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Protease and Amylase Activities-of Javaen barb (*Systomus rubripinnis Val.*)

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Abstract. *Studies on morphology, growth, and reproduction have been carried out on wild Javaen barb, but there was no information on its digestive capacity; therefore, the research was conducted to determine protease and amylase activities in the digestive tract. This study used a total of 50 barbs with body weights between 13.56 -128.93g / fish. The measurement of enzyme activity was carried out using the spectrophotometer method. The results showed that differences in fish size resulted in differences in protease activity, but not for amylase. Fish with a small size have a higher protease activity than fish with a larger size. The protease activity did not differ between pH 6.9 to 10.0 but was higher than pH 12.5. Protease activity also did not vary between the anterior and posterior intestine and between 30-50°C. Amylase activity also found no difference between the anterior and posterior intestine, but there was a difference in activity between temperatures of 30-50°C. In conclusion, protease activity occurs in a neutral to alkaline environment, and there were differences in protease activity between different body sizes but not between intestinal segments. Amylase activity occurs throughout the intestine and decreases at temperatures of 50°C.*

Keywords: *digestive enzyme, gut segment, Javaen barb, pH, temperature Hassk*

Citation

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INTRODUCTION

The food consumed by fish will undergo a digestive process in the intestine before being absorbed and utilized. Therefore, the equivalence between the feed consumed and the digestive capacity will determine the utilization of nutrients by fish. The action of its digestive enzymes primarily determines the ability to use these nutrients in feed. There are several factors such as feeding habits, body size, temperature, and pH that affect the activity of digestive enzymes.

Studies on gouramy (*Osphronemus gouramy*) showed that fry had lower protease activity than large ones. In contrast, amylase had higher activity in smaller sizes than large ones (Susilo et al., 2012). However, in *Lutjanus guttatus*, protease activity increased with increasing body size, and protease activity was higher in the posterior intestine (Pena et al., 2015). A different phenomenon was also found in *Labeo rohita*, which showed higher protease and amylase activity in fish with a bodyweight of 227.3±44.5 g compares to smaller or larger fish (Umalatha et al., 2016). It is seen that there is a diversity of enzyme activity related to differences in size and spe-

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cies, and this information becomes urgent to be able to produce harmony between the feed consumed and the capacity of the digestive enzymes.

In *Glyptosternum maculatum*, the optimal temperature and pH for proteases found along the intestines were 50°C and pH 9.0-10.0, while amylase has an optimal temperature of 30°C and pH 7.0 (Xiong et al., 2011). In tilapia, *Oreochromis niloticus*, protease activity is also optimal in alkaline conditions, amylase is active in neutral conditions, while lipase is active in neutral to alkaline conditions (Klahan et al., 2009). Similar to Mekong Giant Catfish, the optimal temperature for acidic protease is 40-60°C, alkaline protease 40-70°C, and at room temperature (25-30°C), amylase and protease activity is dominant in the intestine (Tongsiri et al., 2010). So it seems that fish digestive enzymes have variations in optimal pH and temperature for their activities, but how temperature affects the enzyme activity of Javaen barb needs to be studied further.

Studies on *Etroplus suratensis* and *Oreochromis mossambicus* also showed alkaline protease and amylase activity variations in different intestinal segments. In *E. suratensis*, the posterior intestine found the highest alkaline protease activity, while in *O. mossambicus*, that was in the mid intestine. The middle and posterior intestines found the most increased amylase activity. However, the highest amylase activity was found in the anterior and middle intestine (Sankar et al., 2014).

Studies on *Sparidentex hasta* showed an increased protease activity in the anterior intestine and amylase activity that did not differ between the anterior, middle, and posterior intestine (Jahantigh, 2015). In contrast, *Ctenopharyngodon idella* and *Leporius obtusidens* had the highest alkaline protease and amylase activity in the middle intestine (Gioda et al., 2017). Different fish species exhibit different digestive enzyme activities, and the understanding of this digestive enzyme activity has not been studied well in Javaen barb.

Javaen barb is a freshwater fish belonging to the family Cyprinidae. This fish has a wide distribution in Indochina and the Sunda Islands. This fish has a medium body with a total length of up to 250 mm with the last spine on the dorsal fin with 30 teeth and has a caudal fin with black top and bottom edges (Kottelat et al., 1993). Preliminary studies of Javaen barb have been carried out, such as studies of several aspects of Javaen barb spawning in the Klawing Purbalingga river, Central Java (Suryaningsih et al., 2012), and studies on the growth with different feedings (Susatyo et al., 2016). However, studies on the performance of digestive enzymes, especially proteases and amylase, have never been carried out, so research on this matter is urgent. The results of this study can support efforts to make a feed formula for Javaen barb and the domestication of this fish in the future.

MATERIALS AND METHODS

Javaen barb (*Systomus rubripinnis*), 50 fish with body weight ranging from 13.56 -128.93g were used in this experiment. Javaen barbs were obtained from catches on September-October 2015 in the Banjaran river, Purwokerto, Banyumas with coordinates 109°13'24,497"E and -7°25'7.215"S.

The fish was then isolated from their digestive tract into two parts: the anterior and posterior segments. The digestive tract was further crushed using an electric homogenizer in a cold solution of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl (1:6 w/v). The homogenate obtained was then placed in an Eppendorf tube with a volume of 1.5 ml

and centrifuged using a refrigerator centrifuge at a temperature of 4°C at a speed of 12,000 rpm for 15 minutes. The supernatant was then transferred to a new 1.5 ml Eppendorf tube and stored in a refrigerator at -80°C (Thongprajukaew et al., 2010). It was ready to be used for enzyme activity testing.

Measurement of supernatant protein content was carried out with a Folin-phenol reagent, and albumin was used as a standard (Gangadhar et al., 2017). The calculation of the enzyme activity used the protein content of the supernatant. The buffers used to measure protease activity were 0.1 M phosphate (pH 6.9), 0.1 M Tris-HCl (pH 8.1), 0.1 M Glycine-NaOH (pH 10) and 0.1 M KCl-NaOH (pH 12.5). Temperature incubation of the measurement of protease activity at different pHs was 37°C. The measurement at different temperature incubation (30, 40, and 50°C) used a buffer of 0.1 M Tris-HCl (pH 8.1).

The method used to measure protease activity was the casein hydrolysis method (Furne et al., 2005 with modification method). The first step of the enzyme reaction was mixing 1% (w/v) casein in water (350 µl), buffer (350 µl), and enzyme sample (50 µl) and incubating for 60 minutes at the incubation temperature. To stop the reaction, a total of 750 µl of 8% (w/v) trichloroacetic acid (TCA) was used. For control, TCA was added to the reaction mixture before adding the enzyme extract. For the standard, the same procedure was carried out, except the substrate was replaced with tyrosine. All reaction mixtures were then centrifuged at 6,000 rpm for ten minutes after being deposited in the refrigerator for at least one hour (temperature 4°C). The absorbance was recorded at a wavelength of 280 nm. Tyrosine standard curve used to calculate protease activity. One unit of enzyme activity is the enzyme required to catalyze the formation of 1 µg of tyrosine per minute (Furne et al., 2005).

Amylase activity was measured using the starch hydrolysis method (Furne et al., 2005). The buffer used was 0.1 M phosphate (pH 6.9). The substrate was prepared by dissolving 1% (w/v) starch in 10 mM NaCl solution, then boiled for five minutes. The reaction was started by mixing the substrate (350 µl), buffer (350 µl), and enzyme extract (50 µl). During 15 min at the incubation temperature (30, 40, or 50°C), then was incubated the reaction incubated mixture. After incubation, 750 µl of 1% dinitrosalicylic acid (DNS) solution was added to the reaction mixture and then immersed in boiling water for 5 minutes. The reaction mixture was then cooled. In the blank, the same procedure was carried out, except that the enzyme extract was added immediately after administration of 1% DNS. For the standard, the same process was carried out, except the substrate was replaced with D-maltose. After 20-60 minutes, the reaction mixture measured its absorbance at a wavelength of 540 nm. The standard curve showed the amount of maltose released from this test. The amount of maltose released (mmol) per minute per mg of protein supernatant is amylase activity (Klahan et al., 2009).

Data on the specific activity of protease and amylase ($U.mg^{-1}$protein) at pH, temperature, and gut segments with different fish sizes were analyzed univariately. The significant difference continued with Tukey's HSD test ($P<0.05$). All these statistical analyzes were performed using the Windows version of SPPS 17 software.

RESULTS AND DISCUSSION

The protease activity at different pH showed 47.17±22.17 $U.mg^{-1}$protein (pH: 6.9), 74.54±24.64 $U.mg^{-1}$protein (pH: 8.1), 48.42±18.21 $U.mg^{-1}$protein (pH: 10.0), and

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0.00±0.00 U.mg^{-1}protein (pH: 12.5), in fish with an average weight of 31.2±4.14g, while in fish with an average weight of 117.7±11.23g showed 37.43±24.05 U.mg^{-1}protein (pH: 6.9), 35.26±19.38 U.mg^{-1}protein (pH: 8.1), 33.77±16.73 U.mg^{-1}protein (pH: 10.0), and 0.00±0.00 U.mg^{-1}protein (pH: 12.5) (Figure 1.). Statistical analysis showed a significant difference ($p<0.05$) between the pH of protease activity. At pH 12.5, it seemed that there was no protease activity (Figure 1.), and this was different from the protease activity at pH 6.9-10.0 ($P<0.05$), which had higher protease activity, but there was no difference in protease activity between pH 6.9-10.0 ($P>0.05$). Protease activity in small fish was also higher than that found in larger fish (Figure 1.).

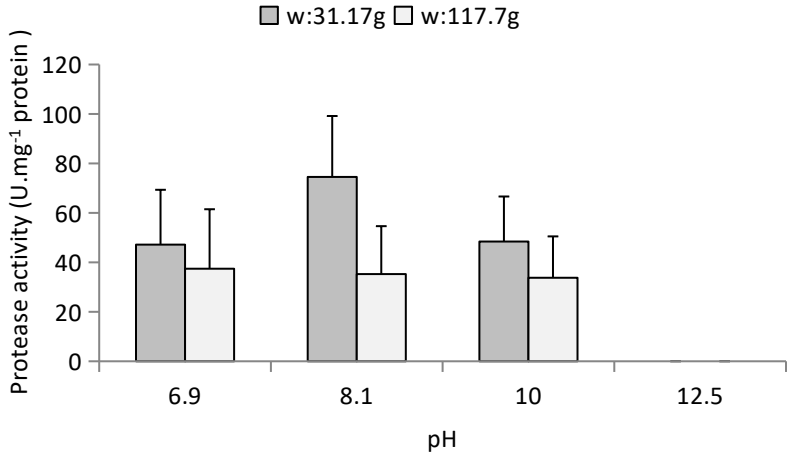

Figure 1. Protease activity at different pH and body weights in Javaen barb

The result of this study also did not differ from previous studies, where protease activity decreased under optimal alkaline tolerance, as happened in seabream (Caruso et al., 2009) and *Odontibutis obscures* (Ye et al., 2013). The results of this study were also not different from previous studies on *Cichlosoma beani* and Cyprinid fishes which showed the stability of alkaline proteases at pH 8.0 – 10.0 (Martinez-Cardenas et al., 2017; Champasri & Champasri, 2017). The presence of Javaen barb protease activity in the pH range of 6.9 – 10.0 is suspected to be active in hydrolyzing proteins in neutral to slightly alkaline conditions, with higher activity found in fish with an average weight of 31.17±4.14g. This condition was in sync with the slightly alkaline intestinal environment found in *Tilapia rendali, Oreochromis mossambicus, Clarias gariepinus, and Salmo salar* (Hlophe et al., 2014; Krogdahl et al., 2015). The alkaline condition of the intestine was possible as an ideal medium for the activity of proteases secreted by the pancreas. However, the protease activity shown in this study may differ from the activity of specific proteases such as trypsin and chymotrypsin. Previous studies have demonstrated in various species, such as *Gymnocypris przewalskii, Mycrophis brachyurus,* and *Mystus nemurus* which found the activity of trypsin and chymotrypsin in the intestine with optimal pH ranging from 9 to 10 (Tian et al., 2019; Martinez-Cardenas et al., 2020; Rahmah et al., 2020). It appears that trypsin and chymotrypsin have optimal activity with a narrower pH range.

Protease activity at different temperatures ranged from 93.26-101.57 U.mg^{-1} pro-

tein in fish with an average weight of 16.5±2.94 g, while in fish with an average weight of 45.0±4.97g, it was 75.83-97.59 U.mg^{-1}protein (Figure 2.). The results of statistical analysis showed no difference in protease activity between different enzyme incubation temperatures (P>0.05), but smaller fish tend to show higher protease activity than larger fish (Figure 2).

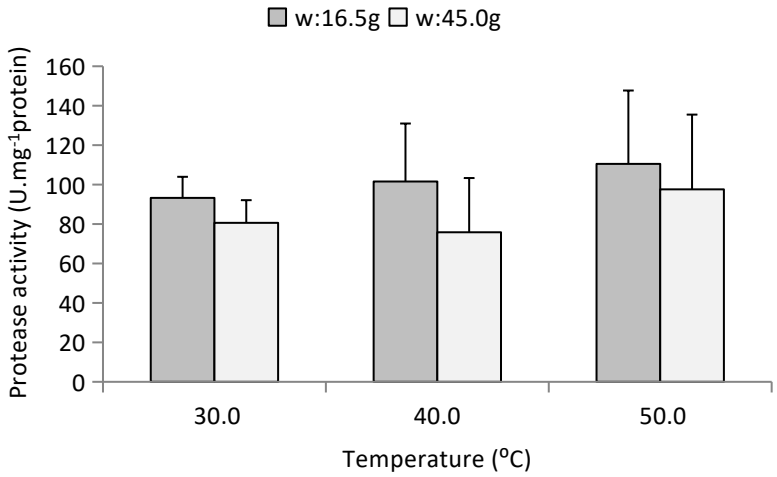

Figure 2. Protease activity at different temperature and body weights in Javaen barb

The protease activity measured in this study did not seem to change significantly (P>0.05) with changes in temperature from 30°C to 50°C (Figure 2.), an indication that protease activity was still in the activation temperature range in the sense that the temperature was still below the optimal temperature. The results of this study are different from those found in previous studies on *Poecilia reticulata, Scorpaena notata,* and *Microphis brachyurus,* which have optimal alkaline proteases temperatures ranging from 40-50°C (Thongprajukaew & Kovitvadhi, 2013; Aissaoui et al., 2017; Martinez-Cardenas et al., 2020). Several previous studies have also shown optimal protease activity at temperatures ranging from 55-75°C, such as in *Barbus callensis, Centropomus undecimalis, and Cichlasoma beani* (Sila et al., 2015; Concha-Frias et al., 2016; Martines-Cardenas et al., 2017). Species differences seem to be the reason for the differences in the results of this study to previous. Therefore, a further experiment by applying temperatures below and above the temperature tested on Javaen barb is to be carried out to obtain differences in the response and optimal temperature of protease.

The protease activity in the anterior segment was found on average 65.10±21.49 U.mg^{-1} protein in fish with an average weight of 31.2±4.14g and 35.69±25.96 U.mg^{-1} protein in 117.7±11.23g, while in the posterior intestine was found to be 48.32±18.26 U.mg^{-1} protein in 31.2±4.14g and 34.43±18.47 U.mg^{-1} protein in 117.7±11.23g (Figure 3.). The statistical analysis results showed that the difference in intestinal segments did not produce a significant difference (P>0.05) in protease activity, but smaller fish showed significantly higher protease activity than that found in larger fish (P<0.05).

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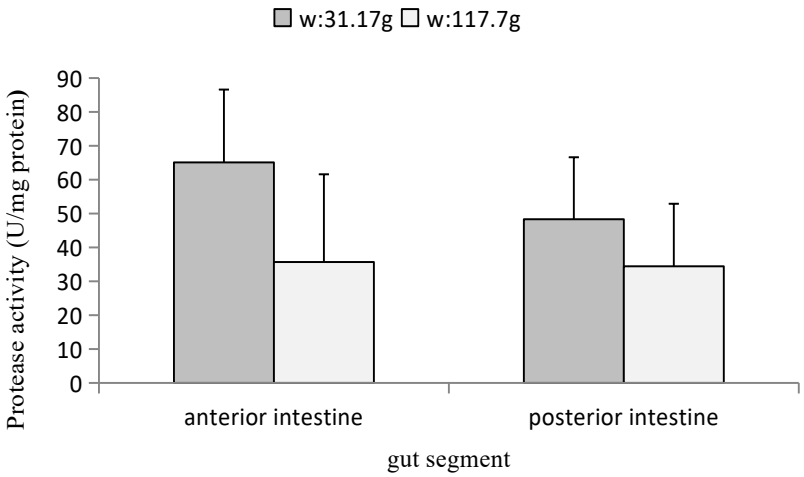

Figure 3. Protease activity at different gut segments and body weights in Javaen barb

Protease activity along the intestine did not differ between the anterior and posterior intestines, which indicated the presence of protein hydrolysis activity along the digestive tract of the Javaen barb. The results of this study were not different from previous studies on *Glyptosternum maculatum, Lota lota*, and *Gymnocypris przewalskii,* which showed similarities in protease activity between the anterior and posterior intestine (Xiong et al., 2011; Izvekova et al., 2013; Tian et al., 2019). In contrast, *Sparindentex hasta, Labeo rohita,* and *Hoplias malabaricus* showed decreased protease activity in the posterior intestine (Jahantigh, 2015; Umalatha et al., 2016; Gioda et al., 2017).

Differences in the size of fish give different responses to protease activity, which indicated higher protease activity in small fish than in large fish, but this did not occur in amylase activity. The results of this study are not different from previous studies on *Oreochromis niloticus and Rasbora lateristriata* (Klahan et al., 2009; Susilo et al., 2018). Changes in protease activity in this study, which are in sync with previous studies, are thought to be related to changes in the growth rate and feeding habits of Javaen barb. Previous studies on *Brycon guatemalensis* showed a decrease in alkaline protease activity as fish increased from juvenile to adult with changes in feeding habits (Drewe et al., 2004). However, the results of this study were different from those found in *Labeo rohita*, which showed hyperbolic changes in protease activity with increasing body size (Umalatha et al., 2016). Differences in species and feeding habits are thought to cause differences with previous studies.

The difference in the incubation temperature of the enzyme resulted in the amylase activity of Javaen barb at an average weight of 16.5±2.94 g ranging from 0.93-1.24 $U.mg^{-1}$ protein and ranging from 0.92-1.15 $U.mg^{-1}$ protein in 45.0±4.97g (Figure 4.). The statistical analysis results showed that the temperature difference resulted in a significant difference ($P<0.05$) in the amylase activity of Javaen barb. Still, the size of the fish did not cause a difference in the amylase activity. Temperatures 30°C up to 40°C did not affect changes in amylase activity, but at higher temperatures (50°C) the amylase activity decreased.

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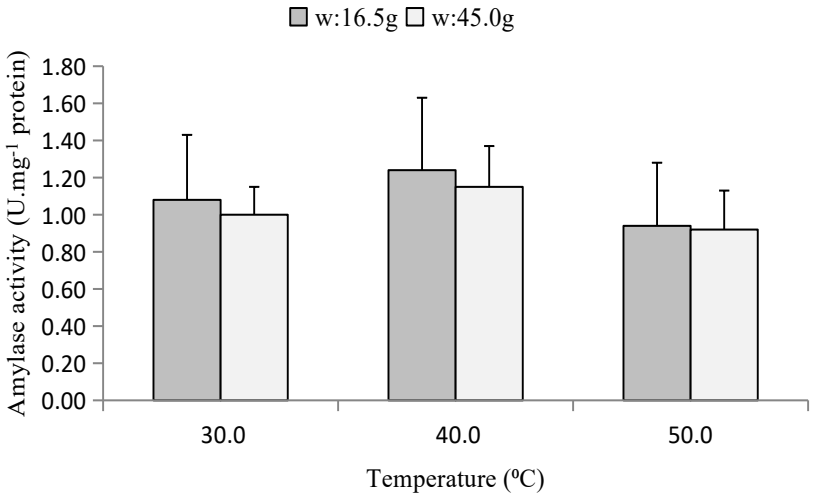

Figure 4. Amylase activity at different temperatures and body weights in Javaen barb

The decreasing amylase activity at a temperature of 50°C was not different from previous studies on *Anguilla japonica, Chelon labrosus,* and *Paralichthys orbignyanus* (Murashita et al., 2013; Pujante et al., 2016; Candiotto et al., 2018). But these results were different from those found in *Betta splendens and Osteochilus hasselti*, which showed a decrease in amylase activity at temperatures ranging from 60-65°C (Thongprajukaew et al., 2010; Champasri & Champasri, 2017). Different species showed different responses of amylase activity to changes in temperature, as shown in this study.

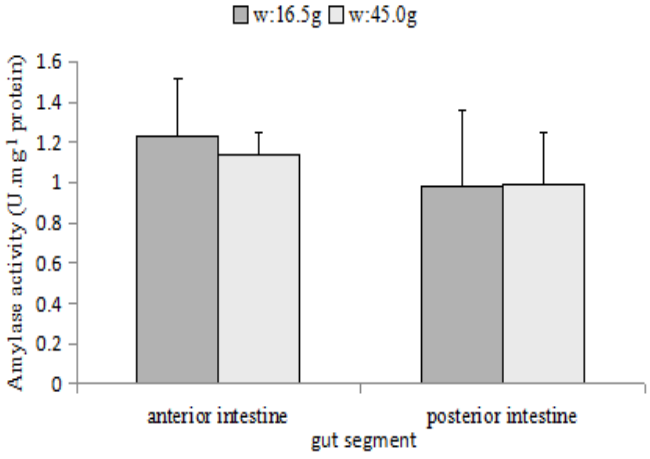

Figure 5. Amylase activity at different gut segments and body weights in Javaen barb

Amylase activity in fish with an average weight of 16.5±2.94 g was 1.23±0.29 U.mg^{-1} protein in the anterior and 0.99±0.38 U.mg^{-1} protein in the posterior intestine, while in fish with an average weight of 45.0±4.97g, it was 1.14±0.11 U.mg^{-1} protein in the anterior

and 0.99±0.26 U.mg^{-1} protein in the posterior intestine (Figure 5). The statistical analysis results showed that the difference in the gut segment and fish size did not produce a significant difference (P>0.05) in the amylase activity of Javaen barb.

The result of this study was not different from previous studies in *Oreochromis mossambicus, Sparidentex hasta, Zenarchopterus buffonis,* and *Labeo rohita* (Sankar et al., 2014 ; Jahantigh, 2015; Abidin et al., 2016; Umalatha et al., 2016), but in *Tilapia rendali* (herbivores) and *Ctenopharyngodon Idella* (herbivores), were decreased amylase activity in the posterior intestine (Hlophe et al., 2014; Gioda et al., 2017). So it seems that Javaen barb has different starch digestion abilities along their digestive tract from herbivores species.

Amylase in the intestine is an enzyme secreted by the pancreas, and the pancreatic enzymes require a neutral to the slightly alkaline environment for optimal activity. In acid or alkaline conditions, amylase will be inactive; therefore, in this study, the measurement of amylase activity was not carried out at acidic or alkaline pH. This is different from proteases which can be found active both at acidic and alkaline pH. Studies on bream (*Abramis brama*), and *Glyptosternum maculatum* have also shown optimal amylase activity at pH 7.0 (Kuz'mina et al., 2011; Xiong et al., 2011).

CONCLUSION

In conclusion, protease activity occurred in a neutral to alkaline environment, and there were differences in protease activity between different body sizes but not between temperature and gut segments. Amylase activity occurs throughout the intestine and decreases at 50°C. The results of this study can be used as basic information to study further the digestive capacity of Javaen barb which is more comprehensive.

AUTHOR CONTRIBUTION

U.S. designed research and wrote the manuscript and, F.N.R. proofreads and language editing and E.S.W. collected and analyzed the data.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest either regarding the order of the authors, research, and research funding.

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