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Isolation, partial purification and characterization of protease enzyme from the head of Nile tilapia fish (*Oreochromis niloticus*).

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ABSTRACT

Fishery processing waste is as environmental problem. Fish waste, such as the head of the Nile tilapia fish (*Oreochromis niloticus*), is underutilized and has a low economic value, even though the waste can be re-processed. The purpose of this study was to isolate and purify protease enzymes from the head of Nile tilapia fish (*O. niloticus*) and to investigate their partial enzyme characteristics. Protease enzymes were isolated using various percentages of ammonium sulfate precipitation, which was followed by partial purification by dialysis. The total protein and specific activity of the enzyme was calculated at each stage of purification. The characteristics of the enzyme, namely optimum temperature, pH and *Km* and k_{cat} were investigated. The result of investigation revealed that the 40% ammonium sulfate extraction showed the highest yield of the enzyme. Partial purification by dialysis can increase its specific activity 21 times from its crude extract form. The optimum temperature of the protease is 38°C, while the optimum pH is 7.5. The protease *Km* and k_{cat} value of this protease were 1.887 mM and 39.768 s^{-1}. The protease enzyme was optimally semi-purified using 40% ammonium sulfate precipitation. Judging from the result, this enzyme categorized as a neutral enzyme. Further analysis is needed to investigate its other characteristics.

INTRODUCTION

Indonesian export of Nile tilapia fish fillets (*Oreochromis niloticus*) as a frozen product to the US market occupies the second position after China. Data from the *National Marine Fisheries Service*, USA, indicated that Indonesian export on fish frozen fillets in 2014 reached 11.608 ton with the value of US$ 78,325,428 (CEA, 2015). The high opportunity for the export of Nile tilapia fillet products from

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Indonesia has triggered an increase in fish fillet production. Nile tilapia fillet processing generates a large amount of waste (Wijaya *et al*., 2015).

Depending of the size of the fish body, waste is approximately 50-70%. Nile tilapia fillet by-product consists of the body parts of the fish, such as the head, bone, skin, and viscera. Until now the availability of by-products are still not being utilized and tend to harm the environment.

The protease enzyme is the most commonly used type of enzyme in everyday life. This enzyme has a very broad aplications. For food applications, this enzyme can be used in the production of cheese. Protease is also commonly used in detergent as well as leather tanning. Moreeover, this enzyme can be used for meat tenderizers. In the bread industry and in the process of making protein hydrolyzates (Ali and Muhammad, 2017). Therefore, it is not surprising that this enzyme dominates the enzyme market. Protease is accounts for 50% of total enzyme sales throughout the world (Vo and Kim, 2010). In the pharmaceutical and health industries, protease is used for diagnostics and treatment. Serine protease, fybrinolytic enzyme can degrade fibrin in the blood vessels and produce more several soluble compounds (Raja *et al*., 2011).

The utilization of industrial waste by increasing the benefits of the waste materials is very important. The utilization of fish processing waste such as bark, bone, fin, and viscera have been proposed already by researchers (Anais *et al*., 2013; Sergio *et al*., 2017). Most of the waste is converted into products such as collagen, gelatin, silage, and fish protein hydrolyzate. Until now, fish head waste has not been much studied or utilized. Just like other waste materials, the Nile tilapia fish head is part of the fillet production waste, which has high potential to be optimized. The products that can be potentially isolated from this by-product, head of Nile tilapia, is enzymes.

Nowadays, the research into this enzyme is still being carried out. The objective of this research was to isolate and semi-purify enzymes from the head of Nile tilapia fish (*O. niloticus*). The characterization of this enzyme was then also investigated.

MATERIALS AND METHODS

Nile tilapia fish (*O. niloticus*) were bought from the nearest market, Pasar Besar, Malang, East Java, Republic of Indonesia. The samples were inserted in polyethylene plastic in which ice was added and were taken immediately to the laboratory at Faculty of Fisheries and Marine Science, Brawijaya University. The fish samples were washed twice with running water. The separated head of the fish was then immediately used.

Isolation and purification of the enzyme

The isolation of the enzyme following the method according to Soottawat *et al*. (2013), with slight modification. In brief, the head waste (100 g) was washed with aquades and homogenized with 200 ml of buffer (10 mM Tris-HCI, pH 8.0 containing $CaCl_2$) for 5 min. Homogenate was centrifuged at 10,000 rpm for 30 min. The supernatant was taken and used as a crude extract sample. Next, the crude extract was fractionated by the method of ammonium sulphate ($(NH_4)_2S0_4$) precipitation in a saturation of 30-60%. The precipitate was centrifuged at 10,000 rpm for 30 minutes. The supernatant was dialyzed using cellophane plastics for 24 hours at 4°C. The results of the next dialysis were tested for characterization.

Protein concentration

The protein concentrations were analysed using the method according to Lowry *et al.* (1951), Bovine serum albumin (BSA) was used as a standard. At each stage of purification of the enzyme, sample absorbance was measured using a 280 nm wavelength.

Enzyme activity

The enzyme calculation followed the method according to Prihanto *et al.* (2016), with modification. In brief, the dialysed solution (0.2 ml) was incubated with 1 ml casein and 3 ml carbonate-bicarbonate buffer, pH 9.0, at 37°C for 30 min. The reaction was stopped by adding 2.0 ml of 5% trichloroacetic acid (TCA). The solution was filtered using Whatman filter paper No. 1. Next 1 ml was mixed with sodium carbonate (Na_2C0_3) 0.4 M and 1 ml of the Folin-phenol's 0.5 N reagent. The entire solution was re-incubated at 37°C for 20 min. The amount of tyrosine in the solution was read by the spectrophotometer with the absorbance of 660 nm. Standard tyrosine was prepared by making a tyrosine solution in TCA with different concentrations. The specific activity of the enzyme was obtained by dividing the enzyme activity (U/ml) with the concentration of protein (mg/ml).

Temperature effect

Temperature effect tests on enzyme activity were performed in ten different temperature ranges namely 25, 30, 35, 40, 45, 50, 55, 60, 65, 70°C.

pH effect

The effect of pH on protease activity was found by testing enzyme activity in the phosphate buffer with different pH. The pH value used was 4-10.

Determination of Km and k_{cat}

The enzyme kinetics, Michaelis–Menten constant (*Km*), and value of the turnover number (k_{cat}) was calculated from the following equation: kcat=Vmax/[E], where [E] is the active enzyme concentration and Vmax is the maximal velocity. The calculation was done using casein as a substrate. *Km* and Vmax, maximal velocity were determined using the Lineweaver-Burk plot.

RESULTS AND DISCUSSION

The result of purification of the enzyme using different saturations of $(NH_4)_2S0_4$ showed different results. The best activity was given by $(NH_4)_2S0_4$ with a saturation level of 40% (Table 1). The difference in activity was strongly influenced by the saturation of $(NH_4)_2S0_4$. The saturation concentration of $(NH_4)_2S0_4$ must be in a proper condition to produce the most optimal purification in isolating the enzyme and retaining its activity. Previous research showed evidence that the addition of $(NH_4)_2S0_4$ will significantly decrease the degree of purification (Narayan *et al.*, 2008).

Table 1: Purification of the enzyme using different saturations of $(NH_4)_2S0_4$

Step	Total protein (mg/ml)	Total activity (U)	Specific activities (U/mg)	Recovery (%)	Purification fold
Crude extract	72.0	42.93	0.60		1.0
$(NH_4)_2S0_4$ 30%	6.6	16.56	2.51	38.57	4.18
$(NH_4)_2S0_4$ 40%	5.3	21.40	4.04	49.85	6.73
$(NH_4)_2S0_4$ 50%	5.0	20.87	4.17	48.61	6.90
$(NH_4)_2S0_4$ 60%	3.9	20.88	5.35	48.64	8.92
Dialysis	2.37	30.21	12.75	70.37	21.25

Temperature effects on enzyme activity

The enzyme produced from the Nile tilapia head had an optimum temperature at 38°C (Fig. 1). This result differs from the optimum temperature characteristics of protease enzymes isolated from the head of the trout (*Salmo gairdnerii*), which had the most optimum activity at 50°C (Michail *et al*., 2006). Proteases produced from the viscera of Bolti fish (*Tilapia nilotica*) had a similar optimum activity to the present research, at 35°C (El-Beltagy *et al*., 2004). The enzyme trypsin and chymotrypsin were successfully isolated from the viscera of Nila fish (*Oreochromis niloticus*) in Thailand with an optimum activity at 60°C (Chaijaroen and Thongruang, 2016). The optimum temperature for protease enzyme activity is reported to be very diverse. This variation can be caused by the location, living habitat and body parts of the species. In general, the enzyme will experience a decrease in activity at temperatures above 70°C as a result of enzyme denaturation by heat.

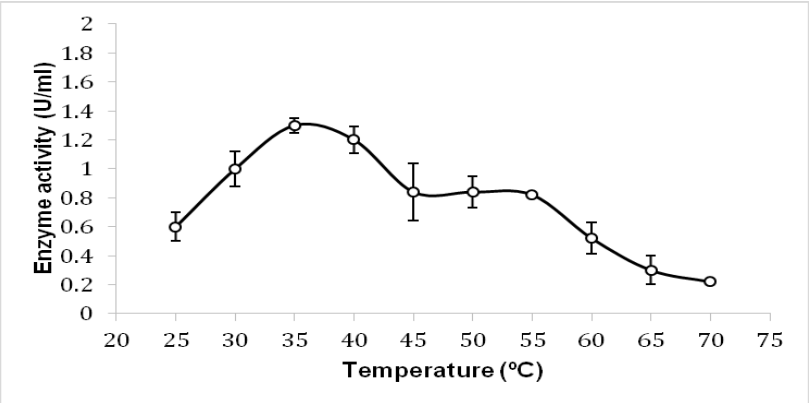

Fig. 1: Effect of temperature on enzyme activity of enzymes extracted from the head of Nile tilapia fish.

Effect of pH on enzyme activity

The effect of pH on the enzyme activity can be seen in Fig. 2. The results show that this enzyme had the most optimum activity at pH 7.5. The results of slightly different research were generated from the viscera of *Labeo horita* fish, which had an optimum activity at pH 8 (Geethanjali and Subash, 2011).

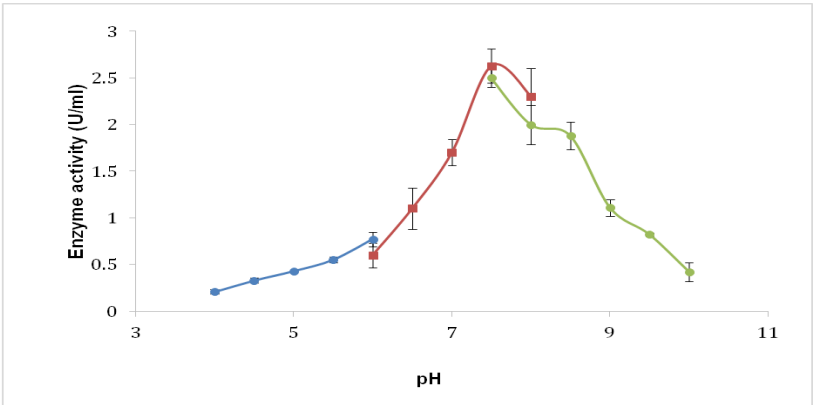

Fig. 2: Effect of pH on enzyme activity of enzymes extracted from the head of Nile tilapia fish (blue = citrate, red = phosphate, green line = tris/HCl buffer)

Different results have been shown in enzymes isolated from sardine viscera (*Sardinella aurita*) which showed optimum activity at pH 3-5 (Khaled *et al*., 2011). The optimum pH of a protease enzyme may also occur under alkaline conditions.

Protease isolated from Venusian barbell viscera (*Barbus callensis*) appears to have an optimal activity in the range of pH 10 (Sila *et al.*, 2012). Decreased enzyme activity generally occurs in conditions that are very acidic or very alkaline. This decrease in enzyme activity may be due to changes in protein conformation that will ultimately affect enzyme prosthetic groups.

Enzyme kinetics

Casein was used for the measurement of *Km* and $k_{cat.}$ The measurement of the kinetics of isolated enzymes from the head of the Nile tilapia showed a *Km* value of 1.887 mM and k_{cat} of 39.768 s^{-1}. This *Km* value was much lower than protease enzymes isolated from brown stripe red snapper *(Lutjanus vitta)* with the value of 0.507 mM for α-N-benzoyl-DL-arginine-q-nitroanilide (BAPNA) and 0.328 mM for α-N-ρ-tosyl-L-arginine methyl ester (TAME) as a substrate (Khantaphant and Benjakul, 2010). Further research on protease from viscera and heads of *Engraulis anchoita* also suggested that BAPNA was more favourable than that of casein (Lamas *et al.*, 2017).

CONCLUSION

Partial purification by dialysis can increase protease enzyme specific activity by 11 times from the crude extract isolated from the head of Nile tilapia fish (*O. niloticus*). The characteristics of the partially purified enzyme shows an optimum temperature of enzyme activity at 38°C, optimum pH of 7.5 with the *Km* values of 1.887 mM and K_{cat} 39.768 s^{-1}. Further information about other characteristics of the enzyme are still required. This protease enzyme was optimally semi purified using 40% ammonium sulfate precipitation.

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