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CHARACTERISATION OF *POMACEA CANALICULATA* EGGS TREATED WITH PROTEASE

NOOR HASYIERAH MOHD SALLEH[1*], NURHADIJAH ZAINALABIDIN[2] AND SITI NOOR HAJAR MD LATIP[3]

[1]Faculty of Chemical Engineering & Technology, Universiti Malaysia Perlis, Kangar, Perlis, Malaysia
[2]Faculty of Mechanical Engineering & Technology, Universiti Malaysia Perlis, Kangar, Perlis, Malaysia
[3]Sustainable Crop Protection Research Group, University Technology MARA, Shah Alam, Selangor, Malaysia

[*]Corresponding author: hasyierah@unimap.edu.my

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ABSTRACT: *Pomacea canaliculata* is a type of freshwater snail that has become a major pest in paddy fields, as it feeds on young paddy leaves and stems, thus, posing a serious threat to paddy production. It was named one of the world's top 100 worst invasive species, with serious consequences for the environment, human health, and the social economy. Their hatchability rate is high, which explains their global distribution worldwide. Therefore, it is vital to manage their hatchability to prevent their population from expanding further by understanding the protection that permits the eggs to survive. *P. canaliculata* eggs are covered with a thin layer of cuticle that is rich in protein to protect the embryo during the hatching process. The biological treatment with protease enzyme successfully hydrolysed the protein cuticle layer, lowering the percentage of hatchability. Disruption of the protein cuticle may have an impact on conductivity, water loss, hatching time, protein content, and other factors. However, documentation of the protease effect on the protein cuticle is scarce. Therefore, the goal of this study is to evaluate the protease treatment on the protein cuticle of *P. canaliculata* eggs physically (conductivity, water loss, and morphological analysis) and chemically (cuticle protein content, protein breakdown, and amino acid profile). Physical characterisation revealed that protease-treated eggs have higher conductivity and water loss than the control egg. Images taken with a light microscope (LM) and a scanning electron microscope (SEM) revealed changes in cuticle structure, which explained the protease-induced cuticle hydrolysis. Chemical characterisation revealed a decrease in cuticle protein content, hydrolysis of protein to a small size, and changes in amino acid composition. The physical and chemical analyses strongly suggested that protease can damage the cuticle protein, thus, preventing the eggs from hatching.

ABSTRAK: *Pomacea canaliculata* adalah sejenis siput air tawar yang telah menjadi perosak utama di sawah padi, kerana ia memakan daun dan batang padi yang muda, sekaligus menimbulkan ancaman serius kepada pengeluaran padi. Ia disenaraikan antara 100 spesis invasif utama dunia dengan kesan serius pada alam sekitar, kesihatan manusia dan sosio-ekonomi. Kadar penetasannya adalah tinggi, meningkatkan penyebaran mereka secara global di seluruh dunia. Oleh itu, adalah sangat penting untuk mengawal populasi ini daripada terus berkembang dengan memahami perlindungan yang membenarkan telur untuk hidup. Telur *P.canaliculata* dilapisi dengan lapisan kutikel nipis yang kaya dengan protein bagi memberi perlindungan untuk embrio semasa proses penetasan. Rawatan biologi dengan enzim protease telah berjaya menghidrolisis lapisan kutikel protein, sekaligus mengurangkan peratusan penetasan. Gangguan terhadap lapisan kutikel protein mungkin memberi kesan pada konduktiviti, kehilangan air, tempoh penetasan, kandungan protein dan faktor lain. Walau

bagaimanapun, kesan protease ke atas kutikel protein adalah kurang. Oleh itu, objektif kajian ini adalah mengkaji rawatan protease ke atas kutikel protein telur *P. canaliculata* secara fizikal (konduktiviti, kehilangan air, dan analisis imej kutikel) dan secara kimia (kandungan protein kutikel, pecahan protein dan profil asid amino). Ciri fizikal menunjukkan telur yang dirawat protease mempunyai konduksi dan kehilangan air tinggi berbanding telur kawalan. Imej yang diambil dengan mikroskop cahaya (LM) dan mikroskop pengimbas elektron (SEM) mendedahkan perubahan dalam struktur kutikel, yang menjelaskan hidrolisis kutikel yang disebabkan oleh protease. Ciri kimia menunjukkan penurunan kandungan protein kutikel, saiz kecil pada hidrolisis protein, dan perubahan pada kandungan asid amino. Analisis fizikal dan kimia mencadangkan bahawa protease merosakkan protein kutikel, oleh itu menghalang telur daripada menetas.

KEYWORDS: *physical characterisation; chemical characterisation; biopesticide; P. canaliculata; eggs*

1. INTRODUCTION

Pomacea canaliculata is one of the most devastating pests in the rice farming sector because of its voracious hunger for young paddy leaves and stems [1]. It was named one of the world's top 100 worst invasive species, with serious consequences for the environment, human health, and the social economy. In February 2023, authorities in Perak, Malaysia, seized 10,125 pax of a banned chemical (fentin acetate) used to control *P. canaliculata*. The arrest of the banned chemical reflects the presence of many *P. canaliculata* in paddy plots and a lack of safety awareness among farmers. The preference of fentin acetate is due to the efficiency and fast action in controlling the *P. canaliculata* even though it is highly toxic to the environment and thus prohibited in the country.

Besides high population, *P. canaliculata* also has a high fecundity and hatchability rate, with females producing 200 to 300 eggs per week, resulting in 8,000 viable eggs per year, with 80% hatchability [1]. Therefore, managing their hatchability is essential to prevent population expansion. Water spraying, egg submersion, plant extracts, enzymes, physical collection, regulating temperature and light, and sustained release of niclosamide-gelatine are some of the techniques used to manage the hatching [2-6]. Control through its eggs is easier than control of the snail itself due to ease of locating eggs based on their bright red colour, fragility at this stage, and immobility. However, hatching control must be implemented quickly before the eggs hatch in 12-14 days.

The eggs are covered by a protective layer for embryo survival during the hatching process. Thus, the disruption of the protective layer covering the eggs consequently exposed the embryos to harsh environments and reduced their chances of hatching. A thin protective layer of tissue covering the egg, known as the cuticle, protects it from being dehydrated or infected [7]. Therefore, disruption of the cuticle will affect the hatching process. It has been shown that cuticle disruption by a biological agent, protease, which specifically targets the cuticle, successfully inhibits 86% of hatchability [5]. The disruption of the cuticle may affect its physical and chemical factors, such as ion conductivity, water loss, protein content, and other factors, which in turn affect hatchability. However, how far the treatment affects those factors is under-discovered. Therefore, an understanding of the relationship between protease and cuticle protease in terms of chemical and physical characteristics is necessary for a better hatching control strategy for *P. canaliculata*.

This paper discusses the physical and chemical changes in the cuticle upon protease application in order to explore its role in suppressing hatchability among *P. canaliculata* eggs.

The physical characteristics of *P. canaliculata* eggs treated with protease included conductivity, water loss, image analysis, and morphological analysis. Chemical characterisation included cuticle protein responses, protein breakdown by SDS-PAGE, and amino acid profiling. The results are important in explaining the role of protease in damaging the protection around these eggs, thus, promoting mortality as one way to manage snail breeding.

2. MATERIALS AND METHODS

2.1 Collection of *P. canaliculata* Eggs

About 50 masses of fresh eggs of *P. canaliculata* were collected weekly from a river bank in Simpang Empat, Perlis, Malaysia. The chosen sampling area has a high population of *P. canaliculata* colonies, as evidenced by collective groups of bright red eggs found on vegetation, leaves, and floating objects (stakes, twigs, and bottles) above the water's surface. Because the eggs were laid at night, the sampling was done early in the morning to minimise discrepancies. These eggs have the following characteristics: a soft eggshell, milky red colour, and mucus on the surface. The eggs that were mucus-free and a little hard on the eggshell were not considered for sampling. During the experiments, the masses of eggs were kept in an open incubator (62 × 40 × 46 cm) filled with tap water and kept at room temperature of 27 ºC to create a humid environment as previously described by Meyer-Willere and Santos-Soto with slight modifications [8]. The humid condition was demonstrated by observing the condensation process.

2.2 Physical Characterisation Studies

2.2.1 Conductivities Studies

The conductivity study was performed according to the method described by Peebles et al. with slight modifications [9]. Approximately 5 g of 3-day-old eggs were placed into a small basket (4 × 2 cm) before immersing in protease solution from *Aspergillus oryzae* (Sigma-Aldrich, USA) ranging from 2.5, 5 and 10 U/mL for 30 min. The protease was diluted using a phosphate buffer solution at pH 7. Each experiment was conducted in triplicate. The eggs were then rinsed several times with distilled water to remove any protease residue. Then, the protease-treated eggs and untreated eggs (control) were immersed in two separate beakers filled with 100 mL of distilled water. The conductivity of each beaker was measured every 2 hrs using a conductivity meter (EcoScan CON 6, Eutech Instruments).

2.2.2 Water Loss in P. canaliculata Eggs

Further assessment of the effect of protease on *P. canaliculata* eggs was conducted by measuring water loss based on slight modifications of the method reported previously [9]. Several clutches of 3-day-old *P. canaliculata* eggs were weighed into 0.5 g each before placing them in 15 × 30 cm containers. These containers were then labelled and treated with 1 mL of water (negative control), 5 and 10 U/mL of protease, and 0.02 M ethylenediamine tetraacetic acid, EDTA (positive control). Based on a preliminary study conducted previously, high protease concentrations had a significant effect on the water loss of *P. canaliculata* eggs. Therefore, a high protease concentration range (0, 5, 10 U/mL) was chosen to observe its effect on water loss in the eggs. This also reflects the actual implementation where high protease is required to maintain efficiency. After 10 min of protease treatment, the samples were dried for several minutes and weighed again. These eggs, labelled as day 1, were placed in a desiccator with 75% relative humidity (RH), which was achieved using saturated Na_2CO_3 (Merck, Malaysia) in distilled water. The desiccator was then placed in an oven at 30 °C. The weight

of the samples was taken daily until day 8 and the eggs were kept in the desiccator until hatching time (approximately 12 days). This experiment was performed in triplicate. The water weight loss was calculated by dividing the daily weight by the initial weight of the eggs.

2.2.3 Image analysis

A single egg was retrieved from the 3-day-old clutch before the clutch underwent treatment with 10 U/mL protease solution in phosphate buffer (0.02 M, pH 7). The untreated egg served as a control. Images of the single egg for both samples were captured from four different angles five times daily to ensure the accuracy of the results. The captured images were then analysed using the JIMAGE 1.4 software. The clutches (protease-treated and controlled) were then placed in the breeding chamber for incubation. The colour changes of both clutches were examined under a dissecting microscope (DBA 200, MOTIC, China) at 4× magnification.

2.2.4 Scanning Electron Microscope

The eggshells of the 10 U/mL protease-treated sample and the control were prepared and loaded on the double tape of the specimen stub. Then, the eggshells were coated with platinum using an auto-fine coater (JFC-1600, Tokyo, Japan), and later, observed using the scanning electron microscope (SEM) under 1,000× magnification.

2.2.5 Statistical Analysis

All data were analysed using Microsoft Excel 2010 with a probability level of less than 5% ($p < 0.05$) was regarded as statistically significant and contributed to the analysis.

2.3 Chemical Characterisation Studies

2.3.1 Response of Cuticle to Protease

The cuticle protein response towards protease was studied by placing 5 g of 3-day-old eggs in a basket (5 × 3 cm), which were treated with different concentrations of 100 mL of protease (0-5 U/mL) and EDTA (0-0.08 M) for 30 min. The eggs were then rinsed using distilled water before being immersed in 100 mL of 0.5% SDS for 10 min [10]. The protein content of the removed cuticle in each treatment was determined directly at an absorbance of 280 nm, with 0.5% SDS as the blank. All analyses were conducted in triplicate. The absorbance data of protease treatment and EDTA as a positive control was reported in %.

2.3.2 Cuticle Protein Analysis Using SDS-PAGE

The hydrolysis of cuticle protein after the protease treatment was evaluated based on SDS-PAGE analysis. The analysis was conducted to prove that the cuticle protein was broken down into a small fraction of protein. In this analysis, 5 g of 3-day-old eggs were loaded into a basket and treated with 5 mL of phosphate buffer (0.02 M, pH 7) in a beaker for 15 min, with gentle stirring and kept at 4 °C. Approximately 1 mL of the supernatant was treated with 0.02 mL of protease (20 U/mL) solution for 10 min at 37 °C. The SDS-PAGE analysis was conducted using a Bio-Rad kit (Bio-Rad Laboratories Inc., California). The samples were solubilised with SDS-sample buffer containing β-mercaptoethanol (BME) and boiled for 5 min at 95 °C before resolving with 10% SDS-PAGE. Precision Plus (Bio-Rad Laboratories Inc., California) was used as the protein marker. Electrophoresis was conducted for 1 hr at 100 V (Biorad Mini-protean II Electrophoresis System, Bio-Rad, California), and was stained with silver staining [11].

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2.3.3 Amino acid Profile of Cuticle Protease-treated Egg

Amino acid profiling of cuticle protein was conducted using the acid hydrolysis method, as reported by Piecyk et al. with several modifications [12]. Approximately 2 g of 3-day-old eggs were treated; one sample with water (control) and one sample with 2.5 U/mL of protease for 30 min, and these samples were later dried. Next, the samples were placed into crucibles before being mixed with 5 mL of 6 M HCl, and these crucibles were heated at 100 °C for 24 hrs. Then, 2 mL of 10% isopropanol was mixed into the hydrolysed eggs before undergoing centrifugation at 8,000 rpm for 10 min. The filtrates obtained underwent amino acid analysis using EZ:faast^{TM} kit (Phenomenex). This treatment was conducted in triplicate.

3. RESULTS AND DISCUSSION

3.1 Physical Characterisation of Protease-treated Eggs

3.1.1 Conductivities of Protease-treated Eggs

Figure 1 shows that the conductivity of protease-treated eggs increases proportionally with protease concentration and is saturated at a protease concentration of more than 5 U/mL. The conductivity of the eggs following 2.5 U/mL of protease treatment was 14.9 µS/hr, then increased to 16.5 µS/hr following treatment with 10 U/mL of protease. The p-value is 0.01178 and is statistically significant. Due to the protease concentration being close between each concentration, only concentrations of 0 and 10 U/mL statistically affect the conductivity reading.

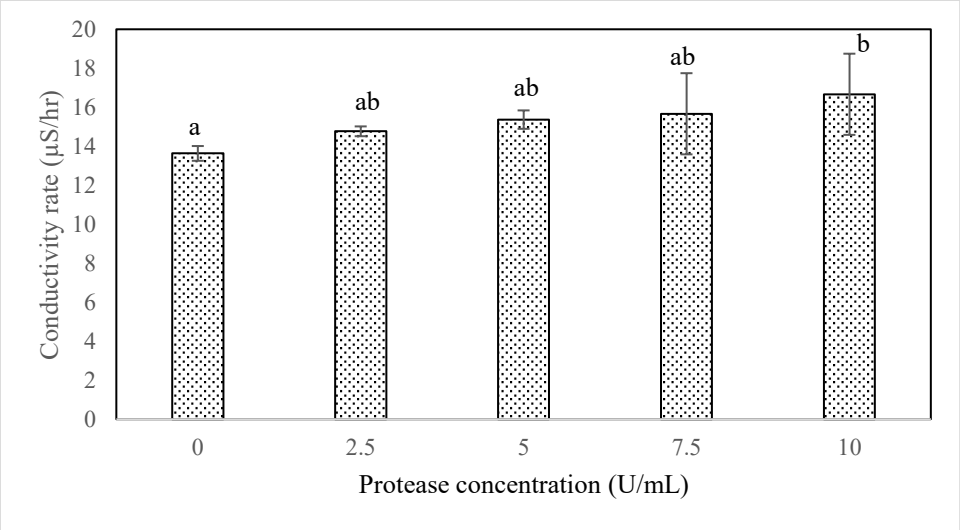

Fig. 1: Conductivity of *P. canaliculata* eggs following protease treatment.
(Note: different superscripts signify significant mean differences)

Conductivity centration increased parallelly with the protease treatment, thus demonstrating the action of protease in hydrolysing the cuticle protein. Initially, the cuticle was plugged into the eggshell pores, however, after protease treatment, it exposed the pores and increased the permeability of the eggs. Subsequently, more ions from the egg liquid, such as Ca^{2+}, K^{+}, Na^{+}, and H^{+} diffused out into the solution and increased the ionic strength. This passive diffusion occurred due to the concentration gradient, in which the ions in the inner part of the eggs have a higher concentration than the outer part, and obeyed Fick's law. A previous

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study demonstrated the salting effect in food preservation, in which the film representing the membrane diffused inorganic salt solution ions, Na^+ and K^+, into the biosolid [13].

It was reported that the increase in electrical conductivity was caused by a number of factors, including structural changes in the tissue and protopectin breakdown [14]. The excessive diffusion of ions from the interior egg liquid to the external environment demonstrated the changes in cuticle protein structure, thus affecting the hatching process.

3.1.2 Water Loss of Protease-treated Eggs

Protease treatment is also found to affect the rate of water loss in the eggs, as presented in Fig. 2. The water loss rate was faster in protease-treated eggs compared to negative control eggs (0 U/mL). An increment of 25% and 61% of water loss rate was recorded in the eggs following treatment with protease and EDTA (positive control), respectively. The protease treatment (0, 5, and 10 U/ mL) statistically affects the water loss, while protease 10 U/mL and EDTA as positive control work in a similar manner. The p-value for the analysis is 7.6×10^{-5} and is statistically significant.

The increment pattern of water loss indicated the effect of protease and EDTA in digesting the cuticle of *P. canaliculata* eggs, which reduced the barrier created by the cuticle and exposed the pores, resulting in high water loss rates. On the contrary, the negative control eggs possessed high-density cuticles that covered and blocked the pores, thus, creating a barrier from excessive water loss. The weight loss of these eggs was entirely dependent on the water vapor diffusion of albumen across eggshells and has been used as an indicator for successful hatchability in a variety of animal models [15].

In this current study, water loss in eggs was primarily caused by water evaporation through the eggshell; thus, removing the barrier formed by the cuticle and accelerating the evaporation of water from the cuticle. These results were consistent with findings reported by Galvez et al. in a study on removing the cuticle layer of an avian egg, which resulted in increased conductance and water loss [16]. Nonetheless, removing the cuticle using acetic acid, vinegar, and citric acid, particularly in the hatchery sector, has helped increase the water loss rate and improve quail egg hatchability. Although removing cuticles using a washing solution is a common practise in modern hatcheries to promote hatching, the conditions are different for snail eggs, which are sensitive to cuticle disruption.

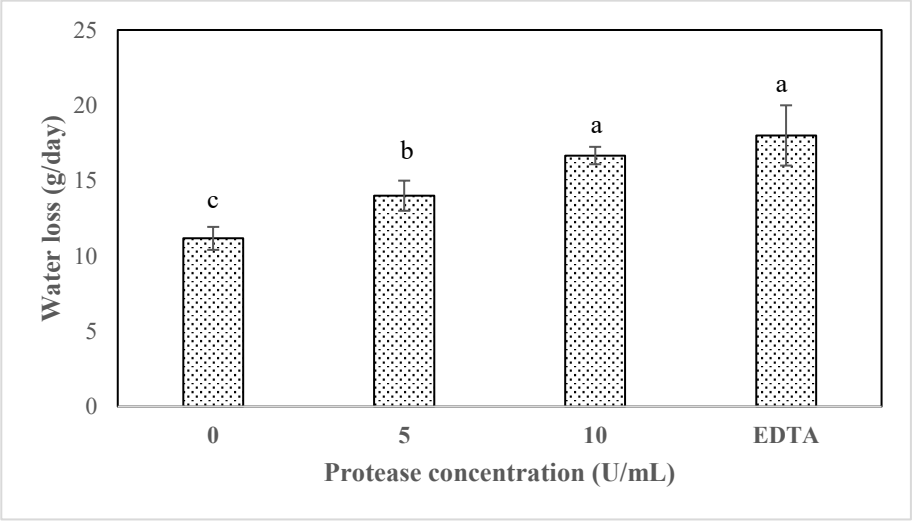

Fig. 2: Water loss in *P. canaliculata* eggs following protease treatment.
(Note: different superscripts signify significant mean differences)

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The permeability of the eggshell influences water vapour diffusion from the egg interior to the external environment. Initially, the eggshell has a tolerable permeability due to the presence of cuticle protein, which coats the wall of the pore interior from the eggshell exterior as a 'pore plug'. Its function is to regulate water vapour and electrical conductivity; however, the coating was removed after protease treatment, exposing the eggshell to the external environment and increasing its permeability. The high permeability thus allows excess water loss and an increase in electrical conductivity, consequently affecting the hatchability.

3.1.3 Image Analysis

The colour changes between the untreated (control) and protease-treated eggs (10 U/mL) were observed under a light microscope, as shown in Fig. 3. The control eggs showed variations in cuticle distribution and were red in colour from day 1 after oviposition until day 7. On day 8, the colour of the control eggs was a bit dull and became more intense as they passed day 9, which turned white on day 10, as an indicator of the hatching process (Fig. 3a).

The control eggs hatched on day 11. However, the eggs treated with protease appeared reddish in colour due to the absence of the cuticle and remained as they were until day 8, and then, some of the eggs turned dull in colour on day 9. The dull colour remained throughout the incubation period without any sign of hatching until day 11 (Fig. 3b).

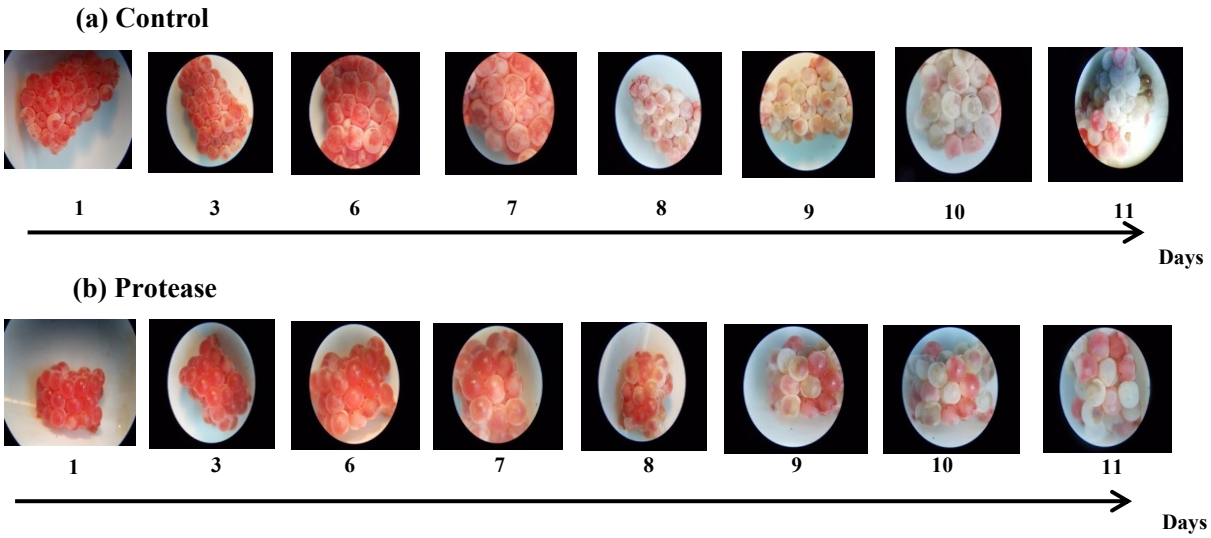

Fig. 3: Colour changes of *P. canaliculata* eggs: (a) control; and (b) protease-treated.

The eggs have a high red perivitelline fluid content during the early stages of embryo development, which provides nutrition for the growth of the embryo and influences the eggs to be red in colour. However, when hatching time approached, the liquidity of the perivitelline fluid began to decrease, thus, the eggshells turned white and became thinner. On the other hand, the volume of perivitelline fluid remained the same in the protease-treated eggs, and the eggshells remained red and thick, as no embryo was developing and no perivitelline fluid was taken up.

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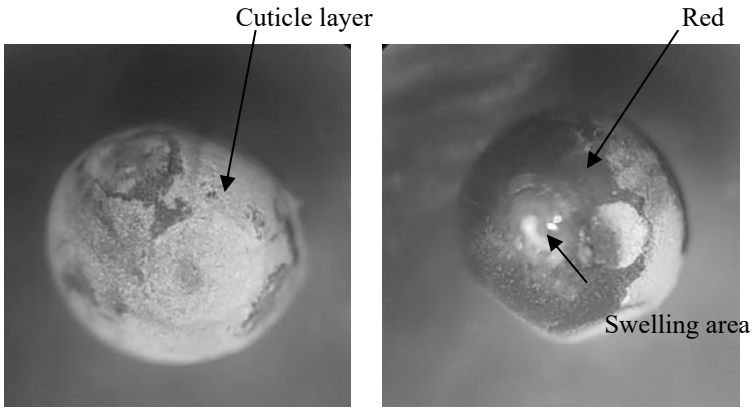
(a) Control egg (b) Protease-treated egg

Fig. 4: The colour of (a) a control egg and (b) a protease-treated egg observed under a light microscope at 40× magnification.

The colour of a single control egg and a protease-treated egg was captured, and the effect of protease treatment is shown in Fig. 4 and 5, respectively. Particle analysis using JIMAGE was conducted to quantify the reddish areas caused by protease treatment in the captured images. The control egg showed a thin white layer of cuticle surrounding the egg. In contrast, due to protease treatment, the cuticle layer was diminished and exposed the protease-treated egg to become more reddish than the control egg, as well as leaving some swelling spots. Hence, protease has played a crucial role in disrupting the cuticle at this point, which promoted ion and water loss, and consequently, affected the hatching process. This observation was comparable to those made by Khan et al. in a study of the effect of *Paecilomyces lilacinus* treatment on *Meloidogyne javanica* eggs [17]. The protease and chitinase excreted by *Paecilomyces lilacinus* hydrolysed the vitelline and lipid layers of the *Meloidogyne javanica* eggs, allowing other metabolites to enter the eggs and resulting in a swelling effect.



(a) Control egg (b) Protease-treated egg

Fig. 5: The effect of protease treatment on *P. canaliculata* egg.

Particle analysis reveals that the red area increases linearly with increasing protease concentration, regardless of the age of the eggs (Fig. 6). Initially, the red area on the untreated eggs was 38%, which gradually increased to 84% following 10 U/mL of protease treatment. A high red zone indicated high cuticle layer digestion, which in turn affected the hatchability of *P. canaliculata* eggs. These results demonstrated that the cuticle layer was successfully thinned by the protease, which also made the red colour of the eggs more obvious.

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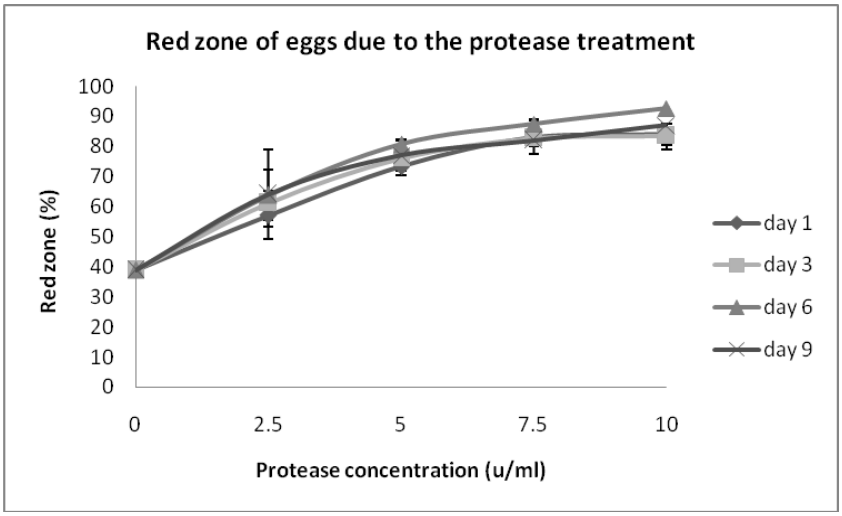

Fig. 6: Effect of protease treatment on the red zone development of *P. canaliculata* eggs.

3.1.4 Scanning Electron Microscope

The effect of protease treatment on cuticle protein is further confirmed through morphological analysis using scanning electron microscopy (SEM), as shown in Fig. 7.

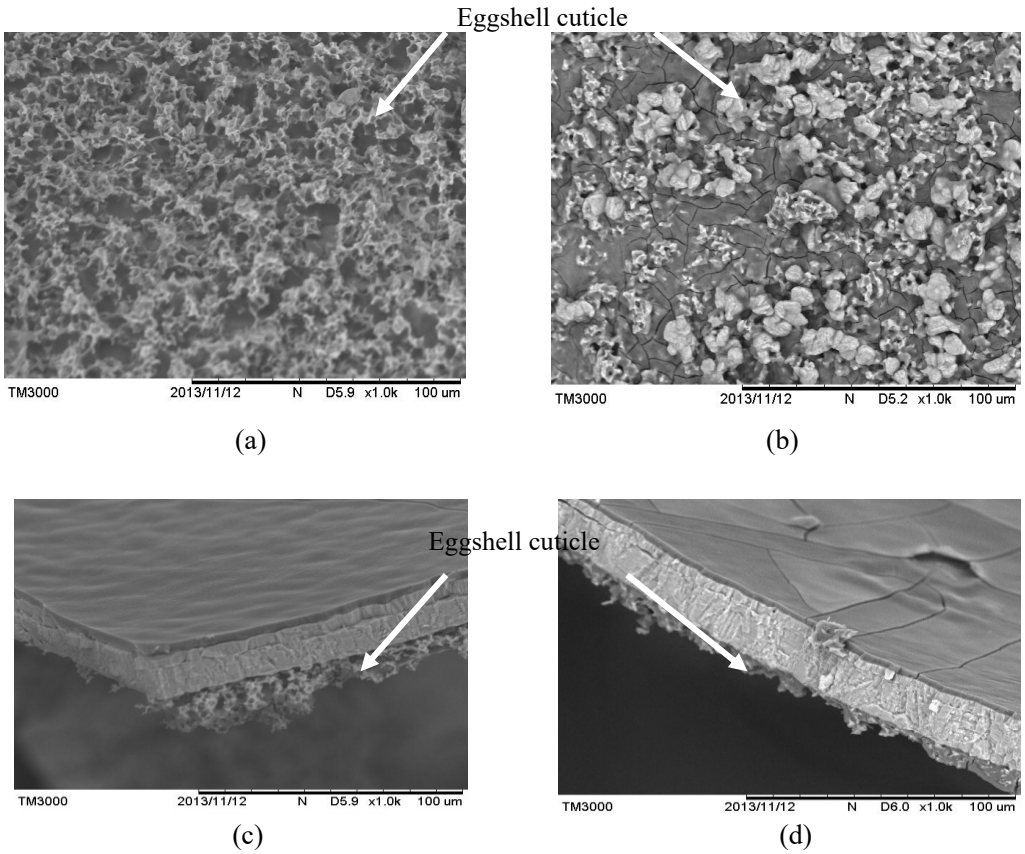

Fig. 7: SEM images of (a) control eggshell, (b) protease-treated eggshell, (c) cross-section of control eggshell, and (d) cross-section of protease-treated eggshell at 1,000× magnification.

The morphology of the control egg shows a homogeneous distribution, fluffy surface, and high cuticle coverage across the eggshell surface (Fig. 7a). The protease-treated egg, on the

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other hand, exhibits a heterogeneous distribution, rough surface, shrinkage, coagulation, low cuticle coverage, and some cracking patterns on the eggshell surface (Fig. 7b). The cross-section image of the eggshell reveals that the control eggshell has a thick layer of cuticle (Fig. 7c), in contrast to the thin layer of cuticle seen on the protease-treated eggs (Fig. 7d). The morphology of the control eggs indicated a healthy cuticle structure. The cuticle protects the embryo from microbial contamination by plugging the pores, while controlling gas exchange and water vapour loss during the hatching process. However, following protease treatment, the cuticle layer was destroyed as a 'pore plug' and became thin, as demonstrated by the cuticle coagulation appearance. The hydrolyzation of the cuticle protein reflects the changes not only in the protein content of the cuticle but also in the electrical conductivity and water loss, which are potent factors for the development of the embryo. The disruption of the cuticle reflects the failure of its role as the main defence agent for the fragile embryo, resulting in poor hatchability. Quanlin et al. reported a similar observation when treating avian eggs with EDTA for cuticle removal, in which the cuticle appeared rough [18].

3.2 Chemical Characterisation in Conductivities Studies

3.2.1 Analysis of Cuticle Response to Protease Treatment

Cuticle protein levels decrease proportionally with increasing protease concentration, as shown in Fig. 9. The cuticle protein concentration has reduced from 100% to 29% following 5 U/mL of protease treatment. The cuticle protein of eggs treated with EDTA also showed a similar reduction pattern as those treated with protease. These analyses showed the function of protease in hydrolysing the cuticle protein. Other hydrolytic enzymes, such as chitinase and collagenases produced by other fungi have been reported to hydrolyse the cuticle of plant-parasitic nematode eggs [19].

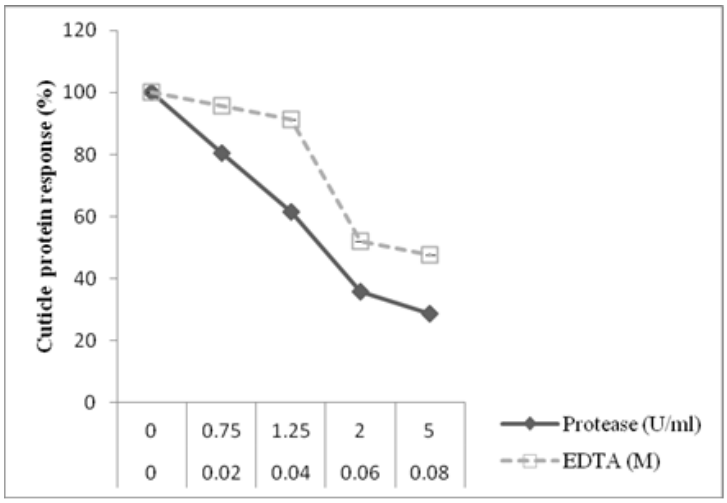

Fig. 9: The cuticle protein response due to protease action.

3.2.2 Analysis of Cuticle Degradation Using SDS-PAGE

To further study protease action on cuticle protein, the SDS-PAGE analysis is conducted, as shown in Figure 10. The marker is shown on the left side of this figure. The analysis results showed that protease appeared in several estimated sizes of 80, 49.1, and 28 kDa. Casein (C) was used as a control since protease is involved in casein hydrolysation to tyrosine. Based on the findings, casein was approximately 20.6 kDa in size and appeared in a thick line, consequently being hydrolysed into a very fine line following protease treatment (C-E). The cuticle protein extracted with phosphate buffer (PBS) revealed several peptides with high

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intensity at protein sizes ranging from 38.4 to 7.1 kDa. However, following protease cleavage (PBS-E), the cuticle protein was broken down to form small peptides in the PBS-E hydrolysates, with lower intensity of low molecular weight peptide fractions, namely 33, 15, and 8 kDa. On the other hand, the cuticle treated with phosphate buffer (prior to protease treatment) was identical to casein, C, at 20.6 kDa. However, following protease treatment, both PBS-E and CE showed no protein band at 20.6 kDa, which were possibly cleaved into even smaller protein sizes. These results clearly show that the cuticle protein of the eggs is completely hydrolysed by protease, as shown in Fig. 10.

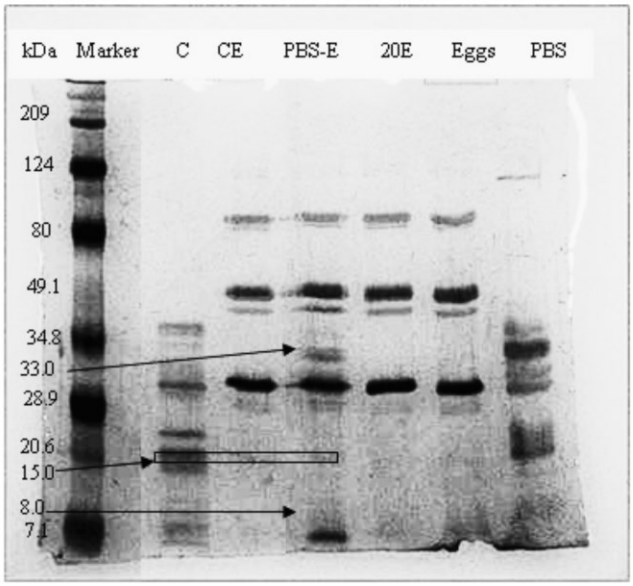

Fig. 10: The bands of SDS-PAGE for casein (C), casein treated with enzyme (CE), cuticle in phosphate buffer, treated with enzyme (PBS-E), 20 U/mL of protease (20E), cuticle protein treated with protease (Eggs), and cuticle extracted with phosphate buffer at pH 7 (PBS).

3.2.3 Analysis of Amino Acid Profile of the Treated Cuticle

The reduction in cuticle protein, as a response to protease, is supported by amino acid analysis results, as shown in Fig. 11.

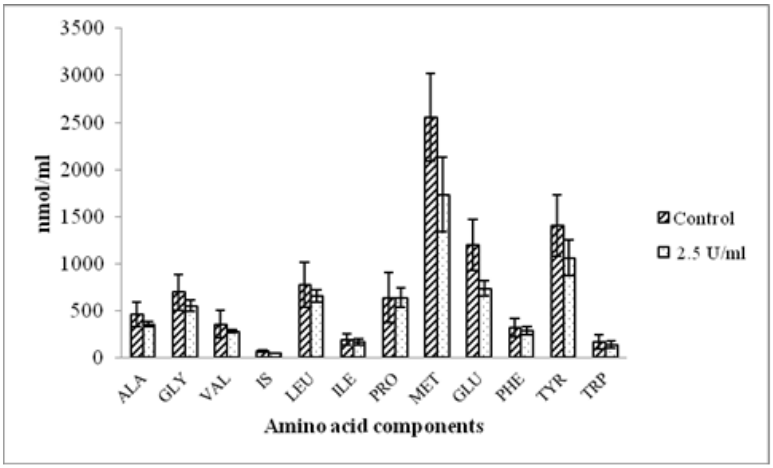

Fig. 11: The amino acid analysis of cuticle protein following protease treatment.

Based on Fig. 11, the cuticle protein of *P. canaliculata* eggs contains 11 major amino acid components, prominently methionine (2,500 nmol/mL), tyrosine (1,400 nmol/mL), and glutamine (1,200 nmol/mL), followed by other amino acids, namely leucine (800 nmol/mL), glycine (700 nmol/mL), and proline (600 nmol/mL). These amino acid groups were reduced in parallel with the increasing protease concentration following treatment, except for proline. The major amino acids that were reduced the most were tyrosine (68%) and methionine (54%). Tyrosine provides an excellent substrate for protease action. This could explain the increased protease action in hydrolysing amino acids in the cuticle protein. These findings showed several similarities with amino acids found in the eggshell of a land snail, *Cornu aspersum*, which contained leucine, tyrosine, lysine, and small amounts of methionine [20]. However, avian cuticles were found to be rich in glycine, lysine, aspartic acid, glutamic acid, tyrosine, and arginine. The diverse compositions of amino acids among different species are important in accommodating the growth requirements of the embryos.

The hydrolysis of cuticle protein by protease is depicted clearly by SDS-PAGE and amino acid profiling. The cuticle protein disintegrated into small fractions and changed in terms of amino acid concentration. The changes in the chemical content of cuticle protein thus play a major role in impaired cuticle function which in turn affects the hatchability. As a result, protease treatment influenced both the physical and chemical properties of cuticle protein during the hatching process. Protease enzyme treatment could be one option for controlling the *P. canaliculata* population through egg management.

4. CONCLUSION

This study showed that the mechanism of protease was not only physically affecting the eggs, but also chemically through the disruption of their cuticle protein. Protease was found to coagulate the cuticle layer on the surface of the eggs, and increased conductivity and water loss rate, while causing swelling and highlighting the red colour of the eggs. The reduction of cuticle protein and protein breakdown showed the effectiveness of protease in digesting cuticle protein, which led to the un-hatchability of the eggs. The damage to the cuticle protein subsequently interrupted the functions of the cuticle in protecting the eggs from microbial penetration, dehydration, and gaseous exchange. As all crucial factors for embryo survival were affected by the disruption of the cuticle, consequently, the hatchability of the eggs was also affected. In conclusion, this study of the mechanism of protease action on *P. canaliculata* eggs has provided more knowledge for the management and control of the snail population. This study, however, was limited to the use of commercial protease on *P. canaliculata* eggs on a lab scale. However, it can be useful to have a basic understanding of the protease action against cuticle protein for field applications. It is suggested that synthesis protease from microbes or plants be used to reduce costs while still providing the same action to the cuticle protein. Future research should focus on the application of synthesized protease in the field, taking into account temperature, intact time, humidity, storage stability, spray method and other factors that may impair protease efficiency in eggs. The protease application is relevant in managing *P. canaliculata* population, beginning with the most vulnerable stage of its lifecycle, the egg. The management of eggs is simpler than the management of matured snails because their eggs are fragile and easy to locate due to their bright red colour. The potential of protease used as biopesticides for crop protection can't be denied. It can be used in liquid form and sprayed on the target, *P. canalculata* egg. Therefore, the protection strategy by protease in managing *P. canaliculata* eggs can be a good alternative and can consequently facilitate the reduction of their distribution, and mitigate the damages done by these snails to protect paddy production from future extinctions.

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