Songklanakarin J. Sci. Technol.
32 (6), 547-554, Nov. - Dec. 2010

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

Interaction of phytase RONOZYME®P(L) and citric acid on the utilization of phosphorus by common carp (*Cyprinus carpio*)

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Received 18 June 2010; Accepted 20 September 2010

Abstract

A feeding trial was conducted for 60 days to study the effects of the combination of microbial phytase and citric acid on phosphorus utilization in *Cyprinus carpio* fingerlings. Four diets designated as diet without phytase or inorganic phosphorus supplementation (T1), with 1.1% MSP (T2), with 0.55% MSP and phytase (T3) and with 0.55% MSP, phytase and citric acid (T4). Four replicate groups of 20 fish were fed two times daily until satiation. Phosphorus digestibility and retention were measured as well as the fish growth performance. It is concluded from the results that the addition of microbial phytase and citric acid enhances the availability of phosphorus from plant sources, improves bone mineralization, growth and feed efficiency. Combining a low dose of citric acid to the phytase significantly increased the positive effects of the enzyme.

Keywords: phytase, common carp, *Cyprinus carpio*, inorganic phosphorus, citric acid, phosphorus retention

1. Introduction

Dietary incorporation of microbial phytase in fish diets increases the digestibility of plant protein sources. The use of exogenous phytase has resulted in an increase of phytate phosphorus utilization in several fish species including rainbow trout (Vielma *et al*., 1998), common carp (Schafer *et al*., 1995), channel catfish (Li and Robinson, 1997) and Nile tilapia (Furuya *et al*., 2001; Tudkaew *et al*., 2008). Several studies reported that phytase has two optimal peaks of activity, one at pH 5.0-5.5 and the other at pH 2.5 (Simons *et al*., 1990). Phytase activity changes along the digestive tract, with most efficient phytate hydrolysis by phytase occurring in the stomach (Yi and Kornegay, 1996). There is some evidence that pH of the gastrointestinal tract affects the bioavailability of minerals in fish (Sugiura *et al*., 1998; Vielma *et al*., 1999). Jongbloed (1987) reported that reducing the intestinal pH through dietary addition of organic acids increased the solubility of phytate phosphorus and thus improved phosphorus absorption. In addition to their effect on intestinal pH, supplementary organic acids can also bind various cations along the intestine and act as chelating agents (Ravindran and Kornegay, 1993), resulting in increased intestinal absorption of minerals (Sugiura *et al*., 1998; Vielma *et al*., 1999). Citric acid (CA) has been reported to increase phosphorus bioavailability by dephosphorylation of phytate in vitro (Zyla *et al*., 1995; Baruah, 2004). Previous studies indicated that dietary addition of CA significantly improved phytate phosphorus utilization in broiler chicks (Boiling *et al*., 2000). In the study by Baruah *et al*. (2005; 2006), it was found that 3% CA combined to a dietary phytase significantly increased phosphorus utilization in *Labeo rohita* juveniles fed soybean meal based diets. Sugiura *et al*. (2001) also found a significant increase in the apparent absorption of magnesium and phosphorus by the addition of 5% CA and phytase in the diet of rainbow trout, *Oncorhynchus mykiss*. In

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carnivorous fish like rainbow trout, production of hydrochloric acid in the stomach assist in lowering the pH in the digestive tract, but in stomachless species like *Cyprinus carpio*, no such mechanism exists. Hence, addition of organic acids or acidifiers may reduce the dietary pH, which in turn may reduce the pH in the intestine. Further, dietary acidification may reduce the rate of gastric emptying (Mayer, 1994), which may also favor the action of phytase. Thus, it was hypothesized that dietary addition of organic acids and phytase (Ronozyme®P(L)) may have a synergistic effect on phosphorus availability. Hence, the present experiment was carried out to study the combination of phytase and citric acid on the utilization of phosphorus in juveniles *Cyprinus carpio* with the expectation that such an approach would be helpful to develop eco-friendly feed in an economically feasible fashion.

2. Materials and Methods

2.1 Diet preparation

The basal diet (diet 1) was formulated to contain 35% crude protein and 18.11 MJ gross energy/kg diet. Dietary nutrient composition was previously reported (Phromkunthong and Gabaudan, 2006; Tudkaew *et al*., 2008) (Table 1). Proximate analysis of experimental diets was performed following the Association of Official Analytical Chemists (AOAC,1995). Solvent extracted soybean meal (45% protein) was used in the diet at 55%, providing the main source of phytate in the diet. This experiment was arranged as a completely randomized design. Experimental diets were formulated as follows: T1 has no added monosodium phosphate (MSP) or phytase; T2 contains 1.1% MSP; T3 contains 0.55% MSP and 750 FYT/kg diet and T4 contains 0.55% MSP, 750 FYT/kg diet and 0.22% citric acid. FYT, phytase unit: one FYT unit is the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH 5.5 and 37ºC. The microbial phytase used is the commercial product Ronozyme®P(L) with 5,000 FYT/g was supplied by DSM Nutritional Products, Bangkok, Thailand. Dry ingredients were mixed in a Hobart mixer and then mixed with water for 15 min before pelleting with a Hobart pelletizer (USA, Model A200T; pellet diameter: 2 mm) and drying at 60ºC for 24 hrs. 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 4ºC 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. Common

Table1. Composition of experimental feed

Ingredient (%)	Diet T1	Diet T2	Diet T3	Diet T4
Fish meal	17	17	17	17
Soybean meal	55	55	55	55
Rice bran	8	8	8	8
Cassava meal	12.9	11.8	12.33	12.13
Fish oil	2	2	2	2
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(L) 5000	0	0	0.015	0.015
Mono-sodium phosphate (MSP)	0	1.1	0.55	0.55
Citric acid	0	0	0	0.22
Cr_2O_3	0.5	0.5	0.5	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 g/kg feed: Na 0.098; Mg 0.758; K 2.298; Ca 1.473; Fe 0.145; Zn 0.02; Mn 0.013; Cu 2.07mg; Co 0.59 mg; I 0.45 mg.

carp was obtained from a commercial farm in the central part of Thailand. 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 the experiment, ten fish were anaesthetized with clove oil 30 mg/l (Velisek *et al*., 2005) and sacrificed for whole body chemical analysis on day 0. Twenty fish were weighed and stocked into each of sixteen 235 l-glass aquaria (4 replicates of 4 treatments). Each aquarium was continuously aerated with two air stones. All fish were fed to satiation at 08:00 h and 16:00 h, except that no feed was given on weighing days. Fish in each tank were anaesthetized, counted and bulk-weighed every two weeks. Fish were sampled every two weeks for weight gain and survival.

2.3 Apparent digestibility coefficient

Apparent digestibility coefficient (ADC) was measured after termination of the growth experiment. Chromic oxide (Cr_2O_3) at 0.5% in the diets was used as an inert dietary marker. The fish were fed the diets containing chromic oxide for two weeks, during which time fecal material was collected by siphoning. Two hours after feeding at 10:00, the tanks were completely cleaned and feces were collected until one hour before the 17:00 feeding. Feces of each treatment were kept at -20ºC and then oven dried at 60ºC for 48 hrs and used in the analysis of chromic oxide and nutrients.

2.4 Chemical analysis

The chemical analyses of raw materials, diets, and feces 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 feces used the method of Furukawa and Tsukahara (1966).

To determine bone ash and phosphorus, previously frozen fish were boiled for about 10 min in 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 105ºC for 24 hrs. 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, 1998). The ash was weighed and subsequently analyzed for phosphorus by molybdovanadate method (AOAC, 1995). Phosphorus in the diet, initial and final whole-body, and feces were analyzed by the molybdovanadate method.

Two individuals were sampled from each tank and 3 ml blood sample was collected from peduncle and centrifuged at 3,500 rpm for 10 min. The resulting serum was used for determination of phosphorus.

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

2.6 Statistical analysis

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

3. Results

Table 2 and Table 3 present the proximate composition of the experimental diets. Proximate composition of diets was similar, except ash and total phosphorus content of diet T2 was higher than those of the other diets. Phytate content of all diets was calculated from raw material analysis to be 0.34% (Table 4). Estimated by difference, non–phytate phosphorus was 0.70% in diet T2 and ranged from 0.52 to 0.60% in the other diets. Therefore, the control diet could be expected to be deficient in available phosphorus while T3 and T4 were expected to be sufficient in available P only if the phytase released P from phytate. Phytase content of diets T1 and T2 showed a basal amount. Phytase content of diets T3 and T4 reflected the addition of exogenous phytase (Table 5). Adding of 0.22% citric acid in T4 feed reduced the pH of

Table 2. Proximate analysis of raw materials (in %), here mean ± standard deviation of three replicates.

Analysis	Moisture	Protein	Fat	Ash	Fiber	P	NFE
Fish Meal	10.06±0.05	62.80±0.08	6.57±0.07	20.27±0.50	-	2.09±0.12	0.32±0.65
Soybean meal	12.68±0.89	44.04±0.02	1.24±0.03	7.12±0.45	7.00±0.28	0.62±0.12	27.68±0.35
Rice Bran	9.50±0.22	12.48±0.13	16.64±0.32	10.96±0.15	7.32±0.19	1.80±0.04	42.75±0.11
Cassava meal	13.24±0.36	2.24±0.02	0.62±0.01	9.15±0.60	2.92±0.01	0.10±0.01	71.60±0.17
MSP	-	-	-	-	-	23.67±0.84	-

Table 3. Proximate analysis of experimental feed (in %), here mean standard deviation of three replicates.

Treatment	Moisture	Protein	Fat	Ash	Fiber	P	NFE
T1	6.07±0.16	35.54±0.84	6.49±0.55	11.99±0.30	5.45±0.35	0.86±0.01	35.23±0.37
T2	8.10±0.39	34.88±0.17	6.38±0.49	12.31±0.29	5.41±0.27	1.04±0.03	34.72±0.23
T3	9.34±0.95	34.84±0.59	6.43±0.48	11.37±0.63	5.42±0.28	0.94±0.01	34.81±0.76
T4	8.30±1.12	35.14±0.68	6.47±0.45	11.50±0.27	5.43±0.02	0.94±0.07	34.52±0.06

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

Treatment	Total P	Total Phytate P[1]	Non phytate P[2]
T1	0.86±0.01	0.343±0.01	0.515±0.02
T2	1.04±0.03	0.343±0.01	0.698±0.03
T3	0.94±0.01	0.343±0.01	0.598±0.01
T4	0.94±0.07	0.343±0.01	0.601±0.05

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

Table 5. Phytase content in four feed formulae, here mean ± standard deviation of two replicates (determined by analysis).

Treatment	Analyzed (mg phytase equiv./kg feed)	pH
T1	0	6.24
T2	0	6.12
T3	956±67	6.10
T4	1,064±36	6.08

Table 6. Average body weight of common carp fed 4 experimental feeds for a 8–week period [1].

Treatment	Week				
	0	2	4	6	8
T1	4.90±0.01a	6.03±0.07a	7.68±0.22a	9.06±0.11a	10.18±0.16a
T2	4.92±0.01a	6.26±0.10b	8.23±0.23b	10.58±0.25c	12.35±0.16c
T3	4.91±0.01a	6.16±0.13ab	8.01±0.14ab	9.87±0.17b	11.14±0.12b
T4	4.91±0.00a	6.22±0.12b	8.09±0.21b	10.05±0.23b	12.02±0.33c

[1]Mean ± standard deviation of four replicates.
Mean within each column not sharing a common superscript are significantly different (p<0.05).

feed from 6.24 to 6.08 (Table 5).

3.1 Growth performance and feed efficiency

Significant difference in average body weight occurred from Week 2 onwards and provided the same trend until Week 8, when T1 weight lagged behind the other three treatments (Table 6). Non–significant difference was shown among T2 and T4 whereas T3 provided intermediate growth (Table 6). The same trends were shown for weight gain and specific growth rate over the eight week–period (Table 7). There were significant differences in feed intake but not for

Table 7. Weight gain, specific growth rate, rate of feed intake and survival rate of common carp fed 4 experimental feeds for a 8–week period[1].

Treatment	Weight gain (%)	SGR (%/day)	Rate of feed intake (%/fish/day)	Survival rate (%)
T1	107.92±3.70a	1.31±0.03a	2.71±0.02bc	100±0.00a
T2	151.19±3.23c	1.64±0.02c	2.62±0.03a	100±0.00a
T3	126.82±2.07b	1.46±0.02b	2.67±0.05ab	97.78±3.85a
T4	144.68±6.77c	1.60±0.05c	2.75±0.03c	98.89±1.92a

[1]Mean ± standard deviation of four replicates.
Mean within each column not sharing a common superscript are significantly different (p<0.05).

Table 8. FCR, PER, ANPU of common carp fed 4 experimental feeds for a 8–week period[1].

Treatment	FCR	PER	ANPU (%)
T1	2.16±0.06d	1.30±0.04a	21.89±0.52a
T2	1.70±0.00a	1.68±0.00d	25.43±0.08c
T3	1.95±0.06c	1.47±0.04b	23.79±0.72b
T4	1.85±0.05b	1.54±0.05c	25.90±0.64c

[1]Mean ± standard deviation of four replicates.
Mean within each column not sharing a common superscript are significantly different (p<0.05).

Table 9. Whole body composition of common carp fed 4 experimental feeds for a 8–week period (%)[1].

Treatment	Dry matter	Protein	Fat	Ash	Phosphorus
Initial fish	21.48±1.29	53.83±1.97	22.16±3.60	10.31±0.22	1.29±0.09
T1	25.51±1.22a	53.95±1.17a	25.97±2.4b	6.56±0.29a	1.05±0.03a
T2	22.37±1.04a	57.96±3.37a	22.12±1.29a	7.67±0.51b	1.48±0.08b
T3	24.12±1.55a	55.14±2.13a	23.34±2.25ab	6.24±0.40a	1.39±0.40ab
T4	23.83±1.05a	58.04±0.90a	20.27±1.65a	6.70±0.05a	1.42±0.07ab

[1]Mean ± standard deviation of three replicates.
Mean within each column not sharing a common superscript are significantly different (p<0.05).

survival (Table 7). Carp, which received monosodium phosphate (T2), phytase (T3) and the combination of monosodium phosphate, phytase and citric acid (T4), had an FCR, PER and ANPU improved compared to the negative control (T1) (Table 8).

3.2 Composition of fish

Whole body fat content decreased from 25.95% to 20.27% (p<0.05) in T4, T2 and T3 compared to T1 (Table 9). The highest ash and P contents in whole body were demonstrated in T2 whereas they were higher in T3 and T4 than those in T1 (Table 9).

3.3 Serum P, bone P, bone ash and fecal P

There was no significant difference in serum P among treatments (Table 10). However, higher content of bone P and bone ash were shown in T2, T3 and T4 compared to T1 (Table 10). Fecal P was highest in T2 (MSP); it decreased with the addition of phytase (T3) and the combination of phytase and citric acid (T4) (Table 10).

Table 10. Serum phosphorus, bone phosphorus, bone ash and fecal P of common carp fed 4 experimental feeds for a 8–week period[1].

Treatment	Serum phosphorus (mg/l)	Bone phosphorus (%)	Bone ash (%)	Fecal Phosphorus (%)
T1	15.65±0.35a	6.20±0.15a	39.17±0.06a	1.83±0.17ab
T2	22.9±3.54a	6.58±0.41ab	44.22±0.66b	1.94±0.13b
T3	18.55±6.43a	6.48±0.69ab	43.89±0.48b	1.61±0.09a
T4	17.60±3.25a	7.20±0.25b	44.94±0.37b	1.52±0.22a

[1]Mean ± standard deviation of three replicates.
Mean within each column not sharing a common superscript are significantly different ($p<0.05$).

3.4 Digestibility

There was no significant treatment effect on dry matter digestibility (Table 11). Phosphorus digestibility were significantly higher in fish fed diets with MSP (T2), phytase and MSP (T3) and phytase, MSP and citric acid (T 4) compared to that of the basal diet (Table 11).

3.5 Phosphorus retention efficiency

There was a dietary effect on phosphorus retention efficiency, which was highest for fish fed the diets with MSP, and lowest for fish fed the basal diet (Table 12).

Table 11. Apparent digestibility coefficients of carp fed 4 trial feeds (%)[1].

Treatment	Dry matter	Phosphorus
T1	64.00±2.79a	23.77±2.03a
T2	60.01±2.34a	38.98±1.97b
T3	61.29±1.59a	33.79±1.94b
T4	61.47±5.31a	37.93±5.31b

[1]Mean ± standard deviation of three replicates.
Mean within each column not sharing a common superscript are significantly different ($p<0.05$).

Table 12. Phosphorus retention of common carp fed 4 experimental feeds for a 8–week period[1].

Treatment	Phosphorus retention (%)
T1	17.13±1.07a
T2	23.42±0.49c
T3	22.10±0.49b
T4	21.55±0.20b

[1]Mean ± standard deviation of three replicates.
Mean within each column not sharing a common superscript are significantly different ($p<0.05$).

4. Discussion

Dietary phytase has been found to affect the hydrolysis of phytate in the digestive tract of several fish species (Schafer *et al*., 1995; Li and Robinson, 1997; Sugiura *et al*., 2001; Tudkaew *et al*., 2008). However, in stomachless fish such as common carp, the lack of acid secretion results in a neutral digestive tract pH. Therefore, the addition of organic acids to the feed may reduce the pH in the intestine and help enhance mineral utilization. In the current study, all measured parameters i.e. growth, feed efficiency as well as phosphorus availability were significantly improved in response to dietary addition of microbial phytase and further increased with the addition of phytase and citric acid (T4). The addition of organic acids or acidifiers reduces the dietary pH as well as the pH in the intestinal lumen, which is likely to enhance the phytase activity (Erdman *et al.,* 1979). Further, dietary acidification may reduce the rate of gastric emptying (Mayer 1994), which may also favor the action of phytase. Dietary addition of microbial phytase combined with an organic acid was more effective in our experiment than the supplementation of the phytase alone. This is in agreement with the work of Suguira *et al*. (2001) in rainbow trout, which indicated that dietary acidification increases the activity of supplemental phytase. Suguira *et al*. (2001) found that in a low-ash diet, the supplementation with both phytase and citric acid resulted in a further increase in P absorption. The authors concluded that the combination of dietary phytase and citric acid is a useful approach to the production of low-pollution feeds.

In our study, supplementation of microbial phytase and citric acid increased the whole body phosphorus content. Moreover, phosphorus availability was also significantly increased with dietary microbial phytase and MSP (T3) and with the combination of microbial phytase and MSP and adding citric acid (T4). Hossain *et al*. (2007) found that organic acids improved the apparent digestibility of protein and amino acids in growing pigs, which might influence mucosal morphology, as well as stimulate pancreatic secretions. Organic acids also serve as substrates in intermediary

metabolism thereby reduce antagonistic interactions including precipitation and co-precipitation between Calcium and phosphates or trace elements at the intestinal brush border (Sugiura *et al*., 1998). Hossain *et al*. (2007) reported that red sea bream fed diet supplemented with 1% citric acid increased absorption of phosphorus in fish meal (Sarker, 2004). The effect of organic acids on availability of mineral elements in fishmeal appeared to be mainly a result of an acidifying effect, which solubilized bone minerals in fish meal, rather than of a chelating effect. This result could be attributed to two related factors: (i) the effect of dietary acidification and solubilization; and (ii) the effect of subsequent chelation of released cations (Sarker *et al*., 2005). The combination of phytase as well as phytase and citric acid in the diet increased phosphorus retention. There are two possible reasons for citric acid to enhance phytase function and hence improve dietary phosphorus utilization. First, combination of citric acid with phytase may increase the solubility of digesta-phosphorus and slow down the gastric emptying rate (Jongbloed *et al*., 1996), thereby improving total phosphorus absorption. Vielma and Lall (1997) observed an improved availability of phosphorus as well as other minerals by adding 10 ml formic acid/kg feed in fish meal-based diets for rainbow trout. Second, microbial phytases have an acidic optimum pH (Simons *et al*., 1990). Acidifying the diet reduced the pH of intestinal digesta and provided a more favorable environment for phytase function.

In conclusion, our trial showed that the addition of citric acid (0.22%) and phytase (750 FYT/kg feed) had a greater effect on total phosphorus bioavailability in a common carp diet than the addition of phytase alone and allowed to reduce the inclusion of inorganic phosphorus while maintaining the performance of the fish. Furthermore, the excretion of fecal phosphorus was reduced with the supplementation of microbial phytase and citric acid.

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