HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF BROMELAIN FROM MD2 PINEAPPLE

RAFIDA BINTI RAZALI

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2020

HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF BROMELAIN FROM MD2 PINEAPPLE

RAFIDA BINTI RAZALI

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2020

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

JUDUL : **HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF BROMELAIN FROM MD2 PINEAPPLE**

IJAZAH : **SARJANA SAINS**

BIDANG : **BIOTEKNOLOGI**

Saya **RAFIDA BINTI RAZALI**, Sesi **2016-2020**, mengaku membenarkan tesis Sarjana ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis ini adalah hak milik Universiti Malaysia Sabah
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/):

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
/	TIDAK TERHAD	

Disahkan Oleh,

ANITA BINTI ARSAD
PUSTAKAWAN KANAN
UNIVERSITI MALAYSIA SABAH

RAFIDA BINTI RAZALI
MZ1611010T

(Tandatangan Pustakawan)

Tarikh : 18 Mei 2020

(Dr. Cahyo Budiman)
Penyelia Utama

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been fully acknowledged.

18 May 2020 .....

Rafida Binti Razali

MZ1611010T

CERTIFICATION

NAME	:	**RAFIDA BINTI RAZALI**
MATRIC NUMBER	:	**MZ1611010T**
TITLE	:	**HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF BROMELAIN FROM MD2 PINEAPPLE**
DEGREE	:	**MASTER OF SCIENCE**
FIELD	:	**BIOTECHNOLOGY**
VIVA DATE	:	**18TH FEBRUARY 2020**

CERTIFIED BY;

CO - SUPERVISORY Signature

1. **MAIN SUPERVISOR**
   Dr. Cahyo Budiman

2. **CO-SUPERVISOR**
   Prof. Vijay Kumar

ACKNOWLEDGEMENT

I would first like to thank my supervisor Dr. Cahyo Budiman of the Biotechnology Research Institute at Universiti Malaysia Sabah (UMS). He is the one who always believed in me to make it all done with flying colors. There can be a hundred people in the room, and 99 of them won't believe in you, but all you need is one and that is him. I have been extremely lucky to have a supervisor who cared so much about my work, and who is always lend an ear to hear my queries and questions. I could not have imagined having a better supervisor for my M.Sc. study. I would also like to thank my co-supervisor Assoc. Prof. Dr. Vijay Kumar, who is always ready for discussion and gave helpful suggestions whenever he can.

Completing this work would have been all more difficult were it not for the continued support and friendship provided by the other members of Protein Research Group, Kak Rai, Herman, Shaf, and Bak. Not forgetting my besties, Kak Rai again, Yna, and Intan, who were always available for a shoulder to cry on and to share a triumph moment with. For my Pasca 2 mates, thank you for the wonderful moments together.

Special acknowledgement for the Yayasan Sarawak, for your generosity in funding the Tunku Abdul Rahman Scholarship. I am honored to be the recipient of this award. And for UMS for financial supports of my research work under UMS Great Grant (GUG0104-1/2017).

And finally, last but no means least, my very profound gratitude, I would like to express to my lovely parents and family, Kak Memey, Abang, Kak Nida, and Dekwin for providing me with unconditional love, strong support and continuous encouragement throughout my study years and developing as a researcher.

This triumph would not have been possible with all of them.
Thank you so much.

Rafida Binti Razali
18 May 2020

ABSTRACT

Bromelain is a complex mixture of proteases mainly found in the stems and fruits of pineapple (*Ananas comosus*). This enzyme belongs to a cysteine protease family member with a wide range of applications. Traditionally, bromelain is widely used as a meat tenderizer. The bromelain-based meat tenderizer products are commercially available which are produced through direct extraction and purification from pineapples. No recombinant bromelain products are so far available in the market. While the production of recombinant bromelain was available in many reports, none of them was successfully produced in fully soluble forms. Previous genomics study on MD2 pineapple revealed that this pineapple strain has 14 genes encoding bromelain with various sizes ranging from 19 kDa up to 211 kDa and therefore classified into small- (MD2-SBro; <20 kDa), medium- (MD2-MBro; 20-60 kDa) and large-sized (MD2-LBro; >60 kDa) bromelain. MD2-SBro primary structure has shown to have no N- or C-terminal additional sequence, meanwhile, MD2-MBro and MD2-LBro demonstrated to have both additional sequences with different sizes. It is therefore assumed that these two bromelains are structurally comparable and may represent each other. This study aims to: (1) determine expressibility and solubility of MD2-SBro and MD2-MBro from MD2-pineapple under heterologous expression using *Escherichia coli*; (2) compare the catalytic and structural properties of MD2-SBro and MD2-MBro overexpressed in *E. coli;* and (3) determine the effects of the selected recombinant MD2-bromelain on the meat tenderization and physicochemical properties. For this purpose, the gene encoding MD2-SBro and MD2-MBro were codon-optimized, chemically synthesized and cloned into pGS-21a and pET-32b(+) plasmids, respectively and transformed into *Escherichia coli* BL21-CodonPlus(DE3). MD2-SBro was expressed in an insoluble form, while MD2-MBro was successfully expressed in a soluble form. MD2-SBro and MD2-MBro were successfully purified to get 14 mg and 20 mg from 1L culture, respectively. Comparative analysis had shown that these bromelains had remarkable difference catalytic properties, whereby catalytic efficiency (k_{cat}/K_M) of MD2-SBro (213 $mM^{-1}s^{-1}$) was found to be lower than that of MD2-MBro (29.13 x 10^5 $mM^{-1}s^{-1}$). Comparative analysis on the three-dimensional models of both proteins demonstrated that while both bromelains have conserved catalytic triads consisting of Cys-His-Asn, the distance of Cys-His in MD2-SBro was found to be not in the appropriate distance for the nucleophilic attack. Besides, the hydrophobicity of the substrate-binding cavity of these bromelains was also found to be different. These are believed to be the main factors causing the differences in their catalytic properties. Given its remarkable higher catalytic activity, MD2-MBro was further characterized and applied as a meat tenderizer. Far-UV circular dichroism of MD2-MBro showed that this protein was indeed in a properly folded state with the helical content of 59.2%. The thermal unfolding curve of this protein was also shown that MD2-MBro has a melting temperature of 54.93 $\pm$ 0.2 ^oC. Further application of MD2-MBro indicated that MD2-MBro had shown to be able to tenderize the meat in a concentration-dependent manner. The shear-force values of the meat in the presence of MD2-MBro fall under the category of tender. The result also suggested that the concentration of 0.01% MD2-MBro was sufficient to tenderize the meat. Nevertheless, the ability of meat tenderizing by MD2-MBro was accompanied by some changes in physicochemical properties of the meat (pH, water holding capacity and cooking loss). Altogether, this study should contribute to the new knowledge on the catalytic properties of different types of bromelain and provide a platform for the production of recombinant bromelain as a meat tenderizer.

ABSTRAK

PENGEKSPRESAN, PENULENAN DAN PENCIRIAN BROMELAIN DARIPADA NANAS MD2

Bromelain adalah campuran kompleks protease yang kebanyakannya terdapat di batang dan buah nanas (Ananas comosus). Enzim ini tergolong dalam kumpulan sisteina protease dengan pelbagai aplikasi dan digunakan meluas sebagai pelembut daging secara tradisional. Produk pelembut daging berasaskan bromelain tersedia secara komersil dihasilkan melalui pengekstrakan langsung dari nanas. Tiada produk bromelain rekombinan sedia ada di pasaran. Walaupun pengeluaran bromelain rekombinan tersedia dalam banyak laporan, tiada satu pun daripada mereka berjaya dihasilkan dalam bentuk larut sepenuhnya. Kajian genomik sebelum ini mengenai nanas MD2 mendedahkan bahawa nanas ini mempunyai 14 gen bromelain dengan pelbagai saiz antara 19 kDa hingga 211 kDa dan dikelaskan kepada bromelain bersaiz kecil (MD2-SBro; <20 kDa), sederhana (MD2-MBro; 20-60 kDa) dan besar (MD2-LBro; >60 kDa). Struktur utama MD2-SBro adalah tidak mempunyai bahagian N- atau C-tambahan, manakala MD2-MBro dan MD2-LBro mempunyai kedua-dua bahagian urutan dengan saiz yang berbeza. Maka diandaikan bahawa kedua-dua bromelain ini mempunyai struktur yang sama dan mewakili antara satu sama lain. Kajian ini bertujuan untuk: (1) menentukan pengekspresan dan kelarutan MD2-SBro dan MD2-MBro dari nanas melalui ekspresi heterolog menggunakan Escherichia coli; (2) membandingkan ciri-ciri pemangkin dan struktur MD2-SBro dan MD2-MBro yang dihasilakan melalui E. coli; dan (3) menentukan kesan MD2-bromelain rekombinan terpilih pada keempukan dan sifat fizikokimia daging. Untuk tujuan ini, pengekodan gen MD2-SBro dan MD2-MBro dioptimumkan kodon, disintesis secara kimia dan diklonkan ke dalam plasmid pGS-21a dan pET-32b(+), dan ditransformasi dalam E. coli BL21-CodonPlus (DE3). MD2-SBro dinyatakan dalam bentuk yang tidak larut, manakala MD2-MBro berjaya dinyatakan dalam bentuk larut, dan telah berjaya ditulenkan untuk mendapatkan 14 mg dan 20 mg daripada 1L kultur. Analisis komparatif menunjukkan bahawa kecengkapan pemangkinan (k_{cat}/K_M) MD2-SBro (213 $mM^{-1}s^{-1}$) didapati lebih rendah daripada MD2-MBro (29.13 x 10^5 $mM^{-1}s^{-1}$). Analisis struktur menunjukkan bahawa kedua-duanya mempunyai triad pemangkin yang terdiri daripada Cys-His-Asn. Walau bagaimanapun, jarak Cys-His di MD2-SBro didapati tidak berada dalam jarak yang sesuai untuk serangan nukleofilik. Selain itu, hidrofobisiti rongga pengikat substrat ini juga berbeza. Ini dipercayai merupakan sebagai faktor utama yang menyebabkan perbezaan pada sifat pemangkin bromelain. Memandangkan aktiviti pemangkin MD2-MBro lebih tinggi, MD2-MBro dicirikan lebih terperinci dan digunakan sebagai pelembut daging. Dichroisma pekeliling far-UV MD2-MBro menunjukkan bahawa protein ini mengandungi kandungan helikal sebanyak 59.2%. Lekuk termal protein ini juga menunjukkan bahawa MD2-MBro mempunyai takat lebur pada 54.93 ± 0.2 °C. Penggunaan MD2-MBro selanjutnya dalam analisis keempukan menunjukkan nilai daya ricih daging jatuh dalam kategori empuk dan kepekatan 0.01% MD2-MBro mencukupi untuk melembutkan daging. Walau bagaimanapun, keupayaan daging yang diperap oleh MD2-MBro diiringi oleh beberapa perubahan dalam sifat fizikokimia daging. Secara keseluruhannya, kajian ini menyumbang kepada pengetahuan baru mengenai sifat pemangkin daripada pelbagai jenis bromelain dan menyediakan satu platform untuk pengeluaran bromelain rekombinan sebagai pelembut daging.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	-	Base pair
E.coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetraacetic acid
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
LB	-	Luria Bertani
K_M	-	Michaelis constant
k_{cat}	-	Turnover number
MD2-SBro	-	MD2 small-sized bromelain
MD2-MBro	-	MD2 medium-sized bromelain
OD	-	Optical density
R.M.S.D	-	Root-mean-square deviation
rpm	-	Revolution per minute
SDS-PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	-	Tetramethylethylenediamine
Trp	-	Tryptophan
Tyr	-	Thyrosine
UV	-	Ultraviolet ray
V_{max}	-	Maximal velocity
WHC	-	Water holding capacity

LIST OF SYMBOLS

%	-	Percentage
µg	-	Micro gram
ml	-	Mili litre
°C	-	Degree celcius
µM	-	Micro molar
mM	-	Mili molar
rpm	-	Revolution per minute
kg	-	Kilo gram
g	-	Gram
M	-	Molar
pH	-	Pouvoir hydrogene
min	-	Minute
kDa	-	Kilo dalton
nm	-	Nano meter
mg	-	Mili gram
cm	-	Centi meter
v	-	Volume
kV	-	Kilo voltage
V	-	Voltage
Ψ	-	Psi
Φ	-	Phi
±	-	More or less
Å	-	Amstrong
α	-	Alpha
β	-	Beta

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Proteases, which are also known as peptidases or proteinases, are one of the enzymes that are found abundantly in the plants. This group of enzymes performs proteolysis which refers to the breakdown of peptide bonds in the polypeptide chain by the assistance of a water molecule (hydrolysis) (Hedstrom, 2002). According to Kraut (1971), during organic evolution, the entire protease groups has been evolved where each enzyme catalytic site became the key factor in driving the biochemical reactions and has emerged as the key tool for the modern commercial industry. All of the proteases exhibit a common action mechanism which act on the carbonyl-carbon bond of the peptide group. However, different strategies are used by every protease to generate a nucleophilic attack on the carbonyl-carbon bond and the core amino acids involved in the catalysis process which act as the active site that defines the specificity and catalytic efficiency of any enzymes. Therefore, the most convincing mode of proteases classification is based on the amino acid involved in hydrolysis. There are six major groups of proteases, including serine proteases (EC 3.24.21), cysteine proteases (EC 3.4.22), threonine proteases (EC 3.4.25), glutamic acid proteases (EC 3.4.19), aspartate proteases (EC 3.4.23), and metalloproteases (EC 3.4.24).

Due to their catalytic properties which are known to be in a broad range of pH or temperature, proteases are widely used in various industrial applications (Murakami *et al.,* 1991; Sanatan *et al.,* 2013). Today, proteases dominate with approximately 60% market share of the total enzyme market worldwide where the major producers are Miles Laboratories, Novo Industries, Genencor International, and

Gist-Brocades (Feijoo-Siota & Villa, 2011). In Malaysia, the needs of proteases are considerably high with a total market value of not less than RM 16 billion. Unfortunately, none of the company from Malaysia is recorded as the producer of proteases. Despite the country is blessed with its mega biodiversity which may serve as a source for local proteases, attempts to consolidate local industry for the production of local protease remain limited.

One indigenous resource of powerful proteases that are widely found in Malaysia is the pineapple (*Ananas comosus*). The proteases in pineapples are known as bromelain, a group of cysteine proteases that depend on the thiol group of a cysteine residue within its active site for catalytic activity (Cooreman *et al.,* 1976; Taussig & Batkin, 1988; Maurer, 2001). Production of pineapple fruits, as reported by Malaysia Pineapple Industry Board in 2013, was more than 80,000 metrics tonnes of pineapples fruit and kept increasing every year suggesting pineapple as an excellent source for proteases.

Bromelains are mainly found in the stems or fruits of pineapples and known to be homologous to the enzymes of the papain family originated from papaya (*Carica papaya*). Along with papain, perhaps bromelains are the most widely known proteases in terms of traditional knowledge. These proteases have been widely used as the traditional meat tenderizer for a long time due to the ability of bromelain in breakdown meat myofibril proteins (Omojasola *et al.*, 2008; Nadzirah *et al.*, 2016). Apart from the stems and fruits, bromelain is also reported present in pineapple peels, cores, crowns, and leaves, with the highest proteolytic activity and protein contents detected in the extract of pineapple crowns (Ketnawa *et al.,* 2012). Cysteine protease is a group of protease which is characterized by the triad catalytic site of Cys-His-Asn/Glu and widely found in plant species (Verma *et al.,* 2016). The biological function of bromelain for pineapple plants remains conflicting, nevertheless, it is believed to follow the main roles of the cysteine proteases in plants. In plants, cysteine proteases are known to play important roles in growth and development and in accumulation and mobilization of storage proteins such as in seeds. Besides, this group of enzyme is also known to be involved in signaling pathways and response to biotic and abiotic stresses (Grudkowska & Zagdanska, 2004).

In addition, bromelain has also reported to exhibit a wide range of therapeutic applications including reversible inhibition of platelet aggregation, sinusitis, surgical traumas (Livio *et al.,* 1978), thrombophlebitis, pyelonephritis angina pectoris, bronchitis (Neubauer, 1961), and enhanced absorption of drugs, particularly of antibiotics (Renzini & Varengo, 1972; Maurer, 2001). A study on oral delivery of this enzyme has also shown that this enzyme can be absorbed in human intestines without degradation and without losing its biological activity (Chobotova *et al.*, 2010; Castell *et al.,* 1997). In fact, Food and Drug Administration has accepted bromelain as a safe food supplement. Apart from the therapeutic applications, for many years, bromelain is used as a meat tenderizer. Traditionally, the meats are seared by thin chunks of pineapple or marinated in blended pineapple before further cooking processes. Some studies have shown that fruits or stems bromelain extract are able to tenderize the meat (Ketnawa & Rawdkuen, 2011).

Due to this versatile properties, bromelain gains wide interest in the industry and currently widely available in the market. In addition, there is a rapid growth of research and commercialization of bromelain worldwide due to its indispensable versatile applications, including food, textile, brewing, cosmetic, and dairy products (Polaina & MacCabe, 2007). In particular, bromelain product as a meat tenderizer is also commercially available, whereby the crude bromelain is extracted from pineapple stems or fruits which then further sprinkled on the meat or re-suspended for marination purpose. The use of bromelain as a meat tenderizer is gaining wide interest as tenderness is considered as the most important meat quality attribute which affects the customer perception (Brooks *et al.*, 2000; Morgan *et al.,* 1991; Mennecke *et al.,* 2007). Accordingly, many interventions have been developed in the meat industry to improve the tenderness of low-value muscles and to ensure the consistent tenderness of high-value muscles (Bolumar *et al.,* 2013).

Nevertheless, local bromelain products remain limited by several issues. While pineapple plantation is widely available in Malaysia, the development of bromelain products through direct extraction of pineapple requires a huge amount of raw materials (stems, fruits, peels or other parts of pineapples). In addition, several steps of chromatography are needed for the production of pure bromelain from pineapples which, to some extent, influences the production costs. Recombinant technology for

bromelain is indeed available and widely studied. However, the production of recombinant bromelain remains limited at the lab scale (Arshad *et al.,* 2014). Besides, no meat tenderizer has ever developed from recombinant bromelain.

Recombinant bromelain refers to a purified of a single cysteine protease, instead of a mixture as presented in the crude bromelain, which is obtained from overexpression of a gene encoding bromelain under, mostly, heterologous expression system. While studies on bromelain extract on the physicochemical properties of meats are widely available, to our knowledge, there is no study on the use of recombinant bromelain for the purpose of meat tenderizing. It is often found that recombinant proteins behave differently compared to their non-recombinant (native) forms (Zhou *et al*., 2016; Clement *et al*., 2015; Ghosh *et al*., 1988). This leads to the assumption that the effect of native and recombinant bromelain on the physicochemical properties of the meat might be different. Nevertheless, the use of recombinant technology under heterologous expression system for the production of bromelain is so far challenged by the issue of expressibility and solubility. To my knowledge, while some reports on the development of recombinant bromelain are available, most of them remain ended up in the insoluble form, fully or partially (George et al., 2014; Amid et al., 2011; Bala et al., 2011a). To note, the production of the bromelain gene was done through a heterologous approach using *Escherichia coli* system. It is believed that the compatibility of bromelain gene with the molecular machinery of *E. coli* is the main issue on the solubility and expressibility of the protein.

Besides, as pineapple harbors many genes encoding bromelain, the selection of the appropriate gene for further overexpression is also challenging. Nevertheless, this approach is considerably shorter and cheaper to obtain the bromelain for various applications. There is no study for return of investment (ROI) of the recombinant bromelain, however, Islam *et al.* (2018) highlighted that while recombinant technology requires a lot of cost at the first place, but, in the long run, this technology is proven to be able to reduce the production cost and bring the products into the affordable prices.

Recent studies on the whole-genome sequence of MD2 pineapple revealed that this pineapple strain has 14 genes encoding bromelains with various sizes (Redwan *et al.*, 2016). Among these 14 genes, which encoded cysteine proteases bromelain with the sizes ranging from 19 kDa up to 211 kDa, they are classified into small- (<20 kDa), medium- (20-60 kDa) and large- (>60 kDa) sized of bromelain. Amino acid sequence analysis of those proteins revealed that those proteins have different primary structures, whereby additional sequences at N- or C-terminal are present in some of the proteins. The smallest bromelain (MD2-SBro) is about 19 kDa without N- or C-terminal additional sequence, meanwhile the medium- and large-sized of bromelain demonstrated to have both sequences with various sizes. Most of the studies are conducted on medium size bromelain, with the size around 30-40 kDa. Among these 14 genes, one gene (GeneBank accession code: OAY85858.1) has shown to be highly similar to the bromelain that was widely reported and thus might serves as a model of medium-sized bromelain of MD2 pineapple (MD2-MBro). It is therefore interesting to see whether MD2-SBro and MD2-MBro exhibited similar catalytic properties and applicable as a meat tenderizer. To note, the primary structures of MD2-MBro and large-sized of bromelain of MD2 pineapple (MD2-LBro) are similar in terms of the presence of N- and C-terminal additional sequences. It is therefore assumed that MD2-MBro may represent MD2-LBro as well.

Further to translate MD2-bromelain genes into the products, solid fundamental study on the genes and the translated proteins is unavoidable. Interestingly, all of the studies on recombinant bromelain are conducted only on the medium-sized of bromelain. There is no study, to my knowledge, on the small-sized bromelain so far. This leads to the urgency of the study on different sizes of bromelain in MD2-pineapple. This study describes the attempts to develop an expression system and purification process of MD2-SBro and MD2-MBro under the heterologous expression using *Escherichia coli*. Comparative analysis on the catalytic properties of these two bromelains is also provided with the support of their structural analysis. Further, a recombinant MD2-bromelain exhibiting the highest catalytic activity is selected for further detail studies and applied as a meat tenderizer. Altogether, this study provided the first evidence on the comparative analysis of the different types of bromelain and also the first study on the recombinant bromelain as a meat tenderizer.