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ENZYME ASSAY TO DETERMINE OPTIMUM CRUDE PAPAIN LEVEL ON PLANT PROTEIN DIETS FOR NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Abstract: **This study was conducted to evaluate the chemical composition, enzymatic activity and optimum crude papain level incorporation in plant protein based diets for Nile tilapia. The crude papain extract (CPE) was collected from unripe paw paw fruits and enzymatic activity determined by tyrosine method. The CPE was used to assay *In vitro* relative protein digestibility (IVRPD) using pH drop method. A diet (Diet 1) was formulated to contain 300g/kg crude protein (CP) using fishmeal (FM), soybean meal (SBM), canola meal (CM) and sunflower meal (SFM). The FM was replaced (10% CP basis) with either SBM, CM, or SFM (Diet 2, 3, & 4, respectively). In a factorial design the four diets were treated with 0.02%, 0.04%, 0.06% and 0.08% of CPE in triplicates and subjected to IVRPD. Crude papain extract and pure papain (standard) had protease activity of 1.9 u/mg and 1.55 u/mg at pH 7.5, respectively. Diet 1 had highest (P<0.05) digestibility (39.96%) and replacement of FM by SBM, CM and SFM reduced the digestibility to 35.84, 34.82 & 33.27%, respectively. Addition of CPE at 0.06% recorded the highest (P<0.05) IVRPD (39.16%). In conclusion, CPE can be used in plant protein based diets for Nile tilapia at 0.06%.**

Keywords: **Chemical composition, Crude papain, Enzymatic activity, *In vitro* protein digestibility, Nile tilapia, pH drop method.**

1. INTRODUCTION

Improving the nutrient digestibility and growth performance has been one of the most important nutritional aspects in animal production, be it in poultry, piggery or pisciculture [17]. In the utilization of dietary nutrients, the digestive enzymes play a vital role in catalyzing the hydrolytic reactions splitting the macromolecules into simple absorbable form of molecules [34]. According to [32, 10], not all compounds in animal feed are broken down by animals' own digestive enzymes, and so some potential nutrients are unavailable to the animal. However, although enzymes are produced by the animal itself or by the microbes naturally present in the digestive tract, specific activities necessary to break down some compounds in feed are not found or are at low levels in the digestive tract. Based on this, animal nutritionists help the animal by identifying these indigestible compounds and feeding a suitable enzyme [26]. Feed enzymes help break down anti-nutritional factors (e.g. fibre, phytate) that are present in many feed ingredients which interfere with normal digestion, resulting in reduced production and lower feed efficiency and can also trigger digestive upsets [13]. Among the exogenous enzymes, Papain crude extract is derived from the sap of unripe papaya fruits or directly from the fruit which are still hanging on the tree [30, 31, 38]. The extract is a proteolytic enzyme that can break down peptide bonds of a protein molecule [9] and its addition in fish feeds can improve nutrient utilization, thereby reducing nutrient losses [33].

In the aquaculture industry, the search for alternative protein sources to replace fish meal, plus concerns regarding the relatively low nutrient digestibility and the presence of an array of anti-nutritional factors in fish meal alternatives, has led to an increasing interest in feed enzymes and research for optimal applications [41]. However, few studies have evaluated enzyme supplementation in feed for aquatic organisms, and many dietary recommendations for aquatic organisms are based on results obtained for non-ruminants animals [15].

However, until now, no enzyme assay has been carried out to get a precise level of crude papain extract for use in Nile tilapia diets. In this regard, the method of *In vitro* protein digestibility can be employed to determine the best suited concentration of crude papain extract in plant protein based diets for Nile tilapia. According to [28], *In vitro* enzyme assays are less expensive, less time consuming and easier method for determining protein digestibility by enzyme. It allows for close observations of the dynamics of the breakdown of protein by using only small amount of raw materials [19]. The current study therefore was conducted to determine chemical composition, enzymatic activity and optimal crude papain enzyme concentration for use in plant protein based diets for Nile tilapia.

2. MATERIALS AND METHODS

Study site and diets

The experiment was conducted at Chuka University Animal nutrition and Biochemistry laboratory. The feed ingredients were sourced from local feed dealers. A control diet (Diet 1) of 300g/kg crude protein was formulated using fishmeal (FM), soybean meal (SBM), canola meal (CM) and sunflower meal (SFM). The test diets were formulated by replacing 10% CP of FM by SBM (Diet 2), CM (Diet 3) and SFM (Diet 4), respectively (Table 1).

Table 1: Ingredient composition (g/kg) and calculated crude protein (g/kg) of diets for Nile tilapia containing either soybean meal (Diet 2), canola meal (Diet 3) or sunflower meal (Diet 4) as replacements of fishmeal (Diet 1)

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	165	90	90	90
Soybean meal	130	240	150	160
Canola meal	165	160	310	150
Sunflower cake	180	190	180	430
Maize grain	180	160	130	100
Wheat bran	180	160	140	70
Total	1000	1000	1000	1000
Calculated crude protein (g/kg)	300.17	300.06	300.15	300.06

Collection of crude papain latex

Latex of *Carica papaya* was collected from locally grown plants in Imenti South District, Meru County, Kenya. Initially, 4 to 6 longitudinal incisions 3 mm deep were made on the unripe mature fruit surface from fruit stalk end to the tip of the fruit by using a stainless steel knife. The exuded latex was allowed to run down the fruit and drip into aluminum tray. The latex was then sun dried (40°C for 14 h) [3]. Using laboratory mortar and pestle, the dried latex was then ground to form a greenish or grey powder known as papain [3, 39, 12].

Amino acid and proximate analysis

The proximate analysis of ingredients and diets were carried out in triplicates as described in [8]. Amino acid analysis of the samples was performed by MPA FT-NIR spectrometer (Bruker, Germany) which is a non-destructive method of analysis. Near-infrared (NIR) spectroscopy is based on the absorption of electromagnetic radiation at wavelengths in the range of 780–2500 nm.

Enzymatic activity testing of crude papain extract

Protease activity was determined using the Hammersten casein as substrate following the procedure by [31]. The samples were passed unto 60 mesh sieve to obtain uniform sample size. Approximately 0.12 g sample was weighed and 10 ml of each buffer was added. The mixture was stirred for 30 min and then centrifuged for 5 min at 12,000 rpm to obtain a clear

supernatant and then diluted with the same buffer. The diluted enzyme solution was allowed to react with the substrate of desired pH for 10 min at 55°C. The reaction was stopped by addition of trichloroacetic acid and the amount of tyrosine released was determined spectrophotometrically using a standard curve at 280 nm. Analysis was based on one unit of protease activity which releases 1.0 micromole of tyrosine.

Determination of in vitro relative protein digestibility using crude papain extract

A 4 by 4 factorial design was adopted in this study considering, in triplicate, 4 diets with the addition of papain enzyme at 0.02%, 0.04%, 0.06% and 0.08%. *In vitro* methods for the protein digestibility assay were conducted using the pH drop method according to [28]. At first the diets (except casein) were finely ground with a mortar and pestle to pass through a 180-µm mesh screen. The diets were soaked with water overnight at 4^0 C. An equivalent amount of each diet that provided 312.5 mg of crude protein, determined by the respective material's proximate analysis was mixed with 50ml of distilled water and 0.02%, 0.04% 0.06% and 0.08% of crude papain enzyme to produce suspension of 8mg crude protein per ml. The mixture was kept at pH 8 with the addition of dilute sodium hydroxide (NaOH) or hydrochloric acid (HCl) and temperature of 37.5^0 C. The pH was recorded at every minute interval for 10 minutes by pH meter (H1 211 pH/ORP Meter, HANNA instruments). Casein was chosen as the reference protein because of its high protein digestibility (about 99%**) [**4]. The protein digestibility (PD) was calculated as the percentage of magnitude of pH drop (-Δ pH) ratio of the ingredient and casein [29] by the following the equation:

$RDP = (-\Delta\ pH_{Ingredients} \div -\Delta\ pH_{Casein})\ x100$ [28]

Where,

-Δ pH is the magnitude of pH decline in each assay

Data analysis

Proximate, enzymatic activity and relative protein digestibility data were subjected to a two way analysis of variance (ANOVA) using SPSS statistical package version 17.0 at P= 0.05 confidence level to determine whether there were significance differences and where the differences occurred, mean separation was done by least significance difference (LSD).

3. RESULTS

Proximate composition

Proximate composition of the four test diets is shown in Table 2. Crude protein values for diet 1, 2, 3 and 4 were near isoproteinous (30.57%, 30.76%, 30.34 and 31.35% respectively: P>0.05). Diet 1 had highest ash content (6.16) but low in crude fibre content (11.06%) with diet 4 recording highest crude fibre content (16.03%).

Table 2: Proximate composition of diets (%) for Nile tilapia containing either soybean meal, canola meal or sunflower meal as replacement of 10% (on cp basis) of fishmeal.

	Diet 1	Diet 2	Diet 3	Diet 4
Proximate composition				
Dry matter	90.90 ± 0.07^{dbc}	91.31 ± 0.16^{dbca}	91.00 ± 0.09^{dbca}	91.56 ± 0.19^{abc}
Crude protein	30.57 ± 0.43^{a}	30.76 ± 0.53^{a}	30.34 ± 0.31^{a}	31.35 ± 0.33^{a}
Ether extracts	7.55 ± 0.27^{cd}	7.67 ± 0.18^{cd}	10.75 ± 0.28^{a}	9.63 ± 0.18^{b}
Ash	6.16 ± 0.03^{abc}	5.60 ± 0.24^{abc}	5.40 ± 0.21^{dbc}	5.81 ± 0.17^{abc}
Crude fibre	11.06 ± 0.08^{d}	12.18 ± 0.12^{c}	13.37 ± 0.17^{b}	16.03 ± 1.00^{a}
Nitrogen free extracts	42.45 ± 0.21^{ab}	42.79 ± 0.65^{ab}	37.44 ± 0.56^{cd}	36.09 ± 0.51^{cd}
Neutral detergent fibre	24.07 ± 0.22^{cab}	24.41 ± 0.31^{abc}	24.41 ± 0.23^{bac}	23.08 ± 0.34^{d}
Acid detergent fibre	8.37 ± 0.25^{cd}	8.23 ± 0.30^{dc}	11.83 ± 0.20^{ba}	11.86 ± 0.47^{ab}

Note. Values are expressed as mean ± SE.$^{a, b, c, d}$ Values in the same row having different superscript letters are significantly different (P<0.05).

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Proximate analysis of dried crude papain extract

Proximate analysis of crude papain is as shown in Table 3. Crude protein content was highest (66.61%) and crude fibre recording least (1.57%). The ash content was 6.89% with ether extract recording 7.69%.

Table 3: Proximate analysis of dried crude papain extract

Dry matter	Crude protein	Ether extract	Crude fibre	Ash	Nitrogen free extract
93.55±.08	66.61±.38	7.69±.36	1.57±.33	6.89±.22	16.98±.62

Amino acid profile of crude papain extract

The concentration of amino acid in the crude papain is shown in Table 4. Glycine (23mg/100g) recorded highest concentration with cysteine (1 mg/100g) being least. Methionine was not detected. Isoleucine and lysine recorded the same concentration (9 mg/100g).

Table 4: Amino acid profile of crude papain extract

Amino acid	Concentration (mg/100g)
Essential amino acid	
Arginine	10
Histidine	2
Leucine	8
Isoleucine	9
Lysine	9
Cysteine	1
Phenylalanine	4
Threonine	7
Tryptophan	3
Valine	14
Non-essential amino acid	
Glycine	23
Tyrosine	15
Glutamine	8
Asparagine	10
Serine	10
Aspartic acid	7
Glutamic acid	7
Proline	8

Protease activity of crude and pure papain

The protease activity of crude papain and pure papain (standard) is shown in Table 5. Crude papain recorded highest activity of (1.9 u/mg) and pure papain (1.5 u/mg).

Table 5: Protease activity of crude and pure papain

Enzyme	pH	Protease activity †(units/mg)
Crude papain	7.5	1.9±0.13
Pure papain	7.5	1.5±0.11

Note. †One unit of protease activity is the amount of enzyme that releases1.0 micromole of tyrosine per minute

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Relative In vitro protein digestibility

Results for the diets and enzyme *In vitro* protein digestibility are as shown in Table 6. Diet 1 recorded highest *In vitro* protein digestibility (39.96%) with diet 4 recording least (33.27%). Diet 2 recorded 35.84% closely followed by diet 3 (34.82%). In the enzyme concentration, 0.06% recorded highest *In vitro* protein digestibility (39.16%) with 0.02% concentration recording least (30.23%). Enzyme concentration of 0.08% was the second in *In vitro* protein digestibility (38.65%) and 0.04% recording (35.86%).

Table 6: Relative *In vitro* protein digestibility of the four diets and crude papain enzyme at different concentrations

	Relative Protein Digestibility
Diet(D)	
1	39.96 ± 1.55^{a}
2	35.84 ± 1.12^{bc}
3	34.82 ± 1.17^{cbd}
4	33.27 ± 0.94^{dc}
Enzyme Concentration (EC)	
0.02	30.23 ± 0.64^{d}
0.04	35.86 ± 0.89^{c}
0.06	39.16 ± 1.16^{ab}
0.08	38.65 ± 1.00^{ba}
Level of significance	
Diet	P<0.05
EC	P<0.05
DXEC	P>0.05

Note. Values are expressed as mean ± $SE^{\cdot\ a,\ b,\ c,\ d}$. Values in the same column between diets or enzyme having different superscript letters are significantly different (P<0.05).

4. DISCUSSION

Chemical composition and enzymatic activity of crude papain

Proximate analysis of dried crude papain showed that crude protein content was high (66.61%) than that obtained by [31]. This variation in proximate composition of crude papain latex is probably due to species difference, soils and stage of latex extraction as reported by [1, 11] that there is increase in protein as fruit matures. Amino acid profile reported in this study was above figures obtained by [27] except for cysteine which was below (2.83mg/100g). However, in both studies, glycine was the predominant amino acid and no methionine was detected in the extract. The variation in amino acid profile of crude papain extract could be as a result of the source of latex, origin and variety of the paw paw tree.

The crude papain in the present experiment recorded higher protease activity (1.9u/mg) than the refined papain (1.5u/mg). This could be attributed to duration and storage conditions of the refined papain prior to use. Papain losses activity with longer storage period. However, in the case of crude papain, it was harvested and activity done after a shorter duration of storage hence could not have lost activity. The protease activity of crude papain at pH 7.5 in this experiment was lower than that recorded by [31] who reported activity of 2.66u/mg at pH 5.5 but (0.285u/mg) at pH 9. However, the activity recorded in this research was higher than (0.95u/mg) recorded by [44]. This variation in protease activity can be attributed to the differences in pH. According to [20, 22], the optimum pH for activity of papain is in the range of 3.0 - 9.0 which varies with different substrate. The conditions of acidity for the optimum action of papain are found to be pH 5. In the present experiment protease activity was tested at near neutral pH (7.5) and according to [31], papain is more active in slightly acidic medium than in a basic hence probable reason for the slightly low activity reported in this study. Several studies supported the idea that papain exhibits its greatest activity at an acidity equal to the concentration of the hydrogen ion of 10^{-5}N [31].

The crude papain latex was sun dried for 14 hrs following the procedure by [3]. This could not have affected the protease activity because papain as a cysteine hydrolase is active under a wide range of conditions and very stable even at elevated temperatures [16]. However, at temperatures over 55^{O}C, papain activity declines due to changes that occur at the active site [48]. Studies done on different methods to dry out raw papain found differences in enzymatic activity which was attributed to the loss of activity due to changes in the enzyme native structure during the drying process [18, 47, 42]. In this study, the latex was collected from greener, mature and unripe fruits. This could have contributed to the higher activity recorded at pH 7.5. The hydrolytic activity of the latex depends upon the state of development of the fruit, the greener the fruit; the more active is the papain [6]. A fully grown yellow fruits contains little latex and almost no enzymes. The high protein content (66.61%) of crude papain recorded in this study could also have led to increased protease activity. According to [1, 11], increase in proteolytic activity in mature fruits is associated with an increase in protein content of the fruit as it ripens.

In vitro protein digestibility

In testing of the *In vitro* protein digestibility, there was a general decrease in pH when different concentrations of crude papain were added to the substrate (diets) at pH 8.0. During proteolysis, protons are released from the cleaved peptide bonds at alkaline pH, resulting in a decrease in pH [37]. There was no significance in the interactions between diets and concentrations of enzyme used (Table 6) and so the interpretation of results was based on the main effects (diet and enzyme concentration). In the present study, ranking of diets by relative protein digestibility, from highest to lowest indicated that control diet provided highest ($P<0.05$) relative protein digestibility estimates (39.96%). This can be attributed to higher amount of fishmeal (165g/kg) in diet 1 compared to (90g/kg) in diet 2, 3 and 4 (Table 1). According to [5], the degree of protein hydrolysis decrease with increasing levels of plant proteins. Fish meal being animal protein ingredient is highly digestible and has low fibre content hence availing sufficient dietary protein as substrate for protease enzyme [25].

There was a general decrease *in vitro* protein digestibility with corresponding increase in crude fibre content of the diets ($P<0.01$). According to [14], enzyme activity is influenced by fibre which reduces *in vitro* enzyme activities. The mechanism of inhibition by most fibres might be due to absorption of enzymes into the fibre matrix [46], or unspecific bindings to the fibres [24]. Thus, in the present study, the activity of papain enzyme could have been inhibited by dietary fibre in the diets and other indigestible residues from food [35]. Therefore the decreasing relative protein digestibility for each assay despite enzyme concentration was related to increase in fibre content of the diets.

In the present study, different concentrations of crude papain (0.02%, 0.04%, 0.06% and 0.08%), were used in order to optimize the enzyme concentration. According to [7, 28], enzyme concentrations influence the sensitivity of *In vitro* assays. Less than optimum concentrations and combinations of enzymes may result in over estimates or under estimates of protein availability in feed ingredients [28]. Successive increase in the concentration of enzyme from 0.02% to 0.06% ($P<0.05$) led to increased relative *In vitro* protein digestibility. However, further increase in enzyme concentration from 0.06% to 0.08% ($P>0.05$) led to smaller increase in relative protein digestibility. Such decrease in reaction rate may be due to end product inhibition as a result of increased Enzyme-Substrate (E: S) ratio [2]. According to [43, 21], proteolysis often leads to an accumulation of digestion products and their subsequent interactions, which ultimately result in inhibition of the enzymatic reactions. Therefore, there is need for optimization of the Enzyme-substrate ratio which yields an accurate account of digestibility as in the present study. The limitations of employing closed *In vitro* assays as in the present experiment, is related to the potential for inhibition of the enzyme activity by reaction products and indigestible food residues [45]. 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" [25].

It's worth noting that, despite different concentration of enzyme, the relative *In vitro* protein digestibility figures for the diets were generally low across the diets compared to the standard ingredient (casein) [4]. This could have been attributed to the cross-binding of proteins from different ingredients which probably yield fewer degradable reaction products as a result of diet formulation process [49]. Also the buffering capacity of different protein sources as in the case of present study interferes with the *In vitro* protein digestibility. According to [40, 36, 50], the components of some food materials interfere with the pH drop due to their buffering capacity. This may have contributed to variation in observed proteolytic activity despite dietary protein in the diet being fixed (300g/kg CP). However, the slightly higher ash content (6.16) in diet 1 was unlikely to affect the *in vitro* protein digestibility. [23] reported no effect of ingredient buffering capacity on *In vitro* protein digestibility, among materials that had high ash content (6-10 times) with greater carbonate buffering capacity than other ingredients tested.

5. CONCLUSION

Based on the enzymatic activity and In *vitro* protein digestibility, it can be concluded that crude papain extract from unripe paw paw fruits can be incorporated in plant protein based diets for Nile tilapia at a concentration of 0.06%. However, the crude fibre content of the diets should be minimal for the enzyme to be more effective. More research need to be carried out on the crude papain extract inclusion in plant protein based diets for Nile tilapia and incorporation of cellulase in order to act on the fibre in the diets.

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