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Full Length Article

Mineral Status of Rohu (*Labeo rohita*) Juveniles Fed an Acidified Phytase Pre-Treated Soybean Meal Based Diet

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Abstract

Fish meal is the first choice for fish feed manufacturers due to its highly important protein profile. But, because of un-forecasted and limited supply of fish meal, researchers are focusing to utilize plants like soybean meal in place of fish meal in fish diet. In this project soybean meal based diets were supplemented with phytase (0 and 1000 FTU/kg) and Citric acid (0 and 2%) with the purpose to enhance availability of nutrients. After the completion of trial, *L. rohita* from each replicate were taken for mineral analysis. The data was statistically analyzed by the two way analysis of variance. Acidification and phytase supplementation increased ($p<0.05$) the whole body minerals including Ca, Mg, Cu, Zn, Mn, Fe, P, Na and K by 10, 31, 14, 9, 19, 20, 24, 28 and 14%, respectively in the *Labeo rohita* juveniles. Which increases its nutritional value of fish in terms of minerals. © 2017 Friends Science Publishers

Keywords: Pretreatment; *Labeo rohita*; Phytase; Citric acid; Soybean meal

Introduction

Aquaculture is fastest growing animal protein producing industry (FAO, 2000). Fish produce nearly 20% of total animal protein and is a source of essential fatty acids. Fish meal is the first choice for fish feed manufacturers due to its highly important protein profile and digestible value (Naylor *et al*., 2000). However, because of un-forecasted and limited supply of fish meal, researchers are focusing to utilize plants proteins in place of fish meal in fish diet (Lunger *et al*., 2007), with growing aquaculture. There are a number of plant protein sources but soybean meal in fish feed industries is used because it has high protein value and essential amino acids (Cheng and Hardy, 2002). However, use of soybean meal in the fish feed is usually restricted due to the anti-nutritional factors like phytate (Imorou *et al*., 2008). Phytate is non digestible form of phosphorous which is usually present in the oil containing seeds (Mullaney *et al*., 2000).

Phytate is considered as an anti-nutritional factors. Due to its chelating ability, it binds essential minerals like Ca, Fe, Cu, Mg and Zn (Satoh *et al.*, 1991) and reduces their availability to fish. It is reactive with various proteins, so decreases the availability of biological proteins and important mineral (Nagashima *et al.*, 1999). It also inhibits the activity of numerous enzymes, as *a*-amylase (Ravindran *et al.*, 1995). Phytase (PHY) named as *myo*-inositol hexaphosphate phosphohydrolase is classified hydrolases class of enzymes, which is produced by bacteria, some plants or plant based ingredients. The unit for Phytase activity is FTU, which is defined as the amount of enzyme that releases 1micromole of inorganic phosphorus in one minute (Cao *et al*., 2007). Fishes being mono-gastric or agastric aquatic animals have limited or unable to produce PHY, so they do not have an ability to hydrolyze the phytates (Higgs *et al.*, 1996). However, adding PHY in aquaculture feed has largely been studied for better biological availability of nutrients from soybean (Cao *et al*., 2007).

The optimum microbial PHY activity takes place at the pH 2.0 (Simons *et al.*, 1990). Phytase activity remain different at different sites of the digestive tract and most prominently remains in the stomach (Yi and Kornegay, 1996). Another approach in fish nutrition is the use of organic acids (citric acid, lactic acid, fumaric acid and propionic acid) in the fish diet. Among organic acids, citric acid significantly increased the phytate dephosphorylation process (Zyla *et al*., 1995; Baruah *et al.*, 2004). The organic acids not only enhance the solubility of phosphorus (P) from phytate but also improve absorption of P by reducing the intestinal pH (Jongbloed, 1987).

Citric acid act as chelating agents by binding various nutrients along the intestine of fish (Ravindran and Kornegay, 1993), which increases the absorption of minerals (Sugiura *et al*., 1998; Vielma *et al*., 1999). Acid production in the carnivorous fish like rainbow trout, helps in utilization of mineral by lowering the pH of the intestinal mucosa. The supplementation of 3% CA to a dietary PHY significantly increased the utilization of phosphorus in the juveniles of *Labeo rohita* (Baruah *et al.*, 2005).

The 5% citric acid and PHY in the feed of rainbow trout, *Oncorhynchus mykiss* also significantly increase the apparent absorption of Mg and P (Sugiura *et al*., 2001). The acidification of diet may lower the gastrointestinal evacuation rate (Mayer, 1994), which also enhances the activity of PHY. Thus, the addition of organic acids and PHY in the diet can synergistically effect the P availability.

In a trial by Hussain *et al*. (2011) reported a significant increase in the mineral digestibility of *Labeo rohita* fed PHY supplemented corn gluten meal (30%). Yildrim and Turan (2010) also stated that PHY supplementation enhanced the growth performance in African catfish *Clarias gariepinus*. Significant effects of different levels of citric acid were reported on the calcium (Ca) and phosphorus (P) content of muscle, scute and serum of Beluga (*Huso huso*) juveniles by Khajepour and Hosseini (2010). Citric acid increased weight gain and improved growth parameters in Beluga fry significantly (Sudagar *et al.*, 2010). Phromkunthong *et al.* (2010) also observed an increased P utilization in common carp (*Cyprinus carpio)* fingerlings using combined supplementation of PHY and CA. This Combination also has positive effects on growth, muscle composition and hematocrite (Hct) of common carp (Khajepour and Hosseini, 2012). Hence, the purpose of present study was the evaluation of the whole body proximate and mineral status of *L. rohita* juveniles fed an acidified PHY pre-treated soybean meal based diet.

Materials and Methods

Fish and Experimental Conditions

Labeo rohita juveniles were acclimatized to experimental conditions for fourteen days in V-shaped tanks (UA system). Fish were treated with 5 g L^{-1} NaCl to make the juveniles free from parasites and fungal infections. Water quality particularly pH and temperature were monitored by thermometer and pH meter (Jenway model 3510), respectively. Dissolved oxygen was measured by D.O. meter (Jenway model 970 Camlab UK) throughout the trial.

Feed Ingredients and Experimental Diets

Four diets were formulated in which 65% soybean meal, 14% wheat flour, 10% rice polish, 5%, fish meal, 3% soybean oil, 1% vitamin premix and 1% mineral mixture were included. The diets consisted of un-incubated soybean meal with no supplementation of phytase and CA, control treatment (T1); un-incubated soybean meal with the supplementation of 2% CA (T2); incubated soybean meal with supplementation of 1000/kg phytase (T3) and incubated soybean meal with both supplementation of 1000/kg phytase and 2% CA (T4).

The process of pre-treatment of the diets was as follows: 1 kg of ingredients were mixed with distilled water (1.5 kg) to make it creamy. This was incubated at 40°C for 16 h and after that was dried by oven at 60°C for 13 h. After drying, the blended dough was again powdered prior to pelleting (Nwanna *et al.*, 2008). Citric acid was added at the levels of 0% and 2% in each PHY supplemented diet resulting in the formulation of four diets. They were further processed through hand machine for making floating pellets. The pelleted diets were stored in the cool environment throughout the experiment. The gross design of experimental diets is available in Table 1.

The juveniles of rohu (*L. rohita)* were fed on experimental diets daily. For each test diets, three replicates were used according to the stocking density of ten fishes per tank. During the experimental period juveniles were fed daily to seeming satiation on the basal diet (Allan and Rowland, 1992). After 90 days of the trial, five fishes from each replicate were taken, killed and dried in an oven and grinded to make fish's carcass for whole body proxate composition and mineral analysis.

Whole body Proximate Analysis

The diet and whole body fish samples for proximate composition were analyzed accessing the standard methods of AOAC (1995). Five fish from each replicate were taken after the experiment and processed for their proximate analysis. Dry matter contents (DM) was measured by oven-drying at 105^{o}C for 12 h. Crude protein (CP) by the formula: N × 6.25, whereas total N was estimated by Kjeldahl apparatus. Ether extract (EE) was calculated by using methods of Bligh and Dyer, 1959 via Soxtec HT2 1045 system. Ash was determined by ignition at 650^{o}C for 12 h in the plug-in furnace (Eyela-TMF 3100, China).

Determination of Minerals

For mineral analysis three replicates of the fish carcass (whole body) and diet samples were analyzed following AOAC (1995). Samples were digested in boiling nitric acid and perchloric acid mixture (2:1). After appropriate dilution Mg, Mn, Zn, Ca and Fe were estimatimated using Hitachi Atomic Absorption Spectrophotometer, Z-8200. P contents were analyzed calorimetrically (Hitachi U2001 UV/VIS spectrophotometer) at 750 nm. Analysis of Na and K was done by means of flame photometer (Jenway PFP-7, UK).

Statistical analysis

Finally, the data of this two factor factorial experiment with three replicates was subjected by two-way analysis of variance. The mean difference were compared by Least significant difference (LSD) at significant level of $P<0.05$ (Snedecor and Cochran, 1991).

Results

Effect of PHY and CA supplementation on whole body proximate composition are presented in Table 1. Effect of PHY and CA supplementation on minerals contents in whole body are presented in Table 2. Supplementation of

Table 1: Composition (%) of experimental diets

Ingredients	SBM1 (%)	SBM 2 (%)	SBM 3 (%)	SBM 4 (%)
Soybean meal	65	65	65	65
Wheat flour	14	12	14	12
Rice polish	10	10	10	10
Fish meal	5	5	5	5
Soybean oil	3	3	3	3
Vitamin premix	1	1	1	1
Mineral mixture	1	1	1	1
Ascorbic acid	1	1	1	1
Citric acid	0	2	0	2
Phytase	0 FTU	0 FTU	1000 FTU	1000 FTU
Total	100	100	100	100

Table 2: Effect of PHY and CA supplementation on whole body proximate composition in the whole body of *Labeo rohita* juveniles fed soybean meal based diet

Diet	CA level (%)	PHY (FTU/kg)	DM (g/kg)	CP (g/kg)	EE (g/kg)	Crude ash (%)
SBM1	0	0	277^{d}	136^{d}	51.333^{d}	3.876^{d}
SBM2	2	0	285.33^{c}	146.333^{c}	45.666^{c}	4.293^{c}
SBM3	0	1000	290.66^{b}	150.333^{b}	43.333^{b}	4.313^{b}
SBM4	2	1000	298.7^{a}	157.666^{a}	38^{a}	4.683^{a}

Means values within columns having different superscripts are significantly different at $p<0.05$; Data are means of three replicates

PSE=pooled SE= $\sqrt{MSE/n}$ (where MSE=mean-squared error)

CA increased the DM, CP and EE contents of the whole body of *L. rohita* juveniles by 3, 7 and 12%, respectively as compare to control diet. Phytase supplementation enhanced DM, CP and EE contents of the whole body of *L. rohita* juveniles by 5, 10 and 18%, respectively as compared to the control diet. Combined supplementation of CA and PHY increased DM, CP and EE contents of the whole body of *L. rohita* juveniles by 8, 16 and 35%, respectively.

Inclusion of CA in diet significantly ($p<0.05$) improved the mineral contents in the whole body of *L. rohita* juveniles including Ca, Mg, Cu, Zn, Mn, Fe, P, Na and K by 10, 32, 13, 9, 19, 20, 24, 28 and 14%, respectively as compared to the control diet (Table.3). The addition of PHY to diet significantly ($p<0.05$) enhanced the mineral contents of the whole body of *L. rohita* juveniles including Ca, Mg, Cu, Zn, Mn, Fe, P, Na and K by 12, 44, 11, 9, 24, 23, 26, 30 and 12%, respectively. Combined supplementation of PHY and CA also increased the mineral digestibility including Ca, Mg, Cu, Zn, Mn and Fe, P, Na and K by 23, 51, 27, 18, 47, 48, 50, 64 and 25%, respectively.

Discussion

In the current study, when *L. rohita* juveniles were fed with CA containing experimental diet, the DM contents were significantly increased ($p<0.05$). That may be due to citric acid, which weakens the phytate complexes, making the nutrients available to fish for ingestion (Jongbloed, 1987). It also lowers the gut pH which favors the digestive enzymes working hence increasing nutrients absorption. Similar to our study, improved DM content was also observed in yellowtail (Sarker *et al.,* 2012) and red sea bream (Hossain *et al*., 2007) in response to dietary acidification through different organic acids.

It has been reported that organic acid supplementation in the diet improves the protein absorption (Partenen and Mroz, 1999). In the present experiment, crud protein level was significantly ($p<0.05$) enhanced in the juveniles having CA acidified diet. However, CA supplementation did not result, in any remarkable improvement in the body crude protein in Common carp (Khajepour and Hosseini, 2012). Difference in the diet composition and formulation and fish species and fish rearing conditions might have contributed to these contradictory results. Sarker *et al.* (2012) described that CA may positively influence the proximate composition of moisture, lipid and ash in yellowtail positively.

Ether extract level, which shows the fat contents in the body of juveniles, in this study was decreased by CA supplementation as compare to the control group. Hossain *et al*. (2007) reported that there was no significant differences in ether extract contents with CA supplementation as compared to other supplementary organic acid in Red sea bream (*Pagrus major*).

Whole body mineralization was also found to be improved ($P<0.05$) in the fish fed CA containing diet as compare to control group. This indicates that CA might have solubilized the phytate-mineral complex present in plant feed ingredients resulting in enhanced body mineralization. Sarker *et al.* (2005) also reported higher carcass mineral content in Red sea bream having CA added plant protein based diet. Improved whole body Fe content were also reported by Vielma *et al*. (1999) in *Oncorhynchus mykiss* by the CA supplementation. Sugiura *et al*. (1998) confirmed that Citric acid is proficient in increasing ingestion of some minerals including P in fish meal.

Table 3: Effect of PHY and CA supplementation on minerals contents in whole body of *Labeo rohita* juveniles fed soybean meal based diet

Diet	CA level (%)	PHY (FTU/kg)	Concentration (mg/g dry weight)					Concentration (µg/g dry weight)			
			Ca	Mg	P	Na	K	Cu	Zn	Mn	Fe
SBM1	0	0	5.57^{d}	0.366^{d}	5.27^{d}	1.253^{d}	4.353^{d}	13.54^{d}	15.33^{d}	0.99^{d}	17.896^{d}
SBM2	2	0	6.133^{c}	0.483^{c}	6.553^{b}	1.61^{c}	4.97^{b}	15.343^{b}	16.696^{c}	1.183^{c}	20.363^{c}
SBM3	0	1000	6.223^{b}	0.526^{b}	6.66^{c}	1.636^{b}	4.896^{c}	15.09^{c}	16.773^{b}	1.22^{b}	20.536^{b}
SBM4	2	1000	6.87^{a}	0.653^{a}	7.913^{a}	2.06^{a}	5.463^{a}	17.31^{a}	18.033^{a}	1.463^{a}	22.976^{a}

Means values within columns having different superscripts are significantly different at p<0.05; Data are means of three replicates

PSE=pooled SE= $\sqrt{MSE/n}$ (where MSE=mean-squared error)

The reason may be that, the P, trace elements and Ca have antagonistic interaction. Which is reduced and these minerals are precipitated by citric acid at the duodenal edge (Sugiura *et al*., 1998). Sarker *et al*. (2005) reported complex carcass organic and inorganic nutrients from the diets supplemented with CA.

In the current study, PHY supplementation significantly (p<0.05) increased the DM content in body as compared to the control. Phytate-protein complex remained insoluble and therefore less digestible by proteolytic enzyme (Ravindran *et al*., 1995). Supplemental microbial PHY may hydrolyze this complex resulting in the release of bounded protein and improve its digestibility, as has been observed in Atlantic salmon having PHY treated diet (Denstadli *et al.*, 2007; Carter and Sajjadi, 2011). However, non-significant differences were reported among PHY treatments in Nile tilapia (Goda, 2007) and Red sea bream (Biswas *et al*., 2007).

Dietary PHY has been testified to increase muscle protein (Lall, 1979; Wee and Shu, 1989). In this study, improved CP level in the fish body was also observed in the group having PHY supplemented diet that can be attributed to improved protein accessibility from PHY complemented diet which might had rehabilitated into body protein. Similar observations were made by Debnath *et al*. (2005) who reported improved (p<0.05) crude protein content in *Pangasius pangasius*. Shapawi *et al*. (2013) also reported significantly improved whole body protein contents in tiger grouper (*Epinephelus fuscoguttatus*) by the supplementation of 2000 FTU/kg PHY. Contrary to our results, dietary PHY supplementation was not reported to cause any marked improvement in body CP content in rainbow trout (Vielma *et al*., 1999), Red sea bream (Biswas *et al*., 2007) and rohu (Xavier *et al*., 2012).

In the current study, when *L. rohita* juveniles were fed PHY supplemented diet, it significantly (p<0.05) decreased EE in body was detected as compared to the control diet. Which increased its market value, as the fish with less fat contents is tastiest and have high protein contents. In a recent study, Shapawi *et al*. (2013) reported significantly lowered lipid content in tiger grouper having PHY treated diet. Likewise, decreased EE was noticed in *P. pangasius* (Debnath *et al*., 2005) and common carp (Rocha *et al*., 2010) while feeding with PHY treated diets Similarly Pezzato *et al*. (2006) and Furuya *et al*. (2008) reported reduced fat contents in the carcass of Nile tilapia with PHY supplementation. Nwanna (2005) reported improvement of fat digestibility in Nile tilapia in 8000 FTU/kg of PHY supplementation.

In this experiment improved (p<0.05) whole body mineralization was observed in our experiment while feeding the fish with PHY supplemented diet. Phytase hydrolyzes the anti-nutritional phytate and enhances the Ca, Mg, Mn and Zn in the bone, plasma and whole body (Vielma *et al*., 1998). Storebakken *et al*. (1998) described that PHY supplementation significantly improved the body P, Ca and Mg contents in Atlantic salmon (*Salmo solar*). Moreover, Debnath *et al*. (2005) reported increased P, Ca, Zn, Cu, Fe and Co contents in the whole body of *P. pangasius* in response to PHY supplementation. Nwanna (2005) also reported that PHY supplementation increased the mineral deposition in African catfish.

It is important to note that PHY and CA showed no interaction for DM and CP contents in the whole body of juveniles in the present study. Similar to our results, Baruah *et al*. (2005) also reported that PHY and CA showed no interaction for DM and CP contents in *L. rohita* juveniles. In the present study, PHY and CA interaction remain insignificant in decreasing the fat contents. Contrary to our findings, Phromkunthong *et al*. (2010) reported significant interaction among these two supplements to decrease the fat content in the fish whole body. The reason may be that CA provided most favorable environment for PHY activity by dropping pH of the fish intestine (Baruah *et al*., 2007). In the present study, whole body mineralization was also not affected by the interaction of PHY and CA similar information was reported by Baruah *et al*. (2007). In contrast to our results, Phromkunthong *et al*. (2010) observed improved availability of P and enhanced bone mineralization in common carp. Likewise, Baruah *et al*. (2007) also reported Ca, K, P and Mn deposition in the body. Discrepancies in the results may be due to the difference in PHY and CA treatments and also in feed formulation.

Conclusion

Acidified and phytase pre-treated soybean meal based diet improved nutrients (DM, CP, EE) and mineral (Ca, Mg, Cu, Zn, Mn, Fe, P, Na and K) profile of rohu (*Labeo rohita*)

juveniles by increasing the digestibility and palatability of feed. Both citric acid and phytase significantly interacted for the improvement of fish quality by improving the nutrients and mineral contents.

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