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Thermostable serine protease inhibitor from Death cap (*Amanita phalloides*)

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ABSTRACT

Background: Protease inhibitor plays an important role in many biological processes in an organism, its selective binding toward protease potentially tuning down some specific biological processes such as enzymatic catalysis regulation, protein signaling as well as protein clearance in order to accomplish homeostasis. Several protein-based protease inhibitors have been isolated and identified, the majority are directed toward serine protease.

Objectives: This study aimed to find a potential protease inhibitor from the local Northern Thailand Death cap (*Amanita phalloides*) together with biochemical characterization of its general properties.

Materials and methods: Death cap extract collected from Phayao Province, Thailand was initially performed trypsin inhibitory activity assay using BSA and alzoalbumin as substrates. Detection of its inhibition activity was assessed by SDS-PAGE and spectrophotometry. Additionally, molecular size was observed by filtering the extract through 3 kDa molecular cut off membrane. Finally, hydrophobic property was verified by passing the filtrate through phenyl sepharose column chromatography.

Results: Death cap contained serine protease inhibitory activity. Molecular size of inhibitor was suspected to be less than 3 kDa. Hydrophobic property of this inhibitor was observed. Interestingly, its inhibitory property retained after heat inactivation at 100 °C for 10 min.

Conclusion: A novel heat-tolerant inhibitor from the water extract of Death cap was characterized to be a small peptide with hydrophobic property, which could be used as a new peptide protease inhibitor targeting to serine protease that benefit for agricultural and medical field.

Introduction

Protease inhibitors are substances that interact with target proteases and abolish protease activities. The inhibitors with native origin are ultimately significant for regulating proteolysis activities in living cells, which are vitally essential.[1,2] In addition to the functions in living cells, these inhibitors have been applied for a variety of applications; in medical field, agriculture and biotechnology.[3,4] Large numbers of protease inhibitors were discovered in a wide range of living organisms including animals, plants and microorganisms.[5] Protease inhibitors can be classified into several classes based on targeted amino acid on their binding pockets such as cysteine protease inhibitors, serine protease inhibitors.[1] Prominently, serine protease inhibitors have shown to be an interesting group of the inhibitors. Their importance role in many biological processes such as regulation of proteolysis reaction in blood coagulation and implication in tumor suppression were established.[6,7] However, only a few serine protease inhibitor have been found in mushrooms.[8]

Death cap (*Amanita phalloides*) is a well-known mushroom which contains toxic substances capable of conferring death to consumers and found extensively in

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both Thailand and other parts of the world. It is classified as ectomycorhiza fungi whose growth symbiotically associated to numerous kinds of trees in the deciduous dipterocarp and pine-dipterocarp forest such as pine, chestnut and deciduous wood tree. Normally appearance of its growth is observed around the root of trees when suitable humidity and temperature exists especially in the raining season. Death cap is morphologically considered the condense form of fruiting body with the distinctive colors such as pale green, yellow or light brown. In Thailand, known as "hed Ra-ngok-hin", it constitutes white gills and stem part without hole compared to "an edible mushroom". [9,10]

This mushroom has been reported to cause death in victims following liver failure. Most research of this mushroom has targeted on RNA polymerase inhibitors which block protein synthesis especially in the liver.[11] However, there are several substances in the mushroom not properly characterization.

The present study aimed to find a new source of serine protease inhibitor from Death cap together with the characterization of this new protease inhibitor such as the hydrophobicity by the phenyl sepharose column chromatography and the molecular mass by molecular cut off.

Materials and methods

Materials

Fruiting bodies of Death cap were collected from Rongkamlaung Forest Park in Phayao Province, Thailand. Centricon was from Merck, Germany. Phenyl-SepharoseCL-4B was from GE Healthcare, Sweden. Trypsin, Azoalbumin, Bovine serum albumin (BSA) and Phenylmethylsulfonyl fluoride (PMSF) purchased from Sigma, USA. All other chemicals were analytical grade.

Mushroom extraction

A 30 gm of the fresh fruiting body of mushroom was grounded with 90 ml of distilled water at room temperature. The homogenate was centrifuged at 10,000xg for 10 min at room temperature. Supernatant was determined for protein content by Lowry method using BSA as standard protein[12] and collected as a crude water extract.

Determination of protease inhibitor activity using SDS-PAGE

A reaction mixture of 30 µL contained 12 µL of 1 mg/mL of BSA solution, 6 µL of distilled water, 10 µL of crude water extract or PMSF as positive control and 2 µL of 1 mg/mL of trypsin (Sigma) solution. Reaction was started by adding the trypsin solution and incubated 37 ºC for 30 min. It was stopped by adding 6 µL of 5x SDS buffer, immediately heated at 100 ºC for 5 min and then centrifuged at 10,000 xg for 5 min. Supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). [13]

Protease inhibitor assay by spectrophotometric method

Method was modified from Tomarelli [14] as follows, 1000 µL reaction composed of 858 µL of "0.5% (w/v) sodium bicarbonate, pH7.4", 100 µL of 1.25% (w/v) Azoalbumin, 40 µL of distilled water or testing sample (mushroom extract etc.) or PMSF as positive control and 2 µL of 1 mg/mL of trypsin solution. Reaction was started by adding the trypsin solution and incubate 37 ºC for 20 min. Then 300 µL of the reaction mixture was taken, immediately mixed with 600 µl of 5% TCA and 450 µL of 0.1M sodium hydroxide was then added. After mixing, the solution mixture was centrifuged at 10,000 xg for 5 min. The supernatant was measured for absorption at 440 nm. To determine the effect of heat, the mushroom extract was incubated for 10 min at 0, 30, 60, or 100 °C before adding to the reaction mixture.

Separation by molecular filtration

Mushroom extract (96 mg protein) was loaded into 3-kDa-cutoff Ultracel-3K molecular filtration device and centrifuged at 10000 xg at room temperature until the retentate decreased to minimal volume. The filtrate was collected. The retentate was adjusted to original volume using 50mM Tris-HCl buffer, pH 8.3. Both the filtrate and the retentate were determined for protein contents and tested for protease inhibitor activity using the spectrophotometric method.

Phenyl Sepharose column chromatography

Phenyl Sepharose CL-4B was washed with distilled water to avoid the preservative agent and broken beads. Sediment bead was used to load on 12x1 mL glass column which washed and equilibrated with 10 volume of 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate. The filtrate (26 mg protein) obtained from molecular filtration was pre-equilibrated with 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate with 1:1 (v/v)ratio before loaded on to the column and then left until sample absorbed into a gel completely. Unbound fraction was collected by washing with 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate 80 mL at 1 mL/min flow rate and Fractions of 2 mL were collected until no absorbance at 280nm was observed. Bound substances were eluted by a stepwise method using 50 mM potassium phosphate buffer, pH 6.5 containing 0.5M ammonium sulfate 60 mL to elute weaker binding peptide and then by 50 mM potassium phosphate buffer, pH 6.5 pH 60 mL to elute stronger binding peptide, the flow through was monitored at 1 mL/min. Fractions of 2 mL were collected until no absorbance at 280 nm was observed. Each peak fraction was pooled, determined for protein content, and for protease inhibiting activity using the spectrophotometric method.

Results

Protease inhibition activity of the crude water extract from Death cap fruiting body

Death cap fruiting body 30 gm was initially extracted with water and obtained 96 mg protein crude water extract which was then assessed for the protease inhibition activity using SDS-PAGE (Figure 1), BSA band at 60 kD was disappeared by trypsin (Lane 5) while the positive control; Phenylmethylsulfonyl fluoride (PMSF) and different concentrations of the crude water extract decreased the activity of the enzyme resulting in the visualized BSA band (Lane 6-8).

Thermo stability of the protease inhibitor

The crude water extract was incubated separately at 0, 30, 60 or 100 °C for 10 min before performing protease inhibition assay by the spectrophotometric method. The result showed that the protease inhibitor in the crude water extract is thermo stable as observed by the retaining protease inhibition activity (20% inhibition) after incubation at 100 °C for 10 min. (Figure 2).

Partial purification of the protease inhibitor

Separation by molecular filtration, the filtrate containing 26 mg protein (27% yield) was obtained from 96 mg protein of the crude water extract. After passing through hydrophobic phenyl sepharose column, an unbound peak (UB) and two bound peaks (B1, B2) were obtained (Figure 3). B1 peak collected from fractions 16-18 contained 5.65 mg protein (5.8% yield) while B2 peak collected from fractions 27-29 had 3.5 mg protein (3.6% yield). All collected peaks were tested for their inhibitions to trypsin. Peak B1 showed the strongest activity with 30% inhibition (Figure 4).

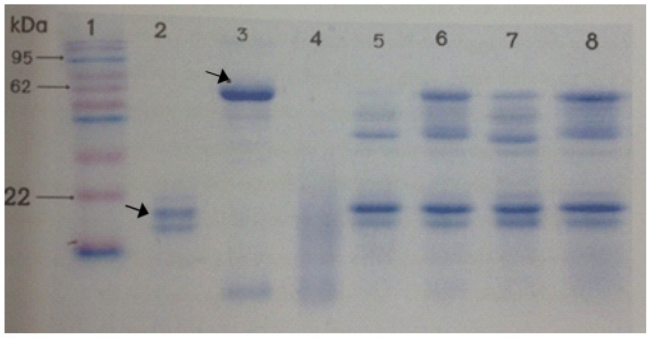

Figure 1. *Detection of protease inhibition activity using SDS-PAGE. Inhibition of trypsin activity was demonstrated on SDS-PAGE (12% polyacrylamide) using BSA as substrate of the enzyme. Protein bands were visualized by Coomassie Brilliant Blue staining. Lane 1: Standard protein maker, Lane 2: trypsin, Lane 3: BSA, Lane 4: Crude water extract (64 ug), Lane 5: BSA plus trypsin, Lane 6: BSA plus trypsin plus 1mM PMSF, Lane 7: BSA plus trypsin plus crude water extract (32 µg), Lane 8: BSA plus trypsin plus crude water extract (64 µg). Position of BSA and trypsin band are indicated by arrow head.*

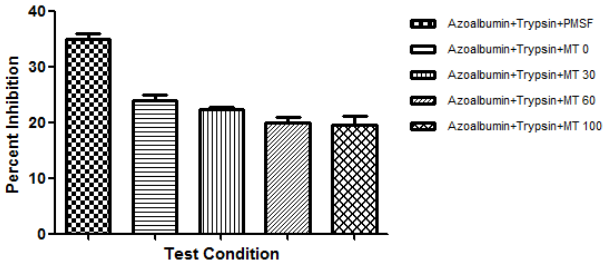

Figure 2. *Thermostability of the protease inhibition factor. Mushroom extract was divided into four parts and then incubated at 0, 30, 60 or 100 °C for 10 min (MT0, MT30, MT60 and MT100, respectively) before using for the protease inhibition assay.*

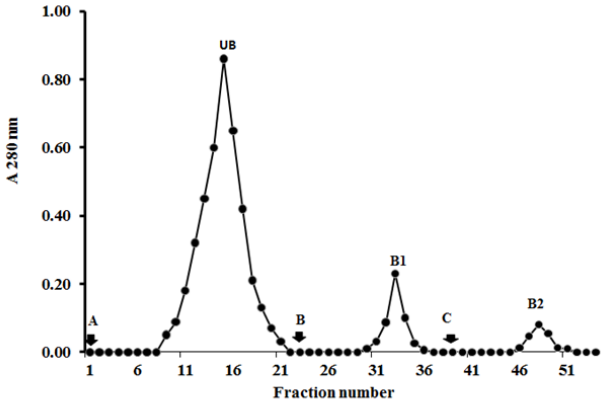

Figure 3. *Phenyl-sepharose column chromatogram of molecular filtrate fraction. Filtrate obtained from molecular filtration was subjected to phenyl sepharose column chromatography following the collection of 2 mL per fraction. Protein concentration of each fraction was determined by monitoring the absorbance at 280 nm. A, B and C indicate the addition of each buffer solution. Different buffers were performed at different stage of elution steps as A: 50 mM potassium phosphate buffer, pH 6.5 containing 1M ammonium sulfate, B: 50 mM potassium phosphate buffer, pH 6.5 containing 0.5M ammonium sulfate and C: 50mM potassium phosphate buffer, pH 6.5. UB means the peak collected from unbound fractions. B_1 and B_2 mean the peaks collected from bound fractions eluted by the buffer solution as indicated in the picture.*

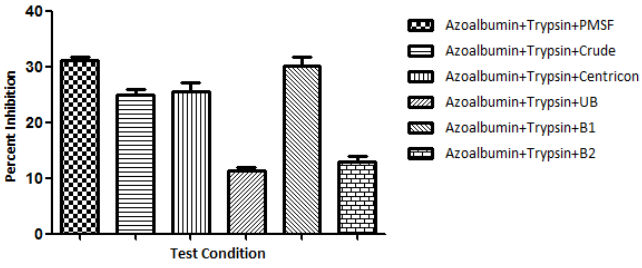

Figure 4. *Inhibition activity of the the purified fractions. Crude water extract, filtrate obtain from molecular filtration (Centricon), unbound fraction and bound fractions were tested for their inhibition of trypsin activity.*

Discussion and Conclusion

Death cap causes the death of people who unintentionally ingest this mushroom which contains toxic substances known as amatoxins and phyllotoxins. Among these toxic substances, the most dangerous substances are amanitins that cause liver failure by inhibition of RNA polymerase II.[15] This mushroom also contains other toxic substances including toxophallin, phallolysin.[16] We are interested to find new protease inhibitor from the Death cap as it contains a varieties of toxic substances.[17] In addition, protease inhibitors have been characterized from different mushrooms.[2,18,19] In this study, the preliminary characterization was assessed by conducting reactions comprising trypsin as serine protease, BSA as protease substrates and PMSF as the positive control for serine protease in accompany with mushroom extracts to observe the potential serine protease inhibitor. The results of SDS-PAGE analysis show that the mushroom crude water extract significantly inhibited

trypsin activity in the concentration dependent manner (Figure 1). Additionally, the spectrophotometric method observing the result of trypsin catalyzed azoalbumin together with its thermostable property confirmed the existence of the inhibitor (Figure 2). In previous report, trypsin inhibitor from *Pleurotus floridanus* was reported to be thermostable up to 80 °C.[19] This study showed the presence of protease inhibitor in Death cap with exceptionally high stability toward heat inactivation (Figure2). Thermal stability of this protease inhibitor is plausibly applied to various applications in the scientific area and industries. This inhibitor is believed to be a small molecule as its passing through 3 kD molecular weight cut off membrane. Stronger inhibition activity was obtained after the partial purification using phenyl sepharose column chromatography. The result also indicates some hydrophobic area on the surface of the inhibitor molecule since it can bind to the column (Figure 3 and Figure 4). Further study will be conducted to find specificity, the molecular structure as well as other properties of this protease inhibitor. This new finding might be applied as the reported protease inhibitor that worked against diseases such as malaria, colds, flu, dengue, cancer, hypertension, Alzheimer as well as AID [4]

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Conflict of interest

The authors declare no conflict of interest.

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