EXTRACTION AND IDENTIFICATION OF ANANAIN-LIKE PROTEASE FROM PINEAPPLE CROWN AND STEM

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DEDICATION

In the name of Allah the Most Gracious and the Most Merciful,

I would like to dedicate my deepest appreciation to the following people, who are very meaningful in my life, people that always inspires and lifting me up, support me emotionally with prayer, love and patience along the journey of this life. Each of them is the biggest spirit for me.

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ABSTRACT

Pineapple (*Ananas comosus*) is the major tropical fruits and belongs to the family Bromeliaceae. The pineapple waste from pineapple industry has contributed to an increase of waste in Malaysia and around the world every year. Hence, there is a demand need to study the utilization of pineapple wastes such as pulp, peel, core, leaves and crown for industrial applications instead of disposing them, which may result in the loss of important biomass and nutrients. The pineapple contains endopeptidase enzymes that constitute the major components of bromelain extract which are stem bromelain, fruit bromelain, ananain and comasain. This study was conducted to extract and purify protease from crown and stem of MD2 pineapple. Protease was extracted and purified using anion exchange chromatography (IEX), gel filtration, desalting method before being identified using LC/MS. Proteolytic activity was determined using Casein Digestion Unit (CDU) and well diffusion method, whereas fibrinolytic activity was determined using fibrin suspension. In the present study, proteolytic assay showed 1 kg crown of MD2 cultivar produced the activity of 126.0 ± 3.86 U/mL, the specific activity of 3937.50 U/mg and total activity of 3.94×10^9 U. In another assessment, 1 kg stem from MD2 cultivar showed the proteolytic activity, specific activity and total activity of 118.5 ± 1.19 U/mg, 5925 U/mg and 5.925×10^9 U, respectively. The proteolytic activity and fibrinolytic activity of purified enzyme from the crown extract were 144.02 U/mL and 0.51×10^{-6} U/mL, respectively. The molecular weight of the purified enzyme was in the range of 25 to 35 kDa at the optimum condition of pH 7 at 37 °C. Meanwhile, a purified enzyme from stem extract was observed to contain high proteolytic activity and fibrinolytic activity which were 20.76 U/mL and 0.000272 U/mL, respectively. Purification of the extract yielded a band at molecular weight of between 20-25 kDa at the optimum conditions of pH 3 and 9 at 60 °C. From LC/MS analysis, the purified enzyme from the crown extract was similar to ananain under accession number A0A199VSS3 (according to Uniprot). It had seven unique peptides and covered 164/356 amino acids (44.9 percent coverage). The ananain (EC 3.4.22.31) is classified in the subfamilies of cysteine protease C1A (clan CA, family C1) that is peptidase family related to papain. In conclusion, protease was successfully extracted and identified as ananain-like protease from crown. However, protease extracted from the stem of MD2 cultivar was not determined. Therefore, further study on this enzyme needs to be explored in the near future.

ABSTRAK

Nanas (*Ananas comosus*) adalah buah tropika utama dan tergolong dalam keluarga Bromeliaceae. Sisa nanas daripada industri nanas telah menyumbang kepada peningkatan sisa di Malaysia dan di seluruh dunia setiap tahun. Oleh itu, terdapat keperluan untuk mengkaji penggunaan sisa nanas seperti pulpa, kulit, inti, daun dan jambul untuk aplikasi industri dan bukan membuangnya, yang boleh mengakibatkan kehilangan biojisim dan nutrien penting. Nanas mengandungi enzim endopeptidase yang merupakan komponen utama ekstrak bromelain iaitu bromelain batang, bromelain buah, ananain dan komasain. Kajian ini telah dijalankan untuk mengekstrak dan menulenan protease daripada jambul and batang nanas MD2. Protease telah diekstrak and ditulenkan menggunakan kromatografi pertukaran anion (IEX), penyaringan, kaedah penyahgaraman sebelum dikenal pasti menggunakan LC/MS. Aktiviti proteolitik telah ditentukan menggunakan Casein Digestion Unit (CDU) dan kaedah resapan agar, manakala aktiviti fibrinolitik ditentukan menggunakan ampaian fibrin. Keputusan kajian mendapati ujian proteolitik menunjukkan 1 kg jambul MD2 kultivar menunjukkan aktiviti 126.0 ± 3.86 U/mL, aktiviti khusus 3937.50 U/mg dan jumlah aktiviti 3.94×10^9 U. Dalam penilaian lain, 1 kg batang dari MD2 kultivar menunjukkan aktiviti proteolitik, aktiviti spesifik dan jumlah aktiviti masing-masing 118.5 ± 1.19 U/mL, 5925 U / mg dan 5.925×10^9 U. Aktiviti proteolitik dan aktiviti fibrinolitik enzim yang ditulenkan daripada ekstrak jambul masing-masing adalah 144.02 U/mL dan 0.51×10^{-6} U/mL. Berat molekul enzim tulen berada dalam julat 25 hingga 35 kDa di keadaan optimum pada pH 7 pada 37 ºC. Sementara itu, enzim yang ditulenkan daripada ekstrak batang dilihat mengandungi aktiviti proteolitik tinggi dan aktiviti fibrinolitik masing-masing iaitu 20.76 U/mL dan 0.000272 U/mL. Penulenan ekstrak menghasilkan jalur pada berat molekul antara 20-25 kDa di keadaan optimum pH 3 dan 9 pada 60 °C. Berdasarkan hasil analisi LC/MS, enzim yang ditulenkan dari ekstrak jambul adalah menyamai dengan ananain dengan nombor aksesi A0A199VSS3 (menurut Uniprot). Ia mempunyai tujuh peptida unik dan merangkumi 164/356 asid amino (liputan 44.9 peratus). Ananain (EC 3.4.22.31) dikelaskan dalam subkeluarga sisteina protease C1A (klan CA, keluarga C1) iaitu keluarga peptidase yang berkaitan dengan papain. Kesimpulannya, enzim berjaya diekstrak dan dikenal pasti sebagai ananain-serupa protease daripada jambul. Walau bagaimanapun, protease yang diekstrak daripada batang kultivar MD2 masih belum ditentukan. Oleh itu, kajian lanjut berkaitan enzim ini perlu dikenal pasti dalam masa terdekat.

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LIST OF ABBREVIATIONS

MPIB	-	Malaysian Pineapple Industry Board
MGI	-	Malaysian Genome Institute
CDU	-	Casein Digestion Unit
ATPS	-	Aqueous Two-Phase System
RME	-	Reverse Micelle Extraction
AEX	-	Anion Exchange Chromatoraphy
GF	-	Gel Filtration Chromatography
UTM	-	Universiti Teknologi Malaysia
LC/MS	-	Liquid Chromatography-Mass Spectrometry

LIST OF SYMBOLS

°C	-	Degree Celcius
kg	-	Kilogram
g	-	Gram
cm	-	Centimetre
mm	-	Milimetre
L	-	Litre
mL	-	Mililitre
%	-	Percent

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Pineapple (*Ananas comosus*) is the most common fruit in the world and belongs to the Bromeliaceae family. *Ananas comosus* is grown in large numbers in tropical countries such as China, Malaysia, Indonesia, Thailand, Philippines, Kenya and India (Ramli *et al.*, 2017). Today, world pineapple production continues to increase, including in Malaysia every year. In Malaysia, the three most common cultivars that are widely planted are Spanish (also known as Maspine, Josapine and Hybrid pineapple), Smooth Cayenne (Sarawak pineapple) and Queen (Hidayat *et al.*, 2012). The introduction of MD2 (hybrid variety) into the pineapple market in Malaysia has increased its demand and a gradual shift from export of Smooth Cayenne to MD2 can also be seen. Moris, Sarawak and Josapine are cultivated mainly for the local fresh fruit consumption whereas MD2 and N36 are usually exported since they have a longer shelf life. Pineapple cultivars MD2 act as a benchmark in the development of future cultivars for fresh consumption. The average pineapple weight is about 1.5 kg each and has a shelf life similar to other fruits such as papaya (Thalip *et al.*, 2015; Falah *et al.*, 2015). According to the Malaysia Pineapple Industry Board (MPIB), in 2017, Malaysia has a total cultivation area of 13, 733 with the production of 394,000 tonnes and the value of RM515 million while exports of RM155 million for all varieties of pineapple. For MD2, the production has increased and reached 3,600 tonnes by the end of the year 2017. The MD2 pineapple production at 3,800 tonnes by 2019 saw an increase of 200 tonnes. Malaysia currently targets RM320 million for the export of pineapple products with a total production of 700,000 metric tonnes by 2020. 360 hectares of MD2 pineapple plants are being grown commercially in Ulu Tiram, Kluang and most recently in Tanah Abang, Mersing, Johor. The current planning is to expand the crop to 1,000 hectares by 2021. The aim is to produce 100 containers together with

1,800 tonnes of pineapple per month including 50 containers or 900 tonnes of pineapple would be exported.

Recent advances in the pineapple industry have led to an increase in waste in Malaysia and around the world every year. The waste proportions are twice the proportion of fruit for flesh, core, peel and crown of *Ananas comosus* (Nor *et al.*, 2015). Disposal of pineapple waste leads to the loss of valuable biomass and nutrients that contribute to industrial uses (Mirabella *et al.*, 2014). Pineapple waste has been discovered as a possible source of proteases like bromelain (Abreu & Figueiredo 2019). Bromelain is a protease enzyme extracted from the pineapple plant that is mostly composed of proteinase and non-enzymatic components (Arshad *et al.*, 2017). The major part of bromelain is from proteases such as stem bromelain (EC 3.4.22.32), fruit bromelain (EC 3.4.22.33), ananain (EC 3.4.22.31) and comosain whereas non-proteases consist of carbohydrates, glucosidases, cellulases, phosphatases, peroxidases and glycoproteins (Sahoo & Das 2017). Furthermore, aside from the pineapple stem and fruit as a source of bromelain, it was revealed that several other parts of the pineapple, such as the core, peel, and crown were also sources of protease enzyme (Ketnawa *et al.*, 2012). This suggested that protease enzyme extracted from pineapple can be utilized to fulfill the demand in industrial processes including foods, textile and medical cosmeceutical industries. Protease enzyme has been shown to aid in the tenderization of meat (Arshad *et al.*, 2017), the relaxation and suppression of shrinkage in baking industries (Sahoo & Das 2017), the improvement of silk and wool quality (Arshad *et al.*, 2014), and the removal of tooth stains in tooth whitening products (Münchow *et al.*, 2016). In addition, topical application of protease enzyme is introduced in the market mainly for debridement of skin wounds and burns (Muhammad *et al.*, 2017), it acts as a fibrinolytic agent which increases fibrinolysis by activating the conversion of plasminogen to plasmin (Kwatra 2019), anti-cancer agents act as a suppressor for carcinogenesis and prevent cancer development (Rathnavelu *et al.*, 2016) and anti-inflammatory agent to treat patients in the third molar extraction postoperative period (Barrera-Núñez *et al.*, 2014). However, enzyme purity is necessary for industrial and commercial applications (Nor *et al.*, 2015). Since a high degree of purity is required, entire downstream processing is necessary, including extraction, purification using ion-exchange chromatography, and

identification of purified enzyme. Therefore, pineapple stems, fruits and waste products such as crowns, leaves, peel and core are extracted and purified with different homogenization processes and addition of different extraction buffers to produce beneficial products (Youryon *et al.*, 2018; Wan *et al.*, 2016).

1.2 Problem Statement

Growing pineapple crops in Malaysia generates millions of tons of plant waste. During harvesting activities, a large amount of leftover from pineapple growing, known as agro-waste was created in the agricultural process. According to Nor *et al.*, (2015), the waste proportions are twice the proportion of fruit for the flesh, core, peel and crown of the Smooth Cayenne pineapple. In addition, 998 million tons of agricultural waste are produced globally each year, with 1.2 million tons of agricultural waste disposed of into landfills in Malaysia each year (Neh and Ali, 2020). Agro-waste is predicted to account for 15% of total waste created in Asia with agricultural waste produced in Malaysia reaching 0.122 (kg/cap/day) in 2009 and expected to reach 0.210 (kg/cap/day) by 2025 (Ngoc and Schnitzer, 2009). Generally, harvest leftovers from crops such as pineapple waste are usually just burnt or left to rot, releasing carbon dioxide and methane gas into the atmosphere. These wastes are disposed of unethically in the environment, due to the high price of transportation and lack of land to be used for waste disposal. Consequently, disposal of pineapple waste causes environmental issues and leads to depletion of valuable biomass and nutrients from fruit processing waste (Mirabella *et al.*, 2014). It was revealed that several other parts of the pineapple, such as the core, peel, and crown were sources of protease enzyme (Ketnawa *et al.*, 2012). These protease enzyme have been shown to be applicable in many industrial processes including the foods, cosmetics and pharmaceutical industries (Nu`n˜ez *et al.*, 2005; Muhammad & Ahmad, 2017). Hence, there is an increasing need to study the sustainable utilisation of pineapple wastes for industrial applications that can be commercialised as an environment-friendly alternative for carbon source utilization.

1.3 Objectives

The objectives of the research are:

(a) To determine the proteolytic activity in stem and crown of pineapple and compare with different cultivars in Malaysia

(b) To purify and characterise the protease extract from pineapple stem and crown from the best cultivar

(c) To determine fibrinolytic activity of protease extract from pineapple stem and crown of the best cultivar

1.4 Research Scope

The area of this research was at Bahagian Pembangunan Teknologi Nanas Lembaga Perindustrian Nanas Malaysia, Pekan Nenas Johor, Laboratory of Plant Biotechnology at T02 in Faculty Science, University Technology Malaysia, Johor Bahru and Malaysian Genome Institute. This research applied the viable method for extraction, purification using ion-exchange chromatography, and identification of purified enzyme from different parts of pineapples specifically crown and stem in different cultivars. Besides that, this study also covered the characterization of protease enzyme extract and the fibrinolytic activity.

1.5 Significance of Study

In this study, significant biomass and nutrients were identified in pineapple waste, such as the crown and stem, for industrial uses. This study determined the best cultivar and pineapple waste, either the crown or stems that generated the maximum proteolytic activity. Finally, the protease enzyme was purified and tested for fibrinolytic activity for industrial usage.

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