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Utilization of papain as feed additive in the fish feed on activity of digestive enzymes, contents of nutrient and minerals of Sangkuriang catfish (*Clarias gariepinus* var. *Sangkuriang*)

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Abstract. Papain is a proteolytic enzyme extracted from the papaya plant (*Carica papaya*) and has an important role in physiological functions. The study aimed to investigate the effects of papain as a feed additive in the fish feed, at dosages of 0 g kg^{-1} (diet A), 1.5 g kg^{-1} (diet B), 3 g kg^{-1} (diet C), 4.5 g kg^{-1} (diet D) and 6 g kg^{-1} (diet E), on the activity of digestive enzymes and on the contents of nutrients and minerals in Sangkuriang catfish (*Clarias gariepinus* var. *Sangkuriang*) for a 56 days observation period. The papain additive in the feed insignificantly increased the activities of α-amylase and trypsin. However, it significantly increased pepsin and lipase in the *C. gariepinus* digestive system. The addition of papain in the diet reduced the activities of alkaline phosphatase and leucyl aminopeptidase, but it did not affect the acid phosphatase enzyme. *C. gariepinus*, which was fed with papain additive, had lower protein content and body mass as well as calcium, iron, copper, and zinc contents.
Key Words: papain, digestive enzymes, mineral, nutrient, body mass.

Introduction. Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) is a freshwater fish commonly cultivated and consumed in Indonesia. As a response to the high demand for the catfish, farmers practice highly intensive system aquaculture. The successful, highly intensive aquaculture of *C. gariepinus* depends on the availability of high quality feed, containing the appropriate protein needed by the fish. Lack of feed efficiency increases the production cost with 50-60% (Hugues et al 2018). Medicines were frequently used as feed additives, in order to increase feed efficiency, growth and as immunostimulants (Rico et al 2013). Restrictions on the use of medicines as feed additives are adopted due to the negative impacts on the environment and health (Hassaan et al 2014; Reverter et al 2014). Since the ban on the use of antibiotics was enforced in European Union in 2006 and more restrictive use was imposed in other countries, natural bioactive substances were adopted (Christaki et al 2012; Hassaan et al 2018). Some of the requirements in the natural material selection as feed additives are to contain safe ingredients and exogenous enzymes (Lin et al 2007).

Papain is a proteolytic enzyme from the proteinases family. Papain is extracted from the leaf, unripe fruit and fruit sap of papaya (*Carica papaya*). According to Singh et al (2011), the papaya leaf contained 9% protein, 5.3% papain and vitamin C (286 mg (100 g)$^{-1}$) and vitamin E (30 mg (100 mg)$^{-1}$). Amri & Mamboya (2012) reported that papain could break down protein into amino acids, therefore, the fish could digest the feed more easily, in turn, it increased feed utilization. Kazerani & Shahsavani (2011) and Patil & Singh (2015) also reported that the papain enzyme increased feed utilization. Mo et al (2016) reported that the papain additive could break down plant-based protein. The study of papain on some fish species, such as Keureling fish (*Tor tambra*) concludes that for a mean weight of 0.30 g fish^{-1}, 27.5 mg of papain enzyme are needed in every kg feed in order to increase feed efficiency and growth (Muchlisin et al 2016). The post-larvae shrimp (*Macrobrachium rosenbergii*) needs feed containing 0.1% papain (Patil & Singh 2015). Some of the papain concentrations needed in feed in order to increase its efficiency and the fish growth are: 10 g papain kg^{-1} of feed for a *Labeo rohita* specimen with the mean weight of 4.0 g $fish^{-1}$ (Khati et al 2015), 1.5% of the feed content in *C. gariepinus* with the average weight of 1.48 g $fish^{-1}$ (Rachmawati et al 2019b), 2% of the feed content in *Cyprinus carpio* with the mean weight of 10 g (Singh et al 2011), 0.24-0.31% of the feed content in *Cherax quadricarinatus* with the mean weight 0.43 g $fish^{-1}$ (Rachmawati et al 2018), 4 g kg^{-1} of feed in *Pangasius hypophthalmus* with the average weight 2.23 g $fish^{-1}$ (Rachmawati & Prihanto 2019a).

It can be concluded from the studies that the need for papain enzyme was different, depending on the fish species and sizes (Patil & Singh 2015). The rationale of the current study is the lack of information on the papain used as feed additive in *C. gariepinus* culture. The purpose of the study was to examine the impacts of papain additive on the digestive enzyme activities, contents of nutrients and minerals in *C. gariepinus*.

Material and Method

Research design. This research is the follow-up of a study carried out in 2019, which examined the same topic about the addition of the papain enzyme in *C. gariepinus* feed (Rachmawati et al 2019b). The 2019 study observed protease enzyme activities (PEA), diet efficiency use (FEU), relative growth rate (RGR), protein efficiency ratio (PER), diet conversion ratio (FCR), and water quality, whereas this study examined the effect of papain additives on the digestive enzyme activities and on the nutrients and minerals content in *C. gariepinus*.

Test fish preparation. *C. gariepinus* specimens used in the study weighed around 5.35±0.24 g $fish^{-1}$, obtained from the Sido Makmur fish farmers in Tambaksari Village, Rowosari Subdistrict, District Kendal, Central Java, Indonesia. The tested fish was acclimated first in fiber plastic containers with the dimension of 1.5 × 1.5 × 1 m located in the Nutrient Laboratory, at the Center for Hatchery and Fresh Water Aquaculture, Muntilan, Central Java, Indonesia, for 2 weeks. During the acclimatization, the fish was given manufactured feed (at satiation) in the morning and afternoon, without any additional papain enzyme. To get rid of the remnant of the metabolism, the fish was let to fast for one day before the study started. Then the fingerling was selected and measured by direct observation in the same category of size, activeness, healthiness, and defectiveness (Rachmawati et al 2017).

Experimental diets. The feed used in the study contained 30% protein (Rachmawati et al 2019b) and Cr_2O_3 was added at a concentration of 0.5% (NRC 2011), as protein digestibility indicator, while the doses of papain additive were 0, 1.5, 3, 4.5 and 6 g kg^{-1} in diet treatments labeled A, B, C, D, and E, respectively. The modification of doses of papain enzyme was done according to the study conducted by Rachmawati et al (2019b). The study recommended that the optimum doses of papain enzyme additive in the diet were 1.67-1.8 g kg^{-1} to obtain the best feed efficiency and growth in *C. gariepinus* with a mean weight of 1.48±0.17 g $fish^{-1}$.

The papain enzyme was acquired from the Center for Brackish Water Aquaculture, Jepara, Central Java, Indonesia. The papain enzyme was diluted with 30 mL pure water for 500 g of feed, at 28°C, and then mixed with the ground feed until the mixture was homogenous. Pellets of 3 mm diameter were formed with the resulting mixture, according to the *C. gariepinus* fingerlings' mouth opening capacity, which were dried at the room temperature, packed in plastic bags and stored storage at 4°C. The tested fish was fed with a quantity of 3% of their biomass weight per day, during the 56 days of observation. 5 treatments were applied, in 3 replications. The size of the specimens was measured was conducted weekly during the study, in order to determine the growth of the catfish. The study used 15 plastic fiber containers with a dimension of

1.5 × 1.5 × 1 m, equipped with recirculation water systems and stocked with 25 *C. gariepinus* fingerlings.

Monitoring activities of digestive enzyme. 5 specimens by treatment were selected to sample the intestine and stomach. They were frozen with liquid nitrogen and stored at -80°C. The method of Wiszniewski et al (2019) was used to investigate the activities of alkaline phosphatase (ALP), phosphatase acid (AcP), leucyl aminopeptidase (LAP), amylase, lipase, trypsin and Pepsin. The observation of the enzymatic activity was repeated 5 times at 25°C.

Proximate analysis and minerals. Proximate analysis of the fish included raw protein, raw fat and ash, which were analyzed based on the AOAC (2016) method. The raw protein (Nx6.25) utilized a semi-automatic Kjeldahl method. The raw lipid analysis used an eter Soxhlet system method. Ash content was obtained by burning the fish in the oven at 550°C for 24 hours. Minerals analyzed, according to Wiszniewski et al (2019).

Water quality parameters. The water quality parameters were monitored daily and they were in the optimal range: dissolved oxygen of 3.2 to 5.6 mg L^{-1}; pH of 7.5 to 8.5; temperature 25.0 to 30.0°C; ammonia-nitrogen (NH_3–N) of 0.001 to 0.001 mg L^{-1} (Boyd 2003).

Observed parameters. The observed parameters were: digestive enzyme activities, based on the method of Wiszniewski et al (2019); the nutrients content, based on the method of AOAC (2016); minerals, based on the method of Wiszniewski et al (2019).

Data analysis. The analysis of variance (ANOVA) was used to analyze the data. When the results were significant at P<0.05 or highly significant at P<0.01, Duncan's Multiple Range test was executed (Steel et al 1996).

Results. The monitoring results on the digestive enzyme activities, as shown in Table 1, indicated that the activities of phosphatase, L-aminopeptidase, amylase and trypsin significantly (P<0.05) decreased for the fish fed with treatment B, C, D, and E, containing papain additive, except for the activities of alkaline. The papain additive also significantly (P<0.05) raised the activities of lipase enzyme, as shown in Table 1, in the treatments: B at 2.04 IU g^{-1}, C at 3.1 IU g^{-1}, D at 3.37 IU g^{-1} and E at 5.0 IU g^{-1} compared to the A treatment (without the papain additive). The activities of pepsin enzyme also raised as more papain enzyme was added into the feed, at 25.15, 21.85, 18.84, and 17.76 IU g^{-1} , in the treatments B, C, D, and E, respectively, compared to the results of the treatment A (without papain additive).

Table 2 displays the nutrient and mineral contents in the body of *C. gariepinus*. The histological influence of the papain additive on *C. gariepinus* was insignificant (P>0.05) related to the lipid content and moisture, but ignificant related to the protein content and the body mass. The specimens treated with the diets containing papain additive had a lower protein content than for the diet without the papain additive. The analysis of the minerals suggested that the papain additive in the feed had an insignificant (P>0.05) effect on calcium, magnesium, iron, copper, and zink of the Sangkuriang catfish. The fish fed with the papain additive (treatments B, C, D and E) had calcium, magnesium, iron, copper and zinc contents lower than without papain additive, as in the treatment A. The contents of natrium and phosphorus in the body of the *C. gariepinus* fed with papain additive were significantly (P<0.05) lower than wihtout papain additive. The results of the proxymate analysis and minerals are displayed in Table 2.

Table 1

Digestive enzymes in the digestive system of *Clarias gariepinus*

	Initial	Treatment				
		A	B	C	D	E
Alkaline phosphatase ALP[a] (IU g^{-1})	49.14±3.27[c]	69.36±4.23[a]	59.28±4.12[b]	57.31±4.28[b]	56.24±3.11[b]	54.56±3.42[b]
Acid phosphatase AcP[a] (IU g^{-1})	3.12±0.24[b]	4.02±0.23[a]	4.14±0.33[a]	4.25±0.26[a]	4.32±0.23[a]	4.43±0.21[a]
Leucyl aminopeptidase LAP[a] (IU g^{-1})	2.98±0.27[a]	2.59±0.21[a]	1.66±0.16[b]	1.63±0.15[b]	1.58±0.23[b]	1.49±0.22[b]
α-amylase[a] (IU g^{-1})	39.57±0.31[b]	49.65±0.67[a]	38.42±0.01[b]	37.23±0.03[c]	35.17±0.02[d]	33.52±0.01[d]
Trypsin[a] (IU g^{-1})	43.77±3.02[b]	68.59±1.52[a]	29.93±1.28[c]	27.62±1.25[c]	25.40±1.03[c]	23.47±1.42[c]
Lipase[a] (IU g^{-1})	4.23±0.27[e]	3.89±0.35[e]	5.93±0.36[d]	6.99±0.13[c]	7.86±0.24[b]	8.89±0.11[a]
Pepsin[b] (IU g^{-1})	5.97±1.13[c]	44.37±1.32[b]	69.52±1.42[a]	66.21±1.56[a]	63.21±1.32[a]	62.13±1.28[a]

Different letters in the same line show significantly different ($p < 0.05$). [a]in the anterior section of the gastrointestinal tract; [b]in the stomach.

Table 2

The results of proximate analysis and minerals of *Clarias gariepinus*

	Treatment A	Treatment B	Treatment C	Treatment D	Treatment E
Proximate analysis of fish composition (g kg^{-1} ww)					
Protein	172.3±8.1[a]	159.2±9.8[b]	156.1±8.3[b]	154.4±8.4[b]	153.3±9.2[b]
Lipid	89.64±4.43[a]	87.32±3.06[a]	85.71±3.19[a]	86.75±3.52[a]	85.24±3.41[a]
Moisture	758.7±10.2[a]	749.2±11.6[a]	745.2±12.9[a]	746.3±11.2[a]	742.5±12.2[a]
Proximate analysis of fish meat composition (g kg^{-1} ww)					
Protein	213.2±4.1[a]	194.3±6.3[b]	191.5±8.3[b]	190.8±7.6[b]	190.1±4.5[b]
Lipid	41.2±2.5[a]	40.9±1.9[a]	40.5±1.2[a]	39.8±1.5[a]	39.2±1.3[a]
Moisture	698.2±12.4[a]	690.3±10.2[a]	687.4±11.3[a]	682.5±12.2[a]	680.7±11.6[a]
Mineral analysis of fish meat composition (mg kg^{-1} ww)					
Potassium	289.22±21.5[a]	288.42±20.1[a]	281.03±20.4[a]	286.23±21.2[a]	282.25±20.2[a]
Calcium	443.16±12.8[a]	266.5±15.4[b]	263.7±16.4[b]	267.5±15.7[b]	268.1±15.4[b]
Magnesium	321.46±4.22[a]	227.46±5.36[b]	222.85±5.19[b]	219.43±5.36[b]	220.71±5.16[b]
Sodium	496.51±5.25[b]	582.29±4.34[a]	589.31±4.37[a]	585.28±4.32[a]	586.43±4.37[a]
Phosphorus	182.18±3.26[b]	215.25±4.31[a]	219.46±3.75[a]	221.14±3.20[a]	223.63±4.21[a]
Manganese	0.08±0.02[a]	0.08 ±0.02[a]	0.07±0.02[a]	0.07±0.02[a]	0.08±0.02[a]
Iron	4.57±0.13[a]	3.25±0.18[b]	3.38±0.11[b]	3.38±0.16[b]	3.28±0.14[b]
Copper	0.56±0.02[a]	0.42±0.05[b]	0.44±0.06[b]	0.45±0.05[b]	0.46±0.06[b]
Zinc	7.24±0.25[a]	5.37±0.23[b]	5.42±0.36[b]	5.51±0.27[b]	5.43±0.19[b]

Discussion. The addition of papain in *C. gariepinus* feed in order to observe the growth parameters, such as relative growth rate (RGR), efficiency of feed utilization (EFU), protein efficiency ratio (PER), feed conversion ratio (FCR), apparent digestibility of protein (ADCp) and survival rate (SR) has been reported by Rachmawati et al (2019b). The study concluded that the papain enriched diet's influence on the protease enzyme activities (reflected by EFU, RGR, PER, FCR, ADCp) was highly significant ($p<0.01$), otherwise it was insignificant ($p>0.05$) on the SR of *C. gariepinus*. The optimum dosages of papain enzyme for FEU, RGR, PER and FCR maximisation in *C. gariepinus* ranged from 1.67 to 1.89%.

The addition of papain as feed additive did not significantly increase the activities of α-amylase and trypsin. Otherwise, it significantly increased pepsin and lipase in the digestive system of the *C. gariepinus* (Table 1). Moreover, the papain-supplemented diet reduced the activities of alkaline phosphate and L-aminopeptidase, but it did not influence the phosphatase acid. This condition indicated that *C. gariepinus* could not excrete papain when added to the feed. The process was similar to the reduction of the secretion of endogenous enzymes, such as trypsin, by protease enzymes. This could explain why endogenous trypsin enzyme in the digestive system decreased for the *C. gariepinus* diet with papain additive, as recorded by He et al (2012) and Kvale et al (2007).

Ribeiro et al (2008) disclosed that digestive enzymes governed the nutrient absorption in the digestive system, being present in the gastrointestinal system. The enzyme activities in the digestive system affected the digestibility process and metabolism of the fish (Hakim et al 2006). Evaluations of the effects of papain additive on the digestive enzyme activities in the fish species were lacking until Rachmawati et al (2019b) reported that *C. gariepinus* with a size of 5-7 cm, fed with papain supplements, had higher activities of protease enzyme in the digestive system compared to those without papain supplementation. Lin et al (2007) stated that amylase activity increased in the intestine of the fish fed with an addition of mixed enzymes (protease neutral, ß-glucan, and xylanase), compared to a diet without enzyme addition. Otherwise, Liu et al (2018) suggested that protease activities in Gibel carp (*Carassius auratus gibelio*) was not affected by the protease enzyme in the feed.

The papain additive in the feed did not influence the fat and moisture level of *C. gariepinus*, but protein tended to decrease as the papain additive in the feed increased. A similar study was conducted by Song et al (2017) and the results showed that there was no difference on the fat content and moisture in the Vannamei shrimp (*Litopenaeus vannamei*) fed with or without a protease enzyme additive. However, the fish fed with the protease enzyme additive had a higher protein content. The content of micro and macro minerals could be observed in the meat of *C. gariepinus*, providing information about the effects of the papain addition on the decomposition minerals in fish.

Conclusions. The papain additive in feed did not significantly increase the activities of α-amylase, trypsin, and enzyme phosphatase acid. However, it significantly increased pepsin and lipase in the digestive system of the *C. gariepinus* and also decreased the activities of alkaline phosphatase and L-aminopeptidase enzyme. The protein content in the fish and body mass of the *C. gariepinus* fed with papain enzyme supplements was lower than that without the papain additive in the feed as well as the contents of calcium, iron, copper, and zinc.

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1	Grzegorz Wiszniewski, Sylwia Jarmołowicz, Mohamed S. Hassaan, Mohamed R. Soaudy et al. "Beneficial effects of dietary papain supplementation in juvenile sterlet (Acipenser ruthenus): Growth, intestinal topography, digestive enzymes, antioxidant response, immune response, and response to a challenge test", Aquaculture Reports, 2022 Publication	2%
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