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Upshots of rapid protein digestibility in legume based grow out diets

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Abstract

Improving protein digestibility in nutrient poor fish feeds through incorporation of dietary enzymes is expected to be achieved with protease. Understanding the role of other dietary enzymes was therefore evaluated to guide appropriate use for optimal fish growth. Protein digestibility of 30, 35, 50 and 55% crude protein (CP) diets was determined with catfish gut enzyme extract, sprouted sorghum, protease and phytase both singly and in a mixture of 500 units of protease and phytase using the pH drop method *in vitro*. Significant ($p<0.05$) digestibilities were recorded in 30 and 35% CP diets incorporated with phytase and in 50 and 55% CP diets incorporated with protease singly. These results showed that protein digestibility was more efficient with protease enzyme in high protein diets while phytase was efficient in low protein diets. This implied that the use of protease was more beneficial in catfish starter feeds and phytase in grower/finisher diets and provided a basis for enzyme selection for production of cost-effective catfish diets.

Keywords: *In-vitro* protein digestion, catfish gut enzyme extract, phytase, protease, sprouted sorghum.

INTRODUCTION

Feed account for 60 to 70% of operating costs in farming of high value fed species like Clarias gariepinus, and without it stock productivity and profitability will remain a cherished desire (World Bank, 2007).

Fish feed quality is compromised by limited use of fishmeal, the most nutritive and digestible protein ingredient traditionally used in fish diets, due to its high cost (US$2/Kg) (World Bank, 2013), associated food insecurity and aquatic degradation (FAO, 2009). This has intensified use of plant protein instead (Gabriel et al., 2007) as they are more accessible and fairly priced (Hecht, 2006). However, almost all practical plant feed ingredients contain invariable amounts of antinutrients of which phytic acid is considered most detrimental. It forms indigestible complexes with nutrients including protein, reducing their utilisation by fish for growth (Gabriel et al., 2007; GarcõÂa-Estepa et al., 1999; Gilani et al., 2005; Hidvegi and Lasztity, 2002; Kumar et al., 2012b).

Table 1. Ingredients used in formulation of experimental diets.

Ingredient	30%	35%	50%	55%
DCP	4	4	0	0
Cassava flour	6	4	0	0
Wheat pollard	8	8	10	2
Whole grain maize	10	10	0	0
Fish meal	20	26	72	96
Soy bean	22	26	13	1
Bush beans	15	8	0	0
L-Lysin	3.5	4	3	1
DL-Methionine	1.5	2	2	0
Cotton seed cake	7	5	0	0
Nile perch oil	3	3	0	0
Salt	0.001	0.001	0.001	0.001
Fish vitamin and mineral premix	0.002	0.002	0.002	0.002
Total	**100.003**	**100.003**	**100.003**	**100.003**

They are difficult to suppress cost effectively using conventional pre-treatments like heating, soaking and germination (Afify et al., 2011). Due to this use of dietary exogenous digestive enzymes especially phytase has been explored as a cheaper pre-treatment strategy of improving protein digestibility in low value plant based feeds for enhanced fish growth (Abdoulaye et al., 2011; Bedford and Partridge, 2010; Gabriel et al., 2007; Kim et al., 2006; Kumar et al., 2012a; Kumar et al., 2012b; Reddy et al., 1989; Serraino and Thompson, 1984; Wheeler and Ferrel, 1971). It has already been proven that the use of phytase in fish diets improves phosphorus absorption by fish reducing water pollution (Tudkaew et al., 2008). However, its contribution to protein digestibility which directly translates into fish growth is not established. As use of multiple enzymes in a single diet gets common in an effort to improve feed efficiency and enterprise profitability (Bedford and Partridge, 2010), the efficiency of other enzymes including protease on improving protein digestibility in phytic acid loaded plant based diets needs to be investigated as there can be antagonistic or additive effects (Dechavez and Serrano, 2012). This study determined rapid protein digestibility in legume based grow out diets (30 and 35% CP) and in fish meal-based larval diets (50 and 55% CP) subjected to catfish gut enzyme extract, sprouted sorghum, protease and phytase enzymes. Information generated provided an insight on the probable appropriate exogenous enzyme for incorporation in fish feeds at different stages of development for improved growth.

MATERIALS AND METHODS

Study area

The *in-vitro* analysis experiment was conducted at the Bioscience laboratory of the National Crop Resources Research Institute (NaCRRI) situated in Namulonge, Wakiso District in Uganda.

Feed formulation and diet development

Four experimental diets were formulated to contain 30, 35, 50 and 55% crude protein with Feedwin software (Table 1). Feeds were pelleted to be stable in water using a pelleting machine locally fabricated in Kampala, Uganda.

Diet proximate composition was cross-examined/confirmed at Makerere University, School of Agriculture and Environmental Sciences, Animal science laboratory following standard procedures of the Association of Analytical Chemists (AOAC, 2002) and presented in Table 2.

Preparation of enzyme extracts (solutions)

Catfish feeding and preparation of digestive enzyme extracts

Fish in four MBAZARDI ponds were fed on diets with crude protein graded at four levels (30, 35, 50 and 55% crude protein diets) for a week. Five catfish (weight 284 ± 4.5 g and length 35.2 ±1.20 cm) were captured randomly using a seine from each of the four pond treatments 30 min after feeding. The caught fish were humanly killed after anesthetizing them with excess clove oil (2.5 ml/L of water) according to guidelines of death as end point by (Homeoffice, 2014). They were then dissected; gut removed together with its contents and kept in a refrigerator at -4°C until when enzyme extraction was conducted according to the flow chart used by Sultana et al. (2010).

Catfish digestive enzyme extraction was conducted following the procedure of (Ali et al, 2009; Sultana et al., 2010). The guts were thawed to 40°C, the region encompassing the stomach and small intestines were cut out and chopped into small sections of 1 to 2 cm long. These small sections from each fish were ground in a beaker placed on ice with an ultra sonic cell lyser (model -150 V/T Biologics Inc) at 60 pulses per minute for 10 min. The slurry formed was diluted with distilled water chilled to 4°C at a ratio of 1:10 (weight/volume). It was then poured into 1.5 ml micro tubes (eppendrof) and centrifuged in a refrigerated centrifuge for 15 min at 12000 RPM. A transparent lipid layer formed on top of the

Table 2. Proximate composition of experimental diets as after independent verification at Makerere University.

Diet description	Dry matter	Ash	Crude protein	Crude fibre	Crude fat	Gross energy (Kcal/Kg as is)
55% CP diet	90.18 ± 0.13	11.01 ± 0.17	54.86 ± 0.35	5.81 ± 0.146	7.71 ± 0.15	4778 ± 0.88
50% CP diet	93.42 ± 0.02	12.80 ± 0.71	50.17 ± 0.72	3.93 ± 0.92	5.45 ± 0.08	4447 ± 18.33
35% CP diet	91.53 + 0.10	8.92 ± 0.28	35.33 ± 0.59	4.41 ± 0.16	7.276± 0.97	4549 ± 15.54
30% CP diet	91.65 ± 0.03	9.07 ± 0.24	30.76 ± 0.17	4.35 ± 1.09	9.00± 1.3	4551 ± 414

Table 3. Amount of protease enzyme dissolved in 1000 ml of distilled water to make a stock solution from which 10 ml worth corresponding activity units was with drawn into 20 ml of feed substrate suspension.

Protease	Enzyme quantity (g) worth 750 activity units	Enzyme quantity (g) worth 1000 activity units	Enzyme quantity (g) worth 1250 activity units
30% CP diet	0.0066	0.009	0.0011
35% CP diet	0.0058	0.0078	0.0097
50% CP diet	0.004	0.0054	0.0067
50% CP diet	0.0036	0.0049	0.0061

Table 4. Amount of phytase enzyme dissolved in 50 and 5 ml of distilled water to make a stock solution from which 2 ml worth corresponding activity units was with drawn into 20ml of feed substrate suspension.

Phytase	Enzyme quantity (g) worth 750 activity units	Enzyme quantity (g) worth 1000 activity units	Enzyme quantity (g) worth 1250 activity units
30% CP diet	0.008	0.0011*	0.0013 *
35% CP diet	0.0069	0.0092	0.0012*
50% CP diet	0.0048	0.0064	0.008
50% CP diet	0.0044	0.0058	0.0073

*Dissolved in 5 ml from which 2 ml were withdrawn.

supernatant was removed using plastic pipettes and discarded. The supernatant was collected in a glass bottle of 50 ml and stored in a deep freezer at -20°C until it was used.

Preparation of sprouted sorghum solution

Sprouted sorghum (*S. bicolor*) grains were dried and ground into flour of fine particles of less than 0.02 mm. An amount of flour equivalent to 10% of the feed used to get 160 mg of crude protein under each category of feed was determined and weighed using a digital scale (Denver Instruments, Germany Model TP-3002). This flour was made into a suspension with distilled water which was mixed with the pre-soaked feed suspension and incubated at 26°C for 10 min.

Preparation of phytase and protease enzymes

The amount of enzyme worth 750, 1000 and 1250 activity units of protease *(fungus Trichoderma reesei*) and phytase (bacteria *Bacilus lincheniformis*) were calculated based on the manufacturer's prescriptions of the enzyme activity (that is, 1 g of protease contained 600,000 activity units and 1 g of phytase contained 5000 activity units).

The amount of enzyme used was measured by sensitive digital scale (Denver Instruments, Germany Model TP-3002). For protease enzyme, 1000 ml of stock solution was made with distilled water at 4°C from which 10 ml worth 750, 1000 and 1250 protease activity units were withdrawn and put into 20 ml of pre-soaked feed substrate following the Tocris morality (Table 3). For phytase enzyme, stock solutions of 50 and 5 ml were made with chilled distilled water from which 2 ml worth corresponding activity units was drawn (Table 4).

Determination of protein digestibility

The pH drop method was used following the procedure described by (Sultana et al., 2010) as adopted from Chisty et al. (2005). Four diets of 30, 35, 50 and 55% crude protein were ground and an amount that provided 160 mg of crude protein weighed (based on proximate composition, that is, 0.53 g for 30%, 0.46g for 35% CP,0.32 g for 50% CP and 0.29g for 55% CP diets. The mount of feed for each protein level was soaked overnight in 20 ml of distilled water at 4°C with casein from bovine milk (90% crude protein, C7078, Sigma-Aldrich, St. Louis, MO, USA) as the standard protein. The 160 mg protein from each diet including casein (in 20 ml) was incubated at 26°C in a water bath (Grant TXF 200) for 3 min. In each case, the suspension pH was first adjusted to pH 8 (optimal pH of protease and phytase enzymes used) using ether Sodium hydroxide or hydrochloric acid as would be appropriate.

Table 5. pH values recorded after every one minute interval during incubation of 1250 protease activity units with 50% crude protein feed suspension substrate in three replicates.

Protease 1250					
Casein	**50%CP**	**Casein**	**50%CP**	**50%CP**	**Casein**
8.09	8.08	8.07	8.00	8.07	8.00
8.02	8.05	8.01	7.98	8.06	7.93
8.00	8.03	8.00	7.96	8.05	7.93
8.00	8.01	7.99	7.95	8.03	7.92
8.00	8.00	7.98	7.87	8.02	7.92
8.00	7.97	7.97	7.82	8.01	7.92
8.00	7.94	7.97	7.71	7.81	7.92
8.00	7.93	7.97	7.64	7.81	7.92
8.00	7.79	7.97	7.62	7.8	7.92
8.00	7.73	7.97	7.62	7.8	7.92
8.00	7.73	7.97	7.62	7.8	7.92

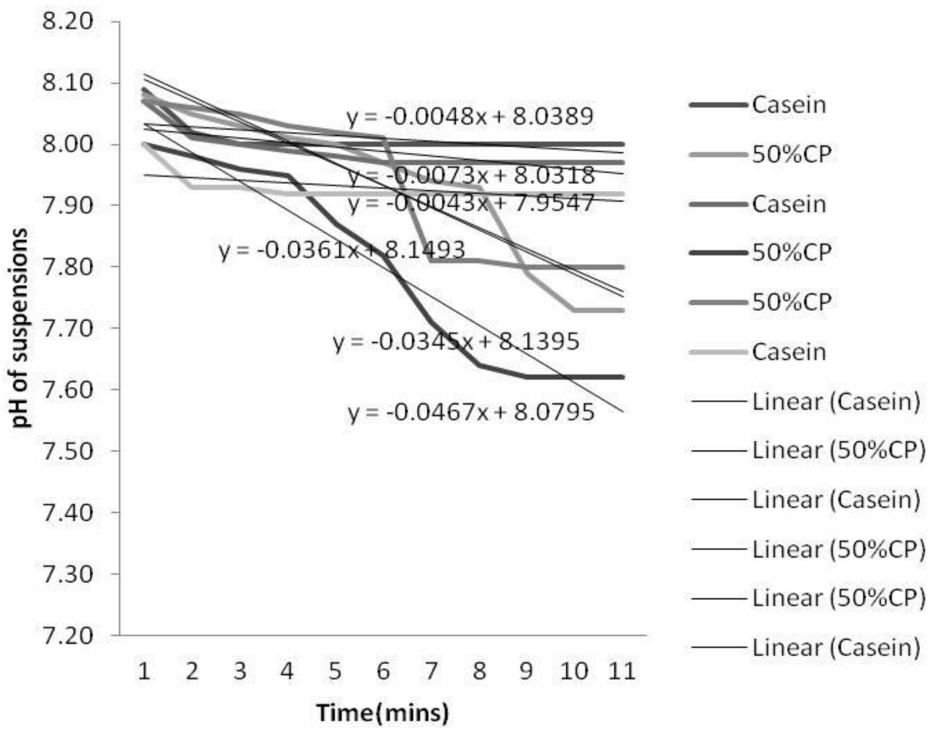

Figure 1. Rates of pH change in casein and diet suspensions per minute (for 10 min).

All the prepared catfish digestive enzyme extracts from each fish (5) and sprouted sorghum (worth 10% of the feed substrate feed), 750, 1000 and 1250 activity units of phytase (*Bacilus lincheniformis bacterium)* and protease (*Trichoderma reesei fungus)* were added to feed substrate suspensions (Table 3 for protease and Table 4 for phytase respectively). The pH readings in each enzyme-feed substrate were in all cases recorded after an interval of one minute for 10 min using a digital pH meter with a protected tip (pH 211, Labor-pH/mV/°C- Meter unit Microprocessor, HANNA instruments), sample data in Table 5.

A graph of pH values for enzyme-casein substrate was plotted against pH values of the enzyme – diet substrate and the slope of the graph used as the rate of pH change with time (Figure 1).The rapid protein digestibility (RPD) was calculated as the ratio of percentage of pH change (-Δ pH) in the enzyme-diet substrate to pH change of enzyme-casein substrate following a formula adapted from that of (Lazo, 1994) as:

$$\boldsymbol{RPD}\,(\%) = [(((-\Delta \boldsymbol{pH\ diet} / -\Delta \boldsymbol{pH\ Casein}) * \mathbf{10}\ )/\mathbf{160}) * \mathbf{100}$$

Where 10 = Number of incubation minutes; 160 = Amount of protein (mg) in feed substrate (Table 6).

Table 6. How protein digestibility was determined from changes in pH of casein and that of the diets (in triplets).

Change in casein pH	Change in diet pH	Ratio of change in diet pH to change in casein pH	Estimated diet digestibility (%)
0.0048	0.0361	7.520833	47.00521
0.0073	0.0467	6.39726	39.98288
0.0048	0.0345	7.1875	44.92188

Table 7. Digestibility regression of diets incorporated with sprouted sorghum, protease, phytase and a combination of 500 units of phytase and 500 units of protease with the fish gut enzymes as the explanatory variable.

Enzyme type	Protein digestibility (%)							
	30 %		35%		50%		55%	
	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value
Fish enzyme	3.07	-	3.13	-	11.57	-	21.20	-
Sprouted sorghum	14.23	0.144	16.93	0.276	13.97	0.822	27.37	0.654
Protease	28.76	<0.001	43.61	<0.001	54.44	<0.001*	59.86	0.002*
Phytase	85.16	<0.001*	76.43	<0.001*	43.84	0.001*	33.20	0.290
Protease and phytase	13.67	0.165	17.67	0.252	7.20	0.682	20.57	0.963

*Significant at $\alpha \leq 0.05$.

Statistical analysis

All data was first entered into Microsoft Excel and later imported into STRATA statistical soft ware (version 14). A simple linear regression (ANOVA) analysis of protein digestibilities was conducted with fish gut enzyme as explanatory variable. Statistical differences were declared at 95% confidence interval ($p \leq 0.05$).

RESULTS

Protein digestibility was significantly higher for Phytase incorporated legume based diets (30 and 35% crude protein) and protease incorporated fish meal based diets (50 and 55% crude protein) than in catfish gut enzyme extract. Protein digestibility in diets incorporated with sprouted sorghum and a mixture of protease and phytase combined was not significantly different from that of catfish gut enzyme extract (Table 7).

Generally there was higher protein digestibility in 55% crude protein diets incorporated with protease and 35% crude protein diets incorporated with Phytase enzyme (Figure 2).Protein digestibility in protease incorporated diets increased with increasing protein concentration while digestibility in diets incorporated to phytase had general decline with increasing protein (Figures 3 and 4 respectively). However the highest digestibility (88.9 and 88.4%) was recorded with 750 units/kg of phytase followed by 70.1% in protease incorporated diets with 1000 units (Figures 3 and 4, respectively).

DISCUSSION

Significantly high protein digestibility in Phytase incorporated legume based diets (30 and 35% crude protein) and protease incorporated fish meal based diets (50 and 55% crude protein) was attributed to limited interference from impurities and antagonistic reactions of other enzymes that could have been present in the crude enzyme extracts from the catfish gut and sprouted sorghum extracts. The recorded protein digestibility with phytase and protease enzymes was however higher than those observed by Ali et al (2009); the while protein digestibility of diets incorporated with fish gut enzyme extract (3.07-21.20%) was lower than what he observed for fish meal (78.08%), soy bean meal (76.08%) and rice polish (35.86%) and Thai koi (*Anabas Testudineus*) gut enzyme extract. This was attributed to differences in gut physiology and composition of test diets with regard to ingredients, nutrient and antinutrient composition of diet ingredients (soy bean, common beans, wheat pollard, cassava, cotton seed cake).

Lack of significance on rapid protein digestibility recorded with incorporating sprouted sorghum and a mixture of protease and phytase at all diet protein levels compared with the catfish gut enzyme extract was thought to be due to antagonistic or proteolytic digestion of phytase by protease enzyme. Similar reports on reduced efficiency of protease in presence of phytase and xylanase enzymes were reported by Ravindran (2013) and Sultana et al. (2010). Degradation of phytase by proteases such as pepsin and trypsin-like enzymes in the fish stomach enzyme extract was also reported for most enzymes except for *Aspergillus niger*, *Escherichia coli* and some *Bacillus* species of which it is not clear whether *Bacilus lincheniformis* is among (Kumar et al.,

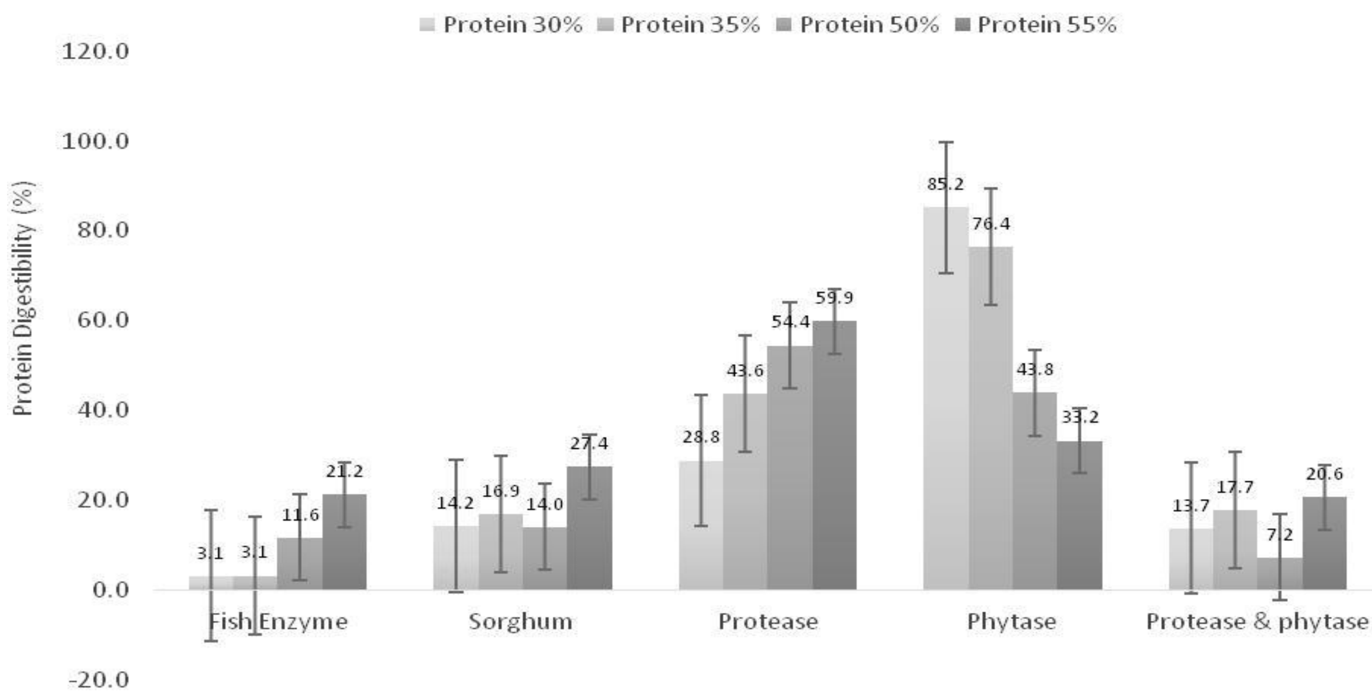

Figure 2. Rapid protein digestibility coefficients (%) of diets incorporated with catfish gut enzyme extract, sprouted sorghum (S. bicolor), protease (fungus *Trichoderma reesei*), phytase (bacteria *Bacillus lincheniformis)* and a combination of 500 units of phytase and protease.

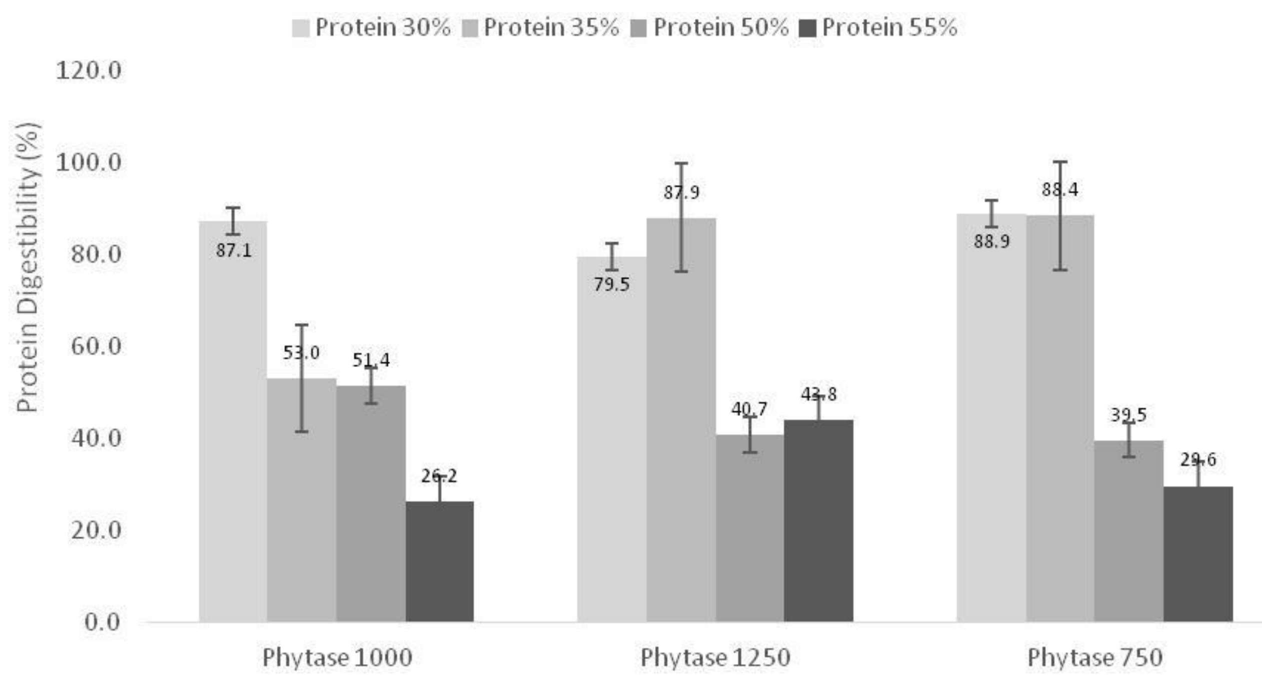

Figure 3. Mean protein digestibility of experimental diets incorporated with 750, 1000 and 1250 activity units of phytase enzyme.

2012b).

This implied that combining enzymes reduces efficiency than when used singly and required to be guided by such limitations or by compressive research on enzyme complementarily to maximize economic benefit of their applications.

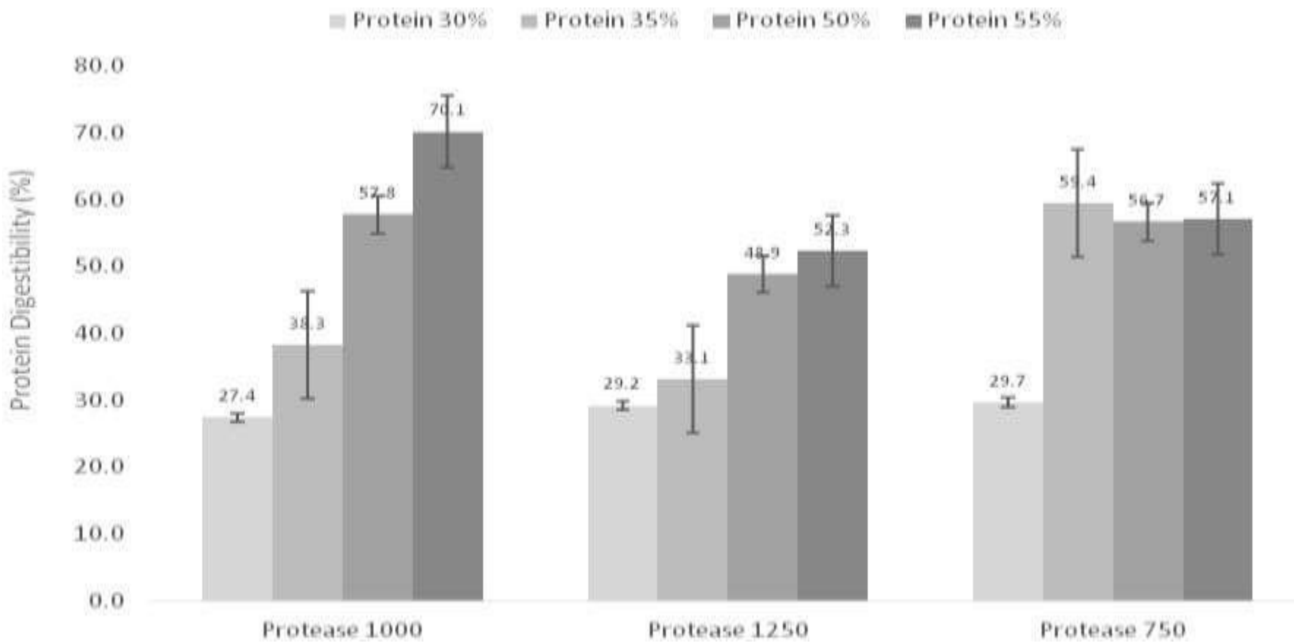

Figure 4. Mean protein digestibility in experimental diets incorporated with 750, 1000 and 1250 activity units of protease enzyme.

The significantly high protein digestibility in the fish meal based 50 and 55% crude protein diets incorporated with protease enzyme was attributed availability of sufficient dietary protein as substrate for protease enzyme than at low protein levels in grow out plant-based feeds (30 and 35%.CP). However, the percentage of protein that remained undigested could have been hindered by antinutrients in the plant protein/material that were included in these diets.

Conversely, the significantly high protein digestibility recorded in legume based grow out diets (30 and 35% crude protein) incorporated with phytase enzymes was attributed to high content of phytic acid bond protein in legume seeds which provided sufficient substrate for phytase enzyme. Most of the protein portions in dicotyledonous legumes which dominated these grow out diets are known to be closely linked to phytic acids with which they form inseparable/indigestible complexes unlike in monocots like corn and wheat where phytic acid is concentrated in germ and aleuronic layer (Chow and Schell, 1980). Breakdown of phytic acid by phytase enzyme should have been responsible for the more protein digestibility than with the case of protease which could have not got sufficient free protein to reduce into amino acids. This is in line with the theory of substrate-enzyme reaction which states that "at relatively low concentrations the rate of enzyme catalyzed reaction increases linearly with substrate concentration but is asymptotic at relatively higher substrate concentrations" (Sousa et al., 2015).

This implied that incorporation of protease is more beneficial in high protein catfish diets such as starter feeds while phytase is beneficial in low crude protein diets such as grower and finisher diets.

Conclusions

Incorporation of exogenous digestive enzymes generally increased protein digestibility in all fish diets than the catfish gut enzyme extract. Incorporation of phytase and protease enzymes however recorded significantly high protein digestibility if incorporated in legume based diets (30 and 35% crude protein) and in fish meal based diets (50 and 55% crude protein) respectively. Mixing protease and phytase enzymes into a single diet significantly lowered protein digestibility than using each enzyme singly. These results demonstrated that protein digestibility was more efficient with protease enzyme in high protein diets while phytase was efficient in low protein ones. This implied that use of protease was more beneficial in catfish starter feeds and phytase in grower/ finisher diets. They therefore provided a basis for selection of appropriate enzymes for production of cost-effective catfish diets at different growth stages.

Recommendations

For practical applicability, results of the study require confirmation with an *in-vivo* catfish feeding experiment with diets used here incorporated with sprouted sorghum; protease and phytase. Research on phytase and protease activities in the catfish enzyme extract and sprouted

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sorghum need to be determined for in depth understanding about the low protein digestibility.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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