Abstract. – Background and Objective: *Artocarpus heterophyllus* (jackfruit) is a latex producing plant. Plant latex is produced from secretory cells and contains many intergradients. It also has been used in folk medicine. This study aimed to purify and characterize the biological activities of a protease from jackfruit latex.

Materials and Methods: A protease was isolated and purified from crude latex of a jackfruit tree by acid precipitation and ion exchange chromatography. The proteolytic activities of protein were tested using gelatin- and casein-zymography. The molecular weight and isoelectric point (pI) of protein were analysed by SDS/12.5% PAGE and 2D-PAGE, respectively. Antimicrobial activity of protein was analysed by broth microdilution method. In addition, the antibacterial activity of protein against *Pseudomonas aeruginosa* ATCC 27853 was observed and measured using atomic force microscopy (AFM) technique.

Results and Conclusions: The purified protein contained protease activity by digesting gelatin- and casein-substrates. The protease was designated as antimicrobial protease-48 kDa or AMP48 due to its molecular mass on SDS-PAGE was approximately 48 kDa. The isoelectric point (pI) of AMP48 was approximately 4.2. In addition, AMP48 contained antimicrobial activities by it could inhibit the growths of *Pseudomonas aeruginosa* ATCC 27853 and clinical isolated *Candida albicans* at minimum inhibitory concentration (MIC) 2.2 mg/ml and Minimum microbicidal concentration (MMC) 8.8 mg/ml. AFM image also supported the antimicrobial activities of AMP48 by the treated bacterial morphology and size were altered from normal.

Key Words: *Artocarpus heterophyllus*, Jackfruit, Latex, Antimicrobial activity.

Introduction

*Artocarpus heterophyllus* or jackfruit is a rubber-producing plant which is widely distributed in tropical areas including Thailand1-4. All parts of jackfruit tree contain sticky white latex which produced from special secretory cells called laticifers5-6. Latex is an aqueous emulsion contains many intergradients, for instance, lipids, rubbers, resins, sugars, proteins including proteolytic enzymes6. In addition, plant latex has been used in folk medicine since it has clot inducing and dissolving properties in human hemostasis, wound healing and antimicrobial activity7-9. At present, there are a few reports of biological properties of jackfruit latex especially latex proteins and proteases. In 1990, Prasad et al4 purified a 79.5-kDa protein from jackfruit latex and characterized as serine-centered protease. However, the medicinal property of this protein was not investigated. This study focused on antibacterial and antifungal activities of a 48-kDa protease (AMP48) purified from jackfruit latex. This study also rendered new information on biological properties of jackfruit latex and also medicinal treatment.

Materials and Methods

Plant Material

The latex was obtained from a jackfruit tree (*Artocarpus heterophyllus*) located in Tambon Suranaree, Muang distric, Nakhon Ratchasima province, Thailand.

Bacterial Strains

Standard American Type Culture Collection (ATCC) strains of Gram-negative bacteria:
Pseudomonas aeruginosa (P. aeruginosa) ATCC 27853, Escherichia coli (E. coli) ATCC 25922 and Gram-negative coccus: Staphylococcus aureus (S. aureus) ATCC 25923 were used in the antibacterial study.

**Fungus Strains**
Clinical isolated strains of Candida (C.) albicans and Candida tropicalis were obtained from Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand. Both strains were used in the antimicrobial study.

**Protein Purification**
The crude latex (4.2 ml) from fruit stem was collected in a clean glass beaker. The sticky white compound was removed by centrifuge (13,000 × g for 30 min) at room temperature. Then the clear supernatant was dialyzed overnight against 25 mM sodium acetate buffer, pH 4.5. Aggregated proteins were removed by centrifugation (13,000 × g for 30 min) at 4°C and the clear supernatant was subjected to further purification by SP Sepharose™ fast flow column chromatography (1.5 cm × 3 cm) (GE Healthcare, Uppsala, Sweden). The proteins were fractionated (2 ml) with a 0-0.5 M step gradient of NaCl in 25 mM sodium acetate buffer, pH 4.5 at flow rate 1 ml/min and every fraction was measured at A_{280}. Then all protein fractions eluted by buffer without NaCl (0 M NaCl) were pooled and dialyzed against 25 mM Tris-HCl buffer, pH 8.8. The purified protein was subjected to purify by Q Sepharose fast flow column chromatography (1.5 cm × 3 cm) (GE Healthcare, Uppsala, Sweden). The proteins were eluted with a 0-0.5 M step gradient of NaCl in 25 mM Tris-HCl buffer, pH 8.8, and at flow rate of 1 ml/min. Eluted fractions (2 ml) were collected and every fractions were measured at A_{280}. The purity and molecular weight of purified protein were analyzed on SDS/12.5%PAGE. Purified protein concentration was determined using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA).

**Two Dimensional SDS-Polyacrylamide Gel Electrophoresis (2D SDS-PAGE)**
Purified latex protein (100 mg) was separated in the first dimension with 7 cm immobilized pH gradient strip pH 3-10 (GE Healthcare, Uppsala, Sweden). The second dimension was separated by 12.5% Tris-glycine sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Protein was examined by staining with colloidal Coomassie brilliant blue G-250. The values of isoelectric point (pI) and molecular weight for protein spot were calculated by ImageMaster™ 2D Platinum software (GE Healthcare, Uppsala, Sweden) using standard proteins with known pI and molecular weight.

**Detection of Proteases**
Proteolytic activities of the purified protein were analyzed using gelatin- and casein-zymography by slightly modifying the method of Shimokawa et al. Briefly, each purified protein sample was mixed with reducing (containing 2-mercaptoethanol) and non-reducing sodium dodecyl sulphate (SDS) gel sample buffers, and applied with and without heating (at 95°C) to a 12.5% polyacrylamide gel containing 0.1% SDS and 1 mg/ml gelatin or casein solution. After electrophoresis, the gel was washed three times in 25 mM Tris-HCl, pH 8.8 containing 150 mM NaCl, 5 mM CaCl₂, 0.02% NaN₃, 0.025% Triton X-100 at room temperature, and then incubated in the same buffer without Triton X-100 at 37 °C for 20 h. Proteins were stained by Coomassie brilliant blue R-250 and protease activity was detected as transparent bands.

**Antimicrobial Activity**
The broth twofold microdilution method in tryptic soy broth was applied for minimum inhibitory concentration (MIC) of purified protein against bacteria and fungus. Briefly, test microbial strains were grown in broth culture and diluted at a density of 1 × 10³ CFU/ml (colony forming unit/ml). Microbial suspension (100 µl) were mixed with medium containing serial twofold dilutions of the purified protein (100 µl) and incubated overnight at 37°C. The mediums contained with and without purified protein were growth control. The buffer (25 mM Tris-HCl, pH 8.8) was mixed with microbial suspension also used as growth control. After incubation, each suspension (10 µl) was transferred and streaked on 5% sheep blood agar plate and incubated overnight at 37°C.
MIC defined as the lowest concentration of purified protein which there was not more than 20% microbial growth\textsuperscript{12}. And Minimum bactericidal concentration (MBC) defined as the smallest concentration of purified protein which could kill 99.9% of the cells in an inoculum\textsuperscript{13}.

**AFM Imaging and Force Measurements**

The cell suspension of (10 µl) with and without purified protein (at concentration 1.1 mg/ml) were dropped on the 9.9 mm mica discs (Pelco\textsuperscript{6}, Ted Pella Inc., Redding, CA, USA) and dried at ambient air and subjected to measure surface tomography with the AFM imaging and force measurements using Park XE-70 atomic force microscopy (Park Systems Inc, Suwan, South Korea). The instrument was controlled by the XEP 1.7.56 software and set in true non-contact mode. The speed scan was set to 0.5 µm/s and dimensions of scan size was 5 × 5 µm\textsuperscript{2}. The images were evaluated by the XEI 1.7.6 software.

**Statistical Analysis**

The sizes of treated and untreated *P. aeruginosa* with purified protein (each group, *n* = 40) were measured under the 10 microscopic fields of AFM imaging. Means, standard deviation (S.D.) of each group of bacterial sizes and all other statistics were calculated using SPSS for windows version 11.5.0 package (SPSS Inc., Chicago, IL, USA). The normality of the data distributions of each group were analyzed using Shapiro-Wilk statistic\textsuperscript{12}. Student’s *t*-test was used for means between group comparisons.

**Results**

A protein was isolated and purified from *A. heterophyllus* (jackfruit) latex using acid precipitation and column chromatography as described above. The purified protein migrated on SDS-PAGE with a molecular mass approximately 48 kDa and its pI was approximately 4.2 (Figure 1). The yield of protein gained from each extraction was approximately 1.446% (total amount 6.204 mg) (Table I). This protein contained protease activity assessed by gelatin- and casein-zymography (Figure 2). In addition, the reducing and non-reducing conditions could not affect the gelatinolytic and caseinolytic of this protein (Figure 2). In contrast, the protease activity of the protein could be destroyed by heating at 95°C (Figure 2). This protein had antibacterial and antifungal activities by it could inhibit the growth of *P. aeruginosa* and *C. albicans* at MIC 2.2 mg/ml, and MMC 8.8 mg/ml. AFM image emphasized the effect of this protein on bacterial growth.

![Image](image_url)
Antimicrobial activity of a 48-kDa protease (AMP48) from *Artocarpus heterophyllus* latex

Table I. Purification of AMP48 from *A. heterophyllus* (jackfruit) latex. The results are the average values of duplicate experiments.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>1. Crude latex protein</td>
<td>429.114</td>
<td>100</td>
</tr>
<tr>
<td>2. Dialysis with 25 mM sodium acetate buffer, pH 4.5</td>
<td>157.500</td>
<td>36.704</td>
</tr>
<tr>
<td>2. SP sepharose cation exchange chromatography</td>
<td>66.700</td>
<td>15.544</td>
</tr>
<tr>
<td>3. Q sepharose anion exchange chromatography (AMP48 fraction)</td>
<td>6.204</td>
<td>1.446</td>
</tr>
</tbody>
</table>

Table II. MIC and MMC of AMP48.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Concentration of AMP48 (mg/ml)</th>
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<tr>
<td></td>
<td>MIC</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 27853</td>
<td>2.2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 25922</td>
<td>No activity</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>No activity</td>
</tr>
<tr>
<td>Clinical isolated Candida albicans</td>
<td>2.2</td>
</tr>
<tr>
<td>Clinical isolated Candida tropicalis</td>
<td>No activity</td>
</tr>
</tbody>
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The morphology of the bacterium changed from normal and its shape was reduced significantly ($p < 0.01$) average from $0.915 \pm 0.036 \mu m \times 3.019 \pm 0.038 \mu m$ to $0.668 \pm 0.035 \times 1.735 \pm 0.069 \mu m$ (Figure 3). Since this protein had molecular weight 48 kDa, contained protease and antimicrobial activities, therefore, the protein was designated as antimicrobial protease-48 kDa or AMP48.

Discussion

This study could isolate and purify a 48-kDa protease (AMP48) from crude latex of a jackfruit tree. The protein could inhibit bacterial and fungal growths. This protein might be a protective agent produced from plant since plant proteins usually are produced to resist potential pathogens and diseases\(^5\). Moreover, AMP48 contained pro-
tease activities by gelatinolysis and caseinolysis. There are many antimicrobial proteases, for example, zingipain isolated from Zingiber ottensii Valeton rhizomes with antiproliferative activities against fungi. In addition, zingipain could also against human malignant cell lines proliferations. These evidences exhibit to use plant protease in medicinal treatments.

AMP48 could against P. aeruginosa and C. albicans in this study. P. aeruginosa is widespread in nature. It can be found in soils, surfacewater, on plants, in human and animal intestines. The main infections are pneumonias in patients on respiratory equipment, infections of burn wounds, postoperative wound infections, chronic pyelonephritis, endocarditis in drug addicts, sepsis, and malignant otitis externa. Moreover, it frequently causes nosocomial infections. Alteration of bacterial morphology, shape and size after treatment of any agent can be used to define the antibiotic mechanism. Therefore, AFM image was used for observing the anti-activity of AMP48. The result of AFM image showed the effect of AMP48 on P. aeruginosa cell by altering bacterium morphology and its shape was significantly reduced after treatment. C. albicans also the important pathogen since at least 70% of all human Candida infections are caused by this microorganism, and a few caused by C. parapsilosis, C. tropicalis, C. guillermondii and C. kruzei. C. albicans is opportunistic mycosis, can cause endogenous infection, primary infection of mucosa and skin with secondary dissemination for instance, in HIV patients. Since AMP48 could against both important pathogenic microorganisms, therefore, it may be applied to use as antibiotic in future.

In conclusion, this work exhibits the antibacterial and antifungal activities of a protease which isolated form jackfruit latex. AFM image also emphasized the antimicrobial activities of AMP48. Further study, the type of AMP48 will be classified. Moreover, its structure, stability, and other biological of this protein will be analyzed. The targets of this protein on microorganisms will be determined. In addition, the test of this protein with clinical samples will be attempted to investigate.

Acknowledgements

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References


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