



The Characterization and Activity of Digestive Enzymes in Different Sizes of Common Lowland Frog (*Rana rugulosa* Wiegmann)

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Abstract The activity of serine and acid protease, amylase and lipase from a 3-day-old whole-body frog, the gastrointestinal tract of a 10-day-old frog, and the stomach, intestine and pancreas of a 45-day-old and a 60-day-old frog (*Rana rugulosa* Wiegmann) were determined. The results showed that the medium-age frog (45 days) appeared to have higher activity of serine protease and lipase than the old frog (60 days) and tadpoles (3 and 10 days), while the old frog had the highest activity of acid protease. The activity of serine and acid protease from the four ages of frogs had the high activity at pH 8-12 and 3 respectively, while the activity of both amylase and lipase were highest at pH 7. The activity of all kinds of enzymes were shown to have the highest activity in the pancreas while the activity of acid protease was found to have the highest activity in the stomach. These results will be used as the basis for suitable feed formulation and feedstuffs for different sizes of common lowland frogs so that optimum nutritional value and cost-effectiveness can be achieved.

Keywords common lowland frog, acid protease, serine protease, amylase, lipase

INTRODUCTION

Amphibian diets indicate that all adult amphibians are carnivores; most feed principally on insects, although many species eat a variety of invertebrates. The anuran larvae are herbivorous, so the diets include such vegetable matter as aquatic invertebrates (Duellman and Trueb, 1994). Some adult anurans, including *Rana* sp., feed on large prey items, e.g. small mammals, bird and other anurans. Hylid frogs (*Acris crepitans*) during post-metamorphic growth ingest increasingly larger prey items. The same trend for anurans, the function of developmental change from herbivorous to carnivorous feeding, varies. For this reason, digestibility is important and necessary. The digestive enzyme system includes protease, amylase and lipase which are basic enzymes necessary for digestion, especially for frog culture involving artificial feedstuffs, such as frogs and catfish feed that are high in nutrients, particularly protein. Amphibians have a gland in the mucosal lining which secretes mucus in which pepsinogen proliferates. Pepsinogen is the precursor of pepsin, released by the main cells in the stomach, and it degrades food proteins into peptides. Pepsin is one of three principal protein-degrading, or proteolytic, enzymes in the digestive system; the others are chymotrypsin and trypsin, which are alkaline, and serine protease which are produced by the pancreas. During the process of digestion, these enzymes, each of which specializes in severing the links between particular types of amino acids, collaborate to break down dietary proteins into their components, which can then be readily absorbed by the intestinal lining. In amphibians, digestive enzymes are generally considered to be absent in buccal secretions, though low concentrations of diastatic amylase have been reported in the secretions of the intermaxillary, lingual and pharyngeal glands in some species of *Rana* (Reeder, 1964). The digestive enzymes in the small intestine are secreted by the

pancreas (Duellman and Trueb, 1994). Lipase activity has been found in extracts of the pancreas, pyloric ceca and upper intestine, but almost non-existent in the stomach and lower intestine. Digestion is completed in the small intestine in which the content of the lumen is mixed with a variety of digestive enzymes by intermittent contraction of the muscular wall (Duellman and Trueb, 1994). The main aim of this study was to determine the activity of digestive enzymes, including protease (acid and serine), amylase and lipase, in four stages of common lowland frogs, given the fact that these are culture stages. The results from this study will be used as a basis to select feedstuffs and develop feeding formulations that are suitable for different stages of common lowland frogs, so that optimal nutritional values and cost-effectiveness can be obtained.

METHODOLOGY

Frog and Sample Preparation

One thousand 3-day-old frogs, five hundred 10-day-old frogs, one hundred 45-day-old frogs and fifty 60-day-old frogs from the Department of Fisheries, University of Phayao were used in this study. The 3-, 10-, 45- and 60-day-old frogs were fed with 52, 40, 35 and 30% protein feed, respectively, twice a day. The whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the intestine, stomach and pancreas of 45- and 60-day-old frogs were collected and weighed 16 hours after feeding. Samples were stored at -80°C until use.

Crude enzyme preparations

The whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the intestine and pancreas of 45- and 60-day-old frogs were prepared by the method of Gimenez et al., (1999) for the serine protease, amylase and lipase assayed and Klomklao et al., (2007) for pepsin assayed.

Enzymatic assay

Characterization of serine protease: Serine protease activity was monitored in triplicate by measuring the increase in cleavage of short chain polypeptides via the method of Bezerra et al. (2005), but with the slight modification of using azocasein as a substrate. Protease specific activity was expressed as units of change in absorbance per min. per mg. of protein of the enzyme extract ($\Delta\text{Abs}/\text{min}/\text{mg}$ protein). Acid protease activity was assayed in triplicate by measuring the increase in oligopeptides via the method of Klomklao et al. (2007), but with the slight modification of using hemoglobin as a substrate. The acid protease specific activity was expressed as units of change in absorbance per min. per mg. of protein of the enzyme extract ($\Delta\text{Abs}/\text{min}/\text{mg}$ protein). Amylase activity was determined in triplicate by the 3,5-dinitrosalicylic acid (DNS) method (Bernfeld, 1951) using starch as the substrate. The amylase specific activity was defined as the mmol. of maltose produced per min. per mg. of protein under the conditions specified. Lipase activity was measured in triplicate by a method modified from Markweg et al. (1995) using 0.01 M para-nitrophenylpalmitate (pNPP) dissolved in iso-propanol as substrate. The lipase specific activity was defined as the μmol of p-nitrophenol produced per min. per mg. of protein under the conditions specified.

Protein concentration: Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

This experiment was conducted according to a completely randomized design (CRD) and the data obtained from the enzyme activity in various pH buffers and ages of frog were subjected to a one-way Analysis of Variance (ANOVA) followed by a Duncan's new multiple range test.

RESULTS AND DISCUSSION

Optimal pH of serine protease

The activity of serine protease from the whole body of 3-day-old frogs was the highest at pH 11, the serine protease activity from the gastrointestinal tract of 10-day-old frogs was the highest at pH 9, and in the intestine and pancreas of 45- and 60-day-old frogs it was the highest at pH 9 and 10, respectively (Fig. 1). The pH range for the maximal activity of serine protease extracted from the pancreas, intestine and the whole body of small tadpoles (3 - 6-day-old frogs) was in the range 9-11. The results were in line with the work of Teo et al. (1990), who showed that the serine protease activity of common Malayan toads displayed a well-defined optimum at pH 7.2. These results were in line with studies of fish, such as Sheng et al. (2006) also reported that alkaline protease activity remained high over a wide range of pH values (6-11). Klomklao et al. (2011) reported that the optimum pH for Na-p-tosyl-L-arginine methyl ester hydrochloride (TAME) hydrolysis was 8.0 and that trypsin was stable over a pH range of 6.0-11.0. The k_{cat} -pH profile of serine proteinase-catalyzed reactions showed a bell-shaped curve, reflecting a maximum activity close to pH 8 (Wong, 1995). Proteases and other enzymes can also be classified and based on their optimal pH (acid or alkaline) and the tissues of production (gastric, pancreatic and mucosal) (Halver and Hardy, 2002).

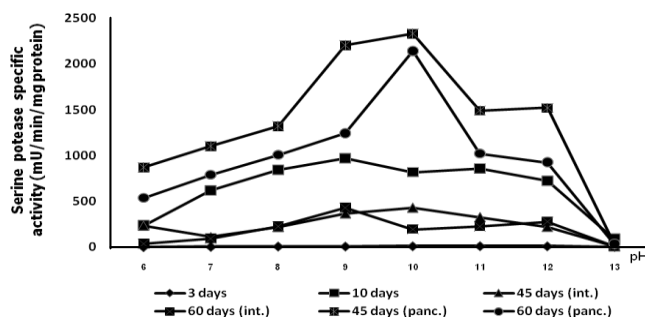


Fig. 1 Serine protease activity (specific activity) in the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the pancreas (panc.) and intestine (int.) of 45- and 60-day-old frogs at pH 6-13

Optimal pH of acid protease (pepsin)

The pepsin activity from the whole body of 3-day-old frogs was the highest at pH 2 and the activity of pepsin from the gastrointestinal tract of 10-day-old frogs and the stomach of 45- and 60-day-old frogs was the highest at pH 3 but not found to be active at over pH 4 (Fig. 2).

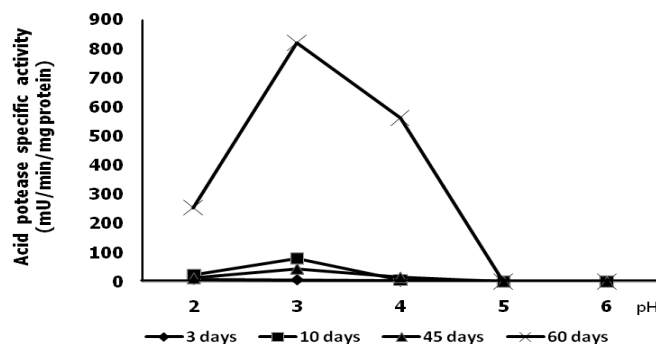


Fig. 2 Acid protease activity (specific activity) in the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs and the stomach of 45- and 60-day-old frogs at pH 2-6

Pepsin functions best in acidic environments; it is often found in an acidic environments, in particular those with a pH of 1.5 to 2, and pepsin denatures when the pH is more than 5.0. Most digestion of pepsin occurs in the stomach as a result of the action of pepsin (De Silva and Anderson, 1995). The results were in line with the work of Teo et al. (1990) who showed that the pepsin activity of common Malayan toads showed a well-defined optimum at pH 2-2.2. The results were similar with those of Tanji et al. (2007) who found that Pepsin-A from the gastric mucosa of African coelacanths, *Latimeria chalumnae* (Actinistia), had the highest activity at pH 2-2.5.

Optimal pH for amylase

The amylase activity from the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs and pancreas and the intestine of 45- and 60-day-old frogs was the highest at pH 7 and sub-optimal at pH 12 (Fig. 3). Amylase or α -amylase is a hydrolysis enzyme produced by the pancreas. By acting at random locations along the starch chain, α -amylase can operate anywhere on the substrate and tends to be faster acting than β -amylase. In animals, like frogs, α -amylase is a major digestive enzyme and its optimum pH range is 6.7-7.0.

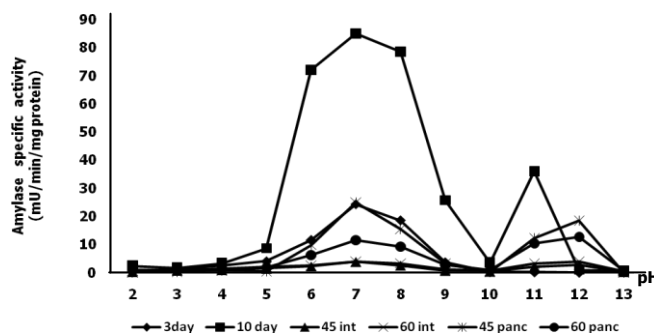


Fig. 3 Amylase activity (specific activity) in the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the pancreas (panc.) and intestine (int.) of 45- and 60-day-old frogs at pH 2-13

De Silva and Anderson (1995) stated that amylase had been identified in pancreatic juices, the stomach, the intestine and bile; however, the main producers were the pancreas and liver, which is consistent with this report, showing high activity of amylase in the pancreas and that it acts in neutral to alkaline environments in the upper intestine. The amylase activity of frogs lacks data, so these results were compared with those for other animals, such as fish. The amylase activity in all ages of frogs was similar to that in the study of Zeng and Cohen (2000) who showed peaks in amylase activity in the salivary glands of *Lygus hesperus* and *L. lineolaris* at pH 6.5. The maltase activity on carbohydrates in the small intestine of African clawed frogs (*Xenopus laevis*) was shown in the report of Sabat et al. (2005) who studied it at approximately pH 6.5 in their study. The optimal pH for amylase activity varies with the enzyme source and values reported for amylase in mammals are in the ranges of 6.0-7.0 and 6.0-8.0 with a temperature tolerance of between 20-40°C for porcine pancreatic α -amylase (Wong, 1995).

Optimal pH of lipase

The lipase activity in the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the pancreas and intestine of 45- and 60-day-old frogs was highest in the range pH 7-9. (Fig. 4). In addition, the highest activity of lipase was detected in the pancreas of 45-day-old frogs. Lipase or pancreatic lipase, also known as pancreatic triacylglycerol lipase, is secreted from pancreas and hydrolyze dietary fat molecules as triglyceride substrates in the digestive system to make monoglycerides and free fatty acids. The principal site for lipase activity is the mucosal layer, but there are no data for anurans. However, the primary site of lipid hydrolysis for most species

appears to be in the pyloric ceca and anterior intestine (Halver and Hardy, 2002), and all fat-digesting enzymes are known to act in alkaline media. The lipase activity data of frogs along with amylase activity have insufficient data so these results were compared with other animals, such as fish. Gjellesvik et al. (1989) showed similar results with cod lipase activity with optimal pH above 8.25, while Sheng et al. (2006) reported maximal activity of lipase in the intestine of hybrid juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) at a pH of between 6.0 and 9.0. All fat-digesting enzymes act in alkaline environment and optimal pH slightly varies from species to species.

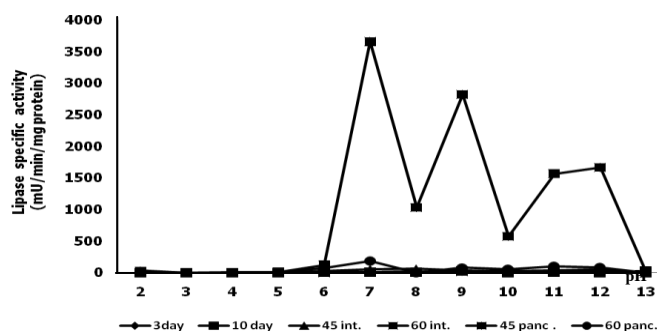


Fig. 4 Lipase activity (specific activity) in the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the pancreas (panc.) and intestine (int.) of 45- and 60-day-old frogs at pH 2-13

Lipase from the intestinal mucosa has an optimal pH of between 7.0 and 7.5, while intestinal esterase is at its most active between pH 8.0 and 9.0 (De Silva and Anderson, 1995), i.e. in agreement with the values reported here.

Digestive enzyme activity in various ages of frogs

Digestive enzyme activity in the whole body of 3-day-old frogs, the gastrointestinal tract of 10-day-old frogs, and the stomach, pancreas and intestine of 45- and 60-day-old frogs is summarized in Table 1.

Table 1 Activity of digestive enzymes extracted from various ages of common lowland frogs

Age (days)	Specific activity (mU mg protein ⁻¹)			
	Serine protease	Acid protease	Amylase	Lipase
3	66.39 ± 9.62 ^d	22.66 ± 1.68 ^d	59.75 ± 10.57 ^c	121.51 ± 16.44
10	5,159.2 ± 165.08 ^c	118.39 ± 17.09 ^c	297.26 ± 29.43 ^a	55.68 ± 5.24
45	11,989.57 ± 677.05 ^a	116.65 ± 3.60 ^b	104.82 ± 3.68 ^b	28,325.45 ± 1616.07
60	9,192.59 ± 250.23 ^b	1,698.77 ± 120.32 ^a	84.29 ± 1.63 ^{bc}	106.09 ± 2.82

^{abcd} Mean values in the same column with different letters are significantly different ($P < 0.01$)

Serine protease activity appears to be significantly higher in the pancreas and 45-day-old frogs had the highest activity ($P < 0.01$). The frogs' serine protease is produced by the pancreas which is most active in alkaline environments in the intestine (De Silva and Anderson, 1995). Pepsinogen is the inactive form of pepsin which is secreted by the esophagus, but it functions in the stomach in pepsin form which attacks protein. The old frogs had the perfect stomach to effect the highest rate of pepsin activity. For this reason, the stomachs of 60-day-old frogs showed the highest pepsin activity. A comparison of amylase activity in the four ages of frogs showed the highest activity in 10-day-old frogs. In general, amylase in animals is produced by the pancreas but *Rana spp.* found that low concentrations of diastatic amylase were secreted by the intermaxillary, lingual and pharyngeal

glands also. Amylase is the one of the carbohydrases and looks like the serine protease produced by the pancreas, so they are called pancreatic enzymes. Furthermore, adult amphibians are carnivores; they principally feed on insects, although many species eat a variety of invertebrates.

The anuran larvae are herbivorous while their diet includes such vegetable matter as aquatic invertebrates (Duellman and Trueb, 1994). Therefore, the young frogs had the highest amylase activity at 10 days old. Lipase activity was significantly higher in the pancreas of 45-day-old frogs ($P < 0.01$). From the results, the activity of protease and lipase, essential for the utilization of proteins and lipids from feed corresponding to high growth rates at early ages, were high in the 45-day-old frogs. Like serine protease and amylase, lipase is produced by the pancreas and acts in alkaline environments in the intestine. The pancreas in frogs is enclosed in the hepato-gastric ligament and lies between the duodenum and the stomach (Duellman and Trueb, 1994). It is a source of digestive enzymes.

CONCLUSION

The study clearly demonstrates variations in digestive enzyme activity (including pepsin and serine protease, amylase and lipase) in different ages of frogs. Serine protease and lipase activity was the highest in base pH (8-13) in medium-age frogs (45 days old), amylase activity was highest at neutral pH (7) in sub-medium frogs (10 days old), and acid protease (pepsin) was the highest at acid pH (3) in the large frogs. Digestive enzyme activity also depends on the organs studied. Protease amylase and lipase activity appears to be high in the pancreas while pepsin is more active in the stomach. The different rates of digestive enzyme activity in various ages of frogs can be used to select suitable feedstuffs for the highest digestibility and lowest cost in each age of frogs. Thus, the results from this study can be used as the basis for devising suitable feeding formulations that can be effectively utilized by different ages of frogs.

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