Cathepsin characterization from crude extract of yellow pike 
(*Congresox talabon*)

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**Abstract.** Fish is a highly perishable food due to internal and external factors. Cathepsin, a proteolytic enzyme available in animal tissues, affects quality deterioration of fish by hydrolizing muscle proteins. Yellow pike fish have an edible portion that is relatively high but has not been used optimally. The aim of the study was to extract the cathepsin enzyme from yellow pike meat. The method used extraction, characterization, and precipitation of ammonium sulfate (NH₄)₂SO₄ fraction at concentrations of 40-50% to 70-80%. The extraction results showed the crude extract activity of cathepsin from yellow pike fish which was 0.19 U/mL. The optimum activity of the cathepsin enzyme at a pH 5 (0.24 U/mL), temperature of 50°C (0.10 U/mL) and metal ions increased cathepsin activity, one of which was CuCl₂ increased cathepsin activity to 0.92 U/mL and FeCl₃ increased activity to 1.41 U/mL. The best precipitate at 40-50% ammonium sulfate concentration with specific activity 1.15 U/mg.

**Keywords:** ammonium sulfate, cathepsin, quality deterioration specific activity, yellow pike

1. **Introduction**

Fish is highly perishable food because its easily damage and deteriorate caused by internal and external factors. Internal factors such as enzyme, bacterial and chemical activities and external factors including poor handling method. Handling techniques to maintain fish quality had been widely reported such as live transportation (Suwandi et al 2013, Manurung et al 2018), gliroxyl use (Ariyani et al 2007), carbonated soft drinks use (Suwandi et al 2008), chitosan use (Suptijah et al 2008), utilization of the cathepsin inhibitors (Nurhayati et al 2011).

The proteolytic enzyme plays an important role in fish quality deterioration by catalyzing protein breakdown through hydrolysis of peptide bonds. Proteolytic enzymes like calpain and cathepsin were involved in damaging the texture of the meat (Jiang 2000). Calpain and cathepsin was reduced the quality of fish, cathepsin is a protease enzyme which is located in the lysosome and the activity of cysteine calpain protease is dependent on calcium breakdown. Cathepsin became active after the dead cell then lead to decreasing pH by the accumulation of lactic acid (Chéret et al 2007). Cathepsin extraction and characterization from various types of fish have been conducted from several species like...
milkfish skin (Nurhayati et al 2010), milkfish meat (Nurhayati et al 2012), catfish meat (Fikri et al 2014), recovery from liquid waste surimi (Nurhayati et al 2015). Yellow pike is one of the fish from Indonesian sea waters that has little information about its deterioration quality.

Yellow pike catch production reached 1,607.6 tonnes in 2011, 1,334.5 in 2012 and 1,062.4 tonnes in 2013 (DKP 2014). Utilization of yellow pike at this time has not been widely reported, one of the efforts that have been made to increase the added value of these fish is by making processed foods such as skin crackers. Berlia et al (2017) reported that from processed foods like skin crackers there was an increased added value reaching 55.20%. Yellow pike as a source of cathepsin has not been widely reported, therefore this research was conduct to study its utilization and the possibility of its added value. The purpose of the study was to determine the characteristics of the enzyme cathepsin from yellow pike.

2. Materials and methods

2.1. Material and equipment
The materials used in this study are yellow pike meat obtained from the Bogor traditional market, distilled water, 0.1 M HCl tris buffer pH 7.4, ammonium sulfate, EDTA (Titriplex III) (Merck, Germany), HCl, haemoglobin (Sigma Aldrich, US), TCA (Merck, Germany), bovine serum albumin (AppliChem, Germany), coomassie brilliant blue (CBB) R-250 staining solution (Bio-Rad Laboratories, US). The tools used are centrifuge (Thermo scientific LYNX 6000), spectrophotometer (Spectro UV-VIS 2500, Germany), pH meter (Hanna), micropipette (Thermo Scientific Vantaa, Finland).

2.2. Methods
2.2.1. Cathepsin extraction. Cathepsin extraction method refers to Dinu et al (2002). Yellow pike fish meat was homogenated with cold distilled water (1:1) then separated using centrifugation at 4°C. The first centrifuge uses 600 g for 10 minutes. The filtrate was further centrifuged at 10,000 g for 10 minutes to obtained pellet. Pellet was added distilled Tris HCl buffers as much as used for extraction then centrifuged at 4,000 g for 10 minutes. The filtrate was considered as a crude extract of the enzyme cathepsin.

2.2.2. Enzyme characterization. Characterization of cathepsin was the measurement of crude extract enzyme activity, determination of optimum temperature, optimum pH, optimum substrate concentration, inhibition of metal ions, purification with ammonium sulfate precipitation concentration of 40-80%. Cathepsin enzyme activity refers to an extract of 0.5 mL was added with hemoglobin substrate 2% pH 2 at 37°C for 10 minutes. The reaction was stopped by the addition of trichloroacetic acid (TCA) 5%, the mixture was filtered with filter paper. The filtrate was added with a 1:1 (v/v) folin ciocalteu reagent then absorbed at 750 nm wavelength. Blank and standard measurements was conducted with the same procedure, where the standard using tyrosine.

Determination of enzyme activity at varied temperature were 30, 40, 50, 60 and 70°C. Determination of optimum pH in the range of 3, 4, 5, 6, 7 and 8. Metal ion inhibition activity using (NaCl, CaCl2, MgCl2, MnCl2, CuCl2 and FeCl3). Determination of protein concentration using the Bradford method with bovine serum albumin (BSA) as standard. The standard concentration used is 0.1-1.0 mg/mL (Dinu et al 2002).

3. Results and discussion

3.1. Crude extract cathepsin activity
Crude extract activity of cathepsin enzyme was 0.186 U/mL, which is lower than the crude cathepsin extracted from milkfish skin with activity of 0.7800-1.1429 U/mL (Nurhayati et al 2010), crude cathepsin extracted from catfish meat 0.278 U/mL (Fikri et al 2014) and smoked catfish with an activity
unit of 0.585-0.880 U/mL (Swastawati et al 2016). Cathepsin B extracted from seabass *Dicentrarchus labrax* showed the higher activity of 2168 U/g (Chêret et al 2007). Species and fresh level of fish could affect the activity of an enzyme. Nonthaput et al (2017) reported that the cathepsin enzyme activity of tilapia significantly increased during fish spoiled period at room temperature. The time delay after death could increase the specific activity of cathepsin. Other factors that affect the activity of the enzyme include temperature, degree of acidity (pH), inhibitors, enzyme concentration, and substrate (Abdurrahman 2008).

3.2. **Crude extract cathepsin characterization**

3.2.1. **pH.** Enzyme characteristic is one of the distinguishing indicators against other enzymes, pH conditions can affect the activity of enzymes. Cathepsin pH level was presented in figure 1. Optimum cathepsin enzyme from yellow pike at pH 5 with the activity of 0.25 U/mL. The pH of cathepsin enzymes varies, carp meat optimum was pH 5 (Makinodan et al 2006), milkfish optimum was pH 4 (Nurhayati et al 2012) meanwhile catfish optimum was pH 6 (Fikri et al 2014). These results indicated that optimal pH of the cathepsin enzyme tends to range between neutral acid. Kolodziedjska and Sikorsi (1996) explained that generally the cathepsin enzyme activated at pH 3-4 but the other has high activity at pH 6-6.5.

![Figure 1. Cathepsin activity of yellow pike on different pH.](image)

3.2.2. **Optimum temperature.** The temperature factor influences the catalysis rate of the enzymatic reaction. Cathepsin enzyme activity at different temperature conditions was presented in figure 2. Optimum cathepsin enzyme temperature was 50°C. The temperature could affect enzyme activity because the main structure of enzymes was protein, the structure of proteins could altered due to heating, increasing temperature could increased enzyme activity to the optimum temperature conditions, but in excess of heat could result in a decreased of enzyme activity. The temperature increased at a certain point could cause interference of the enzyme tertiary structure, structural changes on the active side could inhibit the catalytic activity of the enzyme (Stoker 2010).

3.2.3. **Effect of metal ions on enzyme activity.** Metal ions can interact with enzymes both as activators and inhibitors. As an activator ion can be characterized by increasing the activity of enzymes on the contrary as an ion inhibitor marked by decreasing the activity of the enzyme. Figure 3a shows the activity of the enzyme cathepsin with the addition of metal ions, while figure 3b shows the relative activity of the enzyme.
**Figure 2.** Cathepsin activity of yellow pike fish at different temperature.

**Figure 3.** Effect of metal ions on enzyme activity of yellow pike: (A) enzyme activity, (B) relative enzyme activity.

Decreased activity occurs in the addition of metal NaCl, MnCl₂, MgCl₂ and CaCl₂ ions which were indicated by a range of percentage values of 17-61%, therefore these ions were categorized as inhibitors of the enzyme cathepsin. Reaction with the addition of CuCl₂ and FeCl₃ ions was able to increase the enzyme activity of yellow pike fish, CuCl₂ was able to increase the activity of the enzyme cathepsin by
435% (4.35 times), whereas FeCl$_3$ was able to increase activity 668% (6.68 times). Metal ions could form a complex with the substrate and the active site of the enzyme to form reactive compounds. Metal ions also function as strong electrons attracting electrons at certain stages in the catalytic cycle. These chemical components function as cofactors and in this study Fe$^{3+}$ and Zn$^{2+}$ act as cofactors that accelerate enzyme catalytic reaction.

3.3. Enzyme purification

The initial stage of purification of the crude enzyme extract, namely precipitation of ammonium sulfate salt, the method is relatively easy and relatively inexpensive. The principle of salt deposition is to dissolve proteins where proteins can bind to ionic salts to a certain degree reduce the solubility of proteins in solution so that the protein settles (salting in), conversely the addition of high ionic salt concentrations will make protein solubility decrease due to the density of ionic salts which are more loaded bigger than protein (salting out). Salt and water molecules interact and attract water that surrounds the surface of the protein so that the protein interacts, aggregates then settle (Harris 1898). Ammonium sulfate (NH$_4$)$_2$SO$_4$, is often used for salting out because of its high solubility, which allows solutions of very high ionic strength, low price, and availability of pure material. Additionally, NH$_4^+$ and SO$_4^{2-}$ are at the ends of their respective Hofmeister series and have been shown to stabilize protein structure (Burgess 2009) The specific activity of the cathepsin enzyme is presented in table 1.

| Ammonium sulfate concentration (%) | Enzyme activity (U/mL) | Protein concentration (mg/mL) | Specific activity (U/mg) |
|-----------------------------------|------------------------|------------------------------|-------------------------|
| 40-50                             | 0.09                   | 0.08                         | 1.15                    |
| 50-60                             | 0.01                   | 0.11                         | 0.10                    |
| 60-70                             | 0.21                   | 0.15                         | 1.41                    |
| 70-80                             | 0.03                   | 0.15                         | 0.23                    |

The highest specific activity of purified cathepsin is showed on the fraction of 60-70% with the specific activity score of 1.4096 U/mg. Ammonium sulfate was also used to purify the enzyme Trachurus japonicas, where its activity increases up to three times as much as 3.87×10$^{-4}$ U/mg (Yoshida et al. 2015). This concentration could precipitate the optimum protein in the cathepsin enzyme of yellow pike fish. The addition of ionic salt precipitates various types of proteins, including the enzyme protein cathepsin and non-enzyme protein.

4. Conclusion

The activity of cathepsin enzyme crude extract from yellow pike fish was 0.19 U/mL with the optimum pH 6 and temperature conditions 50°C. The metal ions such as NaCl, MnCl$_2$, MgCl$_2$, and CaCl$_2$ were inhibitors of the cathepsin enzymes, meanwhile CuCl$_2$ and FeCl$_3$ were acted as ion activators. The addition of ammonium sulfate could increase the specific activity became 1.41 U/mg.

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