EFFECT OF WET MASH WITH OR WITHOUT ADDED PHYTASE ON PHYTATE-P AVAILABILITY: *IN VITRO* HYDROLYSIS OF PHYTATE

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ABSTRACT

Dietary wetness is envisaged to augment phytate hydrolysis through enhanced phytate accessibility to phytase, since phytate degradation is water-mediated. In determining the influence of dietary wetness with supplementary phytase on phytate-P availability, four plant-based growers diets were formulated, containing 0.0, 0.1, 0.2 and 0.3g kg^{-1} added phytase and with the required phosphorus being entirely phytate-P. Dietary moisture contents were reconstituted by addition of three levels of 0.0, 30 and 60 % distilled-deionised water on Dry Matter basis (DM). Dietary treatments were arranged in a factorial randomized complete block design with three replications. The released inorganic phosphorus was determined at 6hr intervals throughout the 24hr incubation period at 24ºC. The 30% and 60% dietary wetness with added phytases significantly increased ($P \leq 0.05$) inorganic phosphorus output compared to either 0.0% or wetness without added phytase. At 6hr of incubation, phytase plus 60% wetness had the highest ($P \leq 0.05$) inorganic phosphorus, while at 12hr 30% dietary wetness plus phytase yielded the highest inorganic phosphorus ($P \leq 0.05$). Wetness plus phytase had no pronounced effect ($P \leq 0.05$) beyond 12hr, except at 24hr ($P \leq 0.05$). Increasing supplementary phytase in association with dietary wetness significantly ($P \leq 0.05$)

increased inorganic phosphorus output, however, setting wet diets beyond 12hr, produced gases and dietary discoloration.

Keywords: Phytate-P, Phytase, Dietary-wetness, Inorganic phosphorus, *in vitro*

1. INTRODUCTION

In order for phytate phosphorus to be utilised by monogastric, it has to be hydrolysed into inorganic phosphate, however, the bioavailability of non phytate-P depends to large extent on the amount of inorganic phosphorus in a given finished diet, its value is crucial to avoid excess phosphorus being fed, which ultimately increases excretion of phosphorus into the droppings. But phosphorus bioavailability value might be influenced by feedstuffs high in phytase content, ability of phytase to survive feed processing, as well as its synergy in interacting with the enzymes in the digestive tract. Previous workers have observed that milled grains contain more phytase activity than intact grains (Pointillart, 1993; Fredrikson *et al*., 2001) and that those vital enzymes in ingested food interact synergistically with enzymes within the human body (Prochaska and Piekutowski, 1994). Though the non-enzymatic cleavage of phytate has been suggested, its dephosphorylation is largely a result of phytase activity. There is evidence that wetting food significantly increases food intake, water intake, body weight gain, as well as, it decreases the viscosity of gut contents and enhances development of the villi in the digestive segments and increases crypt cell proliferation rate in the crypts of the epithelium (Yasar and Forbes, 1999). However, the envisaged increase in dietary wet matter content is expected to enhance either the enzymatic hydrolysis of phytate, whether being intrinsic or exogenous phytasem, or the wetness might improve the availability of phytate phosphorus through enhancement in phytate accessibility to the enzyme. Therefore, in determining the influence of phytase and/or dietary moisture (wet mash diet) on phosphorus bioavailability the current study was undertaken to examine the hypothesis; that the wet-mash form

of poultry plant-based diet or increasing dietary wetness enhances diffusion of the phytate out of the aleurone layer of the cereal in order to be accessible to the phytase, then greater phytate digestibility would be expected. In other words: degradation of inositol hexaphosphate (IP6) to lower inositol phosphates (IP_3 –IP_5) by phytase is a water-mediated reaction.

2. MATERIALS AND METHODS

2.1 Diets

Four plant-based diets were formulated to meet the required nutrients for growing chicks (Ministry of Agriculture Fisheries and Food, 1974) and contained increasing levels of phytase (0.0, 0.1, 0.2 and 0.3 g/kg) which were added at the expense of maize starch (Table 1). The diets were formulated to be isonitrogenous, isocaloric, equal in Ca, P, Fe, Zn, Mg, Cu, Mn, sulphur amino acids and lysine, the dietary phosphorus was entirely phytate-P with no added inorganic phosphorus (Table 1).

Table 1: Composition of the Experimental Diets (g kg^{-1})

Ingredient	Phytase			
	0.0	0.1	0.2	0.3
Maize	585.0	585.0	585.0	585.0
Soybean meal	285.0	285.0	285.0	285.0
Maize starch	97.9	97.8	97.7	97.6
Maize oil	2.5	2.5	2.5	2.5
Dl-methionine	0.2	0.2	0.2	0.2
Limestone	22.0	22.0	22.0	22.0
Feed supplement[1]	5.0	5.0	5.0	5.0
Salt	2.4	2.4	2.4	2.4
Phytase	0.0	0.1	0.2	0.3

	1000	1000	1000	1000
Calculated Composition				
CP	187.0	187.0	187.0	187.0
ME, $MJkg^{-1}$	11.9	11.9	11.9	11.9
Lysine	9.0	9.0	9.0	9.0
Methionine	5.0	5.0	5.0	5.0
Methionine+cystine	8.0	8.0	8.0	8.0
Tryptophan	2.0	2.0	2.0	2.0
Calcium	9.0	9.0	9.0	9.0
Total P	4.0	4.0	4.0	4.0

[1]Feed supplement provides per kilogram diet: vitamin A 10 IU, vitamin D_3 3 IU, vitamin E 0.008 IU, copper (cupric sulphuate) 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.

2.2 Experimental procedure

5 g of each diet was weighed into four sets of 9 individual (100 ml) capped-tubes, the moisture content of each set of the four diets was altered by addition of distilled deionised water, such that each 3 replicates of the same diet contained each of the following three levels of moisture content [0.0% (no water added), 30% and 60 % moisture] in a factorial arrangement, each treatment was replicated 3 times (3 replications x 3 levels of moisture × 4 enzyme concentrations). Then, the 36 tubes were incubated at 24ºC, in a Cooled Incubator (BHD).

2.3 Determination of inorganic phosphorus

The released inorganic phosphorus was determined in duplicated samples of each replicate, at every 6 h interval throughout the 24 h incubation period, according to a Manual of the Analytical Methods as used by the Agricultural Development and Advisory Service of the Ministry of

Agriculture, Fisheries and Food (UK) for determination of inorganic phosphorus in feedstuffs (Analysis of Agricultural Materials, 1986).

The six hourly, 1 g samples were collected from each treatment replicate after they had been homogenised and then subjected to extraction of inorganic phosphorus using 0.75M tri-chloroacetic acid, the extract was decolourised with charcoal, and then the concentration of phosphorus in the extract was determined spectrophotometrically as phospho-vanado-molybdate complex. 0.75M trichloroacetic acid: 123g of trichloroacetic acid dissolved in water then diluted to 1l. Ammonium molybdate - ammonium metavanadate reagent:25g of ammonium molybdate and 1.25g of ammonium metavanadate was added into approximately 300ml of water with warming (to dissolve the compounds), cooled then diluted to 500ml. Hydrochloric acid approximately 5M: was prepared by diluting 215ml hydrochloric acid, approximately 36% m/m HCl, to 500 ml. Phosphorus stock standard solution: 0.879g of oven-dried potassium dihydrogen orthophosphate was dissolved in water, with addition of 1ml of approximately 36% hydrochloric acid and diluted to 200ml. Then 1 drop of toluene was added to the solution. Phosphorus working standard solution: 0, 2, 4, 6, 8 and 10µg/ml of phosphorus solutions were prepared on the day of determination.

2.3.1 Extract preparation

50ml of 0.75M trichloroacetic acid and approximately 5ml of charcoal were added to the 1g of the ground (1mm) sample. The mixture was shaken for 1h and filtered through a 110mm Whatman No. 40 filter paper with rejection of the first few drops. Standard curve and sample reading**:** t**o** 10ml of each phosphorus working standard solutions 5 ml of approximately 5M hydrochloric acid and 5ml of ammonium molybdate - ammonium metavanadate reagent were added, the mixture was diluted to 50ml, mixed and allowed to stand for 5min. The absorption was determined by

using spectrophotometer (UV-2101PC. UV-VIS Scanning Spectrophotometer- SHIMAD-ZU) at 400nm against a blank, to give a concentration calibration. Thereafter, concentration of inorganic phosphorus in 10 ml of sample aliquot was read in a spectrophotometer at 400nm following the same treatments as in the standard curve (with final acidity between 0.25 and 0.75M as hydrochloric acid). The experimental treatments were arranged into a factorial experiment, Data obtained were subjected to analysis of variance using Genstat 5 (1995), and the treatment means were separated by LSD test at ($P \leq 0.05$) probability level (Gomez and Gomez, 1984).

3. RESULTS AND DISCUSSION

The effect of increasing dietary wetness or moisture content, as well as, supplementary phytase on phosphorus availability in plant-based diet, are presented in Table 2.

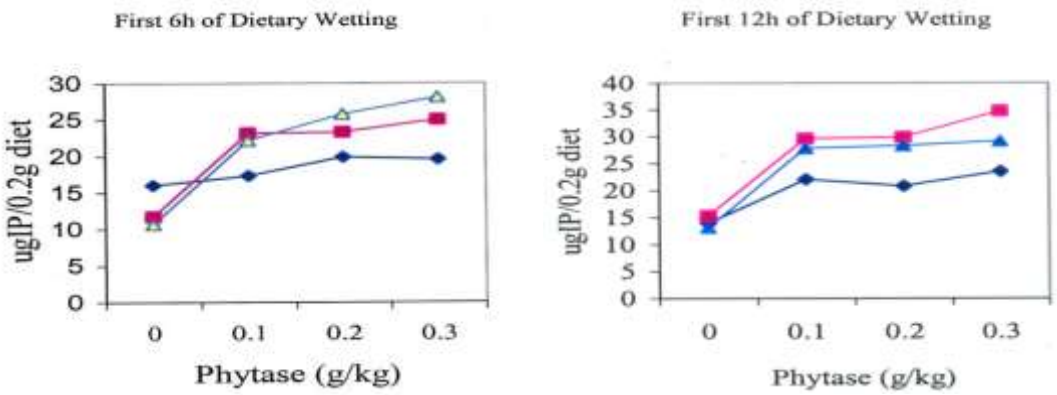
Table 2. Effect of supplementary phytase and dietary moisture on P bioavailability

Moisture	Phytase	μg of released IP 0.2g^{-1} sample			
%	g/kg	6h	12h	18h	24h
0	0.0	16.04 b	13.94 a	16.97 ab	14.63 a
30	0.0	11.70 a	15.32 ab	15.23a	14.28 a
60	0.0	11.19 a	13.07 a	15.15a	14.44 a
0	0.1	17.30 bc	21.97 cd	24.45c	17.95 b
30	0.1	23.11 ef	29.59 fg	35.84e	26.41 d
60	0.1	22.10 ed	27.74 def	33.02de	32.05 ef
0	0.2	19.86 cd	20.74 bc	21.31bc	18.29 b
30	0.2	23.26 ef	29.82 fg	30.70de	30.31 e
60	0.2	25.67 fg	28.22 def	30.27d	32.33 ef
0	0.3	19.54 bcd	23.44 cde	21.33bc	22.71 c
30	0.3	24.93 fg	34.69 g	33.33de	37.15 g
60	0.3	27.25 g	29.06 efg	34.40de	34.06 f

Moisture $LSD_{0.05}$	1.77	3.13	2.61	1.40
Phytase $LSD_{0.05}$	2.05	3.61	3.01	1.62
Moisture + Phytase $LSD_{0.05}$	3.55	6.25	5.21	2.81

Values are means of three replicates; Means in the same column having different letters differ significantly at $P \leq 0.05$.

As well as, a graphical presentation of results are presented in Figure 1. It reveal that increasing both dietary moisture and supplementary phytase levels, resulted in a significant increase ($P \leq 0.05$) in the amount of inorganic phosphorus released at 6, 12, 18 and 24 hours compared to the control diet. Both 30 and 60 percentages of wetting in association with increasing phytase level showed a pronounced increase($P \leq 0.05$) in inorganic phosphorus output when compared to the diets with either 0.0% added moisture or diets that contained phytase in exclusion of increasing wet matter content.



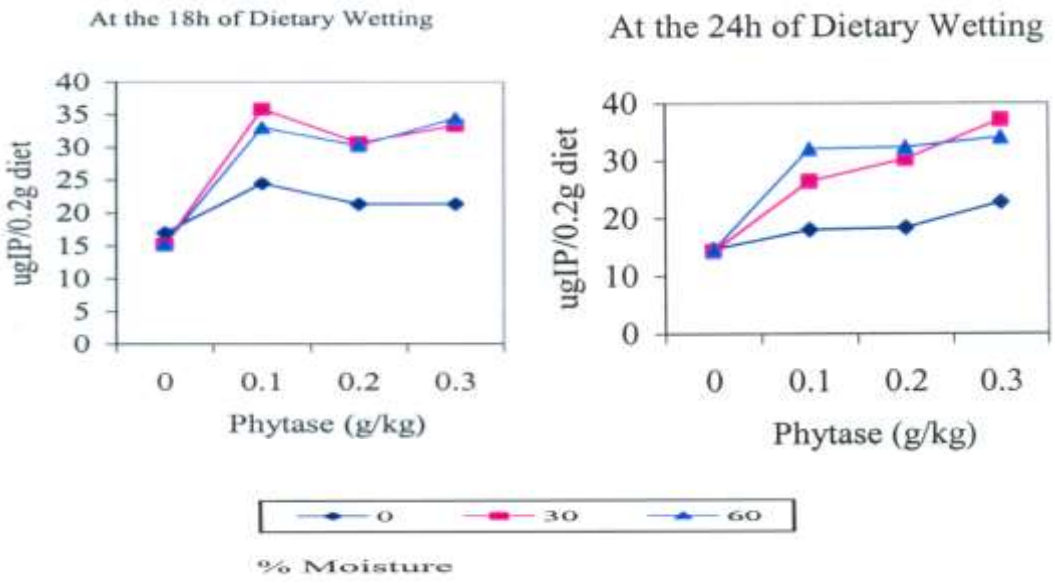


Figure 1: Effect of increasing dietary moisture content and phytase level on dietary phytae-P availability.

During the first six hours of incubation, diets which contained both added phytase and 60% wetness levels, showed higher phytase activity ($P \leq 0.05$); in the second six hours (12 hr) of further incubation, the 30 % wetness plus added phytase yielded higher inorganic phosphorus, followed by the diet that contained phytase plus 60% wetness when compared to the control ($P \leq 0.05$). It was observed that at both 6 and 12 hr of incubation, the released amount of inorganic phosphorus tended to increase with the increase in phytase associated with added water or dietary wetness (Table 2). It seems that after the first 12hrs, increasing phytase level has little effect on the amount of inorganic phosphorus released. Although, 0.1gphytase with either 30 or 60% water showed higher release of inorganic phosphorus than without added moisture, increasing phytase level beyond that had no significant increase ($P \leq 0.05$) in the amount of inorganic phosphorus with either 30 or 60 % moisture content. It was further noticed that incubation of the dietary treatments beyond 12 hr resulted in development of gases as well as discoloration of the diets (light pinkish).

Although the current investigation was limited to twenty four hours (Table 2), it was consistent with the findings of the overnight soaked pulses (Schlemmer *et al*., 1995), where the degradation of the phytic acid was principally by enzymatic hydrolysis (phytase) during the overnight soaking treatment. This study suggests that dietary wetness or the wet-mash form of diet aids in generating more phosphorus, even at the least supplemented phytase level which was less than the manufacturer's recommendation of 0.02% which again was consistent with the previous findings in which incubation of soybean meal with crude phytase preparation before feeding, culminated in chicks utilising the hydrolysed phytate phosphorus as efficiently as the phosphorus from inorganic sources (Nelson, 1976; Newkirk and Classed, 1997; Jubarah and Davis, 2006). Although, those study findings were under different conditions, the previous authors have indicated that even with high phytase levels (340,000 U/kg of meal) detectable phytate (15% phytic acid) still remained over 1h incubation. However, the inability of phytase to hydrolyse all the phytate in the reported study was likely to be related to factors that affect phytase's access to phytate or phytic acid. However, this study did not involve feeding trials, the wetness of the diet seems to have enhanced phytase access to phytate, as well as, it might have added value to the intrinsic phytase in degrading phytate, in addition, the setting time prior to feeding wet-mash diet seems crucial in phytate-P bioavailability and as it seems the gut transit time might be inadequate for phytate hydrolysis.

Previous studies have demonstrated that substantial portion of dietary phytate probably exists in phytase resistant forms during *in vivo* digestion; only relatively small amounts of the phytate are hydrolysed in the digestive tract even when phytase is added to the diet, and that phytate in soybean meal was reported to be more soluble than that in sesame meal (de Boland *et al.,* 1975; Schöner *et*

al., 1993; Simons *et al.*, 1990). Furthermore, Bartnik and Szafranska (1987) showed that, some feed ingredients have endogenous phytase activity, most cereals contain phytases, but their activity varies; rye, wheat and barley contain high levels of phytase activity, whereas, corn, oats, sorghum and oilseeds contain little or none of the enzyme. The same studies have also indicated that endogenous plant phytase is effective in feeds, as chicks were able to utilise phytate phosphorus for bone calcification, furthermore, wheat phytase could act on phytates from other ingredients. Thus it is possible to avoid the use of inorganic phosphate supplements by employing a combination of these ingredients (Temperton and Cassidy, 1964; Temperton, 1965; Scheuermannvon *et al*., 1988). Also, previous studies seem to suggest that phytate in soya-maize plant derived diet could be hydrolysed by inherent or intrinsic phytase, and that the variation in phytate solubility might be responsible for the differences in the extent of phytate from different plant feed sources could be hydrolysed.

Feeding wet mash diet is a worthwhile practice as a feeding system, especially when incorporating ingredients known to have high intrinsic phytase activity, which is backed up by the previous findings, and should be noted here as wheat related, and that wheat phytase is inactive at pH 3 and whether or not this inactivation is reversible when pH increases to 6 - 7 in the small intestine is not known. Therefore, if it is irreversible, then any breakdown of phytate by plant phytases must occur prior to or within the proventriculus, before the low pH inactivates the enzyme (Eeckhout and de Paepe, 1991; Ravindran, 1995) which is supportive to the current findings as far as *in vitro* hydrolysis of phytate or phytic acid prior to feeding is concerned. However, the current study seems to infer that wet mash dietary form or slight dampness of the diet might improve phytase (either intrinsic or exogenous) accessibility to dietary phytate. But given the present findings,

increasing dietary moisture might entail daily preparations of the diet or ration, since losses of nutrients and microbial growth is inevitable if wet mash is to be held beyond 12 hr of its moisture reconstitution. It is also noteworthy that since the optimum pH for plant phytase activity is in the range of 4.0 to 6.0, though some of its activity may be retained at pH 3.0 (Eeckhout and de Paepe, 1991; Irving, 1980). It is unlikely that a significant amount of the enzyme activity will survive the highly acidic conditions (pH 1 to 2.5) of the proventriculus (Eeckhout and de Paepe, 1991; Irving, 1980; Hill, 1971). As such, enzymatic hydrolysis of phytic acid or phytate during setting of wet mash diet and prior to feeding as observed in this study, could be essential for the phytate biological value of intrinsic phytases rich ingredients. Moreover, aleurone layer, which is the major depot of phytin in cereal, has been reported to be particularly resistant to digestion and often appears intact at the terminal ileum of the chicken (Bedford and Autio, 1996). If the aleurone cellwalls are not ruptured in the process of grinding or pelleting, then it is unlikely that their content of phytate will be readily accessible to phytase activity since the enzyme was reported to be far larger than the expected pore sizes present in the plant cell wall (Bedford and Schulze, 1998). Thus, the current findings are supportive of the explanation that, if phytate has to diffuse out of aleurone layer of the cereal grain foodstuff in order to be accessible to phytase, then greater phytate digestibility would be expected and that could be accomplished through dietary wetness, as dietary wetness enhances nutrients solubility (Yasar and Forbes, 2000; 2001). As illustrated in this study, increase in both dietary wet-matter and phytase level, resulted in a significant increase in the amount of inorganic phosphorus released at the first and second 6 hours of incubation when compared to the diets with either 0.0% added water or added phytase in exclusion of added water. In wetting phytase supplemented plant-based diet, the wet mash setting-time has to be limited to 6 or less than 12 hrs

or else the wet mash has to be dried or pelted to ensure adequate hydrolysis of phytate and prevent development of gases and discoloration of the wet mash.

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