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Original Article

The supplementation of phytase RONOZYME P on the growth and the utilisation of phosphorus by sex-reversed red tilapia (*Oreochromis niloticus* Linn.)

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Abstract

The effect of phytase supplementation to a low fish meal based diet on growth performance and phosphorus utilisation was investigated in sex-reversed red tilapia. Diets were prepared without phytase or inorganic phosphorus supplementation, with phytase, with supplemented inorganic phosphorus and with both phytase and supplemental inorganic phosphorus. Available phosphorus was set below requirement and the total phosphorus set to meet requirement for tilapia. After 8 weeks, there were significant differences in weight gain and protein utilisation between diets. There was an effect of phytase addition and inorganic phosphorus supplementation on bone phosphorus and whole-body phosphorus (p<0.05). A significant effect was also observed on phosphorus digestibility, phosphorus retention efficiency and phosphorus load of the water. Phosphorus digestibility and retention efficiency were significantly (p<0.05) higher, and phosphorus load of the water was significantly (p<0.05) lower in fish fed the phytase supplemented diet compared with diets containing supplemental inorganic phosphorus and the basal diet. In conclusion, phytase increased phosphorus availability, therefore reducing the need to add inorganic phosphorus and reducing phosphorus waste from low fish meal based diets for tilapia.

Keywords: phytase, tilapia, *Oreochromis niloticus*, inorganic phosphorus, phosphorus retention, phosphorus load

1. Introduction

Fish meal is important as a source of essential amino acids and phosphorus in fish feeds. This protein source is more expensive than some commonly used plant protein sources. However, about two-thirds of plant phosphorus is present in form of phytates (Lall, 1991), which are unavailable for the fish due to lack of an intestinal phytase to hydrolyze bound phosphate to bioavailable orthophosphate (Yan *et al*., 2002). The availability of indispensable minerals such as calcium, magnesium, zinc, manganese, copper and iron may also be adversely affected (Papatryphon *et al*., 1999). Dietary phytates also result in reduced protein and amino acid digestibility due to formation of phytate-protein complexes (Liu *et al*., 1998: Sugiura *et al*., 2001). Low digestibility of phytates in fish leads to a great amount of phosphorus excretion in fish farming waters.

Dietary incorporation of microbial phytase in fish diets may be used to increase digestibility of plant protein sources. The use of exogenous phytase has resulted in an increase of phytate phosphorus utilisation in several fish species including rainbow trout (Vielma *et al*., 1998), common carp (Schaefer *et al*., 1995), channel catfish (Li and Robinson, 1997) and Nile tilapia (Furuya *et al*., 2001). In a previous experiment with Nile tilapia (Phromkunthong and Gabaudan, 2006), apparent phosphorus digestibility coefficients increased in fish receiving dietary phytase, as did

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bone phosphorus and serum phosphorus.

In this study, total phosphorus was set above the tilapia requirement but available phosphorus was set below the requirement (NRC, 1993). The treatments were the basal diet, the basal diet with added phytase, the basal diet with added inorganic phosphorus, and the basal diet with both phytase and inorganic phosphorus. The objective of the trial was to assess the potential of phytase to enhance growth performance and phosphorus utilisation in sex-reversed red tilapia.

2. Materials and Methods

2.1 Diet preparation

The basal diet (diet 1) was formulated to contain 30% crude protein and 18.11 MJ gross energy/kg diet (Table 1). Dietary nutrient composition was previously reported (Phromkunthong and Gabaudan, 2006). Proximate analyses of experimental diets were performed using AOAC (1995). Soybean (45% protein) was used in the diet at 45%, providing the main source of phytate in the diet. This experiment was arranged as a completely randomized design with phytase level (0 and 750 FYT/kg diet) and di-calcium phosphate (DCP) level (0, 0.5 and 1.5%). Experimental diets were formulated as follows: T1 had no added phytase or DCP; T2 contained 1.5% DCP; T3 contained 750 FYT/kg diet and T4 contained 0.5% DCP and 750 FYT/kg diet. The microbial phytase used was the commercial product Ronozyme P(L) with 5,000 FYT/g supplied by DSM Nutritional Products, Bangkok, Thailand. Dry ingredients were mixed in a Hobart mixer and then homogenized with water for 15 min before pelleting with a Hobart pelletizer (USA, Model A200T; pellet diameter: 2 mm) and drying at 60 C for 24 h. Phytase was diluted in distilled water and sprayed onto the diet to achieve a concentration of 750 FYT/kg diet. The diets were stored at 4 C until fed. The dietary phytase content was determined by Biopract GmbH, Germany.

2.2 Growth experiment

The experiment was performed at the Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Sex-reversed red tilapias were obtained from a private farm in Phattalung Province. Fish were held in a 2,000 l fiberglass tank at low density and fed the basal diet (T1) for one week prior to the trial.

At the beginning of experiment, ten fish were anaesthetized with MS 222 and sacrificed for whole-body chemical analysis on day 0. Twenty fish were weighed and stocked into each of 12 235 l glass aquaria (3 replicates of 4 treatments). Each aquarium was supplied by 2 air stones under continuous aeration. All fish were fed to satiation at 0800 h and 1600 h, except that no feed was given on weighing days. Fish in each tank were anaesthetized, counted and bulk-weighed every two weeks. Treatments were sampled every 2 weeks for an 8 week interval for weight gain and survival.

2.3 Apparent digestibility coefficient (ADC)

Apparent digestibility coefficient (ADC) was measured after the growth experiment was terminated. Chromic oxide (Cr_2O_3) at 0.5% was used as an inert dietary marker. The fish were fed the diets containing chromic oxide for 2 weeks,

Table 1. Composition of experimental feed

Ingredient (%)	Diet T1	Diet T2	Diet T3	Diet T4
Fish meal	10	10	10	10
Soybean meal	45	45	45	45
Rice bran	18	18	18	18
Cassava	20.90	19.40	20.88	20.39
Fish oil	1.5	1.5	1.5	1.5
Vitamin mixtures[1]	1	1	1	1
Choline chloride	0.6	0.6	0.6	0.6
Mineral mixtures[2]	3	3	3	3
RONOZYME P 5000	0	0	0.015	0.015
Di-calcium phosphate (DCP)	0	1.5	0	0.5
TOTAL	100	100	100	100

[1] Vitamin mixtures contain the following vitamins per kg feed: Thiamine (B1) 10 mg; Riboflavin (B2) 20 mg; Pyridoxine (B6) 10 mg; Cobalamin (B12) 2 mg; Retinal (A) 4,000 IU; Cholecalciferol (D3) 2,000 IU; Menadione sodium bisulfite (K3) 80 mg; Folic acid 5 mg; Calcium pantothenate 40 mg; Inositol 400 mg; Niacin 150 mg; Tocopherol (E) 50 IU); Ascorbic acid (C) 500 mg; Biotin 3 mg

[2] Mineral mixtures deliver the following in mg/kg feed: Na 0.098; Mg 0.758; K 2.298; Ca 1.473; Fe 0.145; Zn 0.02; Mn 0.13; Cu 2.07mg; Co 0.59 mg; I 0.45 mg

during which time faecal material was collected by siphoning. Two hours after feeding at 10:00, the tanks were completely cleaned and faeces were collected until one hour before the 17:00 feeding. Faeces of each treatment were kept at -20 C and then oven dried at 60 C for 48 h and used in the analysis of chromic oxide and nutrients.

2.4 Chemical analysis

The chemical analyses of raw materials, diets, fish whole body and faeces were performed according to standard methods: dry matter, ash (AOAC, 1995), crude fat (Bligh and Dyer, 1959), crude protein (Kjeldahl using a selenium catalyst, N 6.25). Analysis of chromic oxide in diets and faeces used the method of Furukawa and Tsukahara (1966).

To determine bone ash and phosphorus, previously frozen fish were cooked for about 10 min in boiled water until the flesh and bone were easily separated. Soft tissues were carefully removed from the vertebrae. Isolated vertebrae were rinsed with distilled water and dried in an oven at 105 C for 24 h. After drying, they were ground with a mortar and pestle and then defatted with solvent (chloroform : methanol 1:1), dried and ashed in a muffle furnace (Vielma and Lall, 1998b). The ash was weighed and subsequently analyzed for phosphorus by molybdovanadate method (AOAC, 1995). Phosphorus in the diet, initial and final whole-body, and faeces was also analyzed by the molybdovanadate method.

Two individuals were sampled from each tank and 3-ml blood sample were collected from peduncle and centrifuged at 300 rpm for 5 min. The resulting serum was used for determination of phosphorus and alkaline phosphatase.

2.5 Phosphorus retention efficiency and load

Nutrient retention efficiency of phosphorus (NR) was calculated as :

NR phosphorus (%) =
100 [(FBW Nf) - (IBW Ni)] / (feed intake Ndiet)

Where FBW is the final body weight and IBW is the initial body weight of fish, N is the concentration of nutrient (P) in the fish at the start (Ni) and end (Nf) of the experiment (Storebakken *et al*., 1998). Phosphorus load was calculated as:

Table 2. Proximate analysis of raw materials (%)

Analysis	Fish Meal	Soybean meal	Rice Bran	Cassava	DCP
Moisture	9.80	9.40	9.40	9.48	-
Protein	72.30	45.50	13.09	2.70	-
Fat	7.51	1.65	14.66	0.25	-
Ash	10.77	6.93	17.53	4.12	-
Crude fiber	-	6.54	6.46	3.14	-
P	1.91	0.67	1.60	0.03	19.43
Phytate P	-	0.403	1.29	0.02	-

Table 3. Proximate analysis of experimental feed (%)

Experimental feed	Dry matter	Protein	Fat	Ash	Crude fiber	P	Cr_2O_3
T1 (Basal)	94.66	30.59	5.69	10.78	5.10	0.79	0.50
T2 (DCP)	93.66	31.72	5.60	12.01	4.78	1.11	0.50
T3 (Phytase)	91.85	30.39	5.70	10.56	4.70	0.82	0.50
T4 (DCP&Phytase)	91.73	30.53	5.74	11.00	4.98	0.88	0.51

Table 4. Non phytate phosphorus of experimental diets (%)

Treatment	Total P	Total Phytate P [1]	Non phytate P [2]
T1 (Basal)	0.79	0.45	0.34
T2 (DCP)	1.11	0.45	0.66
T3 (Phytase)	0.82	0.45	0.37
T4 (DCP&Phytase)	0.88	0.45	0.43

[1] **Total phytate P = calculation from phytate P of each ingredient**
[2] **Non phytate P (%) = total P- total phytate P**

Nutrient load (g P/kg) =
(Nutrient fed (g) - Nutrient deposited (g))/weight gain (kg)
(Vielma *et al*., 2002)

2.6 Statistical analysis

Mean values were reported ± standard deviation (SD). Data were analyzed using ANOVA (CRD) and the differences among averages compared using Duncan's multiple range test. Differences were considered significant at $p<0.05$.

3. Results

Proximate composition of dietary ingredients is presented in Table 2 and Table 3 presents the proximate composition of the experimental diets. Proximate composition of diets was similar, except for dry matter content, which was slightly higher in diets T1 and T2. Total phosphorus content of diet T2 was higher than phosphorus content of the other diets. Phytate content of all diets was calculated from raw material analysis to be 0.45% (Table 4). By difference, non phytate phosphorus was adequate in diet T2 (0.66%) and deficient in the other diets (0.34% - 0.43%). Phytase content of diets T1 and T2 showed a basal amount. Phytase content of diets T3 and T4 were higher, reflecting the addition of exogenous phytase (Table 5).

3.1 Growth performance and feed efficiency

No significant difference in average body weight occurred until week 8, when T1 weight lagged behind the other three treatments (Table 6). The same trends were shown for weight gain and specific growth rate for the entire 8 week period (Table 7). There were no significant differences in feed intake or survival (Table 7).

No significant treatment differences occurred in feed conversion ratio (FCR) or protein efficiency ratio (PER) (Table 8). Apparent net protein utilisation (ANPU) were highest for T3 (phytase treatment) (Table 8).

3.2 Composition of fish

There were no significant treatment differences in whole body dry matter, crude protein and total lipid (Table 9). There were significant dietary effects on whole body phosphorus and ash (Table 9). Fish fed diets with phytase or inorganic phosphorus or both had significantly higher whole body phosphorus and whole-body ash content (Table 9). Bone ash and bone phosphorus were also higher in fish receiving inorganic phosphorus and/or phytase (Table 10).

Table 5. Phytase content in four feed formulae (Determined by analysis)

Treatment	Analyzed (mg phytase equiv./kg feed)
T1 (Basal)	93
T2 (DCP)	72
T3 (Phytase)	914
T4 (DCP&Phytase)	949

Table 6. Average body weight of sex reversed red tilapia fed 4 experimental feeds for a 8 week period [1]

Treatment	Week				
	0	2	4	6	8
T1 (Basal)	14.72±0.10	22.10±1.37	31.92±1.90	43.62±2.48	57.49±1.07a
T2 (DCP)	14.78±0.04	21.92±0.81	32.20±2.18	45.71±1.34	60.17±1.10b
T3 (Phytase)	14.65±0.11	21.97±2.35	33.87±0.39	44.99±0.24	60.46±0.93b
T4 (DCP&Phytase)	14.74±0.05	21.84±0.61	32.12±1.99	45.01±1.61	60.34±1.59b

[1]**Mean ± standard deviation of three replicates**

Table 7. Weight gain, specific growth rate, and survival rate of sex reversed red tilapia fed 4 experimental feeds for a 8 week period1

Treatment	Weight gain (%)	Specific growth rate (%/day)	Rate of feed intake (%/fish/day)	Survival rate (%)
T1 (Basal)	290.68±8.56a	2.43±0.04a	3.32±0.19	93.33±2.89
T2 (DCP)	307.21±2.13b	2.51±0.01b	3.14±0.21	95.00±5.00
T3 (Phytase)	312.74±5.65b	2.53±0.02b	3.03±0.28	91.67±5.77
T4 (DCP&Phytase)	309.46±11.68b	2.51±0.05b	3.08±0.27	96.67±2.89

[1]**Mean ± standard deviation of three replicates**

3.3 Phosphorus in serum and alkaline phosphatase

Serum phosphorus in fish fed diets containing mineral phosphorus, phytase, or both was higher (25.2-27.9 mg/l) than serum phosphorus in fish fed the basal diet (19.0 mg/l; Table 10). Serum alkaline phosphatase was higher in fish receiving mineral phosphorus (19.0 mg/l), phytase (19.0 mg/l), or both (26.0 mg/l) compared to fish receiving the basal diet (17.0 mg/l; Table 10).

3.4 Digestibility

There was no significant treatment effect on dry matter digestibility (Table 11). Phosphorus digestibility was significantly higher in fish fed diets with phytase (62.91%) or phytase and inorganic phosphorus (63.5%), intermediate for inorganic phosphorus (49.89%) and lower for basal diet (41.19%) (Table 11).

Table 8. FCR, PER, ANPU of sex reversed tilapia fed 4 experimental feeds for a 8 week period[1]

Treatment	FCR	PER	ANPU(%)
T1 (Basal)	1.66 ±0.08	1.97± 0.09	31.37± 1.48[a]
T2 (DCP)	1.55 ±0.10	2.04± 0.13	32.00± 2.07[a]
T3 (Phytase)	1.44± 0.15	2.31± 0.24	38.08± 3.89[b]
T4 (DCP&Phytase)	1.54± 0.12	2.13± 0.16	34.08 ±2.61[ab]

[1]Mean standard deviation of three replicates

Table 9. Whole body composition of sex reversed tilapia fed 4 experimental feeds for a 8 week period (%)[1]

Experimental feed	Dry matter	Protein	Fat	Ash	Phosphorus
T1 (Basal)	24.67 ±0.68	59.34 ±0.14	20.87 ± 1.12	12.69 ± 0.42[a]	1.84 ± 0.176[a]
T2 (DCP)	24.10 ± 0.93	59.73 ± 0.88	21.12 ± 0.73	15.86 ± 0.43[c]	2.55 ± 0.109[c]
T3 (Phytase)	25.78± 1.83	59.22 ± 1.08	24.52± 0.83	14.50± 0.29[b]	2.18± 0.190[b]
T4 (DCP&Phytase)	24.02± 0.63	59.94 ± 0.01	23.04± 4.15	15.40± 0.51[c]	2.39 ± 0.089[bc]

[1] Mean ± standard deviation of three replicates, Initial whole body phosphorus is 2.93; Initial whole body protein is 50.63

Table 10. Phosphorus blood serum, alkaline phosphatase activity and bone of sex reversed tilapia fed 4 experimental feeds for a 8 week period[1]

Treatment	Phosphorus in serum (mg/l)	Alkaline phosphatase (U/l)	Bone phosphorus (%)	Bone ash (%)
T1 (Basal)	19 ± 0.41[a]	17± 0.0	7.85 ± 0.24[a]	45.58± 0.67
T2 (DCP)	25.2± 0.42[b]	19± 0.0	8.82 ± 0.28[c]	51.31± 2.63
T3 (Phytase)	27.9± 0.57[b]	19± 0.0	8.10 ± 0.21[ab]	48.83± 1.56
T4 (DCP&Phytase)	27.9± 0.14[b]	26± 0.0	8.37± 0.64[b]	49.55± 2.60

[1] Mean ± Standard deviation of two replications

Table 11. Apparent digestibility coefficients of ingredients with and without phytase supplementation (%)[1]

Treatment	Dry matter	Phosphorus
T1 (Basal)	62.64± 1.30	41.19 ± 1.11[a]
T2 (DCP)	61.57± 0.30	49.89 ± 2.40[b]
T3 (Phytase)	62.41± 0.47	62.91 ± 0.99[c]
T4 (DCP&Phytase)	62.11± 1.02	63.50± 1.25[c]

[1]Mean ± standard deviation of three replicates

Table 12. Phosphorus retention and phosphorus load of sex reversed red tilapia fed 4 experimental feeds for a 8 week period

Treatment	Phosphorus retention (%)	Phosphorus load (g P/kg weight gain)
T1 (Control)	27.43 ±1.11[a]	6.34 ±0.63[bc]
T2 (DCP)	34.94 ±2.24[b]	7.93 ±1.03[c]
T3 (Phytase)	43.07 ±4.34[c]	3.73 ±1.17[a]
T4 (DCP&Phytase)	40.86±2.97[c]	4.86 ±0.99[ab]

3.5 Phosphorus retention efficiency and load

There was a dietary effect on phosphorus retention efficiency, which was highest for fish fed the diets with phytase, and lowest for fish fed the basal diet (Table 12). There was also a dietary effect on phosphorus loading of the water, which was lowest in the fish fed the diet with phytase or combination with phytase and DCP (Table 12).

4. Discussion

In this study, dietary exogenous phytase added to practical tilapia diets was substituted for added mineral phosphorus without negative effect on growth or metabolism. Similar results have been obtained with several fish species fed phytase-supplemented diets. Increased phosporus availability of fish fed phytase has been reported for salmonids (Rodehutscord and Pfeffer, 1995; Brown, 1993; Riche and Brown, 1996; Lanari *et al*., 1998; Forster *et al*., 1999; Sugiura *et al*., 2001), carp (Sch efer *et al*., 1995), striped bass (Papatryphon *et al*., 1999), tilapia (Furuya *et al*., 2001, Portz *et al*., 2003; Phromkunthong *et al*., 2004; Phromkunthong and Musakopat, 2005; Phrom-kunthong and Gabaudan, 2006) and hybrid catfish (Phrom-kunthong *et al*., 2005). Positive effects were also seen when phytase was used to pre-treat selected dietary ingredients fed to salmonids (Cain and Garling, 1995; Vielma *et al*., 2002). Generally, growth improvements were observed in plant protein based diets. When an all plant meal diet is used, available phosphorus content is lower and amino acid profile poorer than in fish meal-based diets. Additionally, phytate may bind to amino acids in the fish stomach and decrease amino acid availability.

In this study, reduced growth of fish fed diet T1 (negative control) compared to diet T2 (basal diet with phosphorus supplementation) indicated that the basal diet was phosphorus limited. The total phosphorus (0.79%) and available phosphorus (0.34%) were insufficient for optimal growth of tilapia and also resulted in reduced SGR and ANPU in addition to lower tissue mineral densities. Tilapia dietary requirement for phosphorus has been estimated at 0.45%- 0.50% (Vielma and Lall, 1998a; Robinson *et al*., 1987; NRC, 1993). Supplementation of exogenous phytase into the basal diet improved these biological indices to the level of the mineral phosphate treatment, or in some cases even better. Apparent phosphorus digestibility and phosphorus retention were highest for diets T3 and T4, both of which were supplemented with exogenous phytase. Confirmation of the improved phosphorus availability conferred in this trial by dietary phytase was provided by biological parameters: higher bone P, higher serum P concentration and higher serum alkaline phosphatase.

Dietary phytase has also been found to increase availability of other phytate-bound nutrients. Phromkunthong and Gabaudan (2006) found that tilapia fed phytase-supplemented diets demonstrated marked increases of serum Zinc. Lei *et al*. (1993) reported that supplementation of phytase to animal feeds may improve bioavailability of protein and trace minerals such as zinc which could in turn lead to improved weight gain.

Growth is only affected by phosphorus deficiency when the whole-body phosphorus content falls below a critical level (Nordrum *et al*., 1997). This relationship has been shown in carp (Sch efer *et al*., 1995), catfish (Jackson *et al*., 1996), striped bass (Papatryphon *et al*., 1999) and Atlantic salmon (Sajjadi and Carter, 2004). Using an excess amount of phosphorus levels leads to lower digestibility (Rodehutscord *et al*., 2000). In one study phosphorus digestibility in rainbow trout dropped when levels higher than needed were fed (Riche and Brown, 1996). Dietary phosphorus absorption is regulated by blood phosphorus level (Lall, 1991); so when the blood phosphorus level is saturated, absorption and apparent digestibility decreases. Insufficient available phosphorus intake leads to the mobilization of phosphorus from the bone and transfer to soft tissues and metabolic processes (Baeverfjord *et al*., 1998).

Phosphorus retention efficiency in the present study was significantly higher in fish fed phytase-containing diets, demonstrating improved total phosphorus utilisation. When dietary phosphorus concentration increases above the requirements, retention decreases (Jahan *et al*., 2003). Conversely, increased phosphorus utilisation in the phytase treatments resulted in reduced phosphorus loading. Rainbow trout fed phytase-supplemented diet also had lower phosphorus loading (Cain and Garling, 1995; Lanari *et al*., 1998; Vielma *et al*., 1998, 2002). Using phytase in plant-meal-based diets increases phosphorus digestibility, reduces need for mineral phosphorus supplementation, and reduces phosphorus load of the water. Hence, the inclusion of phytase in the diets can be used as a tool for aquaculture waste management (Cain and Garling, 1995; Vielma *et al*., 2002).

5. Conclusion

Phytase improved growth in tilapia fed plant based diets with suboptimal available phosphorus. Apparent phosphorus digestibility and phosphorus deposition were also increased in tilapia fed phytase, and phosphorus loading of the water was reduced. Therefore, using phytase in plant-meal-based diets will reduce the need for inorganic phosphorus supplementation in diets, which will lead to reductions in phosphorus discharge from fish farms.

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