POTENTIAL OF *IN VITRO* α-AMYLASE AND PROTEASE ASSAYS IN EVALUATING BROILER DIETS SUPPLEMENTED WITH MULTI-ENZYME PRODUCT

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ABSTRACT

To determine the potential of *in vitro* duodenal α-amylase and protease assays in evaluating broiler diets with in-feed enzyme supplementation, a total of 320 straight-run day-old broiler chickens were divided into four groups and randomly assigned to one of four dietary treatments (T1 is with breed-recommended nutrient levels, T2 is with reduced nutrient levels, T3 is T1+multi-enzyme, and T4 is T2+multi-enzyme) having eight replications in a 2 x 2 factorial arrangement with nutrient levels and enzyme addition as main effects. At the end of each of three feeding phases, representative samples of birds were sacrificed for collection of duodenal digesta for the *in vitro* α-amylase and total protease activity assays. Growth parameters were also calculated. Generally, there were no significant effects of diet on growth performance parameters, and on the α-amylase and total protease activity in all feeding phases. This study suggests that *in vitro* duodenal α-amylase and protease activity assays can potentially be used to predict the effects of in-feed multienzyme on the growth performance and, thus, the quality of enzyme-supplemented broiler feeds.

Keywords: α-amylase activity, duodenal digesta, feed evaluation, protease activity, SDS-PAGE

INTRODUCTION

Many researchers focused on improving broiler performance by conducting different feeding strategies to improve feed utilization. Feed contributes about 70% in the cost of intensive poultry production system (Chang, 2007). Furthermore, the profitability of the farm is highly dependent on the relative cost and nutritive value of the feeds. The ability of the animals to digest different components of the feed raw materials is one of the factors being considered in formulating the feed ration. Although there are many advances in the animal industry, the animals still could not optimally utilize the potential nutritional value of feedstuff leading to a greater production cost (Barletta, 2010).

Some compounds found in feeds cannot be digested by the animals which also hinder its digestive functions. The reason is that animals are incapable of producing necessary enzymes to degrade them. Thus, to aid the animal digestion, it is important to identify these indigestible compounds and add suitable enzymes produced by certain microorganisms under specific conditions to the diet to convert them into a more digestible form (Khattak

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et al., 2006). This method is one of the recent advances in poultry nutrition. Examples of these enzymes are proteases, carbohydrases, and phytases which are responsible for the hydrolysis of dietary protein, carbohydrates, and phytic acids respectively (Bedford and Partridge, 2001)

Little is known on the effects of the dietary multi-enzyme combination on the duodenal α-amylase and total protease activities and their relationship on the growth performance of broilers, hence, this study was conducted. *In vitro* α-amylase and protease assay has the capability of measuring specific enzymatic activities even with small sample size. The result of this study could determine the potential of *in vitro* assays; a more rapid and less tedious method, in evaluating broiler performance fed diets supplemented with feed enzymes, in comparison with feeding trials with larger sample size.

MATERIALS AND METHODS

Three hundred (320) straight-run day-old broiler chickens (>40 g) were randomly divided and assigned to one of four dietary treatments with 8 replicates per treatment and 10 birds per replicate cage in a 2 x 2 factorial arrangement with enzyme and nutrient levels with enzyme addition as main effects. The dietary treatments were corn and soybean meal-based diets with the following specifications: Treatment 1 (T1) – positive control diet with breed-recommended nutrient levels, Treatment 2 (T2) –reduced nutrient levels (see Table 1), Treatment 3 (T3) – T1 with 375 g multi-enzyme combination product (MECP)/ ton of feed, and Treatment 4 (T4) – T2 with 375 g MECP/ ton of feed.

Feeds and clean drinking water were supplied ad libitum. Birds were fed with booster, starter and finisher diets at 1-10, 11-24 and 25-40 days, respectively. Nutrient content of the different experimental diets is shown in Tables 1 to 3. Health programs including immunization and vitamin supplementation were strictly implemented. Sixty (60) broilers

Table 1. Nutrient content of experimental broiler booster diet.

Ingredients	**T1**	**T2**	**T3**	**T4**
ME, kcal/kg	3035.00	2958.00	3035.00	2958.00
Crude Protein, %	21.00	20.60	21.00	20.60
Crude Fat, %	5.72	3.35	5.75	3.37
Crude Fiber, %	2.59	2.67	2.59	2.67
Calcium, %	0.90	0.75	0.90	0.75
Total Phosphorus, %	0.75	0.57	0.75	0.57
Available Phosphorus, %	0.45	0.28	0.45	0.28
Digestible Lysine, %	1.18	1.15	1.18	1.15
Digestible Methionine, %	0.45	0.44	0.45	0.44
Digestible Met + Cyst, %	0.88	0.87	0.88	0.87
Digestible Threonine, %	0.77	0.74	0.77	0.74
Digestible Isoleucine, %	0.79	0.78	0.79	0.78
Digestible Valine, %	0.89	0.88	0.89	0.88
Sodium, %	0.16	0.14	0.16	0.14

were randomly selected for slaughter at the end of the feeding trial. Data determined were average body weight and body weight gain, feed intake, feed conversion ratio, and dressing percentage.

Duodenal digesta samples were collected from 3 birds per treatments that were slaughtered 2 hours after morning feeding. Digesta samples were diluted with 5x 0.01M Phosphate Buffer Saline (PBS) pH 7.2, transported in iced box and stored in -20 degrees until further processing. Proteins in the samples were isolated and partially purified through

Table 2. Nutrient content of experimental broiler starter diet.

Ingredients	**T1**	**T2**	**T3**	**T4**
ME, kcal/kg	3108.00	3020.00	3108.00	3020.00
Crude Protein, %	19.00	18.75	19.00	18.75
Crude Fat, %	6.66	4.81	6.69	4.82
Crude Fiber, %	2.56	2.70	2.55	2.70
Calcium, %	0.84	0.69	0.84	0.69
Total Phosphorus, %	0.71	0.56	0.71	0.56
Available Phosphorus, %	0.42	0.25	0.42	0.25
Digestible Lysine, %	1.05	1.03	1.05	1.03
Digestible Methionine, %	0.42	0.41	0.42	0.41
Digestible Met + Cyst, %	0.80	0.79	0.80	0.79
Digestible Threonine, %	0.69	0.66	0.69	0.66
Digestible Isoleucine, %	0.70	0.69	0.70	0.69
Digestible Valine, %	0.81	0.80	0.81	0.80
Sodium, %	0.16	0.14	0.16	0.14

Table 3. Nutrient content of experimental broiler finisher diet.

Ingredients	**T1**	**T2**	**T3**	**T4**
ME, kcal/kg	3180.00	3081.00	3180.00	3081.00
Crude Protein, %	18.00	18.00	18.00	18.00
Crude Fat, %	7.50	5.98	7.53	5.99
Crude Fiber, %	2.52	2.72	2.52	2.71
Calcium, %	0.76	0.61	0.76	0.61
Total Phosphorus, %	0.66	0.52	0.66	0.52
Available Phosphorus, %	0.38	0.21	0.38	0.21
Digestible Lysine, %	0.95	0.95	0.95	0.95
Digestible Methionine, %	0.39	0.39	0.39	0.39
Digestible Met + Cyst, %	0.74	0.74	0.74	0.74
Digestible Theonine, %	0.65	0.65	0.65	0.65
Digestible Isoleucine, %	0.64	0.64	0.64	0.64
Digestible Valine, %	0.73	0.73	0.73	0.73
Sodium, %	0.16	0.14	0.16	0.14

ammonium sulfate precipitation, membrane dialysis, and lyophilization. Protein concentration of the samples was then determined using Bradford assay following the protocol from the Bio-rad's Quick Start Bradford Protein Assay. Profiling and molecular weight determination of the proteins in the sample were estimated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970).

Alpha-amylase activity of the purified protein samples was determined through a method based on Dinitrosalicylic (DNS) calorimetric assay for reducing sugar following the protocols by Santos *et al*. (2016), Sigma-Aldrich's Enzymatic Assay of α-AMYLASE (EC 3.2.1.1), and Worthington's Amylase, Alpha Assay protocols. All samples were diluted to attain 50 µg/ mL protein concentration. Three sets of conditions were prepared: (1) 50 µL of 1% starch and 50 µL PBS pH 7.2; (2) 50 µL duodenal protein with 50 µL PBS pH 7.2; and (3) 50 µL duodenal protein with 50 µL 1% starch. After addition of 100 µL DNS reagent, samples were boiled for 10 minutes. After cooling to room temperature, 1 mL of distilled water was added followed by reading of absorbance as 540 nm. The unit of activity was defined as the amount of reducing sugar as glucose released per µg protein in 1 min at pH 7.2 at 37°C.

Bovine serum albumin (BSA) was used as a substrate to react with the total protease present in the duodenal samples. Four set-ups were prepared: 1) combination of 20 µL BSA and 20 µL duodenal sample, 2) duodenal sample only, 3) BSA only, 4) blank set-up with only 40 µL PBS. All set-ups were heated at 37°C water bath and allowed to react for 10 minutes. The reaction was stopped using 20 µL 5% trichloroacetic acid (TCA). To determine the amount of BSA hydrolyzed, 1 mL Bradford reagent was added on the set-ups, was stood for 5 minutes at room temperature and was read at 595nm. Total protease activity was defined as mg of BSA hydrolyzed per mL of duodenal digesta in 1 minute, at pH 7.2 and 37°C.

All data collected were subjected to analysis of variance (ANOVA) of 2x2 factorial in completely randomized design (CRD) using SAS (SAS Institute, 1994). Comparison of treatment means was done using Tukey's Honest Significant Difference (HSD) for growth performance parameters and Least Significant Difference (LSD) for Bradford and enzyme activity assays. The level of statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

The computed molecular weights (MW) of pure pancreatic α- amylase are 54.6 (Figure 1A) and 54.56 kDa (Figure 1B). Values are near the average MW of digesta α-amylase (54.8 and 54.06 kDa). Molecular weight of the α-amylase ranges from 50 to 60 kDa (Vihinen and Manantsala, 1989; Worthington Biochemical Corporation, 2015; Lehrner *et al*., 1975). Also, observed the molecular weight of the partially purified α-amylase from Pseudomonas sp. was at 62 kDa (Varalakshmi, 2012).

Trypsin, chymotrypsin, elastase, carboxypeptidase, and two sub-units of aminopeptidase bands shown in Figure for starter and finisher diets, showed marginal difference to the theoretical values (Trypsin =23 kDA, Chymotrypsin =25.6 kDA, Elastase =26 kDA, Carboxypeptidase =34.7 kDA, 2 sub-units of Aminopeptidase =66 and 94 kDA).

The specific amylase activity of the partially purified duodenal protein of broilers fed diets with multi-enzyme supplementation in all feeding stage showed no significant interaction within treatments (Table 4). Pinheiro *et al*. (2004) and Zhu *et al*. (2014) observed an increase in pancreatic alpha activity upon feed restriction and feeding with low metabolizable energy (ME) diets on 14-day-old broilers. The increase in enzyme activity could be one of the bird's mechanisms to adapt to low energy diets. Starch conversion rates of booster and starter

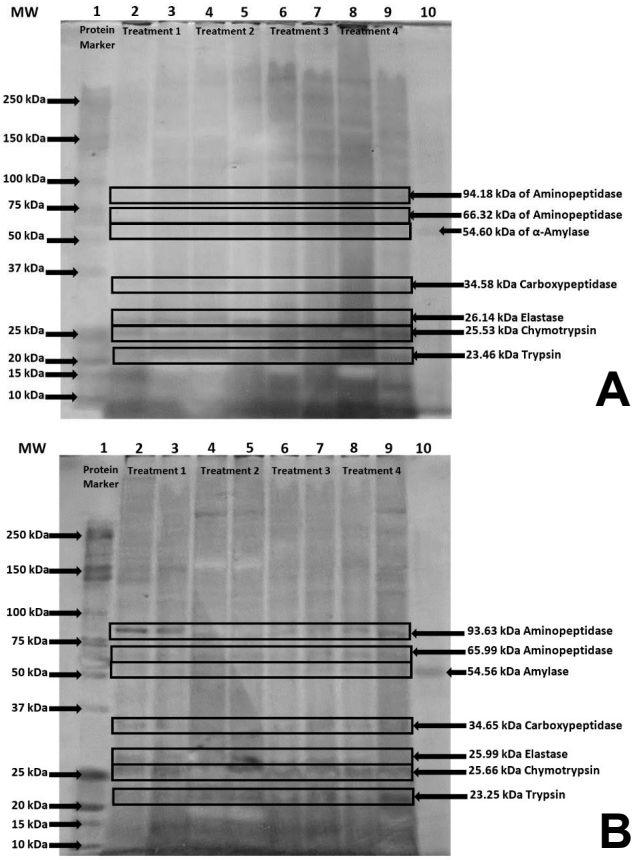

Figure 1. A. Protein profile of the duodenal crude protein of starter broilers supplemented with multi-enzyme combination determined by SDS-PAGE (resolving gel at 11%). Lane 1, Bio-Rad's Precision Plus Protein ™ Standards (MW ranges from 10 to 250 kDa); Lane 2 and 3 consist Treatment 1; Lane 4 and 5, Treatment 2; Lane 6 and 7, Treatment 3; Lane 8 and 9, Treatment 4; and Lane 10 contains pure pancreatic α-amylase. B. Protein profile of the duodenal crude protein of finisher broilers supplemented with multi-enzyme combination determined by SDS-PAGE (resolving gel at 11%). Lane 1, Bio-Rad's Precision Plus Protein ™ Standards (MW ranges from 10 to 250 kDa); Lane 2 and 3 consist Treatment 1; Lane 4 and 5, Treatment 2; Lane 6 and 7, Treatment 3; Lane 8 and 9, Treatment 4; and Lane 10 contains pure pancreatic α-amylase.

broilers fed with negative control decreased by half with supplementation of enzyme. This suggests that supplementation of multi-enzyme decreases the duodenal α-amylase activity of broilers fed with low ME diets. In the presence of exogenous enzyme, there was no need for birds to secrete more endogenous enzyme to compensate for the low ME.

It can be observed that positive and negative controls have very close glucose release values in the finishing stage. The same result was observed by Pinheiro *et al*. (2004) on 42-day broilers fed with restriction, which suggests that broilers' enzymatic response already adapted at this stage. This also suggests that the enzymatic activity of broilers follows a curve wherein there is maximum enzyme activity on early age but will eventually decrease at maturity.

Table 4. Average body weight, body weight gain, feed intake, feed conversion ratio and dressing percentage of broilers fed treatment diets.

Parameters	Treatment				SEM	P-value		
	T1	T2	T3	T4		Diet x MEC	Diet	MEC
Feed intake, g								
0 to 10 days	197	224	203	208	5.88	0.22	0.08	0.60
11 to 24 days	942[b]	1004[a]	1011[b]	945[a]	18.99	0.03	0.95	0.90
25 to 38 days	1628	1662	1624	1685	14.50	0.77	0.30	0.84
0 to 38 days	2767	2891	2838	2838	12.59	0.35	0.35	0.89
Body weight, g								
0 to 10 days	46	46	46	45	0.17	0.27	0.26	0.93
11 to 24 days	154	164	165	159	2.48	0.42	0.85	0.76
25 to 38 days	684	721	755	709	14.72	0.30	0.90	0.46
Body weight gain, g								
0 to 10 days	110	120	121	116	2.38	0.41	0.82	0.71
11 to 24 days	534	563	595	556	12.24	0.20	0.85	0.30
25 to 38 days	910	880	904	917	7.83	0.45	0.76	0.58
0 to 38 days	1535	1542	1599	1566	14.60	0.95	0.60	0.95
Feed conversion ratio								
0 to 10 days	1.84	1.93	1.72	1.83	0.04	0.90	0.35	0.30
11 to 24 days	1.78	1.79	1.71	1.73	0.02	0.99	0.81	0.36
25 to 38 days	1.79	1.89	1.80	1.84	0.02	0.37	0.03	0.56
0 to 38 days	1.81	1.88	1.78	1.83	0.02	0.72	0.17	0.39
Dressing percentage								
With giblets	80.87	80.4	80.45	79.38	0.32	0.53	0.24	0.16
Without giblets	76.11	75.66	75.85	74.14	0.44	0.28	0.07	0.13

[ab]Means within a row with different superscripts are significantly different at $P<0.05$.

The average proteolytic activity of the duodenal samples of broiler chickens fed with and without multi-enzyme addition at different ME level diets is presented in Table 5. The total protease activity is expressed as the μg bovine serum albumin (BSA) hydrolyzed/ min. Results show that there were no significant differences among treatments in all feeding phase. Thus, variation in ME level and the added multi-enzyme combination product in the diets had no effect on the total protease activity of the duodenal samples of the broiler chickens.

The result in the enzymatic assays is in line with the results of the feeding trial for broiler chickens. No significant interaction between treatments and feeding stage were observed in all other growth performance parameters except for the average feed intake at starter stage ($P<0.05$). The significant increase in feed intake can be attributed to the reduction in energy content of the negative control diet. Similarly, Rabie *et al*. (2010) found no significant effects on growth performance in all treatments using a Sicozyme (also a feed enzyme) combination. Furthermore, Nadeem *et al*. (2005) and Alam *et al*. (2003) also observed no significant difference on feed conversion ratio in broiler diets supplemented with multi-enzyme combination product. Consumption of more feeds may be one of the responses

Table 5. Total glucose released and total protease activity at 37℃ of digesta crude protein.

Parameters	Treatments				SEM	P- value		
	T1	T2	T3	T4		Diet x MEC	Diet	MEC
Total glucose released, µg Glu released/µg Protein/min								
0 to 10 days	5.25	11.82	14.40	5.12	2.26	0.11	0.77	0.80
11 to 24 days	2.78	3.53	1.69	2.57	0.68	0.73	0.42	0.35
25 to 38 days	4.90	5.45	6.19	5.34	0.32	0.33	0.83	0.41
Total protease activity, µg BSA hydrolyzed/min								
0 to 10 days	2.73	2.78	2.90	2.59	0.54	0.89	0.93	0.97
11 to 24 days	3.70	3.72	3.85	3.73	0.33	0.29	0.88	0.91
25 to 38 days	3.31	3.36	3.39	3.43	0.44	0.09	0.51	0.46

[ab] Means within a row with different superscripts are significantly different at P<0.05

of the birds to compensate to the deficit energy of the diet (Yu and Robinson, 1992). The study of Bharathidhasan *et al*. (2010) showed a significant increase in body weights only on broilers fed with diets supplemented with 750g and 1000g enzyme per ton of feeds over control.

Multi-enzyme increases the nutrient value of the feeds by improving the digestibility. The gastro-intestinal activity of multi-enzyme-supplemented animals may also result to reduction of endogenous amino acid losses (Zanella *et al*., 1998).

No interaction effect of diets and multi-enzyme combination supplementation on dressing percentage of broilers was observed (Table 4). Zanella *et al*. (1998) also found that enzyme supplementation did not affect the carcass weight of the broilers.

In conclusion, this study showed that multi-enzyme combination supplementation with an inclusion of 375 g per ton of feeds on diets does not significantly affect the α-amylase activity, total protease activity and growth performance of broiler chickens in all feeding stages. Furthermore, the *in vitro* feed evaluation method and feeding trials concluded the same result. Thus, *in vitro* enzymatic assays can be a useful alternative feed evaluation method.

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