Evaluation of Enzymes Supplementation in Diets for Pacific White Shrimp (*Litopenaeus vannamei*)

by

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ABSTRACT

Fish meal is preferred among other protein sources because it is an excellent source of essential nutrients such as protein and indispensable amino acids, essential fatty acids, cholesterol, vitamins, minerals attractants and unidentified growth factors. The cost of fish meal has increased over time because of increased demand, limitations of availability, and growing social and environmental concerns regarding wild fish extraction practices. To develop sustainable and environmentally friendly aquaculture, various plant-based ingredients that contain high protein content are potential alternative sources for fish meal. Among plant protein sources, soybean meal is the most utilized and has received considerable attention as a replacement for fish meal in aquatic animal feeds because of its availability, low price, reasonably balanced amino acid profile and consistent composition. However, the presence of anti-nutritional factors such as phytate and non-starch polysaccharides (NSPs) limited the supplementation level of soybean meal in some aquaculture feeds. In order to deal with the negative effects caused by phytate and NSPs, exogenous phytase and carbohydrase are utilized as feed additives in soybean meal based diet. The second chapter in this research was to evaluate the growth response and apparent digestibility coefficients to practical diets containing grade level of phytase. Results of this study revealed that 2000 IU/kg feed phytase was the recommended addition level in terms of improving phosphorus (P) and protein availability. Growth performance was not affected by phytase supplementation, which indicated that the possibly improved P and protein availability did not pose a significant effect on growth response.

Copper content in the whole shrimp body was significantly increased in the treatment supplemented with 1000 IU/kg feed phytase, which indicates that the addition of phytase to a soybean meal based diet might increase bioavailability of copper, thereby increasing whole body copper deposition. The third chapter of this study investigated the growth performance and apparent digestibility coefficients of Pacific white shrimp responded to carbohydrase incorporation in the soybean meal based diets. Results showed that 0.02% carbohydrase inclusion significantly improved protein digestibility and numerically (P-value=0.0863) increased energy digestibility for more than 3%. However, growth performance was not affected by carbohydrase supplementation, which indicated growth performance responded to the possibly enhanced protein availability was not significant.

Overall, results from these studies reveal that the use of feed additives phytase and carbohydrase should be encouraged in shrimp feed formulation. However, more studies will be needed to further determine the best inclusion level of carbohydrase in terms of protein and energy digestibility and investigate the combined effects of organic acids (as acidifier) and phytase or carbohydrase on the performance of Pacific white shrimp.

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CHAPTER I

INTRODUCTION

Pacific white shrimp *Litopenaeus vannamei*, is native to the Eastern Pacific coast from Gulf of California, Mexico to Tumbes, North of Peru (PeÂrez Farfante and Kensley, 1997). It is one of the most important penaeid shrimp species cultured worldwide and preferred by the U.S customers. The production of Pacific white shrimp increased from 0.15 million tons in 2000 to 3.3 million tons in 2013 (FAO, 2013), and is estimated to increase to 9.2 million tons in 2020 (Tacon and Metian, 2008). Moreover, pacific white shrimp (*Litopenaeus vannamei*) was ranked the first in 2010 in terms of total farmed fish and crustacean production by value, at US $11.23 billion (FAO, 2012).

Feed represents one of the largest variable costs associated with fed culture systems, reducing feed costs can produce considerable savings (Davis and Sookying, 2009). Manufacturing of marine shrimp feed uses 24% to 27% of the world's fish meal (Tacon and Metian, 2008) making them one of the prime consumers. Commercial shrimp feed formulations historically contain from 25% to 50% fish meal, which represents the primary and most expensive protein ingredient (Dersjant-Li, 2002; Tacon and Metian, 2008). Fish meal is preferred among other protein sources because it is an excellent source of essential nutrients such as protein and indispensable amino acids, essential fatty acids, cholesterol, vitamins, minerals attractants and unidentified growth factors (Samocha et al., 2004; Swick et al., 1995). The cost of fish meal has increased over time because of increased demand, limitations of availability, and growing social and environmental concerns regarding wild fish extraction practices (Tacon and Metian, 2008). Due to limited supply and increasing prices, we must shift our emphasis and use

this ingredient only when nutrient requirements of the animal demand its use (Davis and Sookying, 2009).

To develop sustainable and environmentally friendly aquaculture, various plant based ingredients that contain high protein content are potential alternative sources for fish meal (NRC, 2011). Plant based protein sources such as soybean meal, canola meal, corn gluten meal are available worldwide and have a relatively low cost compared to fish meal. Among plant protein sources, soybean meal is the most abundant and has received considerable attention as a replacement for fish meal in aquatic animal feeds because of its availability, low price, balanced amino acid profile and consistent composition (Akiyama, 1989; Akiyama et al., 1991; Amaya et al., 2007; Hardy, 1999; Samocha et al., 2004; Swick et al., 1995; Tacon, 2000). The presence of anti-nutritional factors (ANF) such as phytate and non-starch polysaccharides (NSP), limited the inclusion levels of soybean meal in some aquaculture feeds.

Approximately two-thirds of total phosphorus in plant ingredients is in the form of phytate and consequently has a low variability for monogastric or agastric aquatic animals including shrimps because they lack intestinal phytase for efficient phytate hydrolysis during digestion (Jackson et al., 1996; NRC, 2011). Phosphorus (P) is an important constituent of nucleic acids and cell membranes, a major constituent of the structural components of skeletal tissues, and is directly involved in all energy-producing cellular reactions (NRC, 2011). Dietary deficiency of P impairs intermediary metabolism, which results in reduced growth and feed conversion. Various skeletal malformations associated with reduced mineralization of hard tissues also occur during suboptimal P intake. P is generally observed at low concentrations in natural waters (Boyd, 1979). Therefore, it is unlikely for shrimps to absorb P from natural water, which makes dietary P supplementation necessary. P from shrimp culture effluent has been

identified as one of the major nutrients that contributes to environmental pollution (Pan et al., 2005). P in the solid wastes (faecal material and wasted feed) settling to the sediment can have an impact on the benthic ecosystem of inland and marine waters (Cho and Bureau, 2001). Excessive excretion of P into water can stimulate the growth of algae and phytoplankton, therefore decreasing dissolved oxygen and resulting in water pollution (Boyd, 1990).

Phytate can bind with protein to form insoluble complexes, thus decreasing protein digestibility and utilization (Liu et al., 1998). Moreover, in vitro studies have shown that phytate-protein complex was poorly digested by proteolytic enzymes (Ravindran et al., 1995). Even some enzymes such as pepsin, amylopsin, and amylase may be inhibited by phytate (Cao et al., 2007). Phytate also chelates other minerals such as calcium, magnesium, zinc, iron, and copper to form insoluble complexes to reduce the absorption and bioavailability of these minerals (Papatryphon et al., 1999).

Previous researchers indicated that phytate in plant feed ingredients may be reduced by heat treatment (Eales, 1998). Currently, feed additive phytase has been developed to help counteract the negative impacts of phytate. Phytases, a group of enzymes known as *myo*-inositol-hexaphophate phosphohydrolase, are ideal approaches to degrade phytate to sequentially produce myoinositol penta-, tetra-, tri-, di-, and mono-phosphates, and neutralizes the negative effects of phytate on protein and other nutrients in the diet of monogastric animals (Mitchell et al., 1997). It is well documented that the application of microbial phytase into fish and shrimp feed can improve the phytate-bound phosphorus and nitrogen bio-availability, therefore reducing both P and N discharges into the aquatic environment (Cao et al., 2007; Debnath et al., 2005). Consequently, phytase is increasingly considered as a cost-effective and environmental friendly additive in fish feed formulations. Additionally, it was also reported that the growth and feed

conversion ratio were improved in tilapia (Liebert and Portz, 2005), yellow catfish (Zhu et al., 2014), striped bass (Papatryphon et al., 1999), and Atlantic salmon (Sajjadi and Carter, 2004) when fed with diets supplemented with exogenous microbial phytase.

NSPs are important constituents of a wide variety of grain legumes and cereals (Saini, 1989). In fish, their negative influences may be either because of binding to bile acids or obstructing action against digestive enzyme activity and movement of substrates in their intestine (Storebakken et al., 1998). NSPs such as arabinan, arabinogalactan, and acid polysaccharides, which account for approximately 14 to 18% of the total carbohydrate content of defatted soybean meal, might also bind minerals in the intestine and reduce the digestibility of fat (Krogdahl et al., 2005). However, the presence of digestive enzymes that specifically hydrolyze the β-glycosidic bonds of NSPs seems to be very low or nonexistent (Krogdahl et al., 2005).

Two dominant carbohydrases, xylanase and glucanase, account for more than 80% of the global carbohydrases market. Carbohydrase application in aquaculture nutrition is still relatively new compared to poultry and swine industry. Exogenous carbohydrases have been reported to facilitate a reduction in the degree of polymerization of feed, decreasing its viscosity and liberating carbohydrate oligomers, thus enhancing nutrient utilization (Vahjen et al., 2007). Digestibility of energy yielding nutrients, such as starch and fat, were increased by carbohydrase supplementation since NSPs impair the ability for nutrient absorption by decreasing enzyme accessibility to substrates (Adeola and Bedford, 2004). Moreover, it is possible that carbohydrases can improve nitrogen and amino acid utilization by increasing the access to protein for digestive proteases (Tahir et al., 2008). In addition, energy utilization may be improved by carbohydrase supplementation by shifting absorption of energy-yielding nutrients to the proximal intestine (Castillo and Gatlin, 2015).

The long-term goal of this study is to enhance a sustainable and environmentally friendly feed formulation based on soybean meal for pacific white shrimp *L. vannamei*. Two specific objectives are summarized as follows:

1. Investigate the effects of phytase supplementation in soybean meal based diets on the growth performance, body mineral contents, apparent digestibility coefficients of dry matter, protein, and phosphorus in Pacific white shrimp *L. vannamei*.

2. Determine growth performance and apparent digestibility coefficients of dry matter, protein, and energy in Pacific white shrimp juvenile *L. vannamei* when fed diets contained carbohydrase.

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CHAPTER II

EFFECTS OF DIETARY PHYTASE SUPPLEMENTATION ON GROWTH PERFORMANCE AND APPARENT DIGESTIBILITY COEFFICIENTS OF PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

Abstract

A growth trial and digestibility trial was conducted to evaluate the efficacy of phytase supplemented to practical shrimp feeds. The five weeks growth trial evaluated the effects of phytase supplementation in replete phosphorus (P) diets on the performances and compositions of juvenile Pacific white shrimp*, Litopenaeus vannamei.* The digestibility trial was conducted to study the combined effects of phytase supplementation levels and diet types on apparent digestibility coefficients of pacific white shrimp, *Litopenaeus vannamei*. No significant differences were observed in final biomass, final mean weight, weight gain, feed conversion ratio (FCR) and survival across all the treatments. Shrimp reared on the P deficient diet had significantly higher P retention and lower whole body P levels as compared to shrimp fed the other diets. Digestibility was evaluated in a plant-based diet and a fishmeal based diet. There we no effects of diet type so the data was combined. Phytase incorporation at both 500 and 2000 IU/kg improved protein digestibility whereas P digestibility was enhanced only at 2000 IU/kg phytase supplementation.

1. Introduction

Among all plant protein sources, soybean meal is the most abundant one and has received considerable attention to replace fish meal in aquatic animal feeds because of its worldwide availability, low price, balanced amino acid profile and consistent composition (Akiyama, 1989; Akiyama et al., 1991; Amaya et al., 2007; Colvin and Brand, 1977; Divakaran et al., 2000; Hardy, 1999; Lim and Dominy, 1990; Lim et al., 1998; Samocha et al., 2004; Swick et al., 1995; Tacon, 2000). The presence of anti-nutritional factors such as phytate limited the inclusion levels of soybean meal in some aquaculture feeds.

Phytate, as an anti-nutritional factor (ANF) in soybean meal, poses negative effects on mineral availability and nutrient digestibility such as protein and amino acids digestibility (Kumar et al., 2012). About 60% to 70% of total P in plant protein is in the form of phytate bound P which has a low biological availability in shrimp. Shrimps lack intestinal phytases which is an enzyme required to hydrolyze indigestible phytate allowing the release of P (Cao et al., 2007; Pointillart et al., 1987; Rodehutscord and Pfeffer, 1995a). P is an important constituent of nucleic acids and cell membranes, a major constituent of the structural components of skeletal tissues, and is directly involved in all energy-producing cellular reactions (NRC, 2011). Dietary deficiency of phosphorus impairs intermediary metabolism, which results in reduced growth and feed conversion. Various skeletal malformations associated with reduced mineralization of hard tissues also occur during suboptimal phosphorus intake. Dietary P requirement of *L. vannamei* is influenced both by P level and calcium (Ca) (Cheng et al., 2006; Davis et al., 1993a) as well as the presence of inhibitors such as phytate (Civera and Guillaume, 1989; Davis et al., 1993a). Pan et al. (2005) reported that total P level at 1.33% was required for optimal feed efficiency and weight gain of *L. vannamei*. Civera and Guillaume (1989) reported that due to the presence of

phytic P, the growth rate of *L. vannamei* was significantly reduced. Davis et al. (1993b) indicated that for a basal diet that contained 0.03% Ca, 0.34% P provided adequate phosphorus to *L. vannamei* to achieve normal growth. Optimal weight gain were observed with available P at 0.77%, but an increase to 1.22% available P was needed with 1% supplemental calcium (Pan et al., 2005).

Phytases, a group of enzymes known as *myo*-inositol-hexaphophate phosphohydrolase, are ideal approaches to degrade phytate to sequentially produce myoinositol penta-, tetra-, tri-, di-, and mono-phosphates, and neutralizes the negative effects of phytate on protein and other nutrients in the diet of monogastric animals (Mitchell et al., 1997). Phytases are able to save P resources by converting phytate bound P into available P, which will help to improve P bioavailability, reduce inorganic P supplementation, and thus prevent P pollution in water environment (Sugiura et al., 1999; Yoo et al., 2005a). Using exogenous phytase as a feed ingredient has been demonstrated to be a good approach to increase P digestibility in Korean rockfish (Yoo et al., 2005), Gibel carp (Liu et al., 2012), Tilapia (Liebert and Portz, 2005b), rainbow trout (Sugiura et al., 2001), and salmon (Cain and Garling, 1995). Phytase effectively increases P availability of soybean meal, but less information is available on protein and amino acid utilization (Cao et al., 2007). The results of phytase addition in fish feed are somewhat inconsistent. Positive impacts of phytase addition on protein digestibility were observed in rainbow trout (Vielma et al., 2004), and common carp (Bai et al., 2004).

Information on the use of phytase in Pacific white shrimp is limited. The objectives of this project are to determine the response of Pacific white shrimp juveniles to increasing phytase levels in a soybean meal based feed formulation with replete P and demonstrate apparent digestibility values for practical diets supplemented with moderate and high levels of phytase.

2. Materials and Methods

2.1 Experiment Diets

All test diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid) and were prepared in the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University (Auburn, AL, USA). For the growth trial, six experiment diets were formulated. The basal diet did not contain phosphourus or phyate supplements. Whereas the next five diets contained phytase at increasing levels (0, 500, 1000, 2000, 4000 U / Kg) and replete levels of P (Table 1). Thus evaluating the effects of phytase supplement independent of P requirements. In the digestibility trial, a plant based and fishmeal supplemented practical diets were formulated without a P supplement and 1% chromic oxide. Three levels of phytase (0, 500, 2000 U / Kg) were evaluated (Table 2).

Primary ingredients were analyzed for proximate composition and P levels in the diets formulated. Pre-ground dry ingredient, phytase and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then be blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air dried (<50 °C) to a moisture content of 8-10%. Pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed after drying. Phytase activity in each diet was confirmed (AB Vista Feed Ingredients).

2.2 Growth Trial

The Growth trial utilized 6 dietary treatments with 10 replicates in each treatment. It was conducted in a semi-closed recirculation system at the E.W Shell Fisheries Research Station

Table 1 Formulation and chemical composition of test diets used in the phytase growth trial

Ingredient (As is basis g kg^{-1} feed)	Diet code					
	D_p	D_0	D_{500}	D_{1000}	D_{2000}	D_{4000}
Menhaden fish meal[1]	60.0	60.0	60.0	60.0	60.0	60.0
Soybean meal[2]	495.0	495.0	495.0	495.0	495.0	495.0
Corn protein concentrate[3]	50.0	50.0	50.0	50.0	50.0	50.0
Whole wheat[4]	260.0	260.0	260.0	260.0	260.0	260.0
Menhaden Fish Oil[2]	56.7	56.7	56.7	56.7	56.7	56.7
Trace Mineral premix[5]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0	2.0
Stay C 250 mg/kg using 25%[7]	1.0	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	23.5	0.0	23.5	23.5	23.5	23.5
Lecithin[9]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5	0.5
Corn Starch[4]	18.30	41.80	18.23	18.16	18.02	17.75
Phytase[10]	0.00	0.00	0.07	0.14	0.28	0.55
Proximate analysis[11] (% as is)						
Moisture	8.77	8.63	7.45	8.42	7.07	5.77
Crude protein	35.70	35.39	35.71	35.30	36.03	36.40
Crude fiber	3.23	3.11	3.61	3.30	3.60	3.43
Crude fat	8.01	7.98	8.08	7.99	8.04	8.99
Ash	6.90	4.88	6.83	6.98	6.99	7.12

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, Ohio, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Quantum™ Blue 5 G, AB Enzymes, Darmstadt, Germany.
[11] Analyses conducted by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 2 Formulation of test diets used to determine the digestibility of various phytase supplements.

Ingredient (As is basis g kg^{-1} feed)	Diet code					
	FM_0	FM_{500}	FM_{2000}	SB_0	SB_{500}	SB_{2000}
Menhaden fish meal[1]	120.0	120.0	120.0	0.0	0.0	0.0
Soybean meal[2]	420.0	420.0	420.0	549.5	549.5	549.5
Corn protein concentrate[3]	37.5	37.5	37.5	62.0	62.0	62.0
Whole wheat[4]	303.0	303.0	303.0	280.0	280.0	280.0
Menhaden Fish Oil[2]	51.8	51.8	51.8	60.8	60.8	60.8
Trace Mineral premix[5]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0	2.0
Stay C 250 mg/kg using 25%[7]	1.0	1.0	1.0	1.0	1.0	1.0
CaP-diebasic[8]	0.0	0.0	0.0	0.0	0.0	0.0
Lecithin[9]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5	0.5
Corn Starch[4]	21.20	21.13	20.92	1.20	1.13	0.92
Phytase[10]	0.00	0.07	0.28	0.00	0.07	0.28
Chromium dioxide	10.00	10.00	10.00	10.00	10.00	10.00

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, Ohio, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Quantum™ Blue 5 G, AB Enzymes, Darmstadt, Germany.

(EWS), Auburn, AL, USA. Juvenile shrimp were obtained from the nursery system and selected by hand-sorting to a uniform size. Juvenile shrimp (initial weight 0.22±0.01g) were stocked into 60 tanks with 10 shrimps in each aquarium (75L). A sub-sample of shrimp from the initial stocking were retained for whole body samples to be utilized for later phosphorus and protein retention analysis. A fixed ration of 0.54g/day for the first week, 1.08g/day for the second week, 2.06g/day for the third and forth week, and 2.31g/day for the fifth week was offered over 4 feedings.

Dissolved oxygen (DO), temperature, salinity, and pH were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH). Water samples were taken to measure total ammonia-nitrogen (TAN) and nitrite every week. During the experiment period DO, temperature, salinity, pH, TAN, and nitrite were maintained within acceptable ranges for *L. vannamei* at 6.35±0.22mg/L, 29.6±0.83°C, 4.37±0.11ppt, 7.38±0.33, 0.13±0.12mg/L, and 0.14±0.11mg/L, respectively.

Shrimps were counted to readjust daily feed input on a weekly basis. At the conclusion of 5 weeks growth trial, shrimps were counted and group weighted. Mean final weight, FCR, WG, biomass, and survival were determined. After obtaining the final total weight of shrimps in each aquarium, 3 shrimps were randomly selected and frozen at -20 C for subsequent determination of whole body composition.

Moisture content of shrimp whole body was determined by drying to constant weight at 105°C. Prior to mineral analysis, dried whole shrimps were ground into powder and wet-ashed (Weight dry samples. Add 10 ml HNO_3 and boil gently for 30 to 45 minutes. Then, cool solution somewhat and add 3 ml (60%) $HCLO_4$ boil gently until solution is colorless. Finally, cool slightly, add water and HCL (1:1) to give a final concentration of 1:10). P concentration in initial

and final whole body samples and diets were analyzed using vanadomolybdophosphoric yellow color method. Copper was determined by atomic absorption spectrophotometry according to procedures described by Association of Official Analytical Chemists (AOAC). Crude protein in initial and final whole body samples were determined by the Kjeldhl method (Williams, 1984). Phosphorus and protein retention were calculated as follows:

Phosphorus retention (%)=(final weight×final phosphorus content)-(initial weight×initial phosphorus content)×100/phosphorus offered.

Protein retention (%)=(final weight×final protein content)-(initial weight×initial protein content)×100/protein offered.

2.3 Digestibility Trial

Digestibility trial that contained six treatments with six replicates in each treatments were conducted in a semi-closed recirculating system in EWS, Auburn, AL, USA. Feces from two groups of 10 shrimps will be pooled and collected for 5 days or until adequate samples were obtained. Prior to the beginning of the collection of feces, shrimps were allowed to acclimate to each diet for 3 days. The aquaria were cleaned by siphoning before each feeding. Then the shrimp were offered an excess of feed. Feces were collected by siphoning onto a 500μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight was obtained, and then separately stored in freezer (-20 °C) until analyzed. Apparent digestibility coefficient for dry matter, protein, and phosphorus were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. Chromium concentrations were determined by the method of McGinnis and Kasting (1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540nm.

Phosphorus and protein were determined as previously described. The apparent digestibility coefficient of dry matter (ADMD), phosphorus (ADPh), and protein (ADPr) were calculated according to Cho et al. (1982) as follows:

$$\text{ADMD}(\%) = 100 - \left[100 \times \left(\frac{\%\ \text{Cr2O3 in feed}}{\%\ \text{Cr2O3 in feces}}\right)\right]$$

$$\text{ADPh and ADPr}(\%) = 100 - \left[100 \times \left(\frac{\%\ \text{Cr2O3 in feed}}{\%\ \text{Cr2O3 in feces}} \times \frac{\%\ \text{nutrient feces}}{\%\ \text{nutrient feed}}\right)\right]$$

2.4 Statistical Analysis

All the data was analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from the growth trial were analyzed using one-way ANOVA to determine significant differences ($P<0.05$) among treatments followed by the Student-Neuman-Keuls (SNK) multiple comparison test to determine difference between treatments. Data from digestibility trial were analyzed using two-way ANOVA to evaluate diet type across phytase supplementation levels.

3. Results

3.1 Growth trial

Performances and retention efficiencies of pacific white shrimp, *Litopenaeus vannamei* offered diets contained different phytase levels are shown in Table 3. No significant differences were observed in final biomass, final mean weight, weight gain, feed conversion ratio (FCR), and survival when pacific white shrimps were fed diets contained replete phosphorus and increasing levels of phytase ($P>0.05$). Phosphorus retention was significantly higher in the treatments fed phosphorus deficient diet without phytase supplementation compared to the other

treatments fed phosphorus replete diets with phytase addition ($P<0.05$). Phytase supplementation in the replete phosphorus diets did not significant affect protein retention ($P>0.05$).

Chemical and mineral compositions (Dry weight basis) of whole body shrimp offered varying phytase levels diets are shown in Table 4. Phosphorus content of whole body shrimp was significantly higher in the treatments fed replete phosphorus diets compared to the treatment fed non-phosphorus supplementation diet ($P<0.05$). Copper content of whole body shrimp was significantly improved when shrimps were fed diets contained 1000 IU/Kg feed phytase compared to shrimps fed the basal diet ($P<0.05$). A significantly improved protein content of whole body shrimp was observed in the treatment contained 4000 IU/Kg feed phytase compared to the basal diet and also in the treatments supplemented with 1000 and 2000 IU/Kg feed phytase in contrast with non-phosphorus treatment ($P<0.05$).

3.2 Digestibility trial

Two-way ANOVA of apparent digestibility of dry matter, protein, and phosphorus of pacific white shrimp, offered fish meal based and soybean mean based diets contained different phytase levels are presented in Table 5. No combined effects of diet type and phytase were observed ($P>0.05$). The main effect phytase supplementation significantly affected apparent digestibility coefficient of protein and phosphorus ($P<0.05$).

One-way ANOVA followed by SNK multiple comparisons for the pooled digestibility data are presented in Table 6. Both 500 and 2000 IU/Kg feed phytase supplementation significantly improved protein digestibility ($P<0.05$). 2000 IU/Kg feed phytase incorporation significantly increased phosphorus digestibility ($P<0.05$).

Table 3 Performance and retention efficiencies of juvenile *Litopenaeus vannamei* (0.22±0.01g) offered diets with different phytase levels for five weeks

Diet	Phytase Activity (IU/Kg)	Final Biomass (g)	Final Mean Weight (g)	Weight Gain[1] (%)	FCR[2]	Survival (%)	Protein retention[3] (%)	P retention[4] (%)
D_0	<50	34.2	4.04	1793	1.54	85.0	28.63	20.41[b]
D_{np}	<50	32.8	3.86	1671	1.63	85.0	25.92	27.66[a]
D_{500}	541	34.8	4.00	1775	1.55	87.0	29.59	20.51[b]
D_{1000}	859	33.3	3.97	1718	1.58	84.0	29.63	20.96[b]
D_{2000}	1910	32.0	3.98	1787	1.59	81.0	26.75	20.42[b]
D_{4000}	3930	32.9	4.06	1776	1.56	82.0	27.13	20.80[b]
P-value		0.702	0.705	0.5549	0.572	0.891	0.1607	<0.0001
PSE[5]		0.411	0.029	17.15	0.011	1.20	0.3837	0.2939

[1] Weight gain (%)=(Final weight-initial weight)/initial weight*100%.
[2] Feed conversion ratio (FCR)=Feed offered/(Final weight-Initial weight).
[3] Protein retention (%)=(final weight × final protein content)-(initial weight × initial protein content)×100/protein intake.
[4] P retention (%)=(final weight × final phosphorus content)-(initial weight × initial phosphorus content)×100/phosphorus intake.
[5] Pooled standard error.

Table 4 Chemical and mineral compositions (Dry weight basis) of whole body shrimp offered varying phytase levels diets for five weeks

Diet	Moisture (% as is basis)	Protein (%)	Phosphorus (%)	Copper (μg/g)
D_0	76.21	66.09^{bc}	1.62^{a}	70.43^{b}
D_{np}	77.19	65.64^{c}	1.41^{b}	71.79^{ab}
D_{500}	76.44	68.92^{abc}	1.68^{a}	77.46^{ab}
D_{1000}	76.26	70.24^{ab}	1.74^{a}	79.04^{a}
D_{2000}	76.60	70.09^{ab}	1.74^{a}	74.47^{ab}
D_{4000}	76.34	70.94^{a}	1.76^{a}	75.90^{ab}
P-value	0.1920	0.0038	0.0002	0.0223
PSE[1]	0.0929	0.3581	0.0172	0.5854

[1] Pooled standard error.

Table 5 Two-way analysis output of apparent digestibility of dry matter, protein, and phosphorus of tested diets offered to pacific white shrimp, *Litopenaeus vannamei* supplemented with different diet types and phytase levels

Fish meal (%)	Phytase (IU/Kg)	ADDM[1] (%)	ADPr[2] (%)	ADPh[3] (%)
0	0	71.75	81.82	37.29
0	500	70.90	83.54	40.62
0	2000	73.38	86.33	45.12
12	0	70.46	80.96	37.72
12	500	73.77	85.73	37.49
12	2000	72.86	85.46	48.91
PSE[4]		0.0927	0.1325	0.4332
Phytase		0.0027	0.0083	0.0045
Fish meal		NS	NS	NS
Phytase*Fish meal		0.0025	NS	NS

[1] ADDM: apparent digestibility coefficient of dry matter.
[2] ADPr: apparent digestibility coefficient of protein.
[3] ADPh: apparent digestibility of phosphorus.
[4] Pooled standard error.
NS: not significant (P>0.05).

Table 6 One way ANOVA followed by SNK multiple comparison for the pooled digestibility data (based on the phytase levels)

Phytase (IU/Kg)	ADDM[1] (%)	ADPr[2] (%)	ADPh[3] (%)
0	70.93	81.28[b]	37.13[b]
500	72.33	84.63[a]	39.05[b]
2000	73.32	85.90[a]	47.02[a]
P-value	0.0268	<0.0001	0.0022
PSE[4]	0.2275	0.2207	0.6959

[1] ADDM: apparent digestibility coefficient of dry matter.
[2] ADPr: apparent digestibility coefficient of protein.
[3] ADPh: apparent digestibility of phosphorus.
[4] Pooled standard error.

4. Discussion

Phytases are widespread in nature because they can be found in animals, plants, and microorganisms. They are a group of enzymes known as *myo*-inositol-hexaphophate phosphohydrolase, which are ideal approaches to degrade phytate to sequentially produce myoinositol penta-, tetra-, tri-, di-, and mono-phosphates, and neutralizes the negative effects of phytate on protein and other nutrients in the diet of monogastric animals (Mitchell et al., 1997). Many studies have confirmed that phytase incorporation makes the chelated phytate P available to fish (Baruah et al., 2004; Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995b). Phytate P can be converted to available P by phytase which is able to be utilized by aquatic animals. Apparent P digestibility is considered as the most sensitive criteria for evaluating the effect of phytase on P utilization. In the present study, apparent phosphorus digestibility was significantly enhanced by supplementing 2000 IU/kg feed phytase in the diets. Similar results were demonstrated in Striped bass (Papatryphon and Soares, 2001), Atlantic salmon (Sajjadi and Carter, 2004), Tilapia (Liebert and Portz, 2005a), Korean rockfish (Yoo et al., 2005b), and Gibel carp (Liu et al., 2012). Consequently, we can conclude that 2000 IU/kg feed phytase supplementation in the diet improves P utilization of Pacific white shrimp.

However, effects of phytases supplementation in the replete P soybean meal based diet on P utilization were not significant. In the current study, P retention was not affected by dietary phytase supplementation. In contrast, Vielma et al. (2002) indicated that 500 IU/kg feed phytase significantly improved P retention in rainbow trout. Biswas et al. (2007a) also observed that P retention was significantly increased in Red sea bream when fed with diets contained 1000, 2000, and 3000 IU/kg feed phytase compared to the diets without phytase supplementation. Liebert and Portz (2005a) reported that phytase supplementation (500, 750, 1000, and 1250 IU/kg)

significantly enhanced P retention in tilapia. All these experiments were using diets without inorganic P incorporation. However, our growth trial diets were supplemented with inorganic P to contain replete P. The non-significant effects of phytase on P retention might be caused by the replete inorganic P incorporation. P retention in the treatment fed without inorganic P was significantly higher than the other treatments in the present study. P level in the diets without inorganic P supplementation is 0.7% which is almost half of the P level in other P replete diets (approximately 1.2%). However, the body P level of Pacific white shrimp fed diets without inorganic P addition is 1.41% which is 17% lower than the body P level (average 1.71%) of Pacific white shrimp fed with replete P diets. P retention is calculated by dividing P retained by P offered. Therefore, the significantly higher P retention in the treatment fed without inorganic P can be caused by the lower inorganic P level in the diet.

Whole body P level was commonly used as indicators of diet P status in fish nutrition studies (Luo et al., 2010; Mai et al., 2006; Shao et al., 2008). In the present study, phytase supplementation did not significantly affect whole body P level. Whole body P level in the shrimps fed diets without inorganic P and phytase incorporation was significantly lower. Dietary P level in the control diets and phytase supplementation diets were determined to around 1.2%, while P level (around 0.7%) in the diet without inorganic P addition was lower than the control diet. Therefore, P levels in shrimp body reflected the phosphorus status in the current shrimp diets.

Phytate can non-selectively bind with amino acids and reduce amino acids availability (Singh and Krikorian, 1982). Also, phytate has been to shown to inhibit activities of enzymes including trypsin, pepsin, and alpha-amylase (Liener, 1994). Phytase can improve protein and amino acid availability through the breakdown of phytin-protein complexes and neutralize the

negatives influence of phytate on protein and other nutrients in the feed of monogastric animals (Kornegay and Qian, 1996; Mitchell et al., 1997). In the present study, both 500 and 2000 IU/kg feed phytase significantly improved apparent digestibility coefficient of protein. Similarly, Vielma et al. (2002) indicated that protein digestibility in rainbow trout was significantly improved when fed diets supplemented with 2000 IU/kg feed phytase. Debnath et al. (2005) reported that 500 IU/kg feed phytase supplementation in pangus diets increased apparent protein digestibility compared to diet without phytase. Positive influences of phytase on protein digestibility were also observed in Labeo rohita (Baruah et al., 2005), red sea bream (Biswas et al., 2007a), and Yellow catfish (Zhu et al., 2014). Thus, we can conclude that phytase supplementation levels at 500 and 2000 IU/kg improved the protein digestibility in Pacific white shrimp.

However, protein retention was not significantly affected in the present study when Pacific white shrimp was fed replete P soybean meal based diets supplemented with graded doses of phytase. Similarly, Biswas et al. (2007a) reported that protein retention was not improved in red sea bream fed soybean meal based diets incorporated with graded doses of phytase. Also, Vielma et al. (2000) indicated that protein retention was not enhanced in rainbow trout fed both fish meal and soybean meal based diets contained 1000 IU/kg phytase. Protein retention efficiency is determined by a number of endogenous and exogenous factors, including feed intake, dietary protein and energy levels, dietary amino acid levels, dietary amino acid bioavailability, life history stage, and genetically controlled rate of protein degradation (Halver and Hardy, 2002). Phytase was demonstrated to improve protein availability in the digestibility trial. However, the improved protein availability may not be enough to affect the protein retention, which explains no differences in the protein retention when fed diets with phytase.

Phytate also can chelate with other minerals to decrease their bioavailability to fish and shrimp. Supplementation of phytase can hydrolyze phytate and increase the concentration of minerals in plasma, bone and the whole body (Debnath et al., 2005; Jackson et al., 1996; Liebert and Portz, 2005a; Papatryphon and Soares, 2001; Weerd, 1999). In the present study, copper content in the whole shrimp body was significantly increased in the treatment supplemented with 1000 IU/kg feed phytase compared to the basal diet. Similarly, studies on rainbow trout showed that phytase incorporation improved the apparent absorption of copper in low-ash soybean meal diets (Sugiura et al., 2001). Therefore, the addition of phytase to a soybean meal based diet might increase bioavailability of copper, thereby increasing whole body copper deposition.

Investigations into the effects of different microbial phytases on growth performance of various fish and shrimp species have been conducted. Positive impacts of phytase on growth performance of fish has been reported in Channel catfish (Jackson et al., 1996), African catfish (Weerd, 1999), Stripped bass (Papatryphon and Soares, 2001), Rainbow trout (Vielma et al., 2000), Tilapia (Liebert and Portz, 2005a), and Yellow catfish (Zhu et al., 2014). In contrast, no significant effects of dietary phytase on weight gain of Japanese flounder (Masumoto et al., 2001), *Penaeus monodon* (Biswas et al., 2007b), and Korean rockfish (Yoo et al., 2005b) have been reported. Phytase supplementation studies for Pacific white shrimp were limited. Our experimental diets contained replete inorganic P. Total P levels in P replete diets were tested around 1.2% which is excess for the growth requirements of pacific white shrimp. Thus, growth response is independent of P since diets have replete levels of P. In the present study, growth performance of Pacific white shrimp responded to different phytase supplementation in the replete P diet did not show any significant improvements, which indicating that possible improvement of phosphorus and protein availability did not enhance growth performance.

5. Conclusion

Growth performance, FCR, P and protein retention, whole shrimp body P content were not affected by dietary phytase supplementation in soybean meal based diets with replete inorganic P. Copper content in the whole shrimp body was significantly increased in the treatment supplemented with 1000 IU/Kg feed phytase compared to the basal diet. Phytase incorporation at both 500 and 2000 IU/kg improved protein digestibility. Phosphorus digestibility was enhanced by 2000 IU/kg phytase supplementation. In terms of improving P and protein availability, it is recommended to incorporate phytase at 2000 IU/kg level.

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CHAPTER III

EFFECTS OF DIETARY CARBOHYDRASE SUPPLEMENTATION ON PERFORMANCE AND APPARENT DIGESTIBILITY COEFFICIENTS OF PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

Abstract

This experiment was designed to evaluate the effects of dietary carbohydrase supplementation on the growth performance and apparent digestibility coefficients of *Pacific white shrimp, Litopenaeus vannamei*. Six experimental diets with increasing carbohydrase levels from 0 to 0.05% were randomly assigned to 3 replicates and applied four times per day for 5 weeks. No significant differences were observed in final biomass, final mean weight, weight gain, feed conversion ratio (FCR), and survival across all treatments. However, we can find that weight gain was increased in the treatments fed with diets contained carbohydrase compared to the basal diet. Apparent digestibility coefficients of dry matter, protein, and energy were determined by using 10 g/kg chromic oxide as inert marker with 70:30 replacement techniques. Protein digestibility was significantly improved when fed diets contained 0.02% carbohydrase in contrast with the basal diet. No significant differences were found in apparent digestibility coefficients of dry matter and energy. But energy digestibility was increased from 78.42% to 81.34% (P-value=0.0863) when added 0.02% carbohydrase in the diet compared to the basal diet.

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most prevalent cultured species and accounts for over 66% shrimp aquaculture production in the world (FAO, 2011). Among plant protein sources, soybean meal is the most utilized and has received considerable attention as a primary protein source in aquatic animal feeds because of its worldwide availability, low price, balanced amino acid profile and consistent composition (Akiyama, 1989; Akiyama et al., 1991; Amaya et al., 2007; Colvin and Brand, 1977; Divakaran et al., 2000; Hardy, 1999; Lim and Dominy, 1990; Lim et al., 1998; Samocha et al., 2004; Swick et al., 1995; Tacon, 2000). However, most plant-based meal including soybean meal have a wide variety of anti-nutritional factors (ANF) such as non-starch polysaccharides (NSP), which may reduce nutrient availability, as well as impair performance and health (Francis et al., 2001; NRC, 2011). Digestive enzymes in fish and shrimp that specifically hydrolyze the β-glycosidic bonds of non-starch polysaccharides is very low or even nonexistent (Krogdahl et al., 2005; NRC, 2011). As endogenous enzymes are limited, the use of exogenous enzymes to digest these products may improve the nutritional value of plant based ingredients.

Rovabio^{TM} Eecel LC (Adisseo) is a concentrated solution whose main enzymatic activity is xylanase and β-glucanase obtained from a fermentation broth of Penicillium funiculosum. Xylanase and glucanase are two carbodyrases that dominate more than 80% of the global carbohydrase market (Castillo and Gatlin, 2015). Exogenous carbohydrases have been reported to help reduce the degree of polymerization of feed, decreasing its viscosity and liberating carbohydrate oligomers, thus increasing nutrient utilization (Vahjen et al., 2007).

In pig and poultry industries exogenous dietary enzyme supplements, isolated from plants and bacteria, have been used successfully to overcome the negative effects of the soluble fraction

of dietary NSP (Batterham, 1991; Bedford, 1996; Campbell and Bedford, 1992; Chesson, 1993; Farrell, 1991). Nonetheless, exogenous carbohydrase enzymes supplementation studies in aquaculture species are still new and research data is limited. Positive effects of carbohydrases on the growth performance were observed in Salmon (ali Zamini et al., 2014; Carter et al., 1994), Japanese sea bass (Ai et al., 2007), African catfish (Yildirim and Turan, 2010), and Grass carp (Zhou et al., 2013). In contrast, no significant differences when fed diets contained carbohydrase were found in rainbow trout (Dalsgaard et al., 2012; Farhangi and Carter, 2007; Ogunkoya et al., 2006) and tilapia (Yigit and Olmez, 2011).

Carbohydrase studies conducted with shrimps nutrition are limited. Therefore, the objectives of this study are to investigate the growth response of Pacific white shrimp juveniles to increasing carbohydrase levels in a plant based feed formulation and determined apparent digestibility values for practical diets containing 0.02% carbohydrase.

2. Materials and Methods

2.1 Experiment Diets

All test diets were formulated to have equal protein and lipid levels (35% protein and 8% lipid) and were prepared in the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University (Auburn, AL, USA). For the growth trial, six experiment diets were formulated. The first diet or basal diet did not contain carbohydrase supplements. Whereas the next five diets contained carbohydrase at increasing levels (0.01%, 0.02%, 0.03%, 0.04%, 0.05%) (Table 1). In the digestibility trial, soybean based diets were modified to contain 1% chromic oxide and two levels of carbohydrase (0 and 0.02%), respectively (Table 2).

Table 1 Formulation and chemical composition of diets utilized in carbohydrase growth trial

Ingredient (g kg^{-1} feed)	Diet code					
	D0	D1	D2	D3	D4	D5
Menhaden fish meal[1]	60.0	60.0	60.0	60.0	60.0	60.0
Soybean meal[2]	495.0	495.0	495.0	495.0	495.0	495.0
Corn protein concentrate[3]	50.0	50.0	50.0	50.0	50.0	50.0
Whole wheat[4]	260.0	260.0	260.0	260.0	260.0	260.0
Menhaden Fish Oil[2]	56.7	56.7	56.7	56.7	56.7	56.7
Trace Mineral premix[5]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0	2.0
Stay C 250 mg/kg using 25%[7]	1.0	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	23.5	23.5	23.5	23.5	23.5	23.5
Lecithin[9]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5	0.5
Corn Starch[4]	18.3	18.2	18.1	18.0	17.9	17.8
CHOase[10]	0.0	0.1	0.2	0.3	0.4	0.5
Proximate Analysis[11] (% as is)						
Moisture	8.20	9.13	5.94	6.91	7.48	6.66
Crude protein	35.80	35.17	36.37	35.87	36.07	36.07
Crude fiber	3.13	3.45	3.20	3.42	3.45	3.25
Crude fat	7.60	7.82	8.20	8.44	8.43	8.68
Ash	7.07	6.95	7.11	7.11	7.05	7.06

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, Ohio, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Rovabio™ Excel LC Adisseo USA Inc, Alpharetta, GA, USA.
[11] Analyses conducted by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 2 Formulation of test diets used to determine the digestibility of two carbohydrase supplements

Ingredient (g kg^{-1} feed)	Diet code	
	D_0	D_2
Menhaden fish meal[1]	60.0	60.0
Soybean meal[2]	500.0	500.0
Corn protein concentrate[3]	50.0	50.0
Whole wheat[4]	260.0	260.0
Menhaden Fish Oil[2]	53.4	53.4
Trace Mineral premix[5]	5.0	5.0
Vitamin premix[6]	18.0	18.0
Choline chloride[4]	2.0	2.0
Stay C 250 mg/kg using 25%[7]	1.0	1.0
CaP-diebasic[8]	23.5	23.5
Lecithin[9]	10.0	10.0
Cholesterol[4]	0.5	0.5
Corn Starch[4]	6.6	6.4
Carbohydrase[10]	0.0	0.2
Chromium dioxide	10.0	10.0

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, Ohio, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Rovabio™ Excel LC Adisseo USA Inc, Alpharetta, GA, USA.

Primary ingredients were analyzed for proximate composition and then the diets were formulated. Pre-ground dry ingredient and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air dried (<50 °C) to a moisture content of 8-10%. After drying, pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed.

2.2 Growth Trial

The growth trial utilized 6 treatments with 3 replicates in each treatment. It was conducted in a semi-closed recirculation system at the E.W Shell Fisheries Research Station (EWS), Auburn, AL, USA. Shrimps were obtained from the nursery system and hand-sorting to a uniform size. Juvenile shrimp (initial weight 0.74±0.02g) were stocked into 18 tanks with 15 shrimps in each aquarium. A fixed ration of 2.85g/day for the first week, 3.09g/day for the second week, 3.47g/day for the third week, and 3.86g/day for the forth and fifth week was offered over 4 feedings.

Dissolved oxygen (DO), temperature, salinity, and pH were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH); meanwhile, water samples were taken to measure total ammonia-nitrogen (TAN) and nitrite every week. During the experiment period DO, temperature, salinity, pH, TAN, and nitrite were maintained within acceptable ranges for *L. vannamei* at 6.46±0.13mg/L, 30.1±0.5°C, 5.19±0.23ppt, 7.23±0.15, 0.17±0.08mg/L, and 0.01±0.01mg/L, respectively.

Shrimps were counted to readjust daily feed input on a weekly basis. At the conclusion of 5 weeks growth trial, shrimps were counted and group weighted. Mean final weight, FCR, WG, biomass, and survival were determined.

2.3 Digestibility Trial

Digestibility trial that contained two treatments with six replicates in each treatment were conducted in a semi-closed recirculating system in EWS, Auburn, AL, USA. Feces from two groups of 10 shrimps will be pooled and collected for 5 days or until adequate samples were obtained. Prior to the beginning of the collection of feces, shrimps were allowed to acclimate to each diet for 3 days. The aquaria were cleaned by siphoning before each feeding. Then the shrimp were offered an excess of feed. Feces were collected by siphoning by a 500μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight is obtained, and then separately stored in freezer (-20 °C) until analyzed. Apparent digestibility coefficient for dry matter, protein, and energy were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. Chromium concentrations were determined by the method of McGinnis, Kasting (1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540nm. Gross energy of diets and fecal samples were analyzed with a Semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Protein were determined by micro-Kjeldahl analysis (Ma and Zuazaga, 1942).

The apparent digestibility coefficient of dry matter (ADMD), protein (ADP), and energy (ADE) were calculated according to Cho et al. (1982) as follows:

$$\text{ADMD}(\%) = 100 - \left[100 \times \left(\frac{\%\ \text{Cr2O3 in feed}}{\%\ \text{Cr2O3 in feces}}\right)\right]$$

$$\text{ADE and ADP}(\%) = 100 - \left[100 \times \left(\frac{\%\ Cr2O3\ \text{in feed}}{\%\ Cr2O3\ \text{in feces}} \times \frac{\%\ \text{nutrient feces}}{\%\ \text{nutrient feed}}\right)\right]$$

2.4 Statistical Analysis

All the data were carried out by using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from the growth trial were analyzed using one-way ANOVA to determine significant differences ($P<0.05$) among treatments followed by the Student-Neuman-Keuls multiple comparisons test to determine difference between treatments. Data from digestibility trial were analyzed by two samples T-test to compare the differences of ADP and ADE and the data were expressed as mean±SE.

3. Results

3.1 Growth trial

Performances of pacific white shrimp, *Litopenaeus vannamei* offered six diets contained different carbohydrase levels were shown in Table 3. No significant differences were observed in final biomass (55.7 g to 62.6 g), final mean weight (3.86 g to 5.21 g), weight gain (517% to 575%), feed conversion ratio (FCR) (2.12 to 2.36), and survival (78% to 87%).

3.2 Digestibility trial

Apparent digestibility coefficients of dry matter, protein, and energy of pacific white shrimp, *Litopenaeus vannamei* offered diets supplemented with 0 and 0.02% carbohydrase were presented in Table 4. Apparent digestibility coefficient of protein (ADP) was significantly increased in the diets contained 0.02% carbohydrase in comparison with the basal diet ($P<0.05$). No significant differences were found in apparent digestibility coefficient of dry matter (ADDM)

Table 3 Performance of juvenile *Litopenaeus vannamei* (0.74±0.02g) offered diets contained different carbohydrase levels for five weeks

Diet	Final Biomass (g)	Final Mean Weight (g)	Weight Gain[1] (%)	FCR[2]	Survival (%)
D0	60.5	4.65	517	2.29	86.7
D1	60.6	3.86	566	2.21	84.4
D2	56.6	4.72	553	2.33	80.0
D3	55.7	4.62	543	2.36	80.0
D4	62.6	4.84	568	2.16	86.7
D5	60.8	5.21	575	2.12	77.8
P-value	0.783	0.604	0.837	0.743	0.765
PSE[3]	2.26	0.148	19.6	0.075	3.10

[1] Weight gain (%)=(Final weight-initial weight)/initial weight*100%.
[2] Feed conversion ratio (FCR)=Feed offered/(Final weight-initial weight).
[3] Pooled standard error.

Table 4 Apparent digestibility coefficient of dry matter, protein, and energy of test diets offered to pacific white shrimp, *Litopenaeus vannamei* supplemented with 0 and 0.02% carbohydrase

Diet	ADDM[1] (%)	ADP[2] (%)	ADE[3] (%)
D1	74.37±0.59	86.48±0.68[a]	78.42±1.08
D2	75.31±0.84	92.73±0.42[b]	81.34±0.71

[1] ADDM: apparent digestibility coefficient of dry matter.
[2] ADP: apparent digestibility coefficient of protein.
[3] ADE: apparent digestibility of energy.
Data are expressed as mean±SE.

and energy (ADE). However, ADE was improved by approximately 3% in the diets supplemented with 0.02% carbohydrase compared with the basal diet. More studies will be done to determine the best inclusion level of carbohydrase for improving protein and energy digestibility.

4. Discussion

4.1 Digestibility trial

Supplemental carbohydrase increases energy digestibility by improving energy yielding nutrient digestibility, such as starch and fat, because NSP impair the ability for nutrient absorption by decreasing enzyme accessibility to substrates (Adeola and Bedford, 2004). No significant differences were observed in energy digestibility; however, apparent energy digestibility was numerically (P-value=0.0863) improved for around 3% in the diet with 0.02% supplemental carbohydrase in contrast with the control diet. Similarly, Stone et al. (2003) reported that supplementing Natustarch (α-amylase) and Natugrain-blend (β-glucanase and xylanase) in wheat-based and lupin-based diets did not affect energy digestibility of silver perch. In contrast, apparent gross energy digestibility was significantly improved when rainbow trout fed lupin-based diets contained hemicellulases (Farhangi and Carter, 2007). More studies will be needed to determine the effects of other carbohydrase inclusion levels on the energy digestibility.

Carbohydrases act to improve amino acid utilization by increasing the access to protein for digestive protease (Tahir et al., 2008). In the current study, 0.02% carbohydrase supplementation in the soybean-based diets significantly improved apparent protein digestibility for 6.25% compared to the control diet that did not contain carbohydrase. Although improved protein availability did not affect the growth response, it helps to release 2.2% more protein in

the soybean meal diet contained 35% crude protein, which indeed creates certain economical value. Dalsgaard et al. (2012) reported that supplementing β-glucanase or xylanase to the soybean-based diet increased the apparent digestibility of protein, but no significant differences were observed in protein digestibility in rainbow trout when fed with diets based on sunflower and rapeseed meal. Farhangi, Carter (2007) indicated that apparent protein digestibility was significantly improved when rainbow trout fed lupin-based diets contained hemicellulases, but no significant differences were found in apparent protein digestibility in rainbow trout when fed with diets supplemented with α-galactosidase and a mixture of hemicellulases, α-galactosidase, and protease. No significant differences were observed in protein digestibility of tilapia when fed with canola-based diets incorporated with 1 and 5% cellulase (Yigit and Olmez, 2011). Variations in the outcome of different authors may be result from various carbohydrase enzymes types, physiological difference in digestive systems primarily pH, and sources of plant protein ingredients.

4.2 Growth trail

Few experiments have been carried out on the use of supplementary carbohydrase enzymes in fish and shrimp diets. The effects of carbohydrase on the growth performance of fish and shrimp were somewhat inconsistent. Lin et al. (2007) reported that supplemental exogenous enzymes (protease, β-glucanase and xylanase) at a dose of 0.1 and 0.15% significantly improve growth performance of juvenile hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) fed diets with plant-based feedstuffs (soybean, rapeseed and cottonseed meal). Specific growth rate was significantly improved in tilapia (*Oreochromis niloticus* L.) fingerlings when fed with corn-based diets contained 1 and 2% exogenous enzymes (pepsin, papain and α-amylase) (Goda

et al., 2012). Positive effects of growth performance in carbohydrase enzymes were also observed in Atlantic salmon (Carter et al., 1994), Japanese sea bass (Ai et al., 2007), African catfish (Yildirim and Turan, 2010), Grass carp (Zhou et al., 2013), and Caspian salmon (ali Zamini et al., 2014).

In contrast, Dalsgaard et al. (2012) indicated that no significant differences in growth performance of rainbow trout were observed when high supplementation levels of three different plant based feedstuffs (soybean, rapeseed, sunflower) were incorporated with multi-enzymes complex (β-glucanase (67mg/kg), xylanase (208mg/kg), and protease (228mg/kg). This result was similar with previous reports by Ogunkoya et al. (2006) that indicated a commercial enzyme cocktail (mixture of xylanase, amylase, cellulase, protease, and β-glucanase) supplementation in soybean based diets did not affect growth performance of rainbow trout and Farhangi, Carter (2007) that lupin-based diets supplemented with hemicellulases, protease, galactosidase, and a mixture of those three enzymes did not improve the growth performance of rainbow trout. Also, Yigit, Olmez (2011) indicated that both 1 and 5 g/kg cellulase supplementation in soybean-baed meal and canola-based meal did not affect growth response of tilapia (*Oreochromis niloticus* L.). In the present study, no significant differences in weight gain of pacific white shrimp and feed conversion ratio were found when fed diets contained carbohydrases compared to the control diet, which indicating that growth response to the improved protein availability was not significant. The contradictory effects of carbohydrase enzymes on growth performance may be mainly due to the use of different experimental animal species, various kinds of carbohydrases, the level of plant-based feedstuffs as well as physiological difference in digestive systems primarily pH.

5. Conclusion

Carbohydrase supplementation in Pacific white shrimp diets did not affect the growth performance and feed conversion ratio. Results from the digestibility trial showed that apparent protein digestibility was significantly improved when Pacific white shrimp were fed with diets contained 0.02% carbohydrase compared to the diet without carbohydrase supplementation. More studies will be needed to further determine the best carbohydrase inclusion level for improving energy and protein digestibility.

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CHAPTER IV

SUMMARY AND CONCLUSION

The aquaculture industry is playing an increasingly important role in meeting the demand for fish and shrimp. The continued rapid growth and intensification of aquaculture production depends on the development of sustainable feed formulations to support the expansion of the industry. To develop sustainable and environmentally friendly aquaculture, many researchers have concentrated on the goal of increasing the use of plant-based ingredients and decreasing the use of marine ingredients in shrimp feed formulation. Among plant protein sources, soybean meal is most utilized and has received considerable attention as a primary protein source in aquatic animal feeds because of its worldwide availability, low price, balanced amino acid profile and consistent composition. However, the presence of anti-nutritional factors (ANF) such as phytate and non-starch polysaccharides (NSPs) limited the inclusion level of soybean mal in some aquaculture feeds. The overall goal of this research was to enhance sustainable and environmentally friendly feed formulation based soybean meal for Pacific white shrimp. To be more specifically, the purpose was to investigate the effects of dietary phytase and carbohydrase supplementation on the growth performance and apparent digestibility coefficients of Pacific white shrimp.

Phytases, a group of enzymes known as *myo*-inositol-hexaphophate phosphohydrolase, are ideal approaches to degrade phytate to sequentially produce myoinositol penta-, tetra-, tri-, di-, and mono-phosphates, and neutralizes the negative effects of phytate on protein and other nutrients in the diet of monogastric animals. In the present study, phytase incorporation at both 500 and 2000 IU/kg improved protein digestibility. Phosphorus (P) digestibility was enhanced by

2000 IU/kg phytase supplementation. Thus, we can conclude that phytase supplementation improves P and protein bioavailability. Growth performance, feed conversion ratio (FCR), P and protein retention, whole shrimp body P content were not affected by dietary phytase supplementation in soybean meal based diets with replete inorganic P. Growth performance responded to possible improvement of P and protein availability was not significant. The improved protein availability was enough to affect protein retention of pacific white shrimp. Copper content in the whole shrimp body was significantly increased in the treatment supplemented with 1000 IU/kg feed phytase, which indicates that the addition of phytase to a soybean meal based diet might increase bioavailability of copper, thereby increasing whole body copper deposition.

Carbohydrase application in aquaculture nutrition is still relative new compared to poultry and swine industry. Exogenous carbohydrases have been reported to facilitate a reduction in the degree of polymerization of feed, decreasing its viscosity and liberating carbohydrate oligomers, thus enhancing nutrient utilization. Digestibility of energy yielding nutrients, such as starch and fat, were increased by carbohydrase supplementation since NSPs impair the ability for nutrient absorption by decreasing enzyme accessibility to substrates. In the current study, dietary carbohydrase supplementation at 0.02% level significantly improved protein digestibility, which indicating carbohydrase at 0.02% can help increase protein availability. Growth performance and FCR were not affected by carbohydrase incorporation in soybean meal based diet, which indicating that the possible improved protein digestibility may not be able to enhance the growth response.

These studies have indicated that phytase and carbohydrase are two promising feed additives in plant protein based meal. Carbohydrase supplementation at 0.02% level significantly

improved protein digestibility. Since there is only one level in the carbohydrase digestibility trial, more studies will be needed to investigate the best inclusion level of carbohydrase level. In terms of improving P and protein availability, phytase is suggested to incorporate at 2000 IU/kg. Both phytase and carbohydrase studies in Pacific white shrimp are limited. Future studies will focus on investigating combined effects of organic acids and phytases or carbohydrases on the performance of Pacific white shrimp.

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