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Effect of β-propeller Phytase from *Pichia pastoris* on Energy Partition in Juvenile *Litopenaeus vannamei* Fed a Plant Protein-Based Diet

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Abstract

The main objective of this study was to evaluate the effect of a new isolated exogenous β-propeller phytase (FTEII) obtained from *Pichia pastoris*, on growth, survival and energy partition of juveniles of *Litopenaeus vannamei* fed a plant protein diet. Two treatments were designed for the experiment: a plant protein-based diet without phytase (T1), and a diet comprising pretreated plant protein with β-propeller phytase (T2). The gowth rate monitored over 30 days significantly improved when phytase was added to the diet (T2) compared to control T1 ($p<0.05$), and survival rates were similar between treatments ($p>0.05$). Energy partitioning was affected by basal metabolism (HeE) which was similar in both dietary treatments ($p> 0.05$) but the heat increment of feeding (HiE) was higher with T1 than T2 ($p<0.05$), whereas retained energy (RE) increased in T2 compared to $_T$1 ($p<0.05$). In summary, exogenous phytase added to a plant protein-based diet decreased the negative effect of phytic acid, released phosphorus, and therefore improved weight gain.

Keywords: β-propeller phytase, plant protein, *L. vannamei*, energy partitioning

1. Introduction

Shrimp mineral nutrition is of concern for feed producers for several reasons. First, the mineral mix used in feed production can contains a phosphate form such as $CaHPO_4$ that has low phosphorus bioavailability due to the acid dissociation contrasting for basic/neutral pH in the digestive tract of the shrimp. Regarding fishmeal replacement, the use plant protein such as soybean meal (SBM) that contains phytate can generate lower growth for shrimp (Bulbul, Kader, Ambak, Hossain, & Ishikawa, 2015). There is also an environmental concern due the phosphorus released from intensive farming through the water effluent and can lead to the risk of eutrophication. Nutritional physiology can offer some solutions to overcome this challenge; and in this direction, the energy budget with an emphasis on heat increment of feeding (HiE) was reported for *Penaeus monodon* (Du Preez, Chen, & Hsieh, 1992), and an increase in HiE was observed when feeding *P. japonicus* juveniles with SBM as a replacement for squid meal (Koshio, Kanazawa, & Teshima, 1992). Therefore, HiE is very responsive to feed composition and affects energy partitioning (Jiménez-Yan, Cuzon, García, Taboada, Soto, Brito, & Gaxiola, 2006). Phosphorus requirements remain poorly understood despite previous research on the nutrient requirements of *L. vannamei* (Davis & Gatlin, 1996), and availability of data such as the hemolymph concentration at 107 mg L^{-1} or 3.44 mmol L^{-1} for *Litopeneus stylirostris* (Shimizu, Shike, Klimpel, & Burns, 2001). Moreover, it was reported that phosphorus deficiency caused "molt death syndrome" or some peak of mortality at molt in *L. stylirostris* juveniles raised in floating cages where shrimp retrieved Ca^{2+} but not Phosphorus at a concentration of 0.04 mg L^{-1} (Aquacop, 1983; Aquacop, 2013). These are some of the reasons why special attention was given to formulate feeds when phosphorus content would be in a bioavailable form in plant protein feedstuffs. However, some results reported indicate that *Marsupenaeus japonicus* juveniles could obtain phosphorus from dietary sodium phytate, probably due to the presence of alkaline phosphatase active in its hepatopancreas (Cheng, 1985). Thus far, no clear effect of exogenous phytase in the presence of phytate-containing ingredients has been shown in *L. vannamei*.

Plant protein sources such as soy, wheat, canola, corn, and lupine have been considered as options to achieve a partial or total replacement for fishmeal (Tacon & Foster, 2000; Hardy, 2008). Our research group developed a program of experiments on shrimp nutrition that successfully tested plant meal such as plant protein concentrates as fishmeal substitutes (Maldonado, Cuzon, Guzmán, Brito, Soto, Arena, & Gaxiola, 2009; Suárez, Gaxiola, Mendoza, Cadavid, Garcia, Alanis, Suárez, Faillace, & Cuzon, 2009; Maldonado, Guillen, Pantoja, Arena, Ezquerra-Bauer, Alvarez-González, Cuzon, & Gaxiola**,** 2012; Suarez, Gaxiola, Posso, Garcia, Alanis, Suárez, Faillace, & Cuzon, 2012). However, plant protein sources have limitations related to decreased feed intake due to lack of appetability for shrimp, and by the presence of phytate, an antinutritional factor (AF), which limits bioavailability of phosphorus and other minerals (Lee and Meyers, 1996; Mendoza, Aguilera, & Montemayor, 2000). Phytateor inositol hexaphosphate (*myo*-inositol-1,2,3,4,5,6-hexaphosphate) is classified as a poly-anionic molecule that may chelate strongly with cations such as $Ca^{++,}$ Mg^{++}, Zn^{++}, Cu^{++}, Fe^{+++}, and K^{+}, forming insoluble salts (Kumar, Sinha Makkar, Boech, & Becker, 2011) and reducing absorption of these minerals (Papatryphon, Howell, & Soares, 1999). P excretion is another consequence of the low digestibility of plant ingredients high in phytate and other AF for aquatic organisms. Moreover, P accumulates in the water, causing pollution due to excessive algal bloom, thus killing aquatic organisms by decreasing oxygen availability (Baruah, Sahu, Pal, & Debnath, 2004).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8, EC 3.1.3.26, and EC 3.1.3.72) catalyze the release of phosphate from phytates present in plant protein sources making P and other minerals available (Lei, Porres, Mullaney, & Brinch-Pedersen*,* 2007). At present, phytases are grouped into four major classes: histidine acid, β-propeller, cysteine, and purple acid phytases. Most of the commercially available phytases are histidine acid phytases and possess catalytic activity in a pH range 2.5–6.0. The β-propeller phytase FTEII from *Bacillus* species is an alternative to histidine acid phytases because of its high thermostability, calcium phytate-complex substrate specificity, pH profile, and proteolysis resistance (Oh, Choi, Park, Kim, & Oh, 2004). Several β-propeller phytases, one from *B. subtilis* (Guerrero-Olazarán, Rodríguez-Blanco, Carreon-Treviño, Gallegos-López, Viader-Salvadó, 2010), and others designed by a structure-guided consensus approach, have been produced by the methylotrophic yeast *P. pastoris* (Viader-Salvadó, Gallegos-López, Carreón-Treviño, Castillo-Galván, Rojo-Domínguez, & Guerrero-Olazarán, 2010). Therefore, during the last two decades, optimizing plant-based diets using phytase as a feed additive has been a way to reduce phytates' antinutritional effect (Fox, Lawrence, Saccardi, Davis, Ricque-Marie, Cruz-Suarez, & Samocha 2006; Lei et al., 2007; Lim & Lee, 2009), thereby improving the absorption and retention of minerals and amino acids (Rebollar and Mateos, 1999; Gómez-Villalva, 2005), and enhancing the activity of proteolytic enzymes, survival rate, and weight gain (Ricque-Marie, Cruz-Suarez, Zavala-Chavez, Nieto-Lopez, Guajardo, Tapia-Salazar, McCallum, & Newkirk, 2004; Cao, Ye, Wang, & Guo, 2010; Wang, Yang, Han, Dong, Yang, & Zou, 2009; Gamboa, Aguilera, Gaxiola, Cuzon, Guerrero, & López, 2011). The digestive tract of shrimp has a pH range of 6–8, hence FTEII is a good alternative for either pretreatment with fresh water added to meals or direct inclusion in shrimp feed.

Gamboa-Alvarez (2011) produced a bioenergetic approach related to energy retention (RE) and muscle tissue build-up. Energy partitioning has been shown to increase RE from different metabolic processes, including those related to growth (Suarez et al., 2012; Maldonado et al., 2012). Therefore, this tool allows researchers to predict the impact of feed on biomass and energy efficiency due to nutritional components administered at different stages of shrimp development (Morten & Olsen, 2006).

The principal aim of this study was to evaluate the effect of β-propeller phytase FTEII from *Pichia pastoris* on energy partitioning and weight gain of *L. vannamei* juveniles using FTEII pretreatment on SBM and canola meal included in a plant based-diet.

2. Material and Methods

2.1 Bioassay Facility and Diet Acclimation

Juvenile *L. vannamei* donated by Lamarca shrimp farm in Sisal (Yucatán, Mexico) had an initial average weight of 7.2 ± 0.6 g. The animals were transported to a bioassay room at Unidad Multidisciplinaria de Docencia e Investigación de Sisal (Yucatán, Mexico), and acclimated for 10 days before the trial that lasted for 30 days (when significant differences appeared in wet weight). The experimental design was completely randomized, with two treatments and six replicates. Experimental culture conditions were completely controlled. Twelve 200-L tanks were seeded at a density of 15 shrimp/tank (119 shrimp/m^2). The recirculating water system was closed; this water had previously passed through a deep sand filter bed, a physical filtration system for particle retention at 25, 10, and 5 μm, and a UV filter to remove bacteria. Once inside the wet lab, water circulated through a 20 μm mesh aperture filter cartridge, a skimmer, and a biofilter with zeolite (30% water exchanged every 24 h); the photoperiod was a 12:12 light/dark cycle; and environmental conditions were monitored twice daily (7:00 and 19:00 h) with an

oxymeter Hach©, model HQ40d, Loveland, Colorado, USA. Seawater parameters remained stable (salinity 36 ppt, 28 ± 1 °C and dissolved oxygen 6.50 ± 0.50 mg L^{-1}).

2.2 Diets Composition (Table 1) and Feeding Procedure

Two experimental diets (Table 1) were prepared according to the protocol established for shrimp diets in Nutrition Lab UAS (Sisal). Canola meal and SBM used for the T2 diet were pre-treated with 1.6 U FTEII g^{-1} (1080 U mL^{-1} activity at pH 7.5 and 37 °C) produced at the Institute of Biotechnology, College of Life Sciences, UANL (Viader-Salvado et al., 2010). Both samples were incubated for 16 h at 40 °C and dried for 12 h at 60 °C (Nwana et al., 2008). Analytical data and calculated values are displayed in Table 2.

Shrimp were fed three times daily (07:00, 13:00 and 19:00 h) and the ration calculated from 3% biomass adjusted to take into account uneaten collets. Results were then recorded for weight gain (g), with SGRas100*(ln average FBW-ln IBW/number of days), and percentage survival rate calculated as (final number/initial number) × 100.

Table 1. Basal diet composition (g kg^{-1}). Diet T1: plant protein without exogenous phytase (control) and diet; T2: plant protein pre-treated with FTEII before wet extrusion and analysis values in %

	T1	T2
Menhaden fishmeal	50	50
SPC[1]	270	270
*soybean meal	220	220*
*canola meal	220	220*
L-LYS	9	9
DL-MET	7	7
fish oil[3]	80	80
cholesterol	1	1
wheat starch	93	93
vitamin and mineral mix[2]	25	25
CMC[5]	10	10
zeolite 506[4]	15	15
	1000	***1000***

*pre-treated by FTEII (1080 U mL^{-1}) 1.6 U g^{-11}; [1]Profine© 70% CP; [2]Rovimix©; [3]Cedrosa© cod liver oil. [4]diatomaceous earth (Celite Corporation);[5] carboxymethylcellulose;[6] 100 − (CP + CF + EE + water).

Table 2. Analytical data on diet and calculated values*

	T1	T2
moisture %	5	5
crude protein % DM	36	36
ether extract % DM	8	8
NFE[6]	38	38
cellulose (CF) % DM	6	6
ash % DM	7	7
*PA/PV	9:91	9:91
*P total %	0.6	0.6
*P available %	0.3	0.3
*phytate %	0.3	0.3
after phytase action %	*0.3*	*<0.3*
kJ g^{-1}	16.7	16.7

CF: crude fiber; PA: animal protein; PV: plant protein.

2.3 Indirect Calorimetry and Energy Partition Model

Oxygen consumption followed a methodology previously described by Rosas, Martínez, Gaxiola, Brito, Sánchez, and Soto (1999), using a ten-channel oximeter (Oxy-10 mini, PreSens©, Regensburg, Germany) set for energy partitioning including RE and energy lost in exuviae. An open-system respirometer with seven 500 mL chambers was used; each chamber received one shrimp, plus an empty control chamber. Acclimatization lasted 18 h prior to

measurements in order to reduce handling stress and leave animals in maintenance (HeE). After this time, measurements started from a baseline of 'zero hour'. Subsequently, shrimp received a diet corresponding to one treatment and oxygen levels were recorded at hourly intervals. The temperature range between water inlet and outlet chambers with a 10 mL min^{-1} water flow was 28 ± 1 °C.

Oxygen consumption was calculated as VO_2 = {([O_2] input − [O_2] out) × F} − {([O_2] control input − [O_2] control output) × F}, where VO_2 = oxygen consumption expressed as mg O_2/animal; [O_2] input = concentration in mg $L^{-1}O_2$ in water entering chamber; [O_2] output = concentration in mg $L^{-1}O_2$ in water exiting chamber; and F = flow rate through the chamber in L min^{-1}. Indirect calorimetry was used to measure energy channeled into tissue (g dry weight J^{-1} $sample^{-1}$) given by RE (kJ $animal^{-1}$ day^1). Each chamber received six animals from each treatment, sampled and dried at 60 °C for 24 h, and powdered to prepare pills (0.30 ± 0.05 g). A bomb calorimeter Parr© Model 1341, Illinois, USA, with a steel bucket containing 2 L distilled water at 2 °C lower than room temperature, was used to give a direct measure of energy content: Hg = [(δT x W) − (e_1 + e_2) N}] M, with Hg = sample gross energy (GE); T = water temperature; W = constant to standardize; e_1 = sodium hydroxide to titrate; e_2 = difference between initial and burned wire; N = standard wire calories; and M = pellet weight. Excretion was calculated as (UE + ZE) = RE + HeE + HiE) × 0.08, where energy channeled into growth was derived from conversion of daily growth rate using carcass caloric content; energy lost during molting (H × E) ~1.4 kJ or 25% of the intermolt energy gain (Read and Caulton, 1980); and ZE = energy loss through gills, and UE = energy loss through urine. Predicted surface loss as exuviae was calculated as SE = 0.03 × (RE + HeE + HxE + HiE) with 0.03 as a constant (Bureau et al., 2000).

Based on the results obtained with indirect calorimetry, HiE in mg O_2^{-1} consumed is calculated from the maximum value of O_2 consumed after feeding minus the fasting value; both values were transformed to kJ $animal^{-1}$ day^{-1} with 14.3 J mg^{-1} O_2^{-1} (Lucas, 1996). DE = RE + HeE + HxE + HiE + (UE + ZE) + SE, where DE = digestible energy, RE = retained energy, HeE = maintenance, H × E = energy lost at molt, HiE = heat increment of feeding, (UE + ZE) = excretion, SE = surface loss, exuvia day^{-1} $animal^{-1}$ for 24 h, and HiE times three for three meals a day.

2.4 Statistical Analysis

Water quality, energy partition parameters, survival (% previously transformed to arcsine) and weight gain were analyzed using the t-student test after checking the homogeneity of variances and normality with the bioinformatics package Statistical Analysis Software Program (SAS, 2006). In each case, the t-student test was run at a of significance level of 0.05.

3. Results

Water parameters in the acclimation period were not significantly different between dietary treatments ($p>0.05$).

The characteristics of the diet were such that the ratio PA/PV (animal protein to plant protein) was quite low at 9/91, indicating a strong bias towards plant protein sources. Nitrogen-free extract (NFE) was at a level similar to protein content (Table 2) with crude fiber maintained at a level slightly higher than that in previous fishmeal-based diets. Energy content calculated from coefficients previously established for *L. vannamei* (Cousin, Cuzon, Blanchet, & Ruelle, 1993) led to 16.7 kJ g^{-1}, therefore allowing the same feed intake for shrimp. Phosphorus content was expressed as available phosphorus and total phosphorus from (AEC Table-Commentry, 1978).

Table 3. Energy partitioning (kJ $animal^{-1}$ day^{-1}) with maintenance (HeE), increment of feeding (HiE), no fecal nitrogen excretion (UE + ZE), predicted surface loss exuvia (SE), energy lost during molting (H × E) retained energy (RE), and digestible energy (DE)

	T1	T2
DE	4.0 ± 0.3[a]	4.7 ± 0.3[a]
(UE + ZE)	0.30 ± 0.04[a]	0.32 ± 0.04[a]
HiE	**0.40 ± 0.10[a]**	**0.20 ± 0.03[b]**
HeE	2.30 ± 0.40[a]	2.70 ± 0.50[a]
SE	0.10 ± 0.02[a]	0.10 ± 0.03[a]
HxE	0.20 ± 0.01[b]	0.30 ± 0.03[a]
RE	0.70 ± 0.03[a]	1.10 ± 0.10[b]

[a,b]indicate significant differences between treatments.

Table 4. *Litopenaeus vannamei* from T1 and T2 treatments after 30 days. Mean ± SE of survival rate, IBW, FBW and weight gain

Parameters	T1	T2
survival %	77 ± 14^{a}	90 ± 11^{a}
IBW g	7.2 ± 0.6^{a}	7.2 ± 0.6^{a}
FBW g	8.9 ± 1.0^{a}	9.8 ± 1.0^{b}
weight gain (g week^{-1})	$\mathbf{0.4 \pm 0.2^{a}}$	$\mathbf{0.7 \pm 0.2^{b}}$

[a,b]indicate significant differences between treatments.

Energy partition did not show significant differences for DE or HeE ($p>0.05$), however HiE values differed significantly ($p<0.05$) with the highest level seen with the control diet. Similarly, RE values differed between treatments ($p<0.05$) and T2 produced the highest value ($p<0.05$) (Table 3). When applied to survival rate ($p>0.05$) and weight gain (g week^{-1}), the t-student test showed a significant difference, with the highest value obtained when phytase was present in the diet ($p<0.05$) (Table 4).

4. Discussion

The positive dietary effects of β-propeller phytase were shown when pretreated soybean and canola meals were present in the formulation, in a diet based primarily on carbohydrate. A variety of plant sources are fed to penaeid species with phytase included in the range of 600−3000 FTU (phytase units) g^{-1} to limit fishmeal content (Fox, Lawrence, Saccardi, Davis, Ricque-Marie, Cruz-Suarez, & Samocha 2006; Suarez et al., 2009; Gamboa-Degado et al., 2013). In this study, plant ingredients with phytase helped maintain a weight gain of 0.7 g week^{-1} at high stocking density in clear water. Cheng, Chiu, Guu, Tsai, & Liu (2013) reported a weight gain of 0.2 g week^{-1} with a mixed diet containing some marine protein plus a bacterial phytase fed to *L. vannamei* early juveniles. Biswas, Pal, Sahu, Reddy, Prusty, and Misra (2007) reported survival rates below 80% in *Penaeus monodon* fed SBM but with an emphasis on culture method rather than phytase effect. Civera and Guillaume (1989) reported no adverse effects of phytic acid on *Marsupenaeus japonicus* and *L. vannamei* juveniles, using sodium phytate as a P source, contrasting with the results of the present study which found that *L. vannamei* juveniles fed on T2 had a 90% survival rate, phytate was poorly digested, and weight gain was reduced in the absence of phytase. Davis, Lawrence, and Gatlin (1992) reported increased growth and survival with *L. vannamei* fed on a diet including exogenous phytase. Similar results were reported in *P. monodon* with diets including lysine and/or phytase, with reduced Zn^{++} and PO_4 levels. (Biswas et al., 2007). Histology of the hepatopancreas of *L. vannamei* showed an increase in B-cells' size but the digestive tract still hydrolyzed phytate from plant diets (Afinah, Yazid, & Shuhaimi, 2010; Araujo & Lawrence, 1993) and *P. monodon* (Kumaraguru, Ramesh, & Balasubramanian, 2005). There may be a synergistic effect between lysine and phytase because in the present study 0.9% lysine levels that were similar to previous one would contribute to its positive effect (Biswas, Pal, Sahu, Reddy, Prusty, & Misra, 2007).

Ricque-Marie, Cruz-Suarez, Zavala-Chavez, Nieto-Lopez, Guajardo, Tapia-Salazar, McCallum, and Newkirk, (2004) increased protein and phosphorus digestibility with pea flour (0.3 ppm phytate) plus phytase, taking into account endogenous phytase. Pre-treated SBM with a recombinant phytase C (1000 and 2000 FTU kg^{-1}) can improve growth in *L. vannamei* juveniles (Chen, Pan, Bi, & Zheng, 2005).

When HiE was compared with T2, in the absence of exogenous phytase and with 3.2 mg kg^{-1} phytate, T1 could result in a greater energy demand to metabolize nutrients, but less energy was required in the presence of FTEII active at alkaline pH, which is important as the shrimp digestive tract possesses a slightly basic pH (Ceccaldi, 1990). Phytase action in T2 treatment contributed to the absorption and retention of energy, as T1 was lower in RE. It correlated with observed values for weight gain (Table 4). Therefore, an application of phytase either directly or after pre-treatment of ingredients (Cheng, Chiu, Guu, Tsai, & Liu, 2013) may be an alternative to improve utilization of phytate; it will decrease inorganic P in diet, increase shrimp growth, and reduce possible eutrophication in the environment (Lei and Stahl, 2001). This study showed that phytase was active to a certain extent on metabolism; 80% survival an acceptable range noticeable with a minimum of fishmeal content in diet (5%). Weight gain improved and HiE was lower when phytase was present as was reported for *Penaeus monodon* (Du Preez, Chen, & Hsieh (1992); also *M. japonicus* fed diet in which the SBM replaced squid (Koshio, Kanazawa, & Teshima, 1992. Overall, exogenous phytase will limit the negative effect of phytate present in plant sources and causes the release of bioavailable phosphorus so could replace fishmeal in feed for shrimp, limit eutrophication in ponds, and thereby contribute to sustainable aquaculture.

5. Conclusion

Juvenile *L. vannamei* fed a diet with a mix of plant protein sources (soy protein concentrate, SBM, and canola meal) achieved the greatest weight gain in the presence of FTEII and exhibited a reduction in HiE.

There are several phytases already on the market, and FTEII was active on SBM-based diet by modifying HiE and therefore improving energy partitioning with a higher-end RE resulting in a greater weight gain. In a context of reduction of fishmeal replaced by plant ingredients the role of exogenous enzymes in the group of feed additives will be recommended on a formulation basis and with a great concern about environment protection.

Future studies should focus on elucidating the mechanisms concerning phosphatemia (level of phosphorus in hemolynph), which could be a potential indicator of the assimilation of dietary phosphrus, although there are difficulties in collecting hemolymph owing to the small size of shrimp and the rapid clotting of the sample (Shimizu et al., 2001).

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