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Digestive enzymes activities in *Oreochromis niloticus* fed diet supplemented with recombinant growth hormone

Aktivitas enzim pencernaan pada *Oreochromis niloticus* yang diberi pakan mengandung hormon pertumbuhan rekombinan

Apriana Vinasyiam, Muhammad Agus Suprayudi*, Alimuddin

Department of Aquaculture, Faculty of Fishery and Marine Science, Bogor Agricultural University
Kampus IPB Dramaga Bogor, West Java 16680
Email: agusuprayudi1965@gmail.com

ABSTRACT

The specific activity of the digestive enzymes, namely: pepsin, amylase, lipase, trypsin, and chymotrypsin were studied in Nile tilapia *Oreochromis niloticus* fed diet supplemented with recombinant *Ephinephelus lanceolatus* growth hormone (rElGH). The results showed that fish treated with rE1GH showed lower lipase and chymotrypsin specific activities (P>0.05), while the trypsin/chymotrypsin specific activity (T/C ratio) was found higher compared to control fish. Moreover, higher protein digestibility, higher protein retention and a lower ammonia excretion rate were measured for rE1GH treated fish. Oral rElGH administration enhanced Nile tilapia growth up to 20.04%, without affecting survival. This study suggested that rapid growth performance induced by rElGH was linked with T/C ratio rather than the specific activity of other digestive enzymes.

Keywords: recombinant growth hormone, digestive enzyme, digestibility, *Oreochromis niloticus*

ABSTRAK

Aktivitas spesifik enzim pencernaan pepsin, amilase, lipase, tripsin, dan kemotripsin diamati pada ikan nila *Oreochromis niloticus* yang diberi pakan mengandung hormon pertumbuhan rekombinan ikan kerapu kertang *Ephinephelus lanceolatus* (rElGH)). Hasil menunjukkan bahwa ikan uji pada perlakuan rElGH memiliki aktivitas spesifik enzim lipase dan kemotripsin yang lebih rendah, sedangkan rasio tripsin/kemotripsin (rasio T/C) yang lebih tinggi dibandingan ikan kontrol. Kecernaan protein dan retensi protein bernilai lebih tinggi sementara laju ekskresi amonia bernilai lebih rendah pada ikan perlakuan. Pemberian rElGH secara oral mampu mempercepat laju pertumbuhan ikan nila hingga 20,04% tanpa memengaruhi kelangsungan hidup. Berdasarkan penelitian ini, dapat disimpulkan bahwa laju pertumbuhan cepat yang diinduksi oleh rElGH berhubungan dengan rasio T/C dibandingkan dengan aktivitas spesifik enzim pencernaan lain.

Kata kunci: hormon pertumbuhan rekombinan, enzim pencernaan, kecernaan, *Oreochromis niloticus*

INTRODUCTION

Growth rate is a very important parameter in aquaculture that determines time required to produce marketable size of fish. Different methods oriented by molecular, environmental, and pharmacological approaches have been developed to stimulate growth rate and reduce culture time. Transgenesis (Guan *et al*., 2008), diet composition (Higgs *et al*., 2009), and other methods have been developed and showed significant results in improving growth rate. However, little attention has been paid on using recombinant growth hormone (rGH) (Haghighi *et al*., 2010).

It is generally known that growth hormone affects fish growth through both direct and indirect mechanisms (Reindl *et al*., 2011; Fuentes *et al*., 2013; Talwar *et al*., 2013). Direct pathway leads growth hormone to directly bind its receptor and induce growth in target cells, while in indirect pathway rGH is mediated primarily by insulin-like growth factor-I (IGF-I) produced in liver (Eppler *et al*., 2007, Ohlsson *et al*., 2009). However little is known about the actual relation between growth hormone and digestive enzyme activity.

rGH synthesis has been reported to be succesfully done in Nile tilapia *Oreochromis niloticus* (Acosta *et al*., 2007), giant grouper

Ephinephelus lanceolatus, giant gourami *Osphronemus goramy*, and common carp *Cyprinus Carpio* (Alimuddin *et al*., 2010). Its activity in stimulating the growth has been documented for different fish species such as channel catfish (Silverstein *et al*., 2000), rainbow trout *Oncorhynchus mykiss* (Haghighi *et al*., 2010), Nile tilapia (Alimuddin *et al*., 2010; Bakar *et al*., 2012), eel *Anguilla* sp. (Handoyo *et al*., 2012), and white shrimp *Litopenaeus vannamei* (Subaidah, 2012).

Digestion system has been hypothesized to intensely affect the growth of fish. The quantity of nutrient used for growth is not only determined by the quantity and quality of the feed, but also by fish capability to digest the feed. That capability is strongly related to level and proportion of digestive enzyme which affect nutrient absorption in gut, and energy conversion (Lin & Luo, 2011; Thongprajukaew *et al*., 2011). Present study aimed to investigate the effect of dietary supplementation of rGH on the digestion system using Nile tilapia as a model fish. Observation was conducted on the specific activity of digestive enzymes including protease (pepsin, trypsin, and chymotrypsin), lipase, and amylase in stomach and intestine of fish.

MATERIALS AND METHODS

rGH Preparation

Production of recombinant *E. lanceolatus* growth hormone (rElGH) was performed according to Alimuddin *et al*. (2010). *Escherichia coli* strain BL21 harboring the pCold-ElGH protein expression vector was used as a bioreactor to produce rElGH. In the present study crude protein extract of rElGH was used as a feed supplement. Previous study on quantification of rElGH using CBB (coomassie brilliant blue) staining method and luminescent image analyzer (LAS-4000 mini, Fuji Film) and UN-SCAN-IT gel 6.1) showed that 1 g of crude protein extract of rElGH contain approximately 11,65 mg rElGH pure protein (Irmawati *et al*. 2013).

The presence of rElGH protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, which distinguishes and characterizes proteins based on their molecular weight (Kinoshita *et al*. 2012). A band appearing in the position of 25 kDA (Fig. 1), which was the protein molecular weight of rElGH, characterized the presence of rE1GH (Handoyo *et al*., 2012; Irmawati *et al*,. 2013).

Diet preparation

The rElGH at a dose of 3 mg wet weight/kg feed was determined as the working dose based on the results of our previous study (unpublished). The hormone was mixed with chicken-egg yolk (5 mg/kg feed) as the binder. The mixture was then sprayed onto a commercial floating feed. Chicken-egg yolk was also sprayed to control feed. For digestibility test, Cr_2O_3 was also added as a tracer at a level of 0.6%.

Fish rearing and experimental protocol

Nile tilapia strain NIRWANA used for the study was obtained from BPBIAT Wanayasa (West Java). Prior to experimentation, fish were acclimatized in laboratory environment for two weeks in a 500 L circular tank. Fish were then weighed and placed in 40×50×50 cm^3 aquarium, previously filled with 60 L of fresh water, at a density of 15 individual per aquarium and at initial weight range of 5–6 g/ind. Each aquarium was equipped with aerator, heater, and individual filter. Unconsumed feed was removed daily, and 70% of the water was renewed two to three times a week. The water temperature and pH were maintained at the range of 28–30 ^oC, and 6.91–7.47, respectively.

The study consisted of two treatments, fish fed with and without dietary rElGH supplementation. The rElGH-treated fish was fed with rElGH diet twice a week (with 2–3 days interval, and with non-supplemented feed for the remaining days. Treatment was administered for eigth weeks. Control fish was only fed with non-supplemented diet. Fish were fed twice a day (at 09.00 and 15.00) at a fixed feeding rate 4–5%/day of average fish biomass in both groups. Feed requirement for both groups were adjusted every two weeks by feeding the fish to satiation. Both groups were given the exact same amount of feed to provide the same digestive substrate quantity for the enzyme to the gut.

Specific activity of digestive enzyme

Digestive enzyme activity was measured after eight weeks of culture. Crude enzyme extract was prepared by homogenizing intestine or stomach organ separately in distilled water. The extract was then stored at -80 ^oC until further analysis. Protease analysis was performed according to Walter (1988) using casein as a substrate and tyrosine as a standard. One unit specific activity of protease was defined as 1 mg tyrosine transformed within ten minutes at 37 ^oC.

The specific activity of amylase was determined according to the Bernfeld (1955) method, using starch as a substrate and maltose as a standard. One unit specific activity of amylase was expressed as 1 mg maltose released from starch within three minutes at 20 °C and pH 6.9. Specific activity of lipase was assessed based on the protocol by Borlongan (1990) with olive oil as a substrate. Fatty acid, derived from enzymatic hydrolysis of triglyceride on stabile emulsion of olive oil, was titrated with NaOH. One unit of specific activity of lipase was determined as the volume of NaOH 0.05 N needed to neutralize fatty acid released after 6 hours-long incubation with substrate.

Fish growth, survival, and FCR

Sampling of total biomass per aquarium was conducted every two weeks. Survival was calculated using the following formula: survival rate = number of fish alive/ number of initial fish stock in the aquarium × 100. Food conversion ratio (FCR) was calculated according to Huisman (1987).

Proximate analyses, protein and lipid retention Treatment feed, initial and final fish whole body and feed were analyzed by proximate. Three fishes from each replicate tank were weighed, dissected, pooled together and analyzed. Proximate analysis was conducted according to Takeuchi (1988). The nutritional compositions of the experimental diet were shown in Table 1. Protein and lipid retention were calculated according to procedure applied by Takeuchi (1988) at the closing day of experiment.

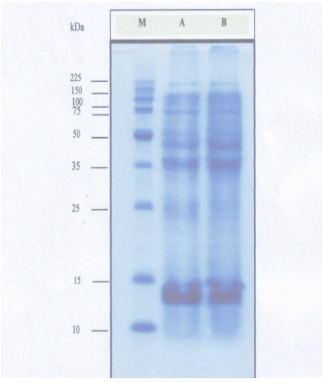

Figure 1. Result of SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of protein recombinant *Ephinephelus lanceolatus* growth hormone (rElGH). M: marker, A: crude protein of inclusion body containing rElGH. Arrow showed rElGH protein band, number in the left showed marker size of protein molecular weight (kDa).

Blood glucose

At the end of the rearing period, blood samples were collected from caudal veins of five individuals per treatment group and kept in Eppendorf tubes using sterile syringes. Prior to blood sampling, fish were anesthetized with 1 g/L ether. Measurement of plasma glucose was performed by UV spectrophotometry.

Liver and muscle glycogen

Samples of liver and muscle were taken at the end of the experiment in triplicate for each replication (15 fishes/group). Glycogen content was determined according to Wedemeyer and Yasutake (1977).

Digestibility test

On the second stage of experiment, fish from the previous experiment were kept to measure different parameters characterizing digestion capacity. Digestibility test was performed using five replicates with a density of 8 fishes/aquarium. Fish were feed with Cr_2O_3-supplemented feed for two weeks. Feces were collected twice daily every 30 minutes after meal and immediately stored in the freezer at temperature of 0 °C until further analysis. Protein and total digestibility were calculated according to Law (1986).

Ammonia excretion rate

On the third stage of experiment, ammonia excretion rate was obtained according to the method described by Yigit and Ergun (2005). Fish from digestibility-test batch were weighed and classified into groups that consisted of 3 fish with similar biomass. Nitrogen excretion was measured using three aquarium replicates with 3 fishes/aquarium. After the completion of feeding activity (within 30 minutes), fish in each group were transferred into other identical tanks filled with 60 L of previously aerated freshwater. No feeding, aeration, nor water renewal were applied during the experiment (24 h).

Samples of water were taken at H0 (Hour 0), H2, H4, H6, and H24. Ammonia and nitrite concentration were determined using Phenate Method (APHA, 1998). The ammonia excretion rate was calculated with the formula: A = [(N2−N1) x V2] / W / T2−1. Where A = ammonia excretion rate, N1 = ammonia concentration at time 1 (µg), N2 = ammonia excretion at time 2 (µg), V2 = volume of the medium at time 2, W = wet weight of the fish (g), T2−1 = time interval between sampling 1 and 2 (h).

Data analysis

Data of both treatments were compared using one-way ANOVA (F-test). The data were analysed using SPSS 18 software. Significant differences were noted if only criteria of p-value were more than 0.05 (P<0.05)

RESULT AND DISCUSSION

Result

There was no significant difference in moisture, ash and fiber content (Table 2) between the two groups of fish (P>0.05). Significantly higher crude protein, crude lipid and NFE contents, was observed in rElGH-treated fish as compared to the control (P<0.05).

No effect was noted for specific activities of most digestive enzymes (P>0.05). Lipase and chymotrypsin specific activity of rElGH-treated fish were lower (P<0.05), while specific activity of T/C ratio was higher (P<0.05) than those of control fish (Table 3). In addition, rElGH-treated fish had protein digestibility 3.65% higher (P<0.05) than those of control treatment, while total digestibility was not significantly different (P>0.05) as compared to the control (Tabel 4).

Glycogen and blood glucose levels were presented in Table 4. The rElGH in present study has tremendously increased muscle glycogen deposition as much as 149.34%. In contrast, control fish showed a 16.97% higher blood glucose level than rElGH-treated fish. No signficant difference was found in liver glycogen concentration between groups (P>0.05). Protein and lipid retention of rElGH-treated fish were respectively 14.56% and 23.53% higher (P<0.05) compared to control fish.

At the end of rearing period (week-8) the individual weight of rElGH-treated fish was significantly higher (P<0.05) than the control (Table 5). The survival levels of rElGH-treated and control fish were not statistically different (P>0.05). Ammonia excretion rate in rElGH treatment was significantly lower (P<0.05) than in the control (Table 5). Dietary rElGH administration could improve FCR (Table 5) by 16.72% (P<0.05).

Discussion

Compare to control fish no effect was noted for specific activities of most digestive enzymes (P>0.05), except those for lipase and chymotrypsin. The lower level of specific activity of lipase in rElGH-treated fish possibly indicated

Table 1. Proximate composition of feed supplemented with recombinant *Ephinephelus lanceolatus* growth hormone (rElGH) and control feed (non-rElGH) in dry basis.

Parameter	Feed + rElGH	Feed non-rElGH
Moisture (%)	12.47	11.07
Ash (%)	8.47	8.82
Crude protein (%)	27.81	28.57
Crude lipid (%)	4.91	5.01
Crude fiber (%)	3.97	42.37
NFE (%)*	4.64	41.89

*NFE = Nitrogen-free extract.

Table 2. Proximate composition (mean ± standard deviation) of recombinant *Ephinephelus lanceolatus* growth hormone (rElGH)-treated and control (whole body).

Parameter	rElGH	Control
Moisture (%)	74.95 ± 0.54a	75.84 ± 0.52a
Ash (%)	3.95 ± 0.06a	3.81 ± 0.07a
Crude protein (%)	14.64 ± 0.10b	14.31 ± 0.19a
Crude lipid (%)	5.97 ± 0.20b	5.60 ± 0.18a
Crude fiber (%)	Not detected	Not detected
NFE (%)*	0.06 ± 0.03a	0.17 ± 0.03b

Different superscript letter in the same row indicated different effect of the treatment (P<0.05). *NFE = Nitrogen-free extract.

Table 3. Specific activity of digestive enzymes (mean±standard deviation) in recombinant *Ephinephelus lanceolatus* growth hormone (rElGH)-treated and control fish.

Parameter	rElGH (µg/mg protein)	Control (µg/mg protein)
Pepsin	0.19±0.02a	0.18±0.02a
Amylase	9.85±0.78a	9.64±1.58a
Lipase	1.56±0.35a	4.95±0.55b
Trypsin	0.05±0.01a	0.06±0.03a
Chymotrypsin ($\times 10^{-3}$)	0.50±0.02a	41.3±12.6b
T/C ratio*	93.40±3.43b	1.57±0.12a

Different letter in the same row indicate significant difference (P<0.05). *T/C ratio = trypsin/chymotrypsin ratio.

Table 4. Digestibility, glycogen, glucose, protein and lipid retention (mean±standard deviation) in recombinant *Ephinephelus lanceolatus* growth hormone (rElGH)-treated and control fish.

Parameter	rElGH	Control
Protein digestibility (%)	89.32±0.99b	86.18±1.23a
Total digestibility (%)	64.66±0.52a	63.50±0.57a
Muscle glycogen (mg/g)	3.94±0.32b	1.58±0.51a
Liver glycogen (mg/g)	1.54±0.64a	1.40±0.53a
Blood glucose (mg/dL)	67.79±6.06a	81.65±11.41b
Protein retention (%)	38.39±1.80b	33.51±3.61a
Lipid retention (%)	91.16±4.36b	73.80±7.12a

Different letter in the same row indicated different effect of the treatment (P<0.05).

Table 5. Individual weight, survival rate (SR), ammonia excretion rate, and food conversion ratio (FCR) (mean±standard deviation) of recombinant *Ephinephelus lanceolatus* growth hormone (rElGH)-treated and control fish.

Parameter	rElGH	Control
Individual weight (g)	33.72±1.23b	29.06±1.16a
SR (%)	90.00±3.33a	86.67±4.71a
Ammonia excretion rate (µg/g fish per hour)	4.40±0.22a	5.20±0.35a
FCR	1.44±0.07a	1.72±0.09b

Different letter in the same row indicated different effect of the treatment (P<0.05).

the relation between lipase and rGH. However, results were not sufficient to explain how growth hormone influences lipase. In contrast, a study held by (Irmawati *et al*., 2013) showed that the application of carp rGH (rCcGH) on giant gourami increases the level of lipase specific activity and the amount of feed intake by the fish. These findings suggested that the secretion of digestive enzymes is determined by the amount of digestion substrate coming to the gut rather than the concentration of recombinant growth hormone in fish.

This study showed that control fish had higher specific activity of chymotrypsin (P<0.05) while no effect was found in trypsin specific activity (P>0.05). Blier *et al*. (2002) found no significant differences between transgenic with higher growth rate and non-trasngenic Coho salmon *O. kisutch* for specific activity of trypsin and chymotrypsin. A similar study on transgenic Atlantic cod (Lemieux *et al*., 1999) found positive correlations between growth rate, trypsin, and chymotrypsin specific activities. The effect of hormonal manipulation on digestive enzyme activity may be species specific.

Chymotrypsin is a digestive protease, which will increase when fish growth is suppressed (Torissen *et al*., 2006). Stress indication has been possibly induced by fish handling during experiment by weight sampling done every two

weeks thus possibly causing a reduction in fish growth (Tahmasebi-Kohyani *et al*., 2012). It is possible that rE1GH reduce stress in fish, thus explaining the higher level of chymotrypsin also blood glucose measured in control fish. Changes on the level of chymotrypsin or blood glucose are determined as physiological effects of stress condition in fish (Barreto & Volpato, 2006; Solati & Falahatkar, 2007; Chan *et al*., 2008).

Another noteworthy parameter was the specific activity of trypsin and chymotrypsin (T/C) ratio. In this research, T/C ratio was linear with tilapia growth. Consistent with this result, previous researches on T/C ratio demonstrated a positive correlation between T/C ratio and growth rate (Blier *et al*., 2002; Torissen *et al*., 2006). These findings suggested that higher growth in tilapia treated with rElGH was not mediated by digestive enzyme activities but might be related to the T/C ratio. Future research is expected to investigate the mechanism of either growth or growth hormone action in relation with T/C ratio.

As the diet was relatively the same, difference in protein digestibility might be due to higher digestion and absorption processes in gut. There was similarity between transgenic and rGH-treated fish as they have more growth hormone than fish without treatments. Study held by Stevens *et al*. (1999) showed that transgenic Atlantic salmon has a larger digestive surface area both in the anterior intestine (surface area 1.5 times control) and in the pyloric caeca (surface area 1.2 times control). Stevens and Devlin (2000) found that transgenic Coho salmon had a 2.2-fold larger surface area of total intestine than non-transgenic fish. These studies suggest that growth hormone can increase the level of protein absorption as the protein digestion.

The rElGH-treated fish displayed a lower level of blood glucose and liver glycogen, but a higher muscle glycogen than compared to the control. The rElGH-treated fish were suggested to be more capable to absorb and utilize carbohydrate in feed to fulfill its energy need, known as protein sparring effect (Hasan & Khan, 2013). Rapid growth required greater energy and higher level of blood glucose to be converted. Thus, the lower level of blood glucose observed in rElGH-treated fish could be explained by the higher conversion rate of glucose into energy.

Few hours after food is ingested, blood glucose level increases and the excess glucose is saved as glycogen in muscle and liver involving insulin hormone (Yilmaz *et al*., 2015). Glycogen acts as an additional energy source which can be converted if glucose level does not fulfill the energy need through glycogenolysis (Leung & Woo, 2012; Västermark & Saier; 2014). Muscle glycogen conversion is only used to fulfill energy need in local muscle where it is saved, while liver glycogen can be used for whole body-energy need (Felip *et al*., 2013; Jørgensen *et al*., 2013; Gaillard *et al*., 2015). Higher muscle glycogen in rElGH treated fish is generally followed by higher liver glycogen. However, in present study similar levels of liver glycogen were observed in both groups as some of the liver glycogen in rElGH-treated fish has been converted into glucose to make energy for the whole body. Muscle glycogen conversion did not occur because the energy from glucose and liver glycogen were still sufficient to fulfill muscle energy need, whereas in control, muscle glycogen was lower because less glucose being converted into it due to lower level of carbohydrat digestion

A higher lipid and protein retention measured in rElGH-treated fish has led to higher lipid and protein deposition than in control fish. In this study, higher protein level measured in flesh and lower ammonia excretion level were found in rElGH-treated fish ($P<0.05$). At the end of the rearing period (week eight), individual weight of rElGH-treated fish was 20.04% higher ($P<0.05$) than those of control. Less ammonia excretion implied that amino acid deamination in the liver was decreased thus optimizing nitrogen used for the fish growth (Guo *et al*., 2012). This study also revealed that the administration of rElGH had no negative effect ($P>0.05$) on the survival of fish.

In conclusion, the present study demonstrated that oral administration of rElGH at level of 3 mg/kg of feed had no effect on specific activity of pepsin, amylase, and trypsin, but showed lower level in lipase and chymotrypsin in Nile tilapia. There might be a possibility that there is no direct relation between growth hormone and digestive enzymes, however growth could be related to the T/C ratio. This study also identified that rElGH treated fish performed a higher protein digestion, and protein retention in the body, but a lower ammonia excretion rate were obtained to support the enhanced growth of the fish.

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