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Full Paper

Growth performance and feed utilisation of common lowland frog (*Rana rugulosa* Wiegmann) fed diet supplemented with protease from papaya peel

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Abstract: This study aims to investigate the effects of papain supplemented diet on the growth performance and feed utilisation of common lowland frogs, as well as the water quality of the frog culture system. A diet with 35% protein and 3,000 Kcal/Kg^{-1} and supplemented with papain extract was fed to the frogs for 90 days. The supplementation of the enzyme extract at 2% enhanced growth performance and feed utilisation of the frogs ($P<0.05$) without affecting the survival rate ($P>0.05$). Also, papain supplementation at 2% can reduce ammonia and nitrite levels in the frog culture system. Thus, water quality parameters, pH and temperature were within a standard range.

Keywords: common lowland frog, *Rana rogulosa*, papain, growth performance

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INTRODUCTION

The common lowland frog (*Rana rugulosa* Wiegmann) is one of the most economically important aquatic animals in Thailand, and it is cultured in all parts of the country, especially the southern, eastern, northern and north-eastern parts [1]. Because they are easy to culture, grow fast and have a good taste, these frogs are raised by frog farmers and exported to Vietnam for culture also [1-3]. Frogs are carnivorous amphibians that require a high-protein diet; however, without proper feed and feeding, they will grow to various sizes and then usually eat one another. Also, without complete protein digestion, a frog culture system can generate a considerable nitrogen waste into the environment. The different grades of feedstuff also affect frog digestion.

Papain is a simple protein which contains only amino acids and is devoid of carbohydrates. All the usual amino acids are present, with the exception of methionine. Papain contains no chromophoric groups other than its constituent amino acids, and it is rich in tyrosine and tryptophan [4]. It is a proteolytic enzyme present in the leaf and raw fruit of the papaya plant (*Carica papaya* L.), and helps break down proteins into smaller fragments [4]. The papaya fruit is a rich source of valuable proteolytic enzymes such as papain, chymopapain, caricain and glycyl endopeptidase, which can greatly aid in the digestive process [5]. Proteases from papaya peel can be extracted with water. The peel proteases are also more stable at pH ≥ 8 and 80°C, as opposed to latex proteases [6]. Papaya peel proteases are composed of papain and chymopapain as the major components [7-9]. Papain is a popular enzyme in meat tenderisers and in supporting protein digestion.

Papain has been added in fish feed to promote growth performance and feed utilisation for many aquatic species such as keureling fish [10] giant fresh water prawn [11] and *Cyprinus carpio* [12]. Moreover, it has been used as a protein hydrolysate in many feedstuffs such as fish viscera protein hydrolysate [13, 14] and soybean meal [15]. The ability of papain to support protein breakdown and accelerate digestion should be beneficial for frog digestion, as it stimulates the digestion of fats and proteins and hence should be useful in improving overall food absorption. Frog digestion depends on digestive enzymes, especially proteases, because the frog is carnivorous and has high protein requirements [3] Thus, with these properties of papain, we investigate its use as a feed additive in frog feed to improve digestion and promote growth.

MATERIALS AND METHODS

Experimental Frogs

Frogs (*Rana rugulosa,* Wiegmann), initial weight 34-45 g/frog, from a private farm were randomly cultured in 0.8-m^2 plastic tanks at a density of 10 frogs per tank. They were acclimated for 1 week with control diet (pellet feed without papain supplementation) before starting the experiment.

Raw Materials and Crude Papain Extract Preparation

The papaya (*Carica papaya* L.) ('Kag Dum'cultivar) was collected at a green stage from a plantation in Phetchaburi province, Thailand. It was washed and air-dried and the peel was separated and chopped into small pieces. The peel was weighed and blended with cold distilled water at 1:1 ratio for 5 min. in a blender and the blend liquid was filtered through a filter cloth and the obtained solution was then centrifuged at 1,000 × g at 4°C for 25 min [16]. The crude papain extract (supernatant) was collected for a papain activity analysis [16] and for mixing with the feed.

Experimental Diet

Frogs were fed a floating pellet diet (35% protein, 3,000 Kcal/kg^{-1} energy). The proximate composition of the diet was determined according to standard methods [17]**.** The crude papain extract was dissolved in distilled water at 0.5, 1 and 2 g/100 mL (0.5%, 1% and 2% respectively) and then each crude papain solution (10 mL) was mixed with 100 g of feed for each trial. After that the pellets were coated with a protein concentrate (Mario bio Product Ltd., Thailand) at 1%, incubated at 37°C for 1 h, and then dried at room temperature for 24 h. The dry pellets were placed in a covered plastic box and stored at room temperature.

Experimental Design

Each of the four treatments (including control) was randomly assigned to a group of 10 frogs (38.23-40.53 g/frog) in triplicate. All groups were fed the prepared diets to satiation twice daily for 90 days. Water in the culture system was completely changed every week throughout the study. During the experiment, mortality was recorded daily and the frogs in each tank were counted and weighed monthly. The growth parameters were calculated as described by Bagenal [18] as follows:

Weight gain (WG, g) = final weight (g) – initial weight (g) ;

Average daily gain (ADG, g/day) = weight gain (g) / time (day) ;

$$\text{Specific growth rate (SGR, \% /day)} = \frac{(\text{Ln final weight (g)} - \text{Ln initial weight(g)})}{\text{time (day)}} * 100$$

Survival rate (SR, %) = (number of surviving frogs /total number of frogs) * 100 ;

Feed conversion ratio (FCR) = feed consumed (g) / weight gain (g) ;

Feed conversion efficiency (FCE, %) = (weight gain (g) / feed consumed (g))*100 ;

Protein efficiency ratio (PER) = live weight gain (g) / protein consumed (g).

At the end of the experiment, 10 frogs from each group (n = 30 frogs/treatment) were used for carcass composition analysis and whole body proximate analysis. The proximate analysis of the bodies was analysed according to standard methods [17]. Water quality was monitored by determining pH, temperature, free ammonia and nitrite levels every week throughout the experiment.

Statistical Analysis

All data were analysed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. A significance level of $P<0.05$ was used.

RESULTS AND DISCUSSION

Papain Activity

In this study the peel of the papaya ('Kag Dum'cultivar) at a green stage was used because it is an agricultural waste. A previous study has indicated that this cultivar has the highest papain content [19]. The activity of the papain extract in this study was determined to be 3.65 U/mg protein/min., which is similar to the previous value (3.16 ± 0.46 U/mg protein/min. [19] as the papaya was collected from the same stage and plantation.

Growth Performance

The results of the growth performance are shown in Table 1. The FW, WG, ADG and SGR of frogs fed the diet supplemented with 2% crude papain extract are highest ($P<0.05$). However, the survival rates are not significantly different among the groups, ranging between 95-100% ($P>0.05$). When the focus is on growth performance, the results indicate that crude papain extract supplemented at 2% can promote outstanding performance in all parameters. The frog yields in all treatments are not significantly different ($P<0.05$) but the yield of frogs fed the diet supplemented with 2% crude papain extract has a trend of being highest.

Table 1. Growth performance of frogs fed diet supplemented with crude papain extract for 90 days

Growth performance	Crude papain extract supplementation (%)				P value
	0	0.5	1	2	
Initial weight (g/frog)	34.70 ± 0.20^{a}	33.50 ± 1.83^{a}	34.60 ± 1.12^{a}	35.40 ± 0.70^{a}	0.9440
FW (g/frog)	200.18 ± 3.38^{b}	187.20 ± 2.61^{d}	193.67 ± 3.91^{c}	206.29 ± 0.02^{a}	0.0365
WG (g/frog)	155.48 ± 0.44^{b}	146.59 ± 1.34^{c}	148.72 ± 2.93^{c}	172.38 ± 2.83^{a}	0.0285
ADG (g/frog/day)	1.73 ± 0.02^{b}	1.60 ± 0.03^{c}	1.65 ± 0.03^{c}	1.90 ± 0.02^{a}	0.0247
SGR (%/day)	1.87 ± 0.02^{b}	1.64 ± 0.02^{c}	1.84 ± 0.02^{b}	1.96 ± 0.03^{a}	0.0002
Survival rate (%)	96.67 ± 5.77^{a}	96.67 ± 5.77^{a}	95.00 ± 7.07^{a}	100.00 ± 0.00^{a}	0.8308
Frog yield (Kg)	19.35 ± 1.15^{a}	18.09 ± 1.08^{a}	18.39 ± 1.36^{a}	20.35 ± 1.03^{a}	0.2024

Note: Means within a row with different superscripts are significantly different ($P<0.05$, n = 30)

Papain plays a key role in the digestive processes, one of which involves the breakdown of tough protein fibres into short chain peptides and free amino acids, precursors for protein synthesis in all organs and tissues. In addition, good health is one factor that promotes growth performance. Papain is known to reduce inflammation in the body, most likely by scavenging damaged and oxidised proteins and breaking them down [7].

Furthermore, proteolytic enzymes such as bromelain, papain, pancreatin, trypsin, chymotrypsin and rutin are essential regulators and modulators of the inflammatory response; they increase the 'appetite' of macrophages and the potency of natural killer cells. Proteolytic enzymes also degrade pathogenic complexes that can inhibit the normal immune function [20]. Although this study did not touch on the immune system response, the results of the study do show a high number of healthy frogs with a survival rate of 95-100%. Zainal et al. [10] studied papain supplementation in keureling fish feed in which papain dosages were tested. They found that the optimum dosage of papain for keureling fish was 27.5 mg/kg^{-1} of feed. Singh et al. [12], found that papain supplementation at an optimal level of 2% in common carp feed resulted in a better growth performance.

Feed Utilisation

The results on feed conversion ratio and feed conversion efficiency of frogs fed diet with papain supplementation at different levels for 90 days show that the diet containing 2% crude papain extract significantly gives the highest value of feed utilisation ($P<0.05$), although the feed intake and PER do not differ significantly among groups ($P>0.05$) (Table 2).

Table 2. Feed utilisation of frogs fed diet supplemented with papain extract for 90 days

Feed utilisation	Papain extract supplementation (%)				P value
	0	0.5	1	2	
Feed intake (g/frog/day)	1.80 ± 0.07[a]	1.79 ± 0.04[a]	1.90 ± 0.24[a]	1.78 ± 0.18[a]	0.4936
FCR	0.96 ± 0.05[b]	1.05 ± 0.01[a]	1.04 ± 0.05[a]	0.91 ± 0.01[c]	0.0457
FCE (%)	103.83 ± 0.36[b]	95.50 ± 0.17[c]	95.86 ± 4.51[c]	110.41 ± 1.56[a]	0.0236
PER	4.37 ± 0.33[a]	4.26 ± 0.08[a]	4.10 ± 0.09[a]	4.39 ± 0.24[a]	0.4039

Note: Means within a row with different superscripts are significantly different ($P<0.05$, n = 30).

Papain improves feed utilisation because it helps break down protein into smaller strings before ingestion [4, 6]. This seems to be beneficial to the frogs, thus leading to a good feed utilisation. Papain has also been used to reduce pain, support gastrointestinal health and reduce inflammation [6]. Other papaya enzymes can also help with the breakdown of other 'troublesome' proteins such as gluten in wheat and casein in milk, which are often implicated in digestive problems. Muss et al. [21] also found that the papaya preparation Caricol® contributed to the maintenance of digestive tract physiology. Patil and Singh [11] found that a diet supplemented with 0.1% papain resulted in a better growth and feed utilisation among post-larvae *M. rosenbergii.* Choi et al. [22] studied the effects of papain and bromelain on the growth enhancement and immunity of grass carp (*Ctenopharyngodon idella*) and found that an addition of those enzymes could enhance the feed utilisation of the fish and improve its growth and immunity, the optimal levels being 1% bromelain or 2% papain.

Carcass Composition and Flesh Quality

The carcass composition of the frogs fed diet supplemented with crude papain extract at different levels for 90 days shows a significant difference in terms of edible flesh, viscera and skin *($P<0.05$)*, but it shows similar results in terms of bone and the hepatosomatic index (HSI) *($P>0.05$)*, as shown in Table 3. The percentages of edible flesh, viscera and skin of frogs fed the feed with 2% crude papain extract are highest, which is related to growth performance and feed utilisation, where this group of frogs utilised more nutrients, especially proteins than did other groups. Although, the PER values are not different among groups, the number and types of amino acids from protein digestion of frogs fed diet supplemented with 2% crude papain extract seemed to be more balanced than those of other groups as balanced amino acids effect a proper protein synthesis in many organs. Furthermore, the highest values of both the edible flesh and viscera from the 2% papain supplementation group seem to indicate that a high amount of protein from the diet is digested by the exogenous enzyme (papain) into short chain peptides and free amino acids. These results are similar to those of Klahan and Sirithanawong [23], who studied the effects of bromelain (a vegetable protease similar to papain) extracted from pineapple crowns on the growth performance and feed utilisation of common lowland frogs, whose diet was supplemented with bromelain extract for 120 days, and found that a feed with a certain amount of the extract gave the highest feed utilisation and percentage of edible flesh.

Table 3. Carcass composition and HSI of frogs fed diet supplemented with crude papain extract for 90 days

Carcass composition (% fresh weight) and HSI	Crude papain extract supplementation (%)				P value
	0	0.5	1	2	
Edible flesh	35.91 ± 0.20^{a}	32.46 ± 0.97^{b}	31.94 ± 0.16^{b}	36.58 ± 0.85^{a}	0.0389
Bone	26.23 ± 0.60^{a}	21.29 ± 2.39^{a}	24.28 ± 2.20^{a}	23.97 ± 3.54^{a}	0.3808
Viscera	18.85 ± 0.00^{a}	14.30 ± 0.68^{b}	14.51 ± 0.20^{b}	17.20 ± 1.06^{a}	0.0043
Skin	11.11 ± 1.30^{ab}	9.96 ± 0.76^{b}	9.63 ± 0.35^{b}	11.58 ± 0.09^{a}	0.0444
HSI	5.84 ± 1.09^{a}	5.64 ± 0.37^{a}	6.20 ± 0.35^{a}	6.72 ± 0.17^{a}	0.4123

Note: Means within a row with different superscripts are significantly different ($P<0.05$, n = 30).

The flesh quality was determined using a proximate analysis (Table 4), which shows that the percentages of protein and fat content of the flesh of frogs fed diet supplemented with crude papain extracts are higher than those in the control group ($P<0.05$). Short chain peptides and amino acids from protein digested with papain at a suitable level seemed to contribute to the synthesis of organs and muscles of the frogs. Furthermore, the results of Choi et al. [22] show that a mixture of 1-2% bromelain and papain can significantly enhance lipid accumulation in grass carp, similar to the observation in this study on a lipid accumulation in the edible flesh of frogs fed diet with papain.

Table 4. Proximate analysis of edible flesh of frogs fed diet supplemented with crude papain extract for 90 days

Proximate analysis (% dry weight)	Crude papain extract supplementation (%)				P value
	0	0.5	1	2	
Protein	85.93 ± 0.27^{c}	87.52 ± 0.21^{b}	89.87 ± 0.44^{a}	87.53 ± 0.21^{b}	0.0009
Fat	5.81 ± 0.12^{b}	7.10 ± 0.15^{a}	6.78 ± 0.41^{a}	6.79 ± 0.04^{a}	0.0188
Ash	5.31 ± 0.06^{a}	5.46 ± 0.01^{a}	4.99 ± 0.09^{a}	5.19 ± 0.19^{a}	0.0523
Fiber	0.10 ± 0.00^{a}	0.09 ± 0.00^{a}	0.10 ± 0.00^{a}	0.09 ± 0.00^{a}	0.4789

Note: Means within a row with different superscripts are significantly different (P<0.05, n = 30).

Water Quality

The effects of different levels of papain supplementation on water quality is shown in Table 5. The results show that there is a significant difference ($P<0.05$) in ammonia, nitrite and pH . This study focuses on nitrogen waste since frogs are carnivorous and have to receive a high amount of protein, which can generate nitrogen waste in water. It can be clearly observed that frogs fed diet with 2% crude papain extract have reduced excretion in the form of ammonia and nitrite, indicating a more perfect protein digestion by exogenous papain supplementation. When dietary protein is digested by papain, free amino acids and short chain peptides are released. After that, the frogs utilise the amino acids as energy source, and free amino acids flooding into the free amino acid pool are used for synthesis of protein, which is accumulated as tissue protein [24]. Frogs excrete nitrogen from amino acid catabolism mainly as ammonia, and ammonia represents a nitrogen waste. The amino group of amino acids is transferred to a glutamic acid form in the amino acid catabolism. Glutamic acid is transported into the mitochondria for deamination as ammonia, which is then further oxidised to nitrite [24, 25].

Table 5. Water quality in culture system for frogs fed with different diets for 90 days

Parameter	Crude papain extract supplementation (%)				P value
	0	0.5	1	2	
Temperature (^oC)	26.33 ± 0.51^a	26.00 ± 0.00^a	26.00 ± 0.00^a	26.33 ± 0.51^a	0.2061
Ammonia (mg/mL)	1.08 ± 0.22^a	1.16 ± 0.07^a	1.01 ± 0.13^a	0.78 ± 0.13^b	0.0171
Nitrite (mg/mL)	0.66 ± 0.12^a	0.54 ± 0.07^{ab}	0.72 ± 0.18^a	0.47 ± 0.08^b	0.0434
pH	7.56 ± 0.05^c	7.83 ± 0.51^b	7.83 ± 0.05^b	7.90 ± 0.00^a	0.0001

Note: Means within a row with different superscripts are significantly different ($P<0.05$; n = 30).

From Table 5, it is evident that the crude papain extract supplemented at 2% is suitable for decreasing both the ammonia and nitrite levels. These results are in contrast with those of Singh et al. [12], who found that papain did not affect the water quality and had no effect on the feeding response of fish, probably because they studied *Cyprinus carpio*, a herbivore that requires low protein.

CONCLUSIONS

Papain extracted from papaya peel is observed to be useful as a frog growth promoter. Crude papain extract supplemented at 2% can improve growth performance and feed utilisation as well as increase the percentage of edible flesh and reduce ammonia and nitrite in a frog culture system.

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