Prececal amino acid digestibility and phytate degradation in broiler chickens when using different oilseed meals, phytase and protease supplements in the feed

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ABSTRACT The purpose of this study was to investigate the effects of phytase and protease supplementation on prececal (pc) amino acid (AA) digestibility, phytate ($InsP_6$) degradation, and ME_n concentration in diets using 3 oilseed meals as main protein sources in broiler chicken feed. The broiler chicken diets, which lacked mineral phosphorus, contained either soybean meal (SBM), SBM and rapeseed meal (SBM/RSM), or SBM and sunflower meal (SBM/SFM) as main protein sources. Diets were not supplemented with enzymes or supplemented with 1,500 or 3,000 FTU phytase/kg, or with 1,600 mg protease/kg. For diets containing SBM as the main protein source, the effects of phytase supplementation with and without monocalcium phosphate were also investigated. Data were obtained during 2 subsequent runs from days 14 to 22 and from days 23 to 31. Each diet was tested using 8 replicates with 4 replicates per run. For pc AA digestibility, no significant interactions were observed between main protein sources, enzyme supplementation, or addition of monocalcium phosphate except for Cys. Supplementation of 1,500 FTU phytase/kg increased pc digestibility of all AA. No differences in pc AA digestibility were observed between 1,500 and 3,000 FTU phytase/kg supplementation treatments. Prececal disappearance of $InsP_6$ and pc P digestibility were greater in the high phytase supplementation treatment. Protease supplementation increased pc digestibility of all AA except for Cys when SBM/RSM was the main protein source. Supplementation of protease and 3,000 FTU phytase/kg increased ME_n concentrations. The effect of phytase on pc AA digestibility was fully expressed at a lower supplementation level than needed for a maximized pc $InsP_6$ disappearance and ME_n concentration.

Key words: broiler chickens, phytase, protease, amino acids, phosphorus

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INTRODUCTION

High nutrient utilization by farm animals is advantageous because it reduces nutrient input and excretion related to the animal product. This reduces the impact of animal husbandry on the environment. Environmentally relevant nutrients in poultry feed are CP and phosphorus (**P**).

Supplementation of feed enzymes can increase the utilization of nutrients by broiler chickens beyond the intrinsic potential of the digestive system. Exogenous phytase has been established as a feed supplement to hydrolyze phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis [dihydrogen phosphate]; **$InsP_6$**) and its salts, aiming to increase the utilization of plant P to animals (Selle et al., 2012). Phytase supplements can additionally increase prececal (**pc**) amino acid (**AA**) digestibility. Studies examining the effects of phytase supplementation on pc AA digestibility are in disagreement, as phytase supplementation increased pc AA digestibility in some studies (e.g., Rutherfurd et al., 2012; Amerah et al., 2014; Sommerfeld et al., 2018), but not in others (e.g., Sebastian et al., 1997; Rodehutscord et al., 2004). Proteases are another additive that can increase pc AA digestibility. Effects of protease supplementation are also divergent. In studies examining broiler chickens

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and turkeys, protease supplementation was reported to decrease (e.g., Walk et al., 2018; Borda-Molina et al., 2019), increase (e.g., Angel et al., 2011; Stefanello et al., 2016; Cowieson et al., 2018; Borda-Molina et al., 2019), or to have no effect (e.g., Boguhn et al., 2011; Kaczmarek et al., 2014; Erdaw et al., 2017; Borda-Molina et al., 2019) on pc AA digestibility.

Distinct features of phytases, such as optimal pH or temperature, can explain variations in efficiency of pc $InsP_6$ hydrolysis and pc P digestibility (Chung et al., 2013). The effects of protease supplementation on pc AA digestibility depended on the protease product (Manangi et al., 2009; Borda-Molina et al., 2019) and supplementation level (Angel et al., 2011; Borda-Molina et al., 2019). However, additional effects on the efficacy of phytase and protease supplementation need to be investigated. Ingredient composition of the feed directly affects the substrate and can modify other conditions that influence enzymes in the digestive tract. For example, different concentrations and locations of $InsP_6$ in seeds can affect the occurrence of protein-$InsP_6$ and protein-cation-$InsP_6$ complexes (Selle et al., 2012). It has been shown that the effect of phytase and protease supplementation on pc CP and AA digestibility can differ among feedstuffs (Ravindran et al., 1999; Rutherfurd et al., 2002). For phytase, this information is based on diets containing the investigated feedstuffs as the sole source of protein (Ravindran et al., 1999; Rutherfurd et al., 2002, 2012). However, little is known regarding the influence of feedstuff on pc AA digestibility in mixed feed supplemented with phytase and protease.

The effects of phytase supplementation on $InsP_6$ hydrolysis and P digestibility in broiler chickens depended on the concentration of calcium (**Ca**) carbonate and supplementation of monosodium phosphate in the diets (Sommerfeld et al., 2018). Similar effects were also described when other sources of mineral P and Ca were used, such as monocalcium phosphate (**MCP**) (Shastak et al., 2014; Zeller et al., 2015b). Reduced gastrointestinal hydrolysis of $InsP_6$ means that more substrate is available for the formation of protein-$InsP_6$ complexes and protein-cation-$InsP_6$ complexes (Selle et al., 2009). Such complexes might affect the efficacy of phytase supplementation on pc AA digestibility. Unlike the influence of MCP on the efficacy of phytase, no such effects have been reported for protease.

Therefore, the primary objective of this study was to investigate the effects of phytase and protease supplementation on pc AA digestibility and $InsP_6$ disappearance in feed when using 3 different oilseed meals as main protein sources. The main protein sources used in this study were soybean meal (**SBM**), a mixture of SBM and rapeseed meal (**SBM/RSM**), and a mixture of SBM and sunflower meal (**SBM/SFM**). High phytase and protease supplementation levels were used to investigate the potential of both enzymes to increase pc AA digestibility. No MCP was included in the diets so that phosphate would not influence the efficacy of phytase. Additionally, we examined whether MCP supplementation has interacting effects with phytase supplementation on pc AA digestibility.

MATERIALS AND METHODS

This study was conducted at the Agriculture Research Station "Unterer Lindenhof" in Eningen unter Achalm, Germany. It was approved by the animal welfare authorities of the Regierungspräsidium Tübingen in accordance with German welfare legislation (Project no. HOH42/16TE).

Experimental Setup

Fifteen dietary treatments were investigated in this study. Data were obtained in 2 subsequent experimental runs. Each diet was tested using 8 replicates (4 replicates of each diet per run) in a randomized block design.

Animals and Housing

Unsexed Ross 308 broiler hatchlings were obtained from a hatchery (Brüterei Süd ZN der BWE-Brüterei Weser-Ems GmbH & Co. KG, Regenstauf, Germany). The birds were raised in floor pens (3 × 4 m) on dedusted wood shavings and provided with a commercial starter diet prior to the experiment (Club Mastkükenstarter 4150020, Deutsche Tiernahrung Cremer GmbH & Co. KG, Mannheim, Germany). The commercial starter diet contained per kg 215 g CP, 10.5 g Ca, 5.5 g P, 12.5 MJ ME, 110 mg coccidiostat monensin sodium, 10 IU endo-1.4-β-xylanase (EC 3.2.1.8), and 750 FTU 6-phytase (EC 3.1.3.26).

Experimental runs lasted from day 14 to d 22 (**run1**) and from day 23 to d 31 (**run2**) of the experiment. During these runs, broiler chickens were housed in metabolism cages (1 × 1 m) on wire frames. Eleven birds were kept in each metabolism cage in run 1 and 9 were kept in each cage in run 2 in order to meet the minimum standard of area per bird weight specified in the welfare legislation. Feed and water were provided for ad libitum consumption throughout the experiment.

For the first day 2 after placement, barn lighting was permanent and the temperature was maintained at 34°C. Afterwards, the lighting regimen was maintained at 18 h light and 6 h dark. Temperature was continuously decreased to 19°C until day 21 of the experiment, and then maintained constant.

Experimental Diets

Diets, which consisted mainly of corn, contained either SBM, a 1:1 mixture of SBM/RSM, or a 1:1 mixture of SBM/SFM as the main protein source (Table 1). Diet formulation was based on analyzed nutrient concentrations of the main protein sources (Table 2) and other feed ingredients. Three diets

Table 1. Composition of the experimental diets (g/kg).

Treatment[1]	SB1	SB2	SB3	SB4	SR1	SR2	SR3	SR4	SF1	SF2	SF3	SF4	SB1+	SB2+	SB3+
Monocalcium phosphate	Without monocalcium phosphate												With monocalcium phosphate		
Main protein source	Soybean meal				Soybean meal + rapeseed meal				Soybean meal + sunflower meal				Soybean meal		
Enzyme[2]	NES	1,500 Phy	3,000 Phy	Prot	NES	1,500 Phy	3,000 Phy	Prot	NES	1,500 Phy	3,000 Phy	Prot	NES	1,500 Phy	3,000 Phy
Corn	575	575	575	575	515	515	515	515	512	512	512	512	572	572	572
Soybean meal	350	350	350	350	200	200	200	200	200	200	200	200	350	350	350
Rapeseed meal	.	.	.	.	200	200	200	200	.	.	.	.	.	.	.
Sunflower meal	.	.	.	.	.	.	.	.	200	200	200	200	.	.	.
Soybean oil	40	40	40	40	55	55	55	55	55	55	55	55	40	40	40
NaCl	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Monocalcium phosphate	.	.	.	.	.	.	.	.	.	.	.	.	6	6	6
Ca carbonate	15	15	15	15	16	16	16	16	19	19	19	19	18	18	18
TiO_2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Premix[3]	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Diamol[4]	6	6	6	6	.	.	.	.	.	.	.	.	.	.	.

[1]SB = soybean meal; SR = soybean meal/rapeseed meal; SF = soybean meal/sunflower meal; 1–4 indicate enzyme supplementation in the sequence as described in footnote no. 2; + indicates monocalcium phosphate supplementation.

[2]NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; Prot = 1,600 mg protease/kg.

[3]Supplied per kg of diet: 12,000 IU vitamin A (retinyl acetate), 2,500 IU vitamin D3 (cholecalciferol), 50 mg vitamin E (DL-α-tocopherol), 1.5 mg vitamin K3 (menadione), 2.0 mg vitamin B1 (thiamine), 7.5 mg vitamin B2 (riboflavin), 3.5 mg vitamin B6 (pyridoxine), 20 μg vitamin B12 (cyanocobalamin), 30 mg niacin, 12 mg pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin, 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.8 mg iodine, 0.15 mg selenium, 125 mg anti-oxidant.

[4]Purified diatomaceous earth mainly consisting of SiO_2.

Table 2. Analyzed nutrient concentrations in the main protein sources (g/kg DM unless otherwise stated).[1]

	Soybean meal[2]	Rapeseed meal[3]	Sunflower meal
DM (g/kg)	879	878	900
CP	553	385	416
Crude fat	24	54	17
Crude ash	76	77	71
Crude fiber	38	142	183
Acid detergent fiber	nm	186	198
Neutral detergent fiber	nm	309	293
Starch	49	63	48
Sugar	111	106	79
Ca	3.0	7.3	3.8
P	6.2	10.1	9.8

[1]nm = not measured.

[2]Trypsin inhibitor activity 2.71 g/kg DM; urease activity < 0.2 mg N/g per minute at 30°C.

[3]Glucosinulates 5.47 mmol/kg DM.

containing SBM as the main protein source (SB1+ to SB3+) also contained MCP. Diamol was used as an inert filler to substitute for MCP in the other 4 SBM diets (SB1 to SB4). Diets without MCP containing SBM (SB1 to SB4), SBM/RSM (SR1 to SR4), and SBM/SFM (SF1 to SF4) were either supplemented with 1,500 or 3,000 FTU phytase/kg (Natuphos® E 5000 G, BASF SE, Germany), supplemented with 1,600 mg protease/kg (Ronozyme® Proact, DSM Nutritional Products AG, Kaiseraugst, Switzerland), or not supplemented with enzymes. Diets with SBM containing MCP (SB1+ to SB3+) were either supplemented with 1,500 or 3,000 FTU phytase/kg or not supplemented. Titanium dioxide (TiO_2) was included as an indigestible marker at a level of 5 g/kg. Analyzed total P concentrations in all experimental diets were at low levels (Table 3). Analyses of the diets showed that for most AA, the recommendations of the Gesellschaft für Ernährungsphysiologie (1999) were exceeded. Intended and measured phytase activities were similar. Experimental diets were prepared by "Research Diet Services" (Hoge Maat 10, 3961 NC Wijk bij Durrstede, The Netherlands).

Experimental Procedures

Birds were selected so that each metabolism cage had an equal mean bird weight at the beginning of the experimental runs. Birds were also weighed on days 14, 19, and 21 in run 1 and on days 23, 28, and 30 in run 2. Feed intake was determined for each cage on the same days. The weight of dead birds and feed intake of the birds in the respective cage up to the point of death were recorded. Total excreta were collected twice daily from day 19 to 21 in run 1 and from day 28 to 30 in run 2 after removing impurities such as feathers or feed from the trays. Excreta and feed residues were immediately frozen at −20°C after being collected. Feed intake was corrected for the feed residues. Dead birds were considered in calculation of ADG and ADFI by taking the day of death into account.

Table 3. Analyzed nutrient concentrations in the experimental diets (g/kg DM unless otherwise stated).

Treatment[1]	SB1	SB2	SB3	SB4	SR1	SR2	SR3	SR4	SF1	SF2	SF3	SF4	SB1+	SB2+	SB3+
Monocalcium phosphate	Without monocalcium phosphate												With monocalcium phosphate		
Main protein source	Soybean meal				Soybean meal + rapeseed meal				Soybean meal + sunflower meal				Soybean meal		
Enzyme[2]	NES	1,500 Phy	3,000 Phy	Prot	NES	1,500 Phy	3,000 Phy	Prot	NES	1,500 Phy	3,000 Phy	Prot	NES	1,500 Phy	3,000 Phy
DM (g/kg)	889	889	889	888	893	890	890	892	894	895	893	893	889	888	888
P	4.1	4.2	4.1	4.2	5.3	5.1	5.2	5.2	5.3	5.3	5.3	5.2	5.6	5.6	5.7
$InsP_6$-P	2.8	2.8	2.7	2.6	3.4	3.4	3.4	3.4	3.7	3.6	3.6	3.5	2.8	2.8	2.8
Ca	9.2	8.9	9.6	9.4	9.6	9.5	9.3	9.6	10.0	10.2	10.0	9.7	10.3	10.5	10.2
Ca/P ratio	2.2	2.1	2.3	2.3	1.8	1.8	1.8	1.8	1.9	1.9	1.9	1.9	1.9	1.9	1.8
Gross energy (MJ/kg DM)	19.2	19.3	19.2	19.2	19.9	19.9	19.9	19.9	19.8	19.8	19.8	19.8	19.3	19.3	19.3
CP	243	244	242	243	231	230	233	231	233	233	235	233	245	245	243
Amino acids															
Ala	12.5	12.7	12.3	12.5	11.8	11.7	11.7	11.6	11.6	11.9	11.8	20.0	12.9	12.9	12.8
Arg	16.6	16.8	16.5	16.7	14.5	14.5	14.6	14.5	16.9	17.0	16.8	17.0	17.0	17.0	16.9
Asx[3]	26.6	26.8	26.4	26.7	21.9	21.9	21.8	21.7	23.8	23.8	23.6	24.1	27.6	27.7	27.5
Cys	3.9	4.0	4.0	4.1	4.4	4.3	4.3	4.2	4.0	4.0	3.9	4.0	4.0	4.0	3.9
Glx[4]	45.0	45.5	44.8	45.3	41.7	41.8	41.7	41.3	44.7	44.5	44.1	45.1	46.8	46.9	46.7
Gly	10.4	10.3	10.2	10.3	10.3	10.3	10.3	10.2	11.3	11.4	11.2	11.4	10.6	10.6	10.5
His	7.2	7.1	7.1	7.3	7.3	7.1	7.0	6.9	6.9	6.9	6.9	7.1	7.8	7.8	7.7
Ile	10.4	10.5	10.1	10.1	8.3	8.7	9.0	8.8	9.4	9.5	9.4	9.2	9.9	9.9	9.8
Leu	21.1	21.2	20.8	21.1	18.7	18.9	18.9	18.7	18.9	18.9	18.8	19.0	21.5	21.5	21.4
Lys	13.5	13.4	13.3	13.4	12.0	12.0	12.1	12.0	11.1	11.1	11.0	11.2	13.7	13.7	13.6
Met	3.8	3.8	3.7	3.8	3.9	4.0	4.0	3.9	4.4	4.4	4.4	4.4	3.8	3.9	3.9
Phe	12.5	12.7	12.4	12.6	10.7	10.8	10.8	10.7	11.6	11.6	11.5	11.6	12.7	12.7	12.7
Pro	14.1	14.2	14.0	14.1	14.0	13.7	13.7	13.8	12.7	12.9	12.9	13.1	14.6	14.8	15.0
Ser	13.1	13.1	13.1	13.2	12.0	11.9	11.7	11.7	12.0	11.9	11.8	12.2	13.8	13.8	13.8
Thr	9.7	9.8	9.7	9.8	9.5	9.5	9.5	9.4	9.3	9.2	9.2	9.4	10.0	10.0	10.0
Tyr	8.5	8.6	8.5	8.5	7.5	7.5	7.6	7.4	7.5	7.5	7.4	7.5	8.6	8.6	8.6
Val	11.2	11.3	10.9	10.9	9.8	10.2	10.5	10.3	10.8	10.8	10.7	10.5	10.8	10.7	10.6
Inositol phosphates[5] and *myo*-inositol (μmol/g DM)															
$InsP_6$	15.0	14.9	14.8	14.1	18.6	18.2	18.5	18.2	20.0	19.7	19.4	19.1	15.0	14.9	15.0
Ins(1,2,4,5,6)P_5	1.2	1.2	1.2	1.1	1.6	1.6	1.6	1.5	1.4	1.4	1.4	1.4	1.2	1.2	1.3
Ins(1,2,3,4,5)P_5	0.5	0.5	0.5	0.4	0.9	0.9	0.9	1.0	0.9	0.9	0.9	0.8	0.6	0.5	0.5
Ins(1,2,3,4,6)P_5	LOQ	LOQ	LOQ	LOQ	0.5	0.5	0.5	0.5	LOQ	LOQ	0.4	LOQ	LOQ	LOQ	LOQ
Myo-inositol[6]	2.5	2.6	2.6	nm	1.9	1.9	2.0	nm	2.3	2.3	2.4	nm	2.4	2.4	2.6
Phytase activity (FTU/kg DM)	60	1,480	3,620	80	60	1,570	3,100	80	100	1,550	3,190	100	<60	1,850	3,600

[1]SB = soybean meal; SR = soybean meal/rapeseed meal; SF = soybean meal/sunflower meal; 1–4 indicate enzyme supplementation in the sequence as described in footnote no. 2; + indicates monocalcium phosphate supplementation.

[2]NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; Prot = 1,600 mg protease/kg.

[3]Asp and Asn together.

[4]Glu and Gln together.

[5]LOQ = below limit of quantification of 0.27 μmol/g DM for Ins(1,2,3,4,6)P_5. Concentrations of other measured inositol phosphate isomers were below the respective detection limits in all diets.

[6]nm = not measured.

At the end of the experiment on days 22 and 31 of run 1 and run 2, respectively, birds were anesthetized with a gas mixture and euthanized by CO_2 exposure (Zeller et al., 2015b). The section of the small intestine between Meckel's diverticulum and 2 cm anterior to the ileocecal-colonic junction was removed. Digesta samples were obtained by flushing the terminal half of the removed section with deionized water as described by WPSA (2013). Digesta were pooled for each cage and immediately frozen at –20°C.

Chemical Analyses

Excreta samples were thawed at 4°C and homogenized. Digesta and excreta were freeze-dried before analyses. For AA, energy, P, Ca, Ti, inositol phosphate, and *myo*-inositol analyses, samples were ground to a powder using a vibrating disc mill (Fritsch Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany). For all other analyses, samples were ground using a centrifugal mill (Retsch ZM200, Retsch GmbH, Haan, Germany) equipped with a 0.5 mm sieve. All analyses were conducted in duplicate except for DM in the excreta, which was determined in triplicate.

The official methods for nutrient analyses in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2007) were followed for DM (no. 3.1) and CP (no. 4.1.1). The Vapodest analyzing system (C. Gerhardt GmbH & Co. KG, Königswinter, Germany) was used for Kjeldahl digestion. Amino acid analysis was conducted according to the method described by Siegert et al. (2017). Samples were hydrolyzed in acidic conditions at 113°C for 24 h after oxidation in an ice bath. Amino acids were separated using the L-8900 Amino Acid Analyzer (VWR, Hitachi Ltd, Tokyo, Japan). The determination of His and Tyr may have been affected by sample hydrolysis (Mason et al., 1980). Asn and Gln form into Asp and Glu, respectively, as side group amide residues are lost during acid hydrolysis (Fontaine, 2003). Thus, Asn and Gln were measured together with Asp and Glu and are referred to as Asx and Glx in this study.

Concentrations of P, Ca, Ti, and inositol phosphates were analyzed following methods described by Zeller et al. (2015a). It was not possible to separate enantiomers of specific isomers using this methodology. Thus, we do not distinguish between D- and L-forms in the results. For *myo*-inositol analysis, samples were derivatized using a 2-step procedure described in Sommerfeld et al. (2018), which involves oximation and silanization. Deuterated *myo*-inositol was used as an internal standard. Measurements were obtained using a gas chromatograph/mass spectrometer (5977A, Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany). Phytase activity in feed samples was analyzed according to ISO 30024:2009.

Calculations and Statistical Analyses

Nutrient accretion and efficiency of retention were calculated according to the following equations:

$$\text{Accretion (g/d)} = \text{intake (g/d)} - \text{excretion (g/d)} \quad (1)$$

and

$$\text{efficiency of retention (\%)} = \text{accretion (g/d)} / \text{intake (g/d)} \times 100 \quad (2)$$

ME_n concentration in the diet was calculated as

$$\begin{aligned} ME_n\,(\text{MJ/kg DM}) = [&\text{intake (MJ/d)} - \text{excretion (MJ/d)} \\ &- 36.5\,(\text{MJ/g}) \times \text{N accretion (g/d)}] \\ &/ \text{feed intake (gDM/d)} \end{aligned} \quad (3)$$

ME concentration was calculated using equation (3) without taking the N accretion into account.

The pc digestibility or disappearance of CP, AA, P, $InsP_6$, Ca, and energy was calculated using the following equation:

$$\begin{aligned} &\text{Pc digestibility/disappearance (\%)} \\ &= 100 - [(TiO_{2\text{Diet}} \times Item_{\text{Digesta}}) \\ &\quad / (TiO_{2\text{Digesta}} \times Item_{\text{Diet}})] \times 100 \end{aligned} \quad (4)$$

where $Item_{\text{Digesta}}$ and $Item_{\text{Diet}}$ are the concentrations of CP, AA, P, $InsP_6$, Ca, and gross energy in the digesta and diets, respectively, and $TiO_{2\text{Diet}}$ and $TiO_{2\text{Digesta}}$ are the concentrations of TiO_2 in the diets and digesta, respectively.

Data were statistically analyzed using the MIXED procedure of the software package SAS for Windows (Version 9.3, SAS Institute, Cary, NC). Two separate statistical evaluations were performed. First, we evaluated the influence of the main protein source on the effects of enzyme supplementation using the results of treatments SB1 to SB4, SR1 to SR4, and SF1 to SF4 (Table 1). Next, we evaluated treatments SB1 to SB3 and SB1+ to SB3+ to investigate the influence of MCP supplementation on the effect of phytase supplementation. Analyses of variance (**ANOVA**) were performed using the following statistical models:

$$\begin{aligned} \text{Evaluation 1}: y_{ijkl} = {} & E_i + P_j + E_i \times P_j + run_k \\ & + block_l + e_{ijk} \end{aligned} \quad (5)$$

and

$$\begin{aligned} \text{Evaluation 2}: y_{iklm} = {} & E_i + M_m + E_i \times M_m + run_k \\ & + block + e_{iklm} \end{aligned} \quad (6)$$

Table 4. Influence of phytase and protease supplementation to diets with soybean meal (SBM), SBM and rapeseed meal (RSM), and SBM and sunflower meal (SFM) as main crude protein sources on growth performance, energy content, prececal digestibility of P and Ca, prececal disappearance of $InsP_6$, and retention efficiency of P and Ca in broiler chickens.

									Prececal digestibility/ disappearance (%)			Efficiency of retention (%)	
			ADG (g/bird)	ADFI (g/bird)	G:F (g/g)	Daily N accretion (g/bird)	ME (MJ/kg DM)	ME_n (MJ/kg DM)	P	$InsP_6$	Ca	P	Ca
Treatments[1]													
SB1	SBM	NES	34.8	70.5	0.51	1.81	14.4	13.7	43^{e}	45^{e}	66^{a}	51	39
SB2		1,500Phy	40.9	74.8	0.56	1.97	14.5	13.7	66^{b}	75^{c}	65^{a}	74	52
SB3		3,000Phy	44.9	76.8	0.60	2.05	14.5	13.8	76^{a}	92^{a}	64^{a}	82	61
SB4		Prot	36.3	68.6	0.54	1.88	14.8	14.0	32^{f}	19^{f}	57^{b}	46	34
SR1	SBM/	NES	38.6	73.1	0.54	1.84	14.2	13.5	32^{f}	23^{f}	52^{d}	42	33
SR2	RSM	1,500Phy	48.0	79.8	0.61	2.10	14.3	13.6	$55^{c,d}$	68^{d}	53^{b-d}	63	51
SR3		3,000Phy	46.1	76.5	0.62	2.09	14.5	13.8	66^{b}	86^{b}	46^{e}	70	56
SR4		Prot	38.3	71.8	0.54	1.89	14.2	13.6	$31^{f,g}$	23^{f}	53^{b-d}	38	28
SF1	SBM/	NES	34.7	69.9	0.51	1.73	13.8	13.1	27^{g}	23^{f}	52^{d}	39	29
SF2	SFM	1,500Phy	41.5	74.6	0.57	1.96	14.1	13.4	53^{d}	64^{d}	$56^{b,c}$	61	49
SF3		3,000Phy	43.6	75.1	0.59	2.00	14.1	13.4	58^{c}	78^{c}	44^{e}	68	53
SF4		Prot	36.6	69.7	0.54	1.85	14.2	13.5	$29^{f,g}$	20^{f}	51^{d}	38	27
Pooled SEM			1.3	1.3	0.009	0.04	0.11	0.11	1.2	2.7	1.6	1.3	1.4
Main effects													
Main protein source (P)	SBM		39.2^{B}	72.7^{B}	0.55^{B}	$1.93^{A,B}$	14.5^{A}	13.8^{A}	–[2]	–	–	63^{A}	47^{A}
	SBM/RSM		42.7^{A}	75.3^{A}	0.58^{A}	1.98^{A}	14.3^{B}	13.6^{B}	–	–	–	53^{B}	42^{B}
	SBM/SFM		39.1^{B}	72.3^{B}	0.55^{B}	1.89^{B}	14.0^{C}	13.4^{C}	–	–	–	51^{C}	40^{C}
	Pooled SEM		0.7	0.7	0.004	0.02	0.05	0.05				0.7	0.7
Enzyme[1] (E)	NES		36.0^{B}	71.2^{B}	0.52^{D}	1.79^{C}	14.1^{B}	13.4^{B}	–	–	–	44^{C}	34^{C}
	1,500Phy		43.5^{A}	76.4^{A}	0.58^{B}	2.01^{A}	$14.3^{A,B}$	$13.6^{A,B}$	–	–	–	66^{B}	51^{B}
	3,000Phy		44.9^{A}	76.1^{A}	0.60^{A}	2.05^{A}	14.4^{A}	13.7^{A}	–	–	–	73^{A}	57^{A}
	Prot		37.1^{B}	70.1^{B}	0.54^{C}	1.88^{B}	14.4^{A}	13.7^{A}	–	–	–	41^{D}	29^{D}
	Pooled SEM		0.8	0.8	0.005	0.03	0.06	0.06				0.8	0.8
ANOVA (*P*-values)	P		<0.001	0.001	<0.001	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	E		<0.001	<0.001	<0.001	<0.001	0.024	0.016	<0.001	<0.001	<0.001	<0.001	<0.001
	P × E		0.248	0.424	0.079	0.752	0.403	0.441	<0.001	<0.001	<0.001	0.331	0.265

[1]SB = soybean meal; SR = soybean meal/rapeseed meal; SF = soybean meal/sunflower meal; 1–4 indicates enzyme supplementation in the following sequence: NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; Prot = 1,600 mg protease/kg.

[2]Not presented because of significant interactions ($P < 0.050$) between main effects.

[a-g]In case of significant interactions ($P < 0.050$) between main effects: different lowercase letters indicate significant differences ($P < 0.050$) between treatments.

[A-D]In case of not significant interactions ($P \geq 0.050$) between main effects: different capital letters indicate significant differences ($P < 0.050$) within the main effects P or E.

where y_{ijkl} and y_{iklm} are the dependent traits, E_i is the fixed effect of enzyme supplementation i (no enzyme supplemented, 1,500 FTU phytase/kg, 3,000 FTU phytase/kg, or 1,600 mg protease/kg), P_j is the fixed effect of main protein source j (SBM, SBM/RSM, or SBM/SFM), M_m is the fixed effect of MCP supplementation m (without or with MCP), run_k is the fixed effect of experimental run k (run1 or run2), $block_l$ is a random block effect, and e_{ijkl} and e_{iklm} are the residual errors. Effects were considered to be significant when $P < 0.050$.

RESULTS

The initial bird weight per cage (mean ± SD) was 700 ± 41 g and 1,428 ± 68 g in run 1 and run 2, respectively. No significant differences were found between the 15 treatments ($P = 0.983$ and $P = 0.999$ in run 1 and run 2, respectively). No health problems were observed during the experiment. Mortality during the experimental runs was low and not related to any treatment (5 out of 1,200 birds in 4 treatments).

Influence of Main Protein Sources on the Effect of Phytase and Protease Supplementation

No significant interactions ($P < 0.050$) were detected between the main protein source and enzyme supplementation for growth performance, N accretion, and ME_n concentrations in the diets (Table 4). Growth performance was similar for SBM and SBM/SFM treatments, but growth was higher ($P < 0.050$) for the SBM/RSM treatment. Supplementation of 1,500 FTU phytase/kg increased ADG and ADFI compared to the treatments without enzyme supplementation ($P < 0.050$), but supplementation of 3,000 FTU phytase/kg did not further increase ADG and ADFI. Protease supplementation had no significant effect on ADG and ADFI. G:F was lowest with no enzyme

supplementation and increased with phytase or protease supplementation, with the highest G:F obtained at 3,000 FTU phytase/kg. Supplementation of protease and 3,000 FTU phytase/kg increased ME_n concentration in the diets ($P = 0.003$ and $P = 0.010$, respectively).

There were no significant interactions between the main protein source and enzyme supplementation for pc digestibility of CP and AA except for Cys ($P < 0.001$) (Table 5). Supplementation of 1,500 FTU phytase/kg increased pc digestibility of CP and all AA (including Cys) in the range of 3 (Asx and Pro) to 6 (Ala, Ile, Leu, and Thr) percentage points ($P < 0.001$). No differences in pc AA digestibility were observed between the phytase supplementation levels. Protease supplementation increased pc digestibility of CP by 2 percentage points and pc digestibility of all AA ($P \leq 0.011$) except Cys in the range of 1 (Arg, Glx, Lys, and Met) to 3 (Ile, Leu, and Tyr) percentage points. Protease supplementation increased pc Cys digestibility for SBM and SBM/SFM ($P < 0.001$), but not for SBM/RSM.

Interactions between the main protein source and enzyme supplementation were significant for pc disappearance of $InsP_6$ and pc digestibility of P and Ca ($P < 0.001$) (Table 4). For all main protein sources, pc $InsP_6$ disappearance and pc P digestibility increased ($P < 0.050$) at both levels of phytase supplementation. The addition of protease had no significant effect on pc $InsP_6$ disappearance and pc P digestibility for SBM/RSM and SBM/SFM, but protease supplementation decreased pc $InsP_6$ disappearance and pc P digestibility for SBM ($P \leq 0.001$). No significant interactions were determined between the main protein source and enzyme supplementation for efficiency of P and Ca retention. Efficiency of P and Ca retention was decreased by protease supplementation ($P \leq 0.009$) and increased with increasing phytase supplementation ($P < 0.001$). Highest and lowest efficiencies of P and Ca retention were determined for SBM and SBM/SFM, respectively, with SBM/RSM being intermediate.

Interactions between the main protein source and enzyme supplementation were significant ($P < 0.050$) for most of the inositol phosphate isomers (Table 6). The interaction between main protein source and phytase supplementation was not significant for digesta *myo*-inositol concentrations. *Myo*-inositol concentrations were higher with supplementation of 1,500 FTU phytase/kg ($P < 0.001$), but no differences were detected between phytase supplementation levels.

Influence of Monocalcium Phosphate on the Effect of Phytase Supplementation

Significant interactions between MCP and phytase supplementation were not detected for growth performance, N accretion, or ME_n concentrations (Table 7). Supplementation of MCP increased growth performance ($P \leq 0.019$). Supplementation of 1,500 FTU phytase/kg had no influence on ADFI, but ADFI increased when the diet was supplemented with 3,000 FTU phytase/kg ($P < 0.001$). ADG and G:F increased ($P < 0.050$) as the level of phytase supplementation increased.

Interactions between MCP and phytase supplementation on pc CP and AA digestibility were not significant (Table 8). Supplementation of MCP increased pc digestibility of CP and AA by 2 (Arg, Glx, Ile, and Lys) to 5 (Cys and Thr) percentage points ($P < 0.001$). Supplementation of 1,500 FTU phytase/kg increased pc CP and AA digestibility by 3 (CP and Arg) to 6 (Thr) percentage points ($P < 0.001$). No differences were detected between phytase supplementation levels.

Significant interactions were observed between MCP and phytase supplementation on pc $InsP_6$ disappearance and pc digestibility of P and Ca ($P \leq 0.044$) (Table 7). Pc $InsP_6$ disappearance and pc P digestibility increased as the amount of phytase supplementation increased. When phytase was not supplemented, pc $InsP_6$ disappearance was lower for diets containing MCP ($P < 0.001$). Interactions between MCP and phytase supplementation were significant for efficiency of P and Ca retention ($P < 0.001$). With no phytase supplemented, addition of MCP increased efficiency of P and Ca retention ($P \leq 0.011$). Phytase supplementation increased efficiency of P and Ca retention ($P < 0.001$) with a more marked increase when no MCP was supplemented.

Interactions between MCP and phytase supplementation were significant ($P < 0.050$) for some inositol phosphate isomers (Table 9). The interaction between MCP and phytase supplementation was not significant for *myo*-inositol concentrations in the digesta. The *myo*-inositol concentration in digesta was lower when MCP was supplemented ($P < 0.001$) and significantly higher with higher level of phytase supplementation ($P < 0.001$).

DISCUSSION

Effects of Phytase

Influence of Main Protein Sources Oilseed meals did not influence the effect of phytase supplementation on pc AA digestibility when used as main protein sources. These results are in agreement with results from a study by Ravindran et al. (1999), which showed that no significant interactions exist between phytase supplementation and protein source (SBM, canola meal, and SFM) on pc AA digestibility. In another study (Rutherfurd et al., 2002), phytase supplementation significantly increased pc digestibility for most AA in RSM, but not in SBM. Possible explanations for these differing results include the types of methods used to determine pc AA digestibility. Basal endogenous AA losses, determined in a separate diet containing enzymatically hydrolyzed casein, were considered in pc AA digestibility calculations in the study

Table 5. Influence of phytase and protease supplementation to diets with soybean meal (SBM), SBM and rapeseed meal (RSM), and SBM and sunflower meal (SFM) as main crude protein sources on the prececal CP and amino acid digestibility (%) in broiler chickens.

			CP	Ala	Arg	Asx[1]	Cys	Glx[2]	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Treatments[3]																				
SB1	SBM	NES	77	77	87	78	65^{f}	84	75	76	81	80	83	83	81	78	76	70	79	78
SB2		1,500Phy	81	83	90	83	$71^{c,d}$	88	80	81	86	85	87	88	87	82	82	77	85	84
SB3		3,000Phy	81	82	90	83	$70^{c,d}$	89	79	81	86	85	87	87	86	82	82	76	85	83
SB4		Prot	80	80	88	80	$73^{a,b}$	86	77	80	83	83	85	86	84	81	80	74	83	81
SR1	SBM/	NES	74	76	83	73	$67^{e,f}$	83	72	76	74	77	77	83	78	73	72	66	75	72
SR2	RSM	1,500Phy	78	80	86	78	$72^{a–c}$	86	76	79	80	82	81	86	83	76	76	71	79	78
SR3		3,000Phy	77	80	87	78	$71^{b,c}$	87	76	78	81	82	82	87	83	76	76	71	80	79
SR4		Prot	74	76	84	74	$68^{d,e}$	83	72	75	77	78	78	83	79	74	72	67	76	74
SF1	SBM/	NES	76	76	87	77	$69^{d,e}$	85	70	75	80	79	80	85	81	76	83	70	77	78
SF2	SFM	1,500Phy	81	82	90	81	$73^{a,b}$	89	74	80	85	84	85	89	86	81	78	75	83	83
SF3		3,000Phy	80	84	91	83	$74^{a,b}$	90	75	82	86	85	86	91	87	82	80	77	84	84
SF4		Prot	78	79	88	79	74^{a}	87	73	78	82	81	82	87	83	80	76	73	80	80
Pooled SEM			0.9	1.2	0.5	0.8	0.9	0.6	0.8	1.0	0.9	0.9	0.9	0.9	0.8	0.9	0.9	1.2	1.0	1.0
Main effects																				
Main protein source (P)	SBM		80^{A}	81^{A}	88^{A}	81^{A}	70	87^{B}	78^{A}	80^{A}	84^{A}	83^{A}	86^{A}	86^{B}	85^{A}	80^{A}	80^{A}	74^{A}	83^{A}	76^{C}
	SBM/RSM		76^{C}	78^{B}	85^{B}	76^{C}	70	85^{C}	74^{B}	77^{B}	78^{B}	80^{B}	79^{C}	85^{C}	81^{C}	75^{B}	74^{C}	69^{B}	77^{C}	76^{B}
	SBM/SFM		78^{B}	80^{A}	89^{A}	80^{B}	72	88^{A}	73^{B}	79^{A}	83^{A}	82^{A}	83^{B}	88^{A}	84^{A}	80^{A}	77^{B}	74^{A}	81^{B}	81^{A}
	Pooled SEM		0.8	0.9	0.4	0.6	0.5	0.4	0.6	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.9	0.7	0.8
Enzyme (E)	NES		75^{C}	76^{C}	86^{C}	76^{C}	67	84^{C}	72^{C}	76^{C}	78^{C}	78^{C}	80^{C}	84^{C}	80^{C}	76^{C}	74^{C}	69^{C}	77^{C}	76^{C}
	1,500Phy		80^{A}	82^{A}	89^{A}	81^{A}	72	88^{A}	77^{A}	80^{A}	84^{A}	84^{A}	84^{A}	88^{A}	85^{A}	79^{A}	79^{A}	74^{A}	82^{A}	81^{A}
	3,000Phy		79^{A}	82^{A}	89^{A}	81^{A}	72	88^{A}	77^{A}	80^{A}	84^{A}	84^{A}	85^{A}	88^{A}	85^{A}	79^{A}	79^{A}	74^{A}	83^{A}	82^{A}
	Prot		77^{B}	78^{B}	87^{B}	78^{B}	72	85^{B}	74^{B}	78^{B}	81^{B}	81^{B}	81^{B}	85^{B}	82^{B}	78^{B}	76^{B}	71^{B}	80^{B}	78^{B}
	Pooled SEM		0.8	1.0	0.4	0.6	0.6	0.4	0.6	0.8	0.7	0.7	0.7	0.7	0.8	0.6	0.7	1.0	0.8	0.8
ANOVA (*P*-values)	P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	E		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
	P × E		0.176	0.240	0.769	0.751	<0.001	0.661	0.586	0.066	0.663	0.469	0.779	0.171	0.639	0.350	0.333	0.350	0.503	0.798

[1]Asp and Asn together.
[2]Glu and Gln together.
[3]SB = soybean meal; SR = soybean meal/rapeseed meal; SF = soybean meal/sunflower meal; 1–4 indicate enzyme supplementation in the following sequence: NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; Prot = 1,600 mg protease/kg.
[a-f]In case of significant interactions ($P < 0.050$) between main effects: different lowercase letters indicate significant differences ($P < 0.050$) between treatments.
[A-C]In case of not significant interactions ($P \geq 0.050$) between main effects: different capital letters indicate significant differences ($P < 0.050$) within the main effects P or E.

Table 6. Influence of phytase and protease supplementation to diets with soybean meal (SBM), SBM and rapeseed meal (RSM), and SBM and sunflower meal (SFM) as main crude protein sources on concentrations of inositol phosphates and *myo*-inositol in digesta (μmol/g DM) of broiler chickens.[1]

			$InsP_6$	Ins(1,2,3,4,5)P_5	Ins(1,2,4,5,6)P_5	Ins(1,2,3,4,6)P_5	Ins(1,2,3,4)P_4	Ins(1,2,5,6)P_4	$InsP_{3x}$[3]	Ins(1,5,6)P_3	*Myo*-inositol[4]
Treatments[2]											
SB1	SBM	NES	24.0^{d}	1.0^{e,f}	0.4^{f,g}	0.5	0.5^{d}	ND	0.4^{b}	LOQ	8.5
SB2		1,500Phy	10.3^{f,g}	2.0^{b,c}	0.6^{e,f}	LOQ	1.6^{c}	0.8^{c}	0.5^{b,c}	0.2	12.3
SB3		3,000Phy	3.4^{h}	0.9^{f}	0.3^{g}	ND	1.6^{c}	0.9^{c}	1.2^{b}	0.2	13.8
SB4		Prot	31.9^{c}	1.2^{d-f}	0.8^{c,d}	0.5	LOQ	ND	ND	0.2	nm
SR1	SBM/RSM	NES	34.9^{b,c}	1.4^{c-f}	1.1^{b,c}	0.7	LOQ	LOQ	ND	LOQ	5.4
SR2		1,500Phy	16.0^{e}	4.4^{a}	1.4^{a}	LOQ	3.8^{b}	2.0^{b}	1.1^{b}	0.2	11.4
SR3		3,000Phy	6.9^{g,h}	2.5^{b}	0.8^{d,e}	ND	3.6^{b}	2.1^{b}	2.9^{a}	0.2	10.9
SR4		Prot	34.4^{c}	1.6^{c-e}	1.1^{b}	0.7	0.2^{d}	LOQ	ND	LOQ	nm
SF1	SBM/SFM	NES	36.4^{a,b}	1.4^{c-f}	0.8^{d,e}	0.7	0.2^{d}	LOQ	ND	LOQ	6.0
SF2		1,500Phy	17.6^{e}	4.5^{a}	1.3^{a,b}	LOQ	3.5^{b}	1.8^{b}	1.0^{b}	LOQ	11.2
SF3		3,000Phy	10.8^{f}	3.9^{a}	1.2^{a,b}	LOQ	5.5^{a}	3.1^{a}	3.9^{a}	LOQ	9.6
SF4		Prot	39.6^{a}	1.8^{c,d}	1.1^{b}	0.7	0.3^{d}	LOQ	ND	LOQ	nm
	Pooled SEM		1.6	0.3	0.1	<0.1	0.5	0.4	0.5	<0.1	0.7
Main effects											
Main protein source (P)	SBM		–[5]	–	–	.	–	–	–	.	11.5^{A}
	SBM/RSM		–	–	–	.	–	–	–	.	9.2^{B}
	SBM/SFM		–	–	–	.	–	–	–	.	8.9^{B}
	Pooled SEM										0.4
Enzyme (E)	NES		–	–	–	0.6	–	–	–	.	6.7^{B}
	1,500Phy		–	–	–	.	–	–	–	.	11.6^{A}
	3,000Phy		–	–	–	.	–	–	–	.	11.4^{A}
	Prot		–	–	–	.	–	–	–	.	nm
	Pooled SEM										0.4
ANOVA (*P*-values)	P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.844	<0.001
	E		<0.001	<0.001	<0.001	0.776	<0.001	0.009	<0.001	0.872	<0.001
	P × E		0.042	<0.001	<0.001	0.915	<0.001	0.008	0.049	0.176	0.160

[1]LOQ = in the majority of samples below limit of quantification (0.27 μmol/g DM for Ins(1,2,3,4,6)P_5, 0.21 μmol/g DM for Ins(1,2,3,4)P_4, 0.27 μmol/g DM for Ins(1,2,5,6)P_4, 0.24 μmol/g DM for Ins(1,5,6)P_3); ND = in the majority of samples below detection limit (0.14 μmol/g DM for Ins(1,2,3,4,6)P_5, 0.14 μmol/g DM for Ins(1,2,5,6)P_4, 0.06 μmol/g DM for Ins(1,2,6/1,4,5/2,4,5)P_3). Concentrations of other measured inositol phosphate isomers were below the respective detection limits in the majority of samples in all treatments.

[2]SB = soybean meal; SR = soybean meal/rapeseed meal; SF = soybean meal/sunflower meal; 1–4 indicate enzyme supplementation in the following sequence: NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; Prot = 1,600 mg protease/kg.

[3]At least one of the following inositol phosphate isomers: Ins(1,2,6)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3.

[4]nm = not measured.

[5]Not presented because of significant interactions ($P < 0.050$) between main effects.

[a-h]In case of significant interactions ($P < 0.050$) between main effects: different lowercase letters indicate significant differences ($P < 0.050$) between treatments.

[A,B]In case of not significant interactions ($P \geq 0.050$) between main effects: different capital letters indicate significant differences ($P < 0.050$) within the main effects P or E.

by Rutherfurd et al. (2002). However, basal endogenous AA losses were not considered in the present study nor in the study by Ravindran et al. (1999). Differences between studies might also be due to the phytase product and supplementation level used. Supplementation levels of 750 FTU phytase/kg for a 6-phytase derived from genetically modified *Aspergillus oryzae* and 1,200 FTU phytase/kg for a 3-phytase derived from genetically modified *Aspergillus niger* were investigated in Rutherfurd et al. (2002) and Ravindran et al. (1999), respectively. In the present study, 1,500 FTU phytase/kg was the lowest supplementation level for a 6-phytase produced by genetically modified *A. niger*.

High $InsP_6$ concentrations in the feed resulted in an increased incidence of binary protein-$InsP_6$ complexes and ternary protein-cation-$InsP_6$ complexes, which reduced pc AA digestibility (Selle et al., 2009). $InsP_6$ concentrations in the supplemented diets differed by 5.0 μmol/g DM in the present study. Previous studies have found that differences in $InsP_6$ concentrations of diets containing SBM, canola meal, and SFM were 6.5 μmol/g (Rutherfurd et al., 2002), and 3.9 μmol/g in diets containing SBM and RSM (Ravindran et al., 1999). Similar $InsP_6$ concentration ranges were observed among feedstuffs across multiple studies; thus, differences in pc AA digestibility are likely due to factors other than variations in $InsP_6$ concentrations. $InsP_6$ concentrations in the diets differed between studies. $InsP_6$ concentrations ranged from 15.0 to 20.0 μmol/g DM in the present study and ranged from 7.3 to 13.5 μmol/g and 5.7 to 9.6 μmol/g in the studies of Ravindran et al. (1999) and Rutherfurd et al. (2002), respectively. Lower $InsP_6$ levels in previous studies are a result of study design. Diets in Ravindran et al. (1999) and Rutherfurd et al. (2002) contained up to 53% of the test ingredient as the sole source of protein and $InsP_6$, while diet formulation in the present study was closer to conditions of the broiler industry. Variation

Table 7. Influence of phytase supplementation to diets without and with monocalcium phosphate (MCP) supplementation on growth performance, energy content, prececal digestibility of P and Ca, prececal disappearance of $InsP_6$, and retention efficiency of P and Ca in broiler chickens.

									Prececal digestibility/ disappearance (%)			Efficiency of retention (%)	
			ADG (g/bird)	ADFI (g/bird)	G:F (g/g)	Daily N accretion (g/bird)	ME (MJ/kg DM)	ME_n (MJ/kg DM)	P	$InsP_6$	Ca	P	Ca
Treatments[1]													
SB1	Without MCP	NES	34.8	70.5	0.51	1.81	14.4	13.7	43^{d}	45^{c}	66^{a}	51^{e}	39^{e}
SB2		1,500Phy	40.9	74.8	0.56	1.97	14.5	13.7	66^{b}	75^{b}	65^{a}	74^{b}	52^{c}
SB3		3,000Phy	44.9	76.8	0.60	2.05	14.5	13.8	76^{a}	92^{a}	64^{a}	82^{a}	61^{a}
SB1+	With MCP	NES	41.6	75.3	0.57	2.03	14.4	13.6	49^{c}	21^{d}	57^{b}	54^{d}	47^{d}
SB2+		1,500Phy	43.4	75.2	0.59	2.05	14.6	13.8	61^{b}	74^{b}	49^{c}	69^{c}	59^{a}
SB3+		3,000Phy	48.8	79.6	0.63	2.14	14.6	13.9	64^{b}	89^{a}	47^{c}	68^{c}	57^{b}
Pooled SEM		1.4	1.4	0.010	0.03	0.08	0.08		2.6	2.3	2.1	0.8	0.9
Main effects													
Mineral P (M)		Without MCP	40.2^{B}	74.0^{B}	0.55^{B}	1.94^{B}	14.5	13.7	–[2]	–	–	–	–
		With MCP	44.6^{A}	76.7^{A}	0.60^{A}	2.07^{A}	14.5	13.8	–	–	–	–	–
		Pooled SEM	1.0	0.8	0.008	0.03	0.06	0.05					
Enzyme[1] (E)	NES		38.2^{C}	72.9^{B}	0.54^{C}	1.92^{B}	14.4	13.7^{B}	–	–	–		–
		1,500Phy	42.2^{B}	75.0^{B}	0.58^{B}	2.01^{AB}	14.5	13.8^{AB}	–	–	–	–	–
		3,000Phy	46.8^{A}	78.2^{A}	0.61^{A}	2.10^{A}	14.6	13.9^{A}	–	–	–	–	–
		Pooled SEM	1.1	1.0	0.009	0.03	0.06	0.06					
ANOVA (*P*-values)	M		<0.001	0.019	<0.001	0.002	0.359	0.407	0.070	<0.001	<0.001	<0.001	<0.001
	E		<0.001	0.001	<0.001	0.004	0.066	0.032	<0.001	<0.001	0.005	<0.001	<0.001
	M × E		0.188	0.268	0.125	0.245	0.434	0.433	<0.001	<0.001	0.044	<0.001	<0.001

[1]SB = soybean meal; 1–3 indicates enzyme supplementation in the following sequence: NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; + indicates monocalcium phosphate supplementation.

[2]Not presented because of significant interactions ($P < 0.050$) between main effects.

[a-f]In case of significant interactions ($P < 0.050$) between main effects: different lowercase letters indicate significant differences ($P < 0.050$) between treatments.

[A-C]In case of not significant interactions ($P \geq 0.050$) between main effects: different capital letters indicate significant differences ($P < 0.050$) within the main effects M or E.

in the incidences of protein-$InsP_6$ complexes as a result of different $InsP_6$ levels may have contributed to the conflicting results between studies.

Another factor hypothesized to influence the effect of phytase supplementation on pc AA digestibility is the level of pc AA digestibility of diets without phytase supplementation (Ravindran et al., 1999). In the present study, differences in pc AA digestibility of up to 9 percentage points were observed between main protein sources without enzyme supplementation. Regardless of these differences, the effect of enzyme supplementation on pc AA digestibility was not influenced by main protein sources.

Phytase supplementation decreased $InsP_6$ concentrations and influenced concentrations of other inositol phosphate isomers in the digesta in a dose-dependent manner, while no further increase of pc AA digestibility was observed with more than 1,500 FTU phytase/kg. It is possible that protein-$InsP_6$ complexes were of minor relevance for pc AA digestibility in diets without phytase supplementation. If so, the higher pc AA digestibility of the treatments with phytase supplementation was caused not only by degraded protein-$InsP_6$ complexes. A more likely possibility is the supplementation of 1,500 FTU phytase/kg dissolved such complexes in a sufficient proportion. In this case, higher phytase supplementation would have no further effect on pc AA digestibility.

Phytase supplementation is also known to increase pc AA digestibility by reducing basal endogenous AA losses (Selle et al., 2012). The proportions of Asx, Cys, Glx, Pro, Ser, and Thr are high in basal endogenous AA losses (Kluth and Rodehutscord, 2009). Among these AA, the increase in pc AA digestibility was on the level of the median of all AA for some (Asx and Cys), while it was higher (Ser and Thr) or lower (Glx and Pro) for others. Phytase supplementation influenced ADFI, which is another influencing factor for basal endogenous AA losses (Adedokun et al., 2011; Adeola et al., 2016). Therefore, phytase supplementation, feed intake, or both may have affected basal endogenous AA losses in the present study. Similar results were obtained from previous research (Borda-Molina et al., 2019). Prececal AA digestibility of feedstuffs excluding basal endogenous AA losses can be examined by the regression approach to determine whether phytase supplementation increases pc AA digestibility by reducing basal endogenous AA losses.

Influence of Monocalcium Phosphate The effect of phytase supplementation on pc AA digestibility did not interact with supplementation of MCP. Similarly, Sommerfeld et al. (2018) determined that Ca carbonate and monosodium phosphate concentrations had no influence on the effect of phytase supplementation on pc AA digestibility. Mineral P was not supplemented in the

Table 8. Influence of phytase supplementation to diets without and with monocalcium phosphate (MCP) supplementation on the prececal CP and amino acid digestibility (%) in broiler chickens.

			CP	Ala	Arg	Asx[1]	Cys	Glx[2]	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Treatments[3]																				
SB1	Without MCP	NES	77	77	87	78	65	84	75	76	81	80	83	83	81	78	76	70	79	78
SB2		1,500Phy	81	83	90	83	71	88	80	81	86	85	87	88	87	82	82	77	85	84
SB3		3,000Phy	81	82	90	83	70	89	79	81	86	85	87	87	86	82	82	76	85	83
SB1+	With MCP	NES	81	82	89	82	71	87	79	82	84	84	86	87	85	82	81	78	84	82
SB2+		1,500Phy	83	85	91	85	75	90	82	85	87	87	89	90	88	85	85	80	87	85
SB3+		3,000Phy	85	86	92	86	76	91	83	86	88	88	90	91	89	87	86	81	88	86
Pooled SEM			0.8	0.9	0.4	0.5	0.9	0.4	0.7	0.7	0.8	0.7	0.6	0.7	0.6	0.6	0.6	0.9	0.7	0.9
Main effects																				
Mineral P	Without MCP		79^{B}	81^{B}	89^{B}	81^{B}	69^{B}	87^{B}	78^{B}	80^{B}	84^{B}	83^{B}	86^{B}	86^{B}	85^{B}	80^{B}	80^{B}	74^{B}	83^{B}	81^{B}
(M)	With MCP		83^{A}	84^{A}	91^{A}	84^{A}	74^{A}	89^{A}	81^{A}	84^{A}	86^{A}	86^{A}	88^{A}	90^{A}	87^{A}	84^{A}	84^{A}	79^{A}	86^{A}	84^{A}
	Pooled SEM		0.7	0.7	0.2	0.4	0.5	0.3	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.7	0.5	0.6
Enzyme (E)	NES		79^{B}	79^{B}	88^{B}	80^{B}	68^{B}	85^{B}	77^{B}	79^{B}	82^{B}	82^{B}	84^{B}	85^{B}	83^{B}	80^{B}	79^{B}	73^{B}	82^{B}	80^{B}
	1,500Phy		82^{A}	84^{A}	91^{A}	84^{A}	73^{A}	89^{A}	81^{A}	83^{A}	87^{A}	86^{A}	88^{A}	89^{A}	88^{A}	84^{A}	84^{A}	79^{A}	87^{A}	84^{A}
	3,000Phy		83^{A}	84^{A}	91^{A}	84^{A}	73^{A}	90^{A}	81^{A}	84^{A}	87^{A}	86^{A}	88^{A}	90^{A}	88^{A}	84^{A}	84^{A}	79^{A}	87^{A}	84^{A}
	Pooled SEM		0.7	0.7	0.3	0.4	0.6	0.3	0.5	0.5	0.6	0.6	0.5	0.6	0.5	0.5	0.5	0.8	0.6	0.7
ANOVA	M		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(*P*-values)	E		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	M × E		0.065	0.091	0.313	0.231	0.467	0.312	0.277	0.376	0.145	0.184	0.128	0.076	0.171	0.309	0.289	0.143	0.204	0.143

[1]Asp and Asn together.
[2]Glu and Gln together.
[3]SB = soybean meal; 1–3 indicate enzyme supplementation in the following sequence: NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; + indicates monocalcium phosphate supplementation.
[A–B]Different capital letters indicate significant differences ($P < 0.050$) within the main effects M or E.

Table 9. Influence of phytase supplementation to diets without and with monocalcium phosphate (MCP) supplementation on concentrations of inositol phosphates and *myo*-inositol in digesta (μmol/g DM) in digesta of broiler chickens.[1]

			$InsP_6$	Ins(1,2,3,4,5)P_5	Ins(1,2,4,5,6)P_5	Ins(1,2,3,4,6)P_5	Ins(1,2,3,4)P_4	Ins(1,2,5,6)P_4	$InsP_{3x}$[3]	Ins(1,5,6)P_3	*Myo*-inositol
Treatments[2]											
SB1	Without MCP	NES	24.0^{b}	1.0^{c}	0.4	0.5	0.5^{c}	ND	0.4^{c}	LOQ	8.5
SB2		1,500Phy	10.3^{c}	2.0^{b}	0.6	LOQ	1.6^{b}	0.8	0.5^{c}	0.2	12.3
SB3		3,000Phy	3.4^{d}	0.9^{c}	0.3	ND	1.6^{b}	0.9	1.2^{c}	0.2	13.8
SB1+	With MCP	NES	35.8^{a}	1.4^{b,c}	1.2	0.6	0.2^{c}	LOQ	ND	LOQ	4.3
SB2+		1,500Phy	12.6^{c}	4.0^{a}	1.3	LOQ	5.3^{a}	3.1	3.8^{b}	0.2	6.3
SB3+		3,000Phy	5.2^{d}	2.0^{b}	0.7	ND	4.3^{a}	2.6	6.5^{a}	0.2	8.2
	Pooled SEM		1.3	0.3	0.1	<0.1	0.5	0.4	0.7	<0.1	0.7
Main effects											
Mineral P (M)	Without MCP		–[4]	–	0.4^{B}	.	–	.	.	.	11.5^{A}
	With MCP		–	–	1.1^{A}	.	–	.	.	.	6.3^{B}
	Pooled SEM				<0.1						0.4
Enzyme (E)	NES		–	–	0.8^{A}	0.6	–	.	.	.	6.4^{C}
	1,500Phy		–	–	0.9^{A}	.	–	1.9	2.2	0.2	9.3^{B}
	3,000Phy		–	–	0.5^{B}	.	–	1.7	3.9	0.2	11.0^{A}
	Pooled SEM				0.1	<0.1		0.4	0.6	<0.1	0.5
ANOVA	M		<0.001	<0.001	<0.001	0.040	<0.001	<0.001	<0.001	1.000	<0.001
(*P*-values)	E		<0.001	<0.001	<0.001	.	<0.001	0.404	0.017	0.166	<0.001
	M × E		<0.001	0.013	0.055	.	<0.001	0.207	0.107	1.000	0.346

[1]LOQ = in the majority of samples below limit of quantification (0.27 μmol/g DM for Ins(1,2,3,4,6)P_5, 0.21 μmol/g DM for Ins(1,2,3,4)P_4, 0.24 μmol/g DM for Ins(1,5,6)P_3); ND = in the majority of samples below detection limit (0.14 μmol/g DM for Ins(1,2,3,4,6)P_5, 0.11 μmol/g DM for Ins(1,2,3,4)P_4, 0.06 μmol/g DM for Ins(1,2,6/1,4,5/2,4,5)P_3). Concentrations of other measured inositol phosphate isomers were below the respective detection limits in the majority of samples in all treatments.

[2]SB = soybean meal; 1–3 indicate enzyme supplementation in the following sequence: NES = no enzyme supplemented; 1,500Phy = 1,500 FTU phytase/kg; 3,000Phy = 3,000 FTU phytase/kg; + indicates monocalcium phosphate supplementation.

[3]At least one of the following inositol phosphate isomers: Ins(1,2,6)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3.

[4]Not presented because of significant interactions ($P < 0.050$) between main effects.

[a–d]In case of significant interactions ($P < 0.050$) between main effects: different lowercase letters indicate significant differences ($P < 0.050$) between treatments.

[A–C]In case of not significant interactions ($P \geq 0.050$) between main effects: different capital letters indicate significant differences ($P < 0.050$) within the main effects M or E.

diets used for evaluation or enzyme effects, though mineral P supplementation is common in practical feed formulation. Supplementation was avoided because mineral P is known to affect inositol phosphate hydrolysis (Shastak et al., 2014; Zeller et al., 2015a; Sommerfeld et al., 2018). The effect of mineral P on inositol phosphate hydrolysis is thought to lead to less pronounced effects of phytase supplementation on pc AA digestibility between protein sources. Supplementation of MCP reduced $InsP_6$ disappearance when no phytase was supplemented but MCP supplementation was without effect on $InsP_6$ disappearance when phytase was supplemented. Regardless, MCP influenced the effect of phytase on concentrations of lower inositol phosphate isomers and *myo*-inositol. No change in pc AA digestibility was observed when more than 1,500 FTU phytase/kg was supplemented in diets with and without MCP supplementation. This supports the conclusion that the effects of inositol phosphates on pc AA digestibility were no longer relevant when 1,500 FTU phytase/kg was supplemented.

The mechanism by which phytase increased pc AA digestibility might differ with the presence or absence of MCP supplementation, even though the determined effect was similar. Possible mechanisms include the direct effects of higher P and Ca concentrations in diets supplemented with MCP. Concentrations of mineral P and Ca had no effect on pc AA digestibility in the study of Sommerfeld et al. (2018). In contrast, Martinez-Amezcua et al. (2006) reported that the supplementation of monopotassium phosphate in a P-deficient diet increased pc AA digestibility to a similar or higher level compared to phytase supplementation. These authors hypothesized that the increase in pc AA digestibility may have been caused by more P being available for metabolic processes. This may have enabled higher nutrient absorption due to an increased functionality of membranes and active AA or peptide transporters. In support of this, Centeno et al. (2007) found that pc digestibility of most AA increased when dicalcium phosphate or phytase was added to a P-deficient diet, while Ca concentrations remained constant by varying Ca carbonate concentrations. Another mechanism of pc AA digestibility is that Ca can compete with proteins for the active sites of $InsP_6$ due to the acid-binding activity of Ca (Selle et al., 2009). This may prevent the formation of $InsP_6$-protein complexes or degrade these complexes at low pH in the anterior digestive tract and thereby increase pc AA digestibility. As a countervailing effect, Ca can bind and thus decrease the solubility of proteins (Selle et al., 2009). For the present study, the mechanism of potentially decreased AA absorption due to P deficiency helps to explain the effect of phytase in the diets without MCP supplementation. The influence of Ca from MCP is difficult to derive in the present study because of the opposing

possible consequences of Ca for pc AA digestibility. A further possible mechanism is the influence of ADFI on pc AA digestibility. Whether pc AA digestibility is elevated or reduced by increasing ADFI depends on other consequences of ADFI, such as lumen fill (Siegert et al., 2018) and the proportion of endogenous AA losses relative to undigested AA from the feed (Kong and Adeola, 2014). As summarized by Sommerfeld (2018), mineral P supplementation increased ADFI in most studies. Supplementation of MCP influenced ADFI in the present study and most likely pc AA digestibility. The actual impact, however, cannot be assessed based on the available data.

Effects of Protease

Protease supplementation increased pc digestibility of CP and all AA except for Cys, regardless of main protein source. The pc digestibility of Cys increased with protease supplementation for SBM and SBM/SFM, but not for SBM/RSM. It was hypothesized that the effect of protease supplementation on pc AA digestibility is influenced by dietary composition because feed ingredients provide the substrate for enzymes to act upon. No such effect was determined in the present study except for one AA. Results of studies investigating the effect of ingredient composition on the effect of protease supplementation are contradictory. Toghyani et al. (2017) found no difference in the effect of protease supplementation on pc CP digestibility in diets with SBM or SBM/canola meal as the main protein sources. In another study (Dalólio et al., 2016), the origin of full-fat soybeans had no influence on the effect of protease supplementation on pc AA digestibility. Mahmood et al. (2018) found no difference in the effect of protease supplementation on pc CP digestibility of different poultry by-product meal levels at the expense of SBM. In other studies, the effect of protease supplementation on pc AA digestibility was influenced when diets were based on wheat or sorghum (Selle et al., 2016) and for diets containing different proportions of corn and SBM (Freitas et al., 2011). Besides the feedstuffs used, differences between studies may be due to differing protease products and dosages. The previously mentioned studies, except for Mahmood et al. (2018), used the same protease product. In other studies, diets were supplemented with 200 mg/kg (Freitas et al., 2011; Dalólio et al., 2016; Toghyani et al., 2017) or 500 mg/kg (Selle et al., 2016) of protease. A previous study showed that supplementing 1,600 mg/kg of the same protease product increased pc AA digestibility, while supplementation of 200 mg/kg had no effect (Borda-Molina et al., 2019). In another study, the potential of the same protease product was reached when 200 mg/kg was supplemented (Angel et al., 2011). This shows that additional factors and interactions among the factors known to influence the efficacy of protease needs to be investigated.

In conclusion, the effect of phytase and protease supplementation on pc digestibility of AA (except for Cys) was not influenced by the oilseed meals used as main protein sources. The highest potential of phytase supplementation to increase pc AA digestibility was reached at the lowest phytase supplementation level (1,500 FTU phytase/kg). Higher phytase supplementation increased pc $InsP_6$ disappearance and pc P digestibility. Protease supplementation increased pc AA digestibility, and this effect was not influenced by the main protein source.

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