Purification and characterization of a cathepsin inhibitor from catfish (*Pangasius* sp.) of Indonesian water

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**Abstract**

Cathepsin inhibitor has been purified to homogeneity from catfish muscle (*Pangasius* sp.). The purification was carried out through ammonium sulphate precipitation, followed by ion exchange chromatography using DEAE-Sephadex A-75, followed by gel filtration on Sephadex G-100. Throughout the purification procedure, cathepsin inhibitory activity was determined against hemoglobin. A single band of molecular weight 16.65 kDa was obtained after the Sephadex G-100 filtration and revealed inhibitory activity against cathepsin as estimated by SDS-PAGE. The purified inhibitor possessed a specific activity of 16.9-fold higher than the initial activity with a 1.85 % yield. The optimum pH of the inhibitor was eight at 40°C. The inhibitor was stable at 10-50°C and at pH 7-9. Ions Mn$^{2+}$ increased the inhibitory activity, while Ca$^{2+}$ and Co$^{2+}$ were slightly repressed. The enzyme inhibitor extracted from this study had similar properties to available commercial inhibitors.

**Introduction**

Degradation of fish quality is initiated by the enzymes activity naturally present in a fish muscle. Proteolytic enzymes, e.g cathepsin, calpain, and collagenase, are major enzymes that cause deterioration of fish quality, such as the accumulation of metabolites, changes in taste, texture softening, and formation of volatile components (Huss, 1995). Cathepsin is a group of cysteine proteases that soften fish muscle (Ladrat *et al*., 2006). Cathepsin L found in most proteinase causes myofibril protein degradation in pacific whitening fish surimi (Morrissey *et al*., 1995) and cannot be removed by conventional bleaching (Hu *et al*., 2007). The addition of protease inhibitors like cystatin into surimi can prevent gel softening by inhibiting endogenous cysteine protease (Morrissey *et al*., 1995). Some protease inhibitors have been studied on fish muscle to understand the role of enzymes in the process of flesh softening during post mortem (Wang *et al*., 2009: 2011). Natural protease inhibitors have been successfully purified into cystatin from Glassfish eggs (Ustadi *et al*., 2005), ovarian fluid from tilapia fish, salmon eggs, fish skin from Atlantic salmon and cod (Olonen, 2004), chum salmon plasma and as trypsin inhibitors from skipjack tuna’s eggs (Li *et al*., 2008).

Catfish (*Pangasius* sp.), as well as other living things, are a source of enzymes and natural inhibitors that can exist in cells (intracellular) and are attached to either the membrane or outside the cell (extracellular) (Choi *et al*., 2002). In an attempt to find cathepsin inhibitors, the source with the highest inhibitory activity was sought in this study from catfish muscle. The inhibitor was purified and characterized and subsequently compared with the available inhibitors.

**Materials and Methods**

**Sample preparation**

Catfishes were collected from a culture pond in Bogor, Indonesia. The samples were transported live and killed upon arrival at the laboratory and stored at -1°C. Cathepsin inhibitor was prepared from catfish muscle in the pre-rigor phase (immediately after death), while cathepsin enzyme was prepared in the post-rigor phase (15 hours after fish death at room temperature) according with Nurhayati *et al.* (2010).

**Extraction and purification of cathepsin inhibitor**

Protease inhibitor from the fish muscle was prepared based on the method of An *et al.* (1995) with some modifications. Fish muscle (100g) was suspended with 100 mL of cold distilled water
(<4°C) and homogenized with Nissei AM-3. The disrupted muscle was centrifuged at 5,000g for 30 min and the obtained supernatant was collected. McIlvaine’s buffer (buffer A) pH 5.5 made from 0.2 M sodium phosphate (Merck) and 0.1 M citric acid (Applichem) was then added into supernatant with an equal volume. The mixture was incubated for 10 min at 80°C and then subjected to centrifugation at 7,000g for 15 min. The obtained supernatant was stored at 4°C and treated as crude extract.

Crude inhibitor extracts were subjected to ammonium sulfate (Merck) precipitation 30%-80% (w/v). The precipitate obtained after centrifugation at 12,000g for 30 min was dissolved in buffer A. Dialysis (MWCO 12kDa) was performed for further purification using buffer B (20 mM Tris base pH 7.5 containing 10 mM sodium azide (Merck) and 10 mM 2-mercaptoethanol (Sigma) (Ustadi et al., 2005).

Extracts of cathepsin inhibitor were purified with ion exchange chromatography by means DEAE Sephadex A-75(Sigma). Ion exchange chromatography column was equilibrated with buffer B. Proteins were eluted from the column using a linear gradient of NaCl (0-0.7 M) in buffer B at flow rate of 1 mL/min. Fractions with the highest inhibitory activity were pooled and further purified with gel filtration on Sephadex G-100 (Sigma). The gel filtration was monitored at 280nm and the eluted fractions with inhibitory activity were then pooled (Cao et al., 2000).

Preparation of cathepsin extract

Extraction of cathepsin followed the method of Dinu et al., (2002). Fish muscle from the post-rigor phase was taken and homogenized in distilled water using Nissei AM-3. The homogenized meat was centrifuged at 600g (Sorvall) for 10 min. The obtained supernatant was centrifuged at 10,000g for 10 min. The pellets were suspended in 0.1 M Tris-HCl buffer, pH 7.4, and centrifuged at 4,000g. The pellets were discarded, while the supernatant was collected and used as the crude extract for the assay of cathepsin inhibitor.

Analysis of cathepsin inhibitor activity

Cathepsin inhibitor activity was determined by measuring the degree of inhibition against cathepsin using hemoglobin as a substrate as described by Dinu et al. (2002). A 1 mL of inhibitor extract was added to 0.1 mL of cathepsin and incubated for 30 minutes at 37°C. 0.5 mL of hemoglobin substrate solution (Sigma) 2% (w/v) was added to the combined solution and after 10 min of incubation at 37°C, the reaction was stopped via the addition of 2 mL TCA 5% (w/v). The acid insoluble materials were filtered and 1 mL obtained supernatant was reacted with 1 mL of folin reagents (Merck) and followed with 20 minutes incubation at 37°C. The absorbance was measured at 750 nm. A blank and a control were prepared with the same procedure, although the cathepsin was replaced with distilled water and tyrosine, respectively. One unit of inhibitory activity was defined as an amount of inhibitor that decreased the cathepsin activity by 50%. The activity of milkfish cathepsin inhibitor was compared with available commercial inhibitor, namely pepstatin 1 mM, PMSF 1 and 5 mM, and EDTA 1 mM.

Protein determination

The concentration of protein was determined by the Bradford method (Bradford and Marion, 1976) using bovine serum albumin (Applichem) as the standard. In addition, during the enzyme purification with column chromatography, the protein elution profile was monitored spectrometrically at an absorbance of 280 nm.

Molecular weight determination by SDS-PAGE

SDS-PAGE was carried out according to the method established by Laemmli (1970). The protein bands were stained with silver nitrate solution. The protein markers were β-galactosidase (175 kDa), MBP-paramyosin (80 kDa), MBP-CBD (chitin binding domain) (58 kDa), CBD-mxe intein-2CBD (46 kDa), CBD-mxeintein (30 kDa), CBD-BmFKBP13 (25 kDa), lysozyme (17 kDa), aprotinin (7 kDa) (Fermentas).

Effect of pH and temperature

Optimum pH of cathepsin inhibitor was determined by varying the pH of reaction mixture from 3 to 10. The activity of inhibitor was analyzed by the previously described method (Dinu et al., 2002). The effect of pH on stability was determined by pre-incubating the inhibitor in various pH for 10 min at 37°C. The influence of temperature on cathepsin inhibitor activity was analyzed by varying the incubation temperature from 10-70°C. Meanwhile, the effect of temperature on inhibitor stability was determined by pre-incubation for 10 minutes at 10 to 70°C.

Effects of metal ions on cathepsin inhibitor activity

The effect of metal ions on the cathepsin inhibitor activity was investigated by adding the metal ions (Na+, Ca2+, Mn2+, Co2+) to the reaction mixture. The final concentrations of each metal ion were 1 mM and 5 mM.
Results and Discussion

In this study, cathepsin inhibitor was extracted from catfish muscle in the pre-rigor phase. The cathepsin enzyme is located inside the lysosomes while the inhibitor is located in cytoplasm and at pre-rigor phase, cathepsin and its inhibitor remain separated (Sentandreu et al., 2002). The extraction method was performed at 80°C to deactivate protease contained in the extract solution. Otto and Schirmeister (1997) reported that cystein inhibitor was stable at high temperatures due to multiple disulfide binding. Purification of cathepsin inhibitor from catfish of Indonesian water carried out through three steps: ammonium sulfate precipitation, ion exchange chromatography and gel filtration. Table 1 summarizes the results for each purification stage. The purification increased the activity 16.9 fold from crude extract with a specific activity rate of 68.15 U/mg. The purification procedure carried out in this research produced similar results with Choi et al. (2002), which involves the ion exchange chromatography technique and gel filtration to purify trypsin protease inhibitors in skipjack tuna fish eggs, and were able to increase the activity 18.18 fold. However, the above procedure gave a lower result compared to Ustadi et al. (2005), whereas the activity of purified a cysteine protease inhibitor was 49.69 fold.

Ammonium sulphate was added to the crude inhibitor extract and the precipitate was dissolved in a buffer solution. The solution was applied to a DEAE Sephacel A-50 (Figure 1) and the active fraction (fraction 10) was pooled, and refractionized to gel chromatography on Sephadex G-100 (Figure 2). The active fraction (fraction 8) was pooled and stored for further analysis.

Protein purification for each step is shown in Figure 3. Each purification step reduced the impurity of protein and a single band was detected in the end of gel filtration. The molecular mass of cathepsin inhibitor from catfish was 15.65 kDa by 10% SDS-PAGE. Cystein protease inhibitor molecular mass can be divided into three families: the steffin, the cystatins; and the kininogens (Sentandreu et al., 2002). The molecular mass of cathepsin inhibitor was similar to the cystatin family, i.e. 12 to 13 kDa. It was slightly smaller than that of a protease inhibitor isolated from Glassfish eggs (Liparistanakai) 16 kDa (Ustadi et al., 2005), sea anemone (Actinia equina) 16 kDa (Lenarcic et al., 1997), but higher than the cysteine protease inhibitor of the eggs from chum salmon (Oncorhynchus keta) 9 kDa (Yamashita et al., 1996).

The effect of temperature on activity and stability of cathepsin inhibitor are shown in Figure 4 and Figure 5, respectively. The temperature affected the cathepsin inhibitor by reducing their inhibitory capacity. The optimal temperature for the cathepsin inhibitor extract to inhibit cathepsin activity was 40°C. Cathepsin inhibitor extract was almost inactive at low temperature, however as the temperature increased, the inhibition activity increased gradually (Figure 4). The inhibition activity decreased at temperatures above 40°C. The inhibition ability of cathepsin inhibitor decreased to 65 percent when the temperature was increased to 70°C. This result showed that cathepsin inhibitor may undergo thermal denaturation when exposed to high temperatures. This phenomenon was similar to the results obtained by Garcia-Carreno et al. (1997) and Alarcon et al. (2001) on protease inhibitor from seed extract. The stability to temperature of catfish muscle cathepsin inhibitor has a different profile compared to Ustadi et al. (2005) on glassfish egg proteinase inhibitor (stable at 50-65°C), Alaska pallock (stable at 5-35°C) and Chum salmon (stable at 5-40°C).

The correlation of cathepsin inhibitor activity and pH is shown in Figure 6. Cathepsin inhibitor possessed the maximum ability at pH 8.0. Other studies showed that protease inhibitors like cystatin

### Table 1. Cathepsin inhibitors decreased cathepsin activity at various stages of purification

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Protein (mg/ml)</th>
<th>Inhibitor activity (U/ml)</th>
<th>Total protein (mg)</th>
<th>Total inhibitor activity (U/ml)</th>
<th>Specific inhibitor activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.0830</td>
<td>1.6450</td>
<td>122458</td>
<td>493506</td>
<td>4.0506</td>
<td>100.00</td>
<td>1.0</td>
</tr>
<tr>
<td>Precipitation</td>
<td>0.3221</td>
<td>1.7222</td>
<td>161029</td>
<td>86111</td>
<td>5.3475</td>
<td>17.45</td>
<td>1.3</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>0.1059</td>
<td>1.9867</td>
<td>0.5294</td>
<td>9.853</td>
<td>18.7625</td>
<td>2.01</td>
<td>4.7</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>0.0268</td>
<td>1.8281</td>
<td>0.1341</td>
<td>0.141</td>
<td>68.1590</td>
<td>1.85</td>
<td>16.9</td>
</tr>
</tbody>
</table>

### Table 2. Effects of metal ions on the activity of the purified cathepsin inhibitor

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>94</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>108</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>98</td>
</tr>
<tr>
<td>Na⁺</td>
<td>104</td>
</tr>
</tbody>
</table>

### Table 3. Comparison of inhibitory activity of catfish muscle cathepsin inhibitor with other inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitory Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish inhibitor</td>
<td>82.51</td>
</tr>
<tr>
<td>Pepstatin 1 mM</td>
<td>94.26</td>
</tr>
<tr>
<td>PMSF 1 mM</td>
<td>83.93</td>
</tr>
<tr>
<td>PMSF 5 mM</td>
<td>83.04</td>
</tr>
<tr>
<td>EDTA 1 mM</td>
<td>91.07</td>
</tr>
<tr>
<td>EDTA 5 mM</td>
<td>92.86</td>
</tr>
</tbody>
</table>
have an inhibitory activity within an alkaline pH range. In the study of Ustadi et al. (2005), protease inhibitor extract from Glassfish eggs had high activity at pH range of 7.0-9.0, with the optimum pH was 8.0. The study of Li et al. (2008) on cysteine protease inhibitors from chum salmon plasma showed that the activity of the inhibitors increased at pH 6.0-9.0, with the optimum pH was 7.0.

Acidic and base solution have a negative effect on the activity of cathepsin inhibitors. Cathepsin inhibitor was unstable when exposed to strong acidic or base solution (Figure 7). The cathepsin inhibitor was the most stable at pH 8.0 and could maintain its inhibition ability by more than half of its maximum stability at pH 4.0-9.0. Choi et al. (2002) reported that inhibitor isolated from tuna eggs was stable at
pH 4-10 and could maintain its inhibition ability up to 80 percent, however it was inactive at a pH below 4 and above pH 10.

Activity of cathepsin inhibitor is influenced by metal ions (Table 2). Metal ions revealed non-significant effects at low concentration, while at higher concentration ions Ca²⁺ and Co²⁺ reduced the activity of the inhibitor while ion Mn²⁺ positively affected the activity of the inhibitor. Different results were obtained by Choi et al. (2002) on trypsin inhibitors. They found Ca²⁺ activated the inhibition activity of the inhibitor, instead of reducing the activity.

Activity of cathepsin inhibitor extracted from catfish muscle was compared to available commercial inhibitors (Table 3). The extract possessed similar activity to PSMF 1 and 5 mM to inhibit cathepsin activity, however lower than pepstatin and EDTA.

Conclusions

The cathepsin inhibitor from catfish muscle were successfully characterized. The molecular weight of inhibitor was estimated to be 15.65 kDa by SDS-PAGE. It was found to be stable to heat and active in neutral pH. Comparing with commercial available inhibitor, the purified cathepsin inhibitor has a similar result.

Acknowledgement

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References

