IN VITRO DIGESTIBILITY OF FISHMEAL REDUCTION DIET IN COMBINATION WITH PROTEASE ENZYME BY NILE TILAPIA (*Oreochromis niloticus*) DIGESTIVE ENZYME

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Abstract:

The *in vitro* digestibility of fishmeal reduction diet in combination with protease enzyme by using Nile tilapia (*Oreochromis niloticus*) digestive enzyme was conducted and assigned in Factorial 2×2×3 in Complete Randomized Design (Factorial 2×2×3 in CRD). Factor A was fishmeal levels of fishmeal 5% and fishmeal 0%. Factor B was protease enzyme supplementation of no protease enzyme supplementation and with protease enzyme supplementation at 100 unit/Kg of feed. Factor C was Tilapia digestive enzyme of no Tilapia digestive enzyme supplementation, enzyme from Tilapia liver, enzyme from Tilapia intestine. Hence, the trial was conducted on 12 treatments and 3 replicates. The results showed that showed the high ability ($p<0.05$) of intestinal enzyme on protein, carbohydrate and phosphorus digestibility. The protease enzyme supplementation at 100 unit/Kg of feed showed no improving ($p>0.05$) of protein, carbohydrate and phosphorus digestibility. The zero fishmeal diet exhibited the better protein digestibility ($p<0.05$) than diet of fishmeal 5% but showed no significantly differences ($p>0.05$) on carbohydrate and phosphorus digestibility.

Keywords: *in vitro* digestibility; Exogenous protease; Tilapia digestive enzyme; Fishmeal reduction diet

Introduction

Digestion is a complex process in animals for maintenance their growth. The study on feed digestibility most focusing on *in vivo* digestibility in animal body which is complicate and costly. Many researchers work on the in vitro methodologies for predicting the potential bioavailability of a given nutrient or chemical substance. Moreover, some researchers work for a better understanding of the different processes, interactions and factors affecting the hydrolysis of protein, lipid and carbohydrate in feeds. The simulation of aquatic animal digestion is based on the methodologies that already developed for terrestrial animals and humans. In the study of fish, most publication have focused on salmonids, rainbow trout, gilthead seabream (*Sparus aurata*), bluefin tuna (*Tunnus thynnus*), common carp (*Cyprinus carpio*) and turbot (*Psetta maxima*) (Gomes *et al.*, 1994). The studies in fish to date have been concentrated on the nutritional quality of protein-rich feed ingredients in particular fishmeal and also have focused on the development of methods to assess carbohydrate hydrolysis (Cousin *et al.*, 1996; Omondi & Stark, 1996; Simon, 2009). A few studies have been conducted on the in vitro digestive evaluation of lipids (Koven *et al.*, 1997). The objective of this study was focus on the ability of Nile tilapia digest enzyme for digestion the diet composed of fishmeal, shrimp meal and animal protein compared to the digestion of fishmeal reduction diet

with increasing plant protein like corn gluten meal and investigated the ability of exogenous protease enzyme adding in fish diet for enhance feed protein digestion.

Materials and Methods

The *in vitro* digestibility of fishmeal reduction diet supplemented protease enzyme by Nile tilapia (*O. niloticus*) digestive enzyme was conducted. The mash feeds were incubated with Nile tilapia digestive enzyme from liver and intestine after that analysis the products liberated from the diets. The modified in vitro digestibility method of Rungruangsak-Torrissen, *et al.* (2002) was applied in this study.

Experimental diets

Two isonitrogeneous diets of 33.5% protein, isolipidic diets of 8.5% lipid of Tilapia feed were formulated. The material compositions presented in Table 1.

Table 1: Material compositions of Tilapia diets

Materials (%)	Diet A	Diet B
Fishmeal	5.0	0.0
Marine protein	2.0	0.0
Animal protein	15.0	10.0
Plant protein	41.0	56.0
-Soybean meal	36.0	36.0
-Corn protein	5.0	20.0
Cassava chip	31.1	26.1
Oil	4.0	6.0
Premix	1.9	1.9
Total	100.0	100.0

Experimental designed

To compare the digestibility of experimental diets from Nile tilapia digestive enzyme, the *in vitro* digestibility was designed in the Factorial 2X2X3 in Complete Randomized Design (Factorial 2x2X3 in CRD). Three factors of fishmeal levels, protease enzyme supplementation and Tilapia digestive enzyme were assigned. Factor A was fishmeal levels of 5%Fishmeal (5%FM) and 0%Fishmeal (0%FM). Factor B was protease enzyme supplementation of no protease enzyme supplementation (0protease) and with protease enzyme supplementation 100 unit/Kg of feed (100protease). Factor C was Tilapia digestive enzyme of no Tilapia digestive enzyme supplementation (no Tilapia enzyme), Tilapia liver enzyme, Tilapia intestinal enzyme. Hence, the trial was conducted on 12 treatments, as following:

Treatments
T1-5%FM-0proteas-no Tilapia Enzyme
T2-5%FM-0proteas-Tilapia liver Enzyme
T3-5%FM-0proteas-Tilapia intestinal Enzyme
T4-5%FM-100proteas-no Tilapia Enzyme
T5-5%FM-100proteas-Tilapia liver Enzyme
T6-5%FM-100proteas-Tilapia intestinal Enzyme

T7-0%FM-0proteas- noTilapia Enzyme
T8-0%FM-0proteas-Tilapia liver Enzyme
T9-0%FM-0proteas-Tilapia intestinal Enzyme
T10-0%FM-100proteas-noTilapia Enzyme
T11-0%FM-100proteas-Tilapia liver Enzyme
T12-0%FM-100proteas-Tilapia intestinal Enzyme

In vitro digestibility of diets

The *in vitro* digestibility of experimental diets was conducted by using Nile tilapia enzyme from live and intestine. Three replicates of each treatment diet were conducted. The experimental diets of fishmeal 5% in T1-T6 and fishmeal 0% in T7-T12 were incubated with and without adding Nile tilapia digestive enzyme for investigated the digestibility of all feed materials in diet of fishmeal 5% with 41.0% plant protein and zero fishmeal with 56% plant protein. The experimental diets without protease enzyme (0 unit/Kg of feed) with fishmeal 5% in T1-T3 and with fishmeal 0% in T7-T9 compare to diets of protease enzyme 100 unit/Kg of feed with fishmeal 5% in T4-T6 and with fishmeal 0% in T10-T12 were incubated with and without adding Nile tilapia digestive enzyme for investigated the efficacy of exogenous protease enzyme supplementation incorporation with Nile tilapia digestive enzyme for digesting all feed materials in diet.

Enzyme extraction

Twelve Nile tilapia with average weight of 58.65±4.75 g/fish was collected the whole digestive tract then separated liver and intestine for enzyme extraction. Each digestive organ was homogenized on ice in Tris HCl pH 7.4 (1:3 *w/v*) using a micro-homogenizer (THP-220, OMNI International, USA). The homogenate was centrifuged at 10,000*g* for 20 min at 4 ^{o}C and then supernatant was collected and kept at -20^{o}C until use for *in vitro* digestibility study. The total protein concentration of crude enzyme extract was determined according to standard method of Lowry *et al.* (1951) using bovine serum albumin as standard protein.

In vitro digestibility condition for nutrient digestion

The experimental diets were crushed to fine particle for study in vitro digestibility. Each sample was weighed then applied into phosphate buffer pH 8. Antibiotic was applied for control the microorganism. The mixture was pretreatment by curing for 1 h at room temperature 25 ^{o}C after that the reaction was stopped by immerged at 100 ^{o}C for 20 minutes and leave it cool down. The solution was divided into 3 parts.

Part 1: Collected the sample of each treatment (0hr.) stored at -20 °c for to be control

Part 2: The solution without adding any Nile tilapia enzyme and incubate for 16 h (16 h)

Part 3: The solution adding Nile tilapia digestive enzyme from liver or intestine and then incubate for 16 h.

Determination of protein digestibility

The *in vitro* digestibility of protein was studied by measuring the reactive amino group using the Ninhydrin assay. A solution of undigested control (0 h) and the digested mixture (16 h) were mixed with cd-ninhydrin reagent then incubated at 84 °C for 5 minutes and suddenly cool down on ice. The mixture was measured at 507 nm and calculated the amino acid concentration by using tyrosine as a standard. The *in vitro* digestibility of protein was expressed as mg Tyrosine/mg sample (Eid & Matty, 1989).

Determination of carbohydrate digestibility

The *in vitro* digestibility of carbohydrate was studied by measuring the increasing of reducing sugar using a colorimetric method with 3,5- dinitrosalicylic acid (DNS). The digested mixture was added DNS then incubate at room temperature (25^{o}C) after that the reaction was stopped by immerged at 100 ^{o}C for 5 minutes and cool down. The mixture was measured absorbance at 540 nm and compare with maltose standard curve. The *in vitro* digestibility of carbohydrate was expressed as mg maltose/ mg sample (Bedford & Classen., 1993; Miller, 1959).

In vitro digestibility condition for phosphorus digestion

The *in vitro* digestibility condition for phosphorus digestion was carried out follow the in vitro digestibility condition for protein and carbohydrate digestion. The experimental diets were crushed and weighed. Acetate buffer pH 5.0 was used for incubated the diets. Antibiotic was applied and the mixture was pretreatment for 1 h at room temperature (25 ^{o}C) then the reaction was stopped by immerge at 100 ^{o}C for 20 minutes and leave it cool down. The solution was divided into 3 parts of control (0 h), incubated solution without adding any Nile tilapia enzyme supplementation (16 h) and solution adding Nile tilapia digestive enzyme from liver or intestine then incubate for 16 hour (16 h).

Determination of phosphorus digestibility was conducted by measuring the orthophosphate liberated from diets. The assay mixture of acetone, H_2SO_4, $(NH_4)_2MoO4$ 2:1:1 was prepared. A solution of undigested control (0 h) and the digested mixture (16 h) were mixed with assay mixture and citric acid then measured at 355 nm. The orthophosphate liberated from diets was calculated by comparing to orthophosphate-phosphorus standard curve. The *in vitro* digestibility of phosphorus was expressed as mg phosphate-phosphorus/mg sample.

Statistical data analysis

All data means from the experiment were subjected to analysis in Factorial 2x2X3 in CRD by statistical software. Duncan's Multiple Range Test was applied to compare the difference between experimental groups (Steel & Torrie, 1980).

Results and Discussion

Protein and amino acid digestibility

The results of *in vitro* digestibility of protein to amino acid of fishmeal reduction diet in combination with protease enzyme by using Nile tilapia (*O. niloticus*) digestive enzyme was presented in Table 1. The result showed that diet of zero fishmeal with plant protein 56% (corn protein 20%) had higher protein digestibility ($p<0.05$) in term of amino acid liberated from the diet than diet of fishmeal 5% with plant protein 41% (corn protein 5%) which compose of cassava chip 31%. The fiber in cassava chip may confound the protein digestion of diet. Focusing on the efficacy of exogenous protease enzyme supplementation, there were not significantly differences ($p>0.05$) on the amount of the amino acid liberated from diet with and without exogenous protease enzyme supplementation due to the high digestibility of corn protein in the diet of zero fishmeal. Comparing the efficacy of enzyme from Nile tilapia digestive organ, liver and intestine, the results showed that Nile tilapia intestinal enzyme exhibited the high efficacy ($p<0.05$) to liberate amino acid from fish feed than activity of

enzyme from the liver and exogenous protease enzyme supplementation in fish diet. These indicated that exogenous protease enzyme and liver protease enzymes have lower ability to digest protein to amino acids due to the digestive protease enzymes from fish liver including pancreatic enzyme most are endoprotease like trypsin, chymotrypsin that can hydrolyze protein to peptide and release a little amino acid which different from protease enzymes from intestinal epithelial that are exoprotease such as aminopeptidase, dipeptidase, tripeptidase which digest peptide to amino acid (Lim and Webster, 2006). The diet of T12 0%FM-100proteas-Tilapia intestinal enzyme was the highest protein digestibility ($p<0.05$) follow by T6 5%FM-100proteas-Tilapia intestinal enzyme, T9 0%FM-0proteas-Tilapia intestinal enzyme and T3 5%FM-0proteas-Tilapia intestinal enzyme, respectively. The protein digestion from diets of fishmeal 5% and 0% both without and with exogenous protease enzyme supplementation including without Nile Tilapia enzyme supplementation were in the same range. It is indicated that the exogenous protease enzyme supplementation in the diet at 100 unit/Kg of feed had low ability to digest protein the diets compare to enzyme from liver and intestine of Nile tilapia. There are many factor related to the low protein digestibility of exogenous protease enzyme supplementation such as the low dosage of protease enzyme enzyme supplementation in the diet, loose of enzyme activity during extruded feed processing, quality of enzyme, etc. Therefore, diet of zero fishmeal with high plant protein of soybean meal and corn protein with protease enzyme was the high protein digestibility diet for Nile tilapia.

Carbohydrate digestibility

In vitro digestibility of carbohydrate in experimental diet by using Nile tilapia digestive enzyme was conducted. The results in Table 1 demonstrated that carbohydrate digestibility in term of reducing sugar content (the sum of glucose, xylose, maltose, and mannose) obtained from diet of fishmeal 5% and 0% showed no significantly differences ($p>0.05$) and also exogenous protease enzyme supplementation in the diets showed no significantly differences ($p>0.05$) on the carbohydrate digestibility of diets. The intestinal enzyme of Nile tilapia exhibited the high ability on the carbohydrate digestibility ($p<0.05$) than liver enzyme and native carbohydrase enzymes in the diets. Nile tilapia is omnivorous fish that has high ability to digest both plant and animal materials. Moreover, Tilapia has long intestine 5-15 times of body length, hence, Tilapia has high ability to digest or ferment fiber in their long intestine to be sugar and energy (Lim & Webster, 2006).

Phosphorus digestibility

The *in vitro* digestibility of phosphorus in experimental diets by using Nile tilapia digestive enzyme was presented in Table 1. The results showed no significantly differences ($p>0.05$) on phosphate-phosphorus obtained from diet of fishmeal 5% and 0%. The diets without and with exogenous protease enzyme supplementation also showed no significantly differences ($p>0.05$) on the phosphorus digestibility. The intestinal enzyme of Nile tilapia exhibited the high ability on the phosphorus digestibility ($p<0.05$) than liver enzyme and native phytase enzymes in the diets. Nile tilapia is omnivorous fish that has a little cellulase and phytase enzyme activity in digestive tract and has long intestine for digest or ferment fiber by water bone bacteria that release fibrolytic enzyme or carbohydrase including phytase to digest fiber and phytate in plant materials then releasing sugar and phosphorus (Lim & Webster, 2006).

Table 1: Amino acids liberated from in vitro digestion of Nile tilapia experimental diets

Treatments	Amino acids (g/100gfeed)	Reducing sugar (g/100gfeed)	Phosphate (g/100gfeed)
T1-5%FM-0proteas-no Tilapia Enzyme	1.11 ^{d}	5.44^{ab}	0.015 ^{d}
T2-5%FM-0proteas-Tilapia liver Enzyme	6.26 ^{c}	4.73^{b}	0.031 ^{bcd}
T3-5%FM-0proteas-Tilapia intestinal Enzyme	10.93 ^{b}	6.18 ^{ab}	0.050 ^{abc}
T4-5%FM-100proteas-no Tilapia Enzyme	1.32 ^{d}	5.65 ^{ab}	0.021 ^{cd}
T5-5%FM-100proteas-Tilapia liver Enzyme	7.10 ^{c}	5.52 ^{ab}	0.045 ^{abc}
T6-5%FM-100proteas-Tilapia intestinal Enzyme	11.37 ^{ab}	8.52 ^{a}	0.064 ^{a}
T7-0%FM-0proteas- noTilapia Enzyme	1.70 ^{d}	6.30 ^{ab}	0.036 ^{abcd}
T8-0%FM-0proteas-Tilapia liver Enzyme	7.83 ^{c}	5.73 ^{ab}	0.050 ^{abc}
T9-0%FM-0proteas-Tilapia intestinal Enzyme	11.36 ^{ab}	6.54 ^{ab}	0.052 ^{ab}
T10-0%FM-100proteas-noTilapia Enzyme	1.71 ^{d}	7.48 ^{ab}	0.036 ^{abcd}
T11-0%FM-100proteas-Tilapia liver Enzyme	7.86 ^{c}	6.15 ^{ab}	0.056 ^{ab}
T12-0%FM-100proteas-Tilapia intestinal Enzyme	12.72 ^{a}	8.68 ^{a}	0.057 ^{ab}
p-Value FM	0.007	0.191	0.066
p-Value Protease Enzyme	0.116	0.060	0.156
p-Value Tilapia Enzyme	<0.001	0.043	0.001
p-Value interaction FM*Protease	0.964	0.909	0.487
p-Value interaction FM*Tilapia enzyme	0.662	0.762	0.250
p-Value interaction Protease*Tilapia Enzyme	0.568	0.465	0.819
p-Value interaction FM*Protease*Tilapia Enzyme	0.503	0.886	0.983

Note : Data with superscript letters a,b,c in the same column indicates significantly difference (P<0.05)

Conclusion

The *in vitro* digestibility of fishmeal reduction with protease enzyme supplementation by using Nile tilapia digestive enzyme extract showed the high ability ($p<0.05$) of intestinal enzyme on protein, carbohydrate and phosphorus digestibility. The protease enzyme supplementation at 100 unit/Kg of feed showed no improvement ($p>0.05$) of protein, carbohydrate and phosphorus digestibility. The zero fishmeal diet exhibited the better protein digestibility ($p<0.05$) than diet of fishmeal 5% but showed no significantly differences ($p>0.05$) on carbohydrate and phosphorus digestibility.

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