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A simple method for Isolation of serine protease inhibitor from Siamese land snail Cryptozona (*Cryptozona siamensis*) mucus

Phichaya Khamai* **Pariyaphat Ngandee** **Ketpaillin Chimong** **Rungthiwa Srimora**
Pannawich Siriwechviriya **Widsanusan Chartarrayawadee** **Kritchai Pooncharean**

Division of Biochemistry, School of Medical Sciences, University of Phayao, Phayao Province, Thailand,

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ABSTRACT

Background: Siamese land snail is the single shell snail which has become widespread very quickly in Thailand and other parts of the world. It is considered to be a pest by most farmers since it destroys parts of plants such as leaves, vegetables and fruit by consuming them and leaving behind the line of sticky mucus over the plants. This mucus is believed to be the snail's defense mechanism, such as lubricant, for wound healing as well as a response against the snail's predators. That the land snail releases mucus in response to the predator has been hypothesized, however, screening for protease inhibitor has still not substantiated this. On the basis of the prevailing assumptions, through biochemical, and biophysical isolation we investigated the proteinase inhibitor functions of the protein from the mucus of the Siamese land snail.

Objectives: This study aimed to find a potential protease inhibitor from the mucus of the local Northern Thailand land snail (*Cryptozona siamensis*) together with the biochemical characterization of its general properties.

Materials and methods: Land snail extract collected from Phayao Province, Thailand, was initially subjected to trypsin inhibitory activity assay using alzoalbumin as substrates. The detection of its inhibition activity was assessed by spectrophotometry. Additionally, the molecular size was observed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Results: Land snail (*Cryptozona siamensis*) mucus contained serine protease inhibitory activity. The molecular size of the inhibitor was suspected to be about 10.5 kD. Interestingly, its inhibitory property was retained after heat inactivation at 100 °C for 20 min.

Conclusion: A novel heat-tolerant serine protease inhibitor from acetone extraction of land snail mucus was characterized as a small peptide with tolerance to organic solvent (acetone) and high temperature, which could be used as a new peptide protease inhibitor targeting serine protease that may benefit to scientific and medical fields.

* **Corresponding author.**
Author's Address: *Division of Biochemistry, School of Medical Sciences, University of Phayao, Phayao Province, Thailand*
** **E-mail address:** : phurngk@gmail.com
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Introduction

Protease inhibitors are substances that bind to and inactivate target proteases activities. They play an important role in many biological processes, such as regulation of proteolysis reaction in blood coagulation, and tumor suppression.[1] There have been reports of their application in the medical field, agriculture, and biotechnology.[2] Several protease inhibitors were discovered in a wide range of living organisms, including animals, plants, and microorganisms.[3] However, only a few protease inhibitors have been reported in snail mucus.[4]

Land snail (*Siamese cryptozona*) in Thailand, known in Thai as "hoi-dak-daan", is a small invertebrate animal in the Mollusca phylum with a circular single shell and brown to reddish-brown color. The shell is built up by an open circular Tubular coiled flat with a mature size of up to 30 mm in width. This snail is found throughout Thailand and other parts of the world. It habit is to hide itself during the day in dark and humid places, such as under the log, tree, and rotting tree. At night or dawn, it moves around on the ground, climbing the tree to find food, feeding on leaves, and vegetable.[5, 6] Along with moving around, the land snail also produces mucus. Different substances in land snail mucus have been studied[4, 7] Land snail mucus has been investigated with reports of several beneficial properties, such as antioxidant[8], anti-inflammation[9], antimicrobial[10], anti-cancer[11], and wound healing.[12] As for the commercial applications, land snail mucus has been developed, distributed and sold in the market such as cosmetic beauty products.[13] Snail mucus contains various valuable substances that are waiting to be discovered. In this study, we are interested in isolating and characterizing the protease inhibitor from *Siamese cryptozona* which may be the defense mechanism against microorganisms or predators.

The rapid mucus extraction procedure was introduced to be simple and less equipment consuming. To harvest the mucus from *Cryptozona siamensis*, a simple plastic bag was used as a moving pathway while it moved along and produce mucus in a critical environment as occurred in the nature. This adaptive procedure was capable of producing the large amount of snail mucus for the research as compared with other method of extraction.[14] Acetone precipitation was performed to isolate proteins instead of salt- induced protein precipitation as acetone could effectively solubilize sticky glutinous mucus from the protein pellet, avoiding clumping phenomenal that usually occur when perform salt- induced protein precipitation. Molecular weight of the protein was determined by SDS-PAGE as well as its activity by inhibition of trypsin catalyzing azoalbumin.[15]

It was found that the peptide showed the trypsin inhibition activity with heat-stable property while the molecular weight was approximately 10.5 kD.

Materials and methods

Materials

Land snails were collected from the ground area of the University of Phayao, Phayao Province, Thailand. Trypsin, Azoalbumin, Bovine serum albumin (BSA), and Phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma, USA. all the other chemicals were analytical grade.

Methods

Snail mucus extraction

In this study, 10-20 mature snails were cleaned by wet cloth and put in a clean 6*8" plastic bag, wrapped around and applied to stress at 60 °C for 5 min. The bag was then open in room temperature around 10 min. The snail will climb up toward the open site of plastic bag in company with mucus secretion. The mucus which flew down to the bottom of plastic bags was collected into test tube and centrifuged at 10,000 x g for 10 min to collected supernatant as a crude extract. (Figure.1A,B,C)

Protein precipitation from the mucus of snail with 20-80% serial acetone precipitation

Crude snail mucus was subject to a 20-80% serial acetone precipitation. Start with 4 ml of mucus with 0.8 ml 100% acetone, mixed and kept in -20 °C for 1 hr. then centrifuged to collected pellet and supernatant. Pellet was re-suspended and kept for protein and activity assay. The supernatant was further precipitated with 40, 60, and 80% acetone concentration, respectively.

Determination of protein concentration

Protein content in the sample was determined by a colorimetric method using Bio-Rad protein assay kit I (Bio-Rad, USA). Briefly, pipetting 20 µl of sample or distil water into 1.5 ml micro centrifuge tube following by 1 ml of Bradford reagent (1X), mix well and leave at room temperature for 5 min. Then measured the absorbance at 595 nm. The concentration of the protein sample was calculated according to standard curve of BSA. The protein was express as a unit of milligrams.

Determination of protease inhibitor molecular weight using SDS-PAGE

The molecular weights of inhibitors were estimated using SDS-PAGE. A reaction mixture contained 5 µg of fractionate protein fraction was mix with 5x SDS buffer, immediately heated at 100 ºC for 5 min and then centrifuged at 10,000 x g for 5 min. The supernatant was subjected to 13% polyacrylamide, SDS-PAGE. The proteins were visualized by Coomassie blue staining.

Protease inhibitor assay by spectrophotometric method

Method was modified from Tomarelli 15 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 (snail mucus extract) or PMSF as positive control and 2 µL of 1 mg/mL of trypsin solution. The reaction was started by adding the trypsin solution and incubate 37 °C for 20 min. Then 300 µL of the reaction mixture was taken, immediately mixed with 600 µl of 5% TCA, and 450 µL of 0.1 M sodium hydroxide was then added. After mixing, the solution mixture was centrifuged at 10,000 x g for 5 min. The supernatant was measured for absorption at 440 nm. To determine the effect of heat, the snail mucus extract was incubated at 100 °C for 10, 20, 40, and 80 min. before adding to the reaction

mixture. The triplicate reaction was carried out in all experiments and the results were evaluated by the mean standard.

Results

Protease inhibition activity of the crude extract from Siamese land snail mucus

Snail 100 gm. was initially used to induce mucus secretion and obtained 40 mg protein crude extract which was then assessed for the protease inhibition activity by trypsin catalyzing azoalbumin.

Partial purification of the protease inhibitor

From crude extract 40 mg protein follow serial concentration 20, 40, 60, and 80% acetone protein precipitation, the recovery protein was 3, 10, 17, and 1mg respectively. All collected sample, were tested for their inhibitions to trypsin. The fraction of 60% acetone precipitation (P60) showed the strongest activity with 57% inhibition (Figure 2).

Determination of protease inhibitor activity using SDS-PAGE.

Following the analysis of fractionated protein by SDS-PAGE. Fraction P60 showed the single major bands of protein approximately 10.5 kD. (Figure. 3).

Thermo stability of the protease inhibitor

Protein from P60 fraction was incubated separately at 100 °C for 10, 20, 40, or 80 min before performing protease inhibition assay by the spectrophotometric method. The protein retaining protease inhibition activity (43% inhibition) after incubation at 100 °C for 20 min. (Figure. 4).

A B C

Figure 1. *Snail mucus extraction. A, Cryptonas siamese snail, B, and C, Cryptonas siamese snail were forced to secrete mucus on a clean plastic bag.*

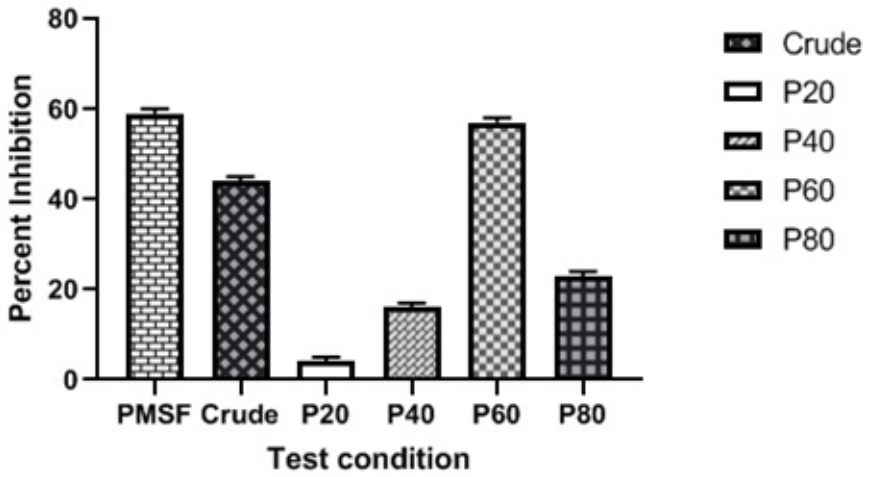

Figure 2. *Inhibition activity of the purified fractions. Crude, 20% (P20), 40 % (P40), 60% (P60), 80 % (P80) acetone precipitation respectively, were tested for their inhibition of trypsin activity.*

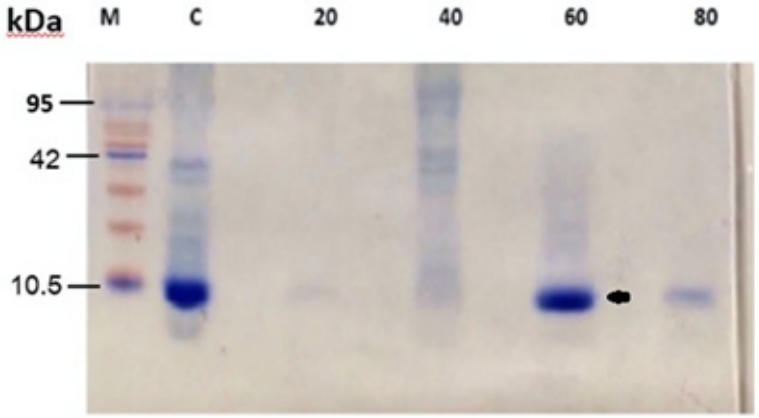

Figure 3. *SDS-PAGE of Cryptonas siamese mucus fractionations. C: Crude, 20, 40, 60, 80 are protein fraction which serial precipitation with 20, 40, 60 and 80% acetone, respectively. Arrow indicated a band of protease inhibitors.*

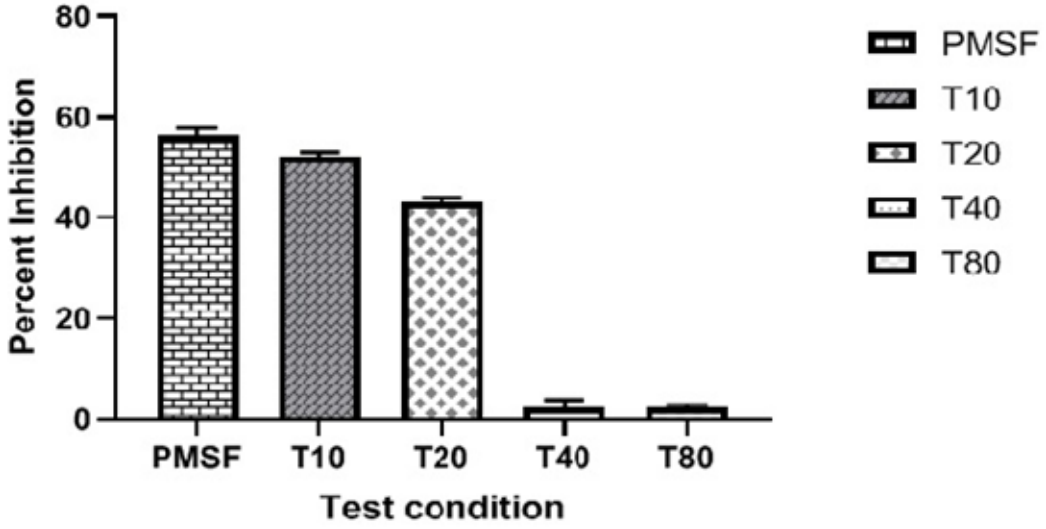

Figure 4. *Thermo stability of the protease inhibition factor. P60 snail extract was divided into four parts and then incubated at 100 °C for 10, 20, 40, or 80 min. (T10, T20, T40, and T80) respectively, before using for the protease inhibition assay.*

Discussion

The mucus of *Cryptonas siamese* contains several worthy substances to investigated and provide necessity to human, for instance; development to gel for wound healing[12], cosmetic cream[13] and other clinical used for human life.[9, 16] In this study, we provided information with a simple method for verify serine protease inhibitor from *Cryptonas siamese*. Including, an easy way to get a mucus from snail in plastic bag, this condition may cause lands snail consider for survival by moving and secreting a lot of mucus to defense itself.[7,17] Protein precipitation from mucus by acetone effectively removed undesirable proteins leaving only clear pellet from a viscous supernatant. This result clearly showed better procedure of protein isolation than salting-out method [10], since high salt usually causes clumping of glutinous mucus and then interfere protein separation. The procedure for evaluating the serine protease inhibition activities was performed simply by colorimetric spectrophotometric method and that for determining molecular size by SDS-PAGE. Following those simple procedures, the serine protease inhibitor in snail mucus was observed. Since several curative products have been made from protease inhibitor especially raised against the virus, this include: NS3; anti-hepatitis C[18], NS3 and NS5 proteins, for anti-dengue drug design.[19] Recently, a potential anti-viral drug discovery against SAR CoV was reported.[20] The present study protein with high protease inhibitor activity was pelleted in the fraction of 40-60% acetone precipitation (Figure. 2) which correlates to Crowell AM work that uses acetone 50-80% to precipitate protein.[21] SDS-PAGE analysis the protein was found the single band of about 10.5 kD (Figure. 3) which is correlated to serine protease inhibitor that reported in *Antheraea mylitta* with the molecular size of 10.4 kD.[22] The present protease inhibitor show high stability toward heat inactivation at 100 °C for 20 min (Figure.4) which is correlated to an evaluated of a serine protease in Death cap mushroom that stability at 100 °C for 10 min[23] and that of small pinto beans which tolerate at 100°C for half an

hour.[24]

This study informs the uncomplicated method to isolate and characterize of protease inhibitor protein from land snail mucus. Next work, the amino acid and DNA sequence, and deep qualifications needed to be verified. This protease inhibitor is plausibly applied to various applications in area of medical, scientific, and industries.

Conclusion

This study highlighted the simple procedure to harvest mucus of *Cryptonas siamese* and also characterized some biochemical properties of isolated protein. The protein contains serine protease inhibitor with molecular size approximately 10.5 kD. The inhibitor is shown to be tolerated to solvent (acetone) and high temperature.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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