Manangi et al. 2008; Delezie et al. 2012*).

It has also been shown that effects of phytase supplementation were more pronounced when Ca was on a lower level (*Tamim et al. 2004; Manangi et al. 2008; Delezie et al. 2012; Amerah et al. 2014*).

In broiler studies by *Shastak et al. (2014), Zeller et al. (2015)*, and *Siegert et al. (2019*), a decrease in prececal $InsP_6$ disappearance was observed when a mineral P source was supplemented to a low P/low Ca diet in the absence of a microbial phytase (Table 1).

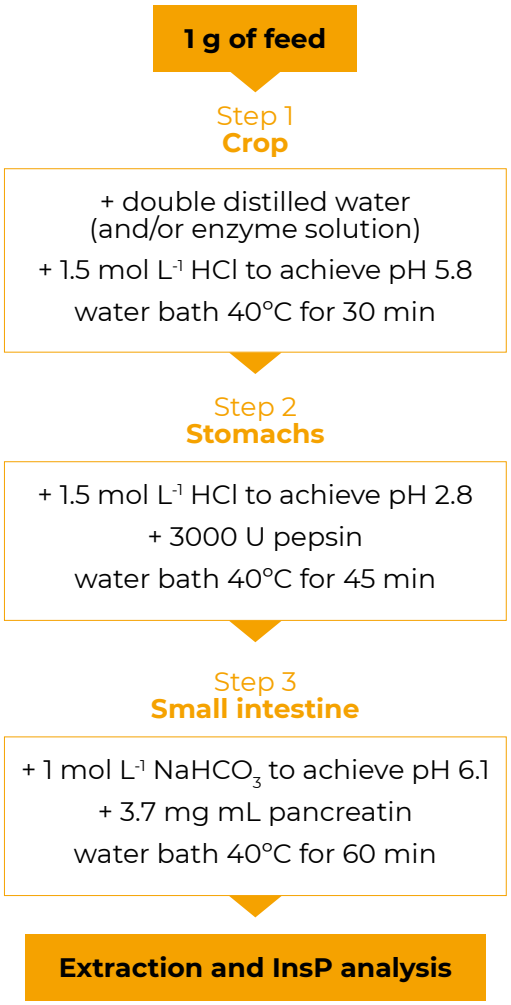
The diminishing effect of P and Ca supplementation was repealed by a very high phytase dosage (*Zeller et al. 2015 and Siegert et al. 2019*).

However, the diminishing effects could still be observed in a slower degradation of lower phosphorylated InsP isomers. In all three studies, P and Ca were supplemented simultaneously which made it impossible to distinguish whether the observed effects derived from the supplemented P, Ca or the combined supplementation.

To address this question, several experiments were conducted in the Animal Nutrition group of the Institute of Animal Science in Hohenheim.

With the aim to implement a fast and cost-effective investigation method and to reduce animal experiments, an *in vitro* assay was established in our laboratory to simulate the poultry's digestive tract and to work on this research question.

IN VITRO ASSAY AND ITS APPLICATION TO STUDY P AND CA EFFECTS ON PHYTATE DEGRADATION



In vitro assays are valuable tools to obtain fast and reproducible results. Animal trials are more time consuming, expensive and need a considerable number of animals.

A three-step *in vitro* assay simulating the crop, stomach and small intestine of poultry as described by *Zyla et al. (1995)* was used as a template. It was modified for our purposes to study the degradation of phytate and the formation of lower phosphorylated InsP isomers.

In each step, a certain retention time, temperature, water content, pH and digestive enzymes are applied or supplemented (see Figure 1).

A mixed broiler feed based on corn and soybean meal low in P and Ca was used as phytate matrix. This contrasts classical *in vitro* assays for phytase characterization in which purified sodium phytate is usually used as a matrix.

Figure 1.
Schematic diagram of the in vitro assay used to study $InsP_6$ disappearance and the formation of lower phosphorylated InsP (from Sommerfeld et al. 2017).

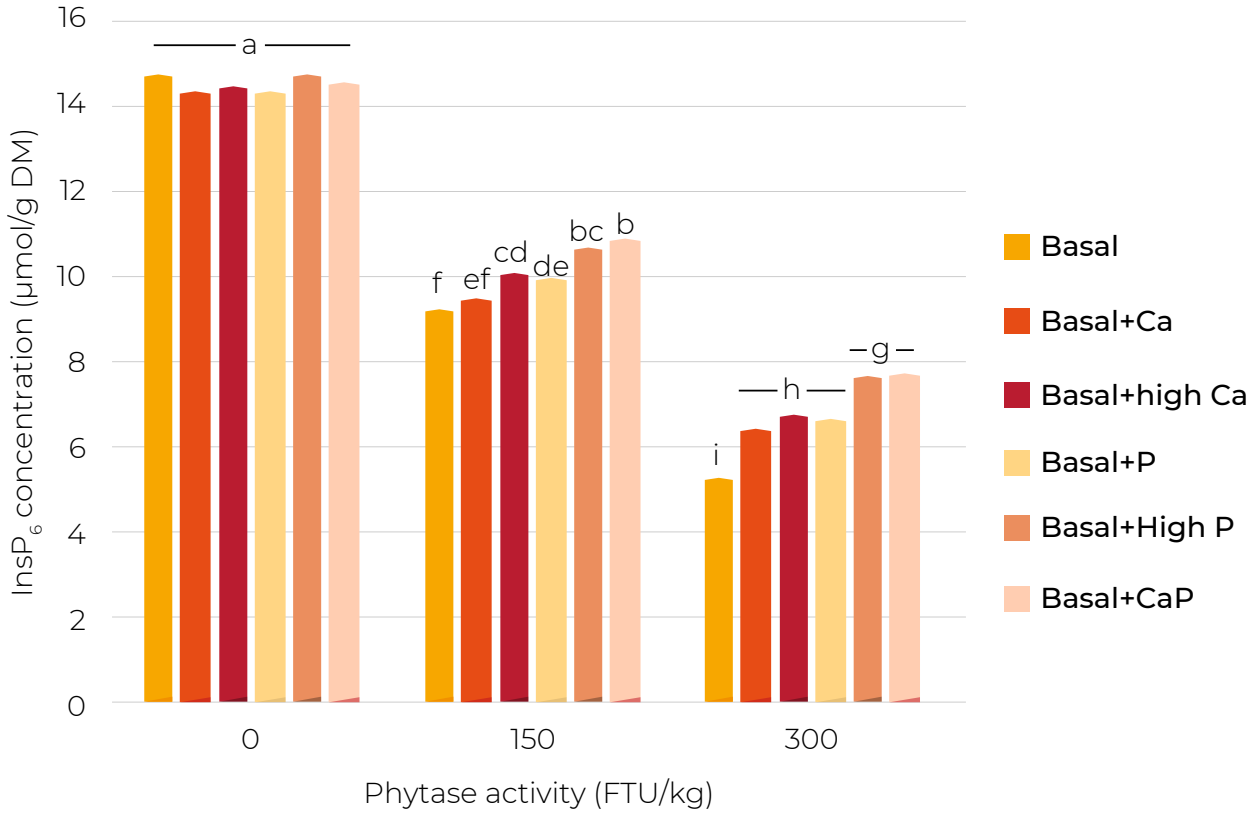


Figure 2.
Concentration of $InsP_6$ in a corn-soybean meal-based diet with three phytase concentrations and with different Ca and P additions after incubation in a three-step in vitro assay (from Sommerfeld et al. 2017)

However, it was shown that phytate degradation in general, and phytase efficacy in particular, depends on the phytate matrix. Therefore, purified sodium phytate is considered not to be the best choice for phytase efficacy studies.

In an experiment that is described in detail in *Sommerfeld et al. (2017)*, first attempts have been made to distinguish between single or interactive effects of P and Ca supplementation in a low P and Ca corn-soybean meal-based broiler feed (herein referred to as Basal).

Different P and Ca levels were achieved by supplementing NaH_2PO_4 (+P and +high P) or limestone (+Ca and +high Ca), or both (+CaP). Three phytase concentrations were applied (0, 150 and 300 FTU/kg). After incubation of the respective feed mixtures, the InsP concentrations in the feed slurries were analyzed.

It was shown that the mineral supplements had no effect when no microbial phytase was supplemented (Figure 2). This occurred since phytate degradation was in general very low without supplemented phytase.

This was expected because the phytase activity of corn and soybean is negligible.

After adding 150 FTU phytase/kg, there was a significant higher $InsP_6$ concentration in +highCa and +P than in the Basal diet.

With +highP or +CaP, the highest concentration, thus lowest degradation of $InsP_6$ was achieved. With 300 FTU phytase/kg, these effects were even clearer.

These results show that mineral supplements can have a diminishing effect on phytate degradation. The diminishing effects of the supplemented P and Ca might be explained by a direct inhibitory effect of P on the added phytase and the formation of Ca-phytate complexes.

Results further demonstrate that the *in vitro* assay reacts in a plausible manner to different supplements. Phytases can be tested prior to an animal trial in an environment similar to the poultry's digestive tract.

This makes it a suitable, standardized tool to preselect treatments for animal trials. This practice avoids unnecessary treatments and reduces the number of test animals.

However, the absolute values of InsP concentrations after incubation do not directly reflect *in vivo* results. As in any *in vitro* assay, many conditions prevailing in poultry's digestive tract cannot be simulated.

Animal related factors like endogenous enzymes from epithelial cells or the microbiota are not considered.

Despite the advantages of this *in vitro* assay, it is still necessary to evaluate dietary effects in the animal model. For that reason, an *in vivo* trial was conducted to separate P and Ca effects on InsP degradation in broiler chicken.

IN VIVO TRIAL TO STUDY P AND CA EFFECTS ON PHYTATE DEGRADATION

In this trial, which is described in detail in *Sommerfeld et al. 2018*, broilers were fed 8 diets based on corn and soybean meal from day 15 to 27. The diets comprised 2 P levels (P-, 4.1 g P/kg DM and P+, 6.9 g P/kg DM), 2 Ca levels (Ca-, 6.2 g Ca/kg DM and Ca+, 10.3 g Ca/kg DM) and 2 phytase levels (0 and 1500 FTU phytase/kg).

All single and interactive effects among these supplements were tested.

The prececal $InsP_6$ disappearance in the absence of microbial phytase (56%) was decreased by the supplementation of P (40%) and even more when P and Ca were added simultaneously (21%; Table 3).

Table 3.
Interaction of P, Ca, and phytase on $InsP_6$ hydrolysis and P disappearance (%) up to the terminal ileum of broiler chickens fed the experimental diets from d 15 to d 27

Phytase	Treatment	$InsP_6$ hydrolysis	P disappearance
0	P-Ca-	56[b]	48
	P-Ca+	54[b]	41
	P+Ca-	40[c]	62
	P+Ca+	21[d]	48
1500	P-Ca-	87[a]	68
	P-Ca+	77[a]	60
	P+Ca-	87[a]	76
	P+Ca+	77[a]	59
	SEM	2.8	1.7
P-value	P	<0.01	<0.01
	Ca	<0.01	<0.01
	Phy	<0.01	<0.01
	P × Ca	<0.01	<0.01
	P × Phy	<0.01	<0.01
	Ca × Phy	<0.01	0.40
	P × Ca × Phy	<0.01	0.41

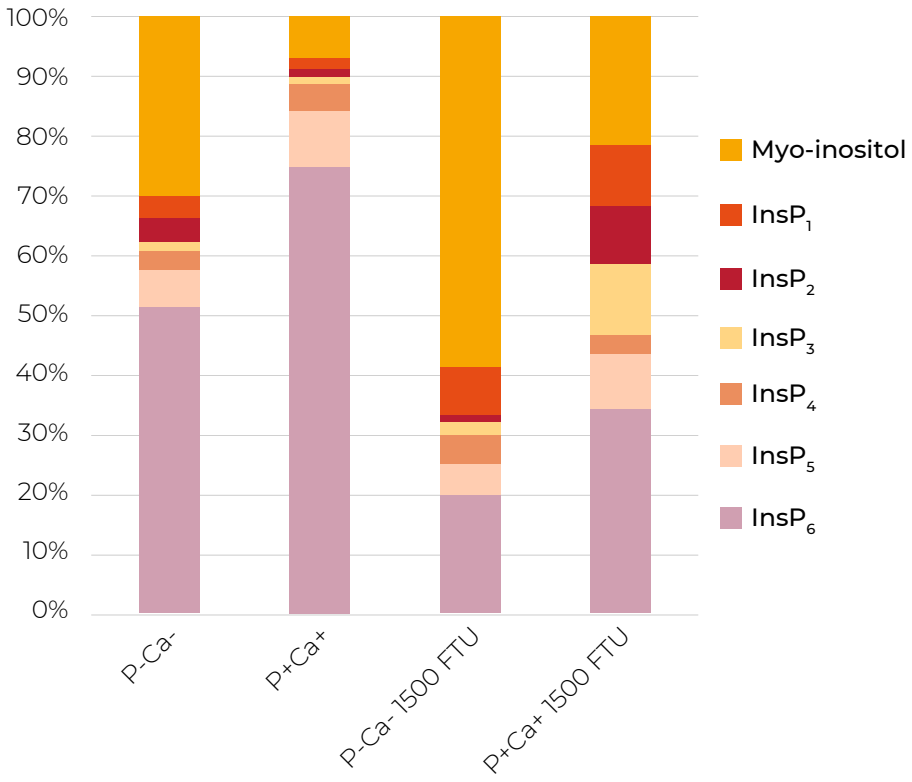


Figure 3.
Relative proportions of the concentrations of InsP esters and myo-inositol in the digesta of the terminal ileum of 27-day-old broiler chickens depending on P, Ca, and phytase supplementation (adapted from Sommerfeld 2018). The sum of the concentrations of InsP esters and myo-inositol on a molar basis is defined as 100%.

When phytase was supplemented, mineral supplements had no diminishing effect (77-87%) on prececal $InsP_6$ disappearance. However, the mineral supplements had clear effects on the degradation of lower phosphorylated InsP (Figure 3).

While the $InsP_6$ disappearance did not differ significantly between the treatments P-Ca- and P+Ca+ in the presence of phytase, the relative proportion of the single InsP and the end product *myo*-inositol (when all P groups are released) differed markedly between those two treatments. P-Ca- had a lower proportion of $InsP_6$ and $InsP_{1-5}$ and consequently a higher proportion of myo-inositol than P+Ca+.

Possible causes include a diminishing effect of mineral supplements especially on the activity of endogenous epithelial and microbiota-derived phosphatases.

This circumstance becomes relevant in the context of efficient P usage and avoidance of unnecessary P excretion. Even if $InsP_6$ has disappeared, a considerable amount of P can still be attached to lower phosphorylated InsP, remain unabsorbed and, hence, is subject to unfavourable effects of poultry farming. This is reflected in the prececal P disappearance that was significantly reduced by Ca supplementation (Table 3), supporting the findings regarding InsP degradation.

CONCLUSIONS

The results of these two trials –*in vitro* and *in vivo*– clearly demonstrate a diminishing effect of P, especially in combination with Ca supplementation on intestinal phytate degradation. Possible causes include affected endogenous phosphatases and formation of mineral-phytate complexes.

Several studies from our group (*Künzel et al. 2019, Shastak et al. 2014, Siegert et al. 2019, Sommerfeld et al. 2018, Sommerfeld et al. 2019, Zeller et al. 2015*) confirm these results and show that even low amounts of supplemented P are detrimental for phytate degradation.

Therefore, a certain amount of potentially usable plant-based P will be left unused and excreted. As not only $InsP_6$, but also lower phosphorylated InsP are affected by mineral supplements, this can have an impact on 1) the utilisation of plant-based phytate-P for the P requirement of the bird and 2) the excretion of unused phytate-P, leading to environmental issues.

In terms of aiming for a sustainable usage of mineral P and the reduction of excreted P due to environmental aspects, supplementation of P and Ca should be kept on a level where the full potential of broiler chickens to degrade phytate can be used without negatively affecting bird health and growth.

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