FINAL REPORT

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Molecular Cloning and Overexpression of Stem Bromelain from *Ananas Comosus* in *Kluyveromyces lactis*

By

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REPORT SUMMARY

Nowadays, numerous therapeutic benefits have been claimed for bromelain. Bromelain, belonging to papain family, is a glycosylated single chain protein. Until recently, the three-dimensional (3D) structure of bromelain remained to be elucidated. The comprehensive information about the thorough structural organization of bromelain is vital for therapeutical application and in understanding their role in the cell and in other related molecular mechanisms. In addition, up to now, the available commercial bromelain in the market were partially produced by pineapple stem and fruit. Nevertheless, none of them are produced and formulated from recombinant forms other than *E. coli*. However, due to safety concerns of the potential bacteria endotoxin contamination, an alternative system needs to be applied. Therefore the purpose of this research work is to screen for bromelain from different variants of A. comosus followed by bromelain sequence analysis and accurate 3D structure determination using bioinformatics tools. Finally, recombinant bromelain construction in cloning and expression systems is performed. This research study will provide the information of the bromelain sequence importance for therapeutical application followed by the accurate predicted structure of bromelain from *A. comosus* that can be used to define the molecular catalysis mechanism of bromelain which is very important for protein engineering purpose in future. In addition, recombinant bromelain construction in GRAS system of *K. lactis* produced which will ensure simplicity, high quality of the enzyme, reduce contamination and also cost effective.

So far, we had one peer-reviewed article published in Journal of the science of food and agriculture, won 1 award and have one paper presented in International conference. Two undergraduate students and one MSc student were also graduated from work related to this project. In conclusion, this project has achieved its target for human capital development.

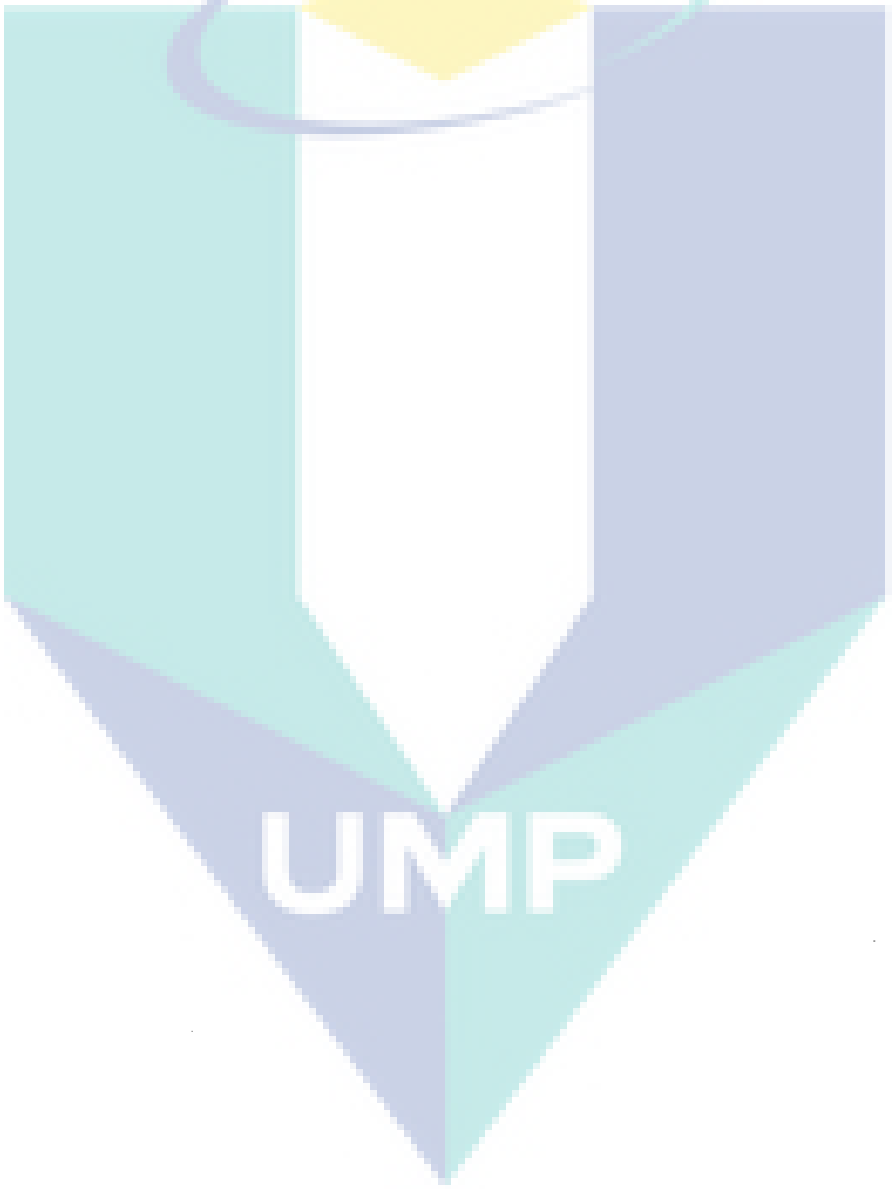
JOURNALS

This project produced ONE (1) peer-reviewed research article:

Aizi Nor Mazila Ramli, Tuan Norsyalieza, Tuan Aznan, Rosli Md. Illias, (2017). Bromelain: from production to commercialization. Journal of the Science of Food and Agriculture. 97(5): 1386–1395.

AWARD

Recombinant bromelain: potential source of pure bromelain product. Creation, Innovation, Technology & Research Exposition (CITREX), April 2017, UMP- **Silver Medal**



ABSTRACT

There are many cultivar of pineapple, *A. comosus* available around the world. Morris, N36 and Sarawak cultivar use throughout this work was amongst cultivars grown in Malaysia. Proteolytic enzyme found in pineapple plant, is known as bromelain. The enzyme catalyzes the breakdown of peptide bond from the polypeptide chain by hydrolysis. Nowadays, numerous therapeutic benefits have been claimed for bromelain. Bromelain, belonging to papain family, is a glycosylated single chain protein consists of cysteine proteinases. A gene encoding bromelain from pineapple (*Ananas comosus*) was isolated from Morris cv. using the Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) techniques. The isolated gene was successfully expressed in the *E. coli*. Analysis of the nucleotide sequence revealed the presence of bromelain coding sequence, 1,056 base pair (bp) which encodes 302 amino acid. Up to now, the three-dimensional (3D) structure of bromelain remained to be elucidated. The comprehensive information about the thorough structural organization of bromelain is vital for therapeutical application and in understanding their role in the cell and in other related molecular mechanisms. Therefore the purpose of this research work is to screen for bromelain from different cultivars of *A. comosus* followed by bromelain sequence analysis and accurate 3D structure determination using bioinformatics tools. This research study will provide the information of the bromelain concentration in different cultivars of *A. comosus*. In addition, details of bromelain sequence importance for therapeutical application followed by the accurate predicted structure of bromelain from *A. comosus* that can be used to define the molecular catalysis mechanism of bromelain will be provided.

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selective interest in the market to fulfil the uniformity and consistency in taste, size and ripeness. According to the previous research of (Yuris and Siow 2014), the antioxidant activity of Morris cv., is higher compared to N36 cv. and Sarawak cv. In addition, these cultivar is only commercialized in Malaysia.

Bromelain is one of the proteolytic enzyme found in the pineapple plant, *A. comosus* (Bhattacharyya 2008). There are 351 amino acids residues estimated to be present in the bromelain of which seven cysteines were reported to be responsible in catalysis of the enzyme. The enzyme is used in therapeutic and industrial applications (Muntari, Ismail et al. 2012). Its therapeutic properties were first introduced in 1957 for various medical treatments. The properties of bromelain has shown to be effective in the platelet inhibition aggregation treatment, fibrinolysis, inflammation healers, regulation of body immunity, potentiation of other drugs, digestive assistance, wound healing and assisting with the improvement of cardiovascular circulatory (Kelly 1996). Apart from that, bromelain is also being used in the baking industries and to tenderize the meat (Arshad, Amid et al. 2014).

In general, the name of stem bromelain and fruit bromelain was used to accordingly distinguish the origin of the enzyme in the plant (Kelly 1996). Direct consumption of fruit bromelain provides various source of vitamins and minerals. Unlike stem bromelain, less studies has been reported on fruit bromelain. In the present work, stem bromelain are the most studied enzyme as an alternative source of bromelain compared to fruit bromelain which usually being eaten fresh. Therefore, the purpose of this research is to reveal an in-depth information regarding fruit bromelain.

1.5 Scope of Study

This research was initiated with the screening of fruit bromelain from different cultivars of *A. comosus* (Morris cv, N36 cv. and Sarawak cv.) by enzyme assay and protein analysis. Next, the gene of fruit bromelain (BAA21848) was isolated using total RNA method. Cultivar with the highest enzymatic activity was selected and used as a template in the gene cloning approach. DNA sequencing was then performed. The gene sequence of fruit BAA21848 was eventually used for the structural analysis and comparison with stem bromelain.

2.2 Pineapple (*A. comosus*)

Back to the early age, Christopher Columbus discovered pineapple (*A. comosus*) on his journey across the world. At that moment, the plant was domesticated by the native community of South America (Ming, Wai et al. 2016). The plant was later on being introduced to the European until it reaches worldwide nowadays (Hajar, Zainal et al. 2012). The pineapple was mainly cultivated vegetatively using the crown section (Fitchet and van de Venter 1988).

2.2.1 *A. comosus* Cultivar

According to Bhattacharya (2016), desirable characteristics of a plant can be identified as a cultivar. Many pineapple cultivars are grown with distinguishable characteristics such as size, flesh color and taste to meet the acceptable standards (Shoda, Urasaki et al. 2012). Gandul cv., Josapine cv. (hybrid), Maspine cv. (hybrid), MD2 cv. (hybrid), Morris cv., Morris Gajah cv., N36 cv. (hybrid), Sarawak cv. and Yankee cv. found in Malaysia are amongst cultivars that possess variation in its characteristic. Morris cv. (Queen) and Sarawak cv. (Smooth Cayenne) is the most notable cultivar used for commercialization around the world so do in Malaysia (Bartholomew, Paull et al. 2002, Lembaga Perindustrian Nanas Malaysia 2017). Whereas, the N36 pineapple is a hybrid selected from a cross between 'Gandul' (Spanish) and the Sarawak (Smooth Cayenne) cultivar.

2.4 Bromelain

Bromelain, a collective name for stem bromelain or fruit bromelain in pineapple plant, *A. comosus*, is a proteinase enzyme that hydrolyze protein into smaller polypeptides or amino acids (Bhattacharyya 2008). In addition, the sulfhydryl, peroxidase, acid phosphatase, several protease inhibitors and organically-bound calcium can also be found in bromelain (Gautam, Mishra et al. 2010). According to Muntari, Ismail et al. (2012) the stem and fruit part of pineapple stores bromelain. It was reported that despite of the level of amino acid composition differences, both stem and fruit bromelain retain similar catalytic action constantly (Mohapatra, Rao et al. 2013).

In order to produce a highly purified bromelain, several processes need to be carried out. The process covers the extraction, purification, drying and packing of bromelain. Above all, purification process was the most important process as it will affects the purity of the end product and the overall processing cost (Arshad, Amid et al. 2014). The extraction of bromelain from pineapple takes place when the desired part of purification was grinded without water using a domestic juicer. The juice extracted was the crude extract of the enzymes. Generally, bromelain is prepared from cooled pineapple juice by centrifugation, ultrafiltration, and lyophilisation (de Lencastre Novaes, Jozala et al. 2016). Consequently, the process yields a yellowish powder.

Bromelain, known since 1875 is mainly used as a phytomedical compound. Besides, bromelain also offers an abundant use for industrial and other applications (Ramli and Aznan 2017). Commercial bromelain is not cheap, the price can cost up to 2400 USD/kg (Muntari, Ismail et al. 2012). The process of bromelain production was very expensive thus making the bromelain value increased in the market.

2.5 Application of Bromelain

Tones of industrial and therapeutic application using this enzyme were made nowadays including pharmaceutical, food industries and others. It was firstly introduced as a therapeutic compound on 1957. Inhibition of platelet aggregation, fibrinolytic activity, anti-inflammatory action, anti-tumor action, modulation of cytokines and immunity, skin debridement properties, enhanced absorption of other drugs, mucolytic properties, digestive assistance, enhanced wound healing, and cardiovascular circulatory improvement was the action of bromelain.

Bromelain was categorized as a food additive and generally accepted as safe by The Food and Drug Administration, USA (Muntari, Ismail et al. 2012). Food industries also using this enzyme for baking, meat tenderization as well as a daily supplement for healthy lifestyle (Muntari, Salleh et al. 2011). At present, bromelain application was divided into three categories: clinically, pharmaceutically and industrially (Muntari, Ismail et al. 2012).

2.5.1 Clinical Use

According to the National Institute of Health (2012) clinical involves individual people or the use of materials from human origin, such as observed behaviour, answers to questions or tissue samples, obtained through direct contact with a particular living person that volunteers and agrees to participate in a research study. Bromelain was clinically being used to treat several inflammatory disorders of the musculoskeletal system. With respect to this matter, a number of clinical studies were made as evidence to support the findings.

2.5.1.1 Treatment of Osteoarthritis

Osteoarthritis is a common musculoskeletal disorder caused by pain and disability in joint (Klein, Kullich et al. 2006). Based on recent statistics of

been done by Engwerda, Andrew et al. (2001). In the study, bromelain can enhance IFN-γ-mediated nitric oxide and TNFα production by macrophages thus, enhancing the innate immune response against unknown threats (Engwerda, Andrew et al. 2001). In another study of immunogenicity, bromelain can also inhibit the T cell signal transduction to block the Raf-1/extracellular-regulated-kinase- (ERK-) 2 pathways to participate in mitogenesis, apoptosis, and cytokine production (Mynott, Ladhams et al. 1999). Moreover, with the treatment of cells using bromelain, the activation of CD4 (+) can be lowered down thus reducing the expression of CD25 (Secor, Singh et al. 2009). An increased levels of CD25 have been shown to correlate with disease severity in individuals with allergic asthma which commonly take place in children (Hoeger, Niggemann et al. 1994, Sujata Pandit Sharma and Brajbhushan 2015).

2.5.1.3 Fibrinolysis

The excessive synthesis of fibrin which causing the blood to clot tremendously was reported by Lotz-Winter (1990). By applying bromelain at higher concentration, the period of inhibition of prothrombin was prolonged (Pavan, Jain et al. 2012). The inhibition caused the prothrombin to be inactive thus lessen the time for the blood to coagulate. Besides that, bromelain is an effective fibrinolytic agent. It stimulates the conversion of plasminogen to plasmin, resulting in increased fibrinolysis by degrading fibrin (Taussig and Batkin 1988).

2.5.1.4 Diarrhoea

Several evidence proposed that bromelain able to prevent some of the effects of *Vibrio cholera* and *E. coli*, an intestinal pathogens (Bitange Nipa Tochi, Zhang Wang et al. 2008). This pathogen produces enterotoxin and causes diarrhoea in animals (Pavan, Jain et al. 2012). To counteract with this pathogen, bromelain interact with the intestinal secretory signalling pathways, that includes several component of

(Ahmed, Hasan et al. 2010). The effectiveness of bromelain most frequently being used in the preparations containing differing complexes of proteolytic enzymes and differing concentrations of bromelain (Brien, Lewith et al. 2004).

2.5.2.1 Surgery

The average number of days for pain and post-surgery inflammation recovery can be reduced by the administration of bromelain before a surgery (Unknown 2010). Besides that, several trials state that the use of bromelain might be effective in reducing swelling, bruising, and pain in women having episiotomy. In 1993, a German government commission approved the use of bromelain to treat swelling and inflammation following surgery, especially sinus surgery (Dighe, Pattan et al. 2010). Bromelain was today used for treating acute inflammation and sports injuries (Brien, Lewith et al. 2004).

2.5.2.2 Potentiation of Antibiotics

The demand for novel effective antimicrobial or antibiotic drugs is high nowadays (Pieren and Tigges 2012). According to Kelly (1996) antibiotic potentiation is one of the primary uses of bromelain. Bromelain works by modifying the permeability of organs and tissues to different drugs (Bhattacharyya 2008). Neubauer (1961) evaluate the administration of combined bromelain together with antibiotic therapy. As a result, no treatment responses take place in 22 patients which only consuming antibiotics whereas 23 patients give out a positive result with the addition of bromelain.

2.5.2.3 Debridement Burns

Debridement is known as the removal of damaged tissue from wounds or second/third degree burns (Pavan, Jain et al. 2012). Bromelain accelerates healing

effectiveness of bromelain was proven to treat eight patients with *Pityriasis lichenoides chronica* a skin disease of unknown etiology.

2.5.3 Industrial use

Proteolytic enzymes, bromelain was widely used industrial enzyme (Kirk, Borchert et al. 2002). It have been used extensively in industrial fields such as in tenderization, baking industry, textile, tooth whitening and cosmetics (Polaina and MacCabe 2007).

2.5.3.1 Tenderization

Meat tenderness refers to the characteristic of meat texture. Basically, meat tenderness is an important factor that affects the consumers' need. However, it is not easy to make the meat tenders due to the complex structure of muscle (Calkins and Sullivan 2007). In order to do this, many approaches have been taken to improve the tenderness of meat such as blade tenderization, moisture enhancement technology and enzymatic treatment (Pietrasik, Aalhus et al. 2010). Previously, meat is keep cool for up to 10 days to allow cathepsins and calphins (proteolytic enzyme) break the toughness of meat (Arshad, Amid et al. 2014). Nevertheless bromelain, papain, and ficin has been use to tenderize meat as these exogenous enzyme has been classified as Generally Regarded as Safe (GRAS) by USDA's Food Safety Inspection Service (ISIS) (Calkins and Sullivan 2007). Compared to other exogenous enzyme, bromelain is the most effective enzyme that improve the meat texture (Sullivan and Calkins 2010). As a matter of fact, bromelain is reported to efficiently hydrolyse a few meat tissue fibre by degrading the connections between the sarcolemma and the myofibrils as observed on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Sunantha and Saroat 2011). In the meantime, bromelain possess a clear proteolytic effect on myosin and other myofibrillar proteins when it was added to a dry chopped sausage (Melendo, Beltrán et al. 1996).

60°C during silk cocoon process can reduced the softening time from 20 hours to only 30 minutes.

2.5.3.4 Tooth whitening

A large number of people seek for dentist regarding their teeth appearance and colour. The colour of the teeth is determined by the coupling effects of intrinsic and extrinsic colorations on the surface of teeth. The main cause of extrinsic discoloration is due to the intake of coloured foods, caffeinated drinks and smoking (Joiner 2004). According to Chakravarthy and Acharya (2012) dentifrices containing papain and bromelain extracts exerts a significant lightening effect compared to the control dentifrice (Colgate Regular). Discoloured teeth have raised the demand for tooth-whitening products such as toothpaste with the ability to remove the extrinsic stains. The stain can be removed using papain and bromelain based on the current evidence discovered by Kalyana, Shashidhar et al. (2011) which reported that higher lightening value after brushing compared to the control dentifrice.

2.5.3.5 Cosmetic industry

With gentle peeling effect to skin, bromelain has been used as an active ingredient in cosmetics product (Ketnawa, Chaiwut et al. 2011). For instance, bromelain can be used in the treatment of skin problems such as wrinkles, acne and dry skin. The action of bromelain works by gently digesting the protein layer of dead cells and replace it with a younger skin cells (Ozlen 1995).

2.6 Structural Studies of Bromelain

Bioinformatics involves the use of computational approach to analyze a biological data (Fenstermacher 2005). According to Luscombe, Greenbaum et al. (2001), there are numerous amounts of biological data were deposited in the Gen

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample Preparation

The fruit and stem used throughout this research were obtained from the local pineapple (*A. comosus*) cultivars around Pahang, Malaysia. The pineapple cultivars were Morris, N36 and Sarawak. Initially, the pineapple fruit and stem were cut into small pieces and the pieces were weighed using the analytical balance (Appendix 1) and grinded in the industrial blender separately. Next, each of the extract obtained was filtered using the whatman filter paper and were centrifuged at 10,000 rpm in 4°C for 20 min to remove left residue. The crude supernatant was collected and stored at -20°C until further use.

3.2 Buffer and reagent preparation

3.2.1 Enzymatic Assay Buffers and Reagents

The pH of reagent A (50mM Potassium Phosphate Buffer) was adjusted to 7.5, at 37°C. In the preparation of reagent B (Casein), 0.65% (w/v) was dissolved in 125ml of reagent A. Dilution of reagent C, tricholoroacetic acid (TCA), D (F-C) Folin

was incubated again at 37°C for 30 minutes. The mixtures were filtered out after 30 minutes using the 0.45µm syringe filter.

Reagent D (F-C) and E (Na_2CO_3) were used to detect the presence of L-tyrosine content in the test sample. Therefore, 5ml of reagent E was added to the sample mixture followed by the addition of reagent D. The mixture were incubated in the water bath at 37°C in 30 minutes. After 30 minutes, the color development was observed. The enzymatic activity of protease activity was calculated using the tyrosine standard curve. The standard curve is prepared by using different concentration of L-tyrosine at range of 0.05 ml to 0.50 ml. At the end, the test sample and standard were measured using the spectrophotometer to obtain the absorbance reading.

3.5 Isolation of DNA sequence from pineapple fruit

The pineapple fruit was grounded in liquid nitrogen to obtain extracts in the form of a fine powder (Figure 3.1). The procedure was done under the free-RNAse environment and stored at -80°C for the total RNA extraction of fruit and stem bromelain.

Figure 3.1 The sample of grounded fruit bromelain in powder form

3.6 Fruit bromelain (BAA21848) isolation and cloning

3.6.2 Fruit bromelain amplification gene via Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify the nucleic acid respectively. An amount of 5 ng of cDNA was used for this PCR amplification. The volume of each component was stated in the Table 3.1. The reaction was carried out in a 0.2 ml PCR tube. A 20 µl of reaction mixture contained: 5 µl genomic DNA of cDNA from fruit bromelain, 2 µl of 10X PCR buffer, 1.2 µl of 3.0 mM $MgCl_2$, 1 Unit of Taq polymerase, 0.5 µM of each forward and reverse primer (FBroF and FBroR) and 1.6 µl of 0.2 mM dNTPs. A PCR Mastercyler (Eppendorf Scientific Inc.) was used to perform the amplification under specific condition (Table 3.2). The reaction was carried out for 34 cycles under the following conditions: 3 minutes initial denaturation at 94°C followed by the PCR cycles consisting of denaturation at 94°C for 30 seconds, annealing temperature at 58°C for 30 seconds and extension at 72°C for 1 minutes 30 seconds. The PCR cycles were followed by an additional final extension at 72°C for another 10 minutes.

Table 3.1 Reagents for the PCR amplification reactions of fruit bromelain

Reagents	Concentration	Volume (µl)
10x PCR Buffer	1x	2
dNTP	0.2mM	1.6
Primer Forward	0.5µM	0.5
Primer Reverse	0.5µM	0.5
dH_2O		8.7
$MgCl_2$	3.0mM	1.2
cDNA template	5-20 ng	5

powder mix with 30mL of TAE buffer and 0.30g of Agarose powder mix with 30mL of TAE buffer. The mixture was heated in the microwave for at least 30s until the agar melt and boiled. The prepared agar was left to cool down at 70°C before it was being added with 0.5µl of GelRed™. The gel were poured down inside the gel casting tray with the casting comb attached to it and was left to solidify for approximately 20 minutes. After the gel had solidified, the casting comb was removed. The gel was then ready for sample loading when it was placed in the 1X TAE buffer tank.

Prior to running the gel, the DNA ladder was loaded into the first well. This ladder was used as ruler to predict the size of the separated nucleic acid. An amount of 2µl of loading dye and 5µl of distilled water, dH_2O was mixed together with the sample to produce a volume of 10µl. The samples were added accordingly into the well. The buffer tank was closed after sample loading and 80V of were applied up to 35 minutes. The visualization of gel was carried out using the Gel Imager™.

3.6.4 Insertion of the target gene into the cloning vector

Cloning of PCR product was done using the pGEM®-T Easy Vector Systems. Initially, TA cloning was carried out using the Taq DNA Polymerase assisted by PCR. The cloning produceed 3'-adenine overhang to each end of the PCR product. The use of pGEM®-T Easy Vector Systems relatively joins the vector and fragment together as the vector possess a 3' T overhangs end. Hence, an efficient ligation can be achieved when the fragment of interest was introduced into the prepared vector.

The pGEM®-T Easy Vector and Control Insert was centrifuged together to collect the contents to the bottom of the tube. The ligation reaction was performed (Table 3.3). Prior to each use, the 2X Rapid Ligation Buffer was vigorously vortexed. The reaction was mixed evenly. The reaction was incubated either at 1 hour in the room temperature or overnight in 4°C to achieve maximum number of transformants.

into the ligation reaction and the other 900 µl was added to the uncut DNA control tube. The inoculums were incubated in the orbital shaker (~150rpm) for 1.5 hours at 37°C. After the incubation, 100 µl of each transformation culture was spread and grown overnight at 37°C onto the LB ampicillin agar spread with IPTG and X-Gal. Successful transformant grown in white was selected. The presence of the fruit bromelain gene were identified by colony PCR method.

3.6.6 Colony PCR of transformed colonies

Colony PCR is a commonly used method to quickly screen for plasmids containing a desired insert directly from bacterial colonies. This method eliminates the need to culture individual colonies and prepare plasmid DNA before analysis. All transformed colony were subjected to colony PCR. The reagents used were shown in Table 3.4. A slight modification of PCR program was done by changing the period of initial denaturation from 3 minutes to 10 minutes to allow effective cell wall breakdown (Table 3.5).

The PCR reaction products were purified using the innuPrep gel extraction kit to eliminate traces of the remaining reagents that can interfere with subsequent downstream applications. Samples were stored at –20 °C.

3.7 Sequencing of DNA

Recombinant plasmid obtained was further subjected to DNA sequencing using two universal primers: T7 polymerase and SP6 polymerase. The plasmid was sent for sequencing service provided by the 1st Base Laboratories, Malaysia.

3.8 Bioinformatics analysis of fruit bromelain

3.8.1 Data mining

The NCBI GenBank and Protein Data Bank (PDB) databases were used to retrieve all the informations regarding fruit bromelain.

3.8.2 Primary sequence analysis

Basic local alignment search tool (BLAST) server is used to perform a homology search corresponding to fruit bromelain (Altschul et al., 1990). The sequence alignment of fruit bromelain with the sequence obtained from NCBI Database was done using the Clustal Omega programme. The catalytic domain of the fruit bromelain protein was identified by InterProScan (Zdobnov and Apweiler, 2001); while the theoretical molecular weight, isoelectric point and amino acid composition of the protein were calculated using the ProtParam tool (reference).

3.8.3 Secondary sequence analysis

Fruit bromelain amino acid sequence (352 residues) was subjected to various sequence prediction analyses, including BLAST-PDB, HHPred, Mod-link+, Phyre2, and PSI-BLAST to identify possible families or conserved domains in the protein.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

Bromelain enzyme can be obtained from the pineapple, *A. comosus*. This enzyme is widely used to assist in various industrial applications. Despite of that, the enzymatic mechanism remains unclear due to less information on its three dimensional structure. All data related to the screening of fruit bromelain from different *A. comosus* cultivars and the molecular work on bromelain with the highest proteolytic content was collected and discussed in this chapter. In addition, the data also includes the structural comparison of fruit bromelain sequence, BAA21848 with other available fruit and stem bromelain retrieved from the NCBI database,

4.2 Bromelain analysis from different cultivars

4.2.1 Sample collection and analysis

The enzymatic activity of bromelain has been reported in several research studies. However, some of the enzymatic activity of bromelain in different *A. comosus* cultivars (cv.) including Morris cv., N36 cv. and Sarawak cv. has yet to be reported through fully. Morris, N36 and Sarawak cultivar as shown in Figure 4.1 and

Table 4.1 Morphological features of *A. comosus* cultivars

Cultivars	Crown size	Fruit shape	Stem size
Morris	Medium	Tapered	Small (2 cm)
N36	Long	Cylindrical	Large (2.0-2.8 cm)
Sarawak	Short	Cylindrical	Large (2.0-2.8 cm)

Regardless on the pineapple physical appearance, different volume of pineapple juice extract is obtained from each of the cultivar. These differences may contribute to different protein content. The highest volume of juice extracts from pineapple fruit, 230.0 mL is obtained from Sarawak cv. (Table 4.2). On the other hand, the volume of juice extracts from pineapple fruit of Morris cv. was 187.5 mL. In contrast to Morris cv., the volume of juice extract in N36 cv. is higher in fruit, 200.0 mL respectively. Based on the observation, the pineapple peel content in Sarawak cv. is less than the other cultivar. Less juice is produced when more pineapple peels present in the fruit, thus causing the space of water content to be reduced. It's difficult to commercialize small fruits which are under marketable value. Sarawak cv. is large in size and weighs approximately around 1.5 to 4.0 kilograms (maximum). Either consumed as fresh fruit, desserts or canned products, Sarawak cv. is known to its sweet taste and crunchy texture (Soloman George, Razali et al. 2016). Despite of that, the N36 cv. is far more resistance to spoilage. Therefore, N36 cv. is suitable to be exported using a reefer container (Hajar, Zainal et al. 2012).

Besides that, the pineapple juice content is also affected by the ripening fruit. The fruit ripening produce more juice than the unripe fruit. This statement can be supported by the other journal that study on the moisture content of the unripe and ripe of mango fruit and the result shown that the moisture content of the fruits increased significantly during ripening from 79.75% to 83.11% (Appiah, Kumah et al. 2011). Thus, high moisture percentage will produced more juice.

Table 4.2 Weight and volume of fruit from each pineapple cultivar; Morris, N36 and Sarawak

0.05	0.063
0.1	0.121
0.2	0.261
0.4	0.504
0.5	0.632

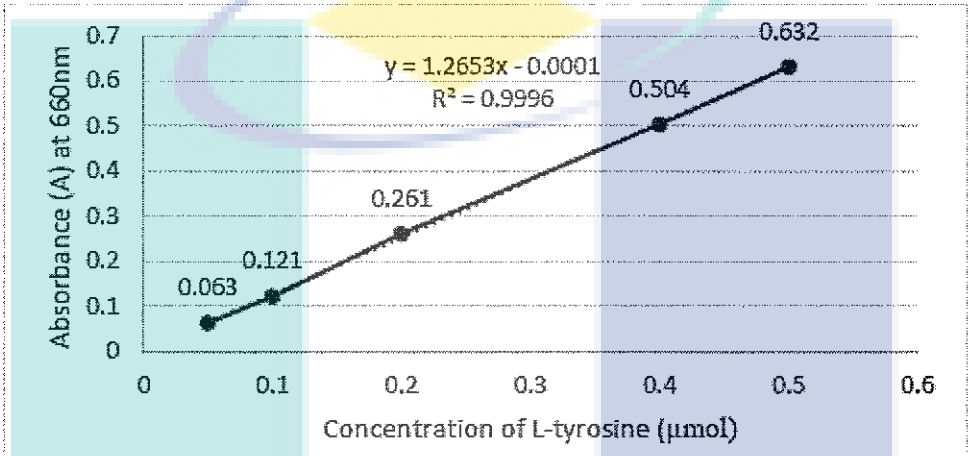

Figure 4.2 The standard curve of enzyme assay using different concentration of L-Tyrosine

The enzymatic activity of fruit bromelain in Morris cv., 1.891 A (average value) is high compared to the other cultivar; the N36 cv. is 1.772 A and Sarawak cv. is 1.593 A (Table 4.4). Besides that, the amount of fruit bromelain (Appendix 2) in Morris cv. is also higher, 0.8220 units/ml than N36 cv. (0.7703 units/ml) and Sarawak cv. (0.6925 units/ml).

The breakdown of casein by fruit bromelain and stem bromelain (generally determined as substrate converted into product) in Morris cv., N36 cv. and Sarawak cv. release L-tyrosine. On the other hand, the enzyme units serve to quantify the amount of an enzyme. The volume of juice content doesn't affect the amount of fruit bromelain and stem bromelain. Hence, it can be presume that there is no relationship between the amounts of enzyme present in the cultivar with the volume of extracts obtained previously.

samples were prepared by cutting the pineapple flesh into pieces. The fruit part was taken out and ground into a fine powder to increase the surface area of samples when the extraction of RNA was performed. From the result, the purity of RNA can be assessed with a wavelength of 260/280 nm. Apparently all sample are nearly pure based on the accepted ratio of RNA purity which falls approximately to 1.8. An ideal 260/280 absorbance reading also strongly implies the RNA is free from any substances which could interfere with its stability (Fleige and Pfaffl 2006).

Table 4.5 The yield and purity of fruit bromelain, total RNA

Sample	Conc.	Wavelength					
		A230	A260	A280	A320	A260/ 280	A260/ 320
F1	108.8	3.91	2.47	1.08	-0.252	2.045	0.654
F2	117.6	3.19	2.67	1.16	-0.269	2.056	0.850
F3	127.6	1.27	2.83	1.22	-0.360	2.019	1.957
F4	145.6	1.76	3.39	1.59	-0.252	1.978	1.811

4.3.2 Total RNA detection using gel electrophoresis

Gel electrophoresis makes use of the electrical field to separate mixture of biomolecules such as DNA, RNA and proteins according to its molecular size. The migration of biomolecule takes place when electrical charges were applied, the samples will move from negative electrode towards positive electrode. The movement of the biomolecule across the gel was measured using 1kb DNA marker. In general, shorter bands migrate faster than the larger bands.

Two out from four samples of fruit total RNA were selected; F3 and F4. The selection was done as both of the sample gives out an optimal absorbance reading of approximately to 1.8 at wavelength of A260/280nm. Agarose gel analysis (Figure 4.3) was then carried out using sample F3 (Lane 1) and F4 (Lane 2) to visualize the RNA bands. The total RNA extract was identified through the

4.3.3 Complementary DNA (cDNA) synthesis of fruit bromelain

A reverse transcription of RNA was carried out to produce a complementary cDNA library. The cDNA is a double stranded DNA synthesized by the messenger RNA (mRNA) template. The fruit bromelain cDNA of *A.comosus* was successfully obtained from the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using a specific primer from the fruit bromelain gene, BAA21848. Up to now, no studies has described about the isolation and cloning of this fruit bromelain gene.

Later, the cDNA was amplified using PCR. To check whether the PCR generated the anticipated cDNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products. Amplification of the cDNA product using a routine PCR manage to obtain desired biomolecules size (Figure 4.4). The size of successfully amplified fruit bromelain is 1056kb. These PCR fragment was then purified to remove primer dimer and other contaminant that can interfere during the ligation of the target gene into the first cloning vector.

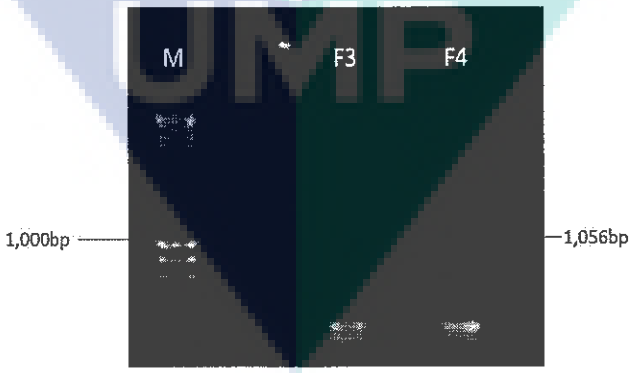

Figure 4.4 Determination of the cDNA amplified fragment of fruit bromelain; M: 1kb DNA Hyperladder (Bioline), Lane 2: F3 and Lane 3: F4

later on oxidized into a blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo (Karasova, Spiwok et al. 2002, Padmanabhan, Banerjee et al. 2011). The growth of the transformed colonies will appear to be white in color whereas, the untransformed ones appear blue. Six white transformed single colonies are selected and cultured from the blue-white screening plate as shown in Figure 4.5.

Figure 4.5 Blue-white screening of the fruit bromelain transformed colonies

4.3.6 Screening of the recombinant plasmid DNA using the colony PCR

The presence of the insert DNA in the vector constructs can be identified using the colony PCR method. Initially, the individual transformants were grown for 16 to 24 hours in LB medium and harvested. Plasmid from the cell were released by a short heating step with a small pick of colony lysed in sterile ddH_2O (Ramli 2012). Other than that, the colony also can be added directly to the PCR reaction and lysed during the initial denaturation step. The released plasmid can serve as template for the amplification reaction. The amplification goes specifically when specific primer was used. In all experimental designs, presence or absence of a PCR amplicon and size of the product are determined by electrophoresis alongside a DNA size marker on an agarose gel. From the agarose gel electrophoresis (Figure 4.6), all colonies (Appendix 3) selected integrates with the gene of interest except for colony F1. The upper band represents the pGEM vector with insert (4,107 bp) while the lower band is fruit bromelain gene (1,056 bp). The size of parent plasmid was 3,051 bp. The PCR product should involve parent plasmid (3,051 bp) and fruit bromelain gene (1,056 bp).

Figure 4.7 The NCBI BLASTX analysis from the purified plasmid of fruit bromelain from the order of nucleotide generated by sequencing

Therefore, a sequence comparison analysis was carried out to identify which sequence is entirely opposite to each other. From the analysis, the coded region translates 352aa of fruit bromelain which is located at 9 to 1038 in bromelain gene. Based on the multiple sequence alignment result the two sequences shared almost a similar protein encoded region. However, there is a minor alteration in the coding sequence (Figure 4.8).

4.4 Data mining of fruit bromelain and stem bromelain using bioinformatics tools

GenBank is a database system responsible to store and assign the submission of nucleotide and amino acid sequence with no cost over the internet prior to any publication. Accessible through the NCBI (www.ncbi.nlm.nih.gov/) server, it provides and encourage access within the scientific community to the most up to date and comprehensive DNA sequence information by data exchange with European Nucleotide Archive (ENA) and the DNA Data Bank of Japan (DDBJ) (Benson, Cavanaugh et al. 2017). Over the pass decades, the database manage to receive thousands of sequence data submission leading to an extensive growth in the database (Pina-Martins and Paulo 2016).

Bromelain is a proteolytic enzyme made up from endopeptidases, glycoproteins, and carbohydrates that catalyse peptide bond from the polypeptide chain by hydrolysis. It was reported that the read length of bromelain starts from 250 to 360 amino acids. The sequence retrieval of bromelain from the NCBI database found out there are 31 amino acid sequences of bromelain from *A. comosus (Table 4.6)*. Regardless of that, 8 out from 31 amino acid sequence has been discard away due to the read length sequence which is less than 200 or more than 360 of amino acid respectively. The amino acid sequence accession number are OAY85828, OAY80102, OAY83410, OAY80099, OAY76881, OAY67114, OAY85856 and OAY85857. Thus, only 23 amino sequences has been selected to be further evaluated with 4 of them is known as stem bromelain and the rest is considered as fruit bromelain. Yet, most of the bromelain amino acid sequences retrieved in the database remains unpublished in any publication. Table 4.6 shows the summary of the bromelain sequences available in the NCBI database.

There are 19 amino acid sequences of fruit bromelain was found, the sequences accession number are AEH26024, AGC54590, AGS78388, BAA21929, BAA21848, BAA22544, BAA22546, BAA22545, BAA22543, O23791, OAY85858, OAY85826, OAY71019, OAY68894, OAY68270, OAY68854, OAY68387, OAY65848 and OAY62650 respectively. Commonly, a

Table 4.6 Summary of bromelain sequence from Genbank NCBI

No.	Type of bromelain	Accession No.	Read length	% identity	Reference
1.	Fruit bromelain	OAY71019	326	95% OAY76881.1 (fruit bromelain)	NA
2.	Fruit bromelain	OAY68894	326	75% OAY68891.1 (fruit bromelain)	NA
3.	Fruit bromelain	OAY68854	359	100% OAY85857.1 (fruit bromelain)	NA
4.	Fruit bromelain	OAY85858	351	98% BAA21848.1 (bromelain)	NA
5.	Cysteine peptidase	AEH26024	352	97% OAY85857.1 (fruit bromelain)	(Wang, Zhang et al. 2014)
6.	Fruit bromelain	OAY68270	319	99% OAY85857.1 (fruit bromelain)	NA
7.	Bromelain	BAA21929	312	100% BAA21848.1 (bromelain)	NA
8.	Bromelain	BAA21848	352	100% OAY85826.1 (100% recovery) 100% ADY68475.1 (82% coverage) 100% BAA21929.1 (88% recovery) 98% OAY85858.1 (fruit bromelain)	NA
9.	Stem bromelain, partial	ADY68475	291	100% BAA21848.1 (bromelain)	(Tap, Majid et al. 2016)
10.	Stem bromelain	P14518	212	94% OAY80104.1 (Ananain)	(Sekhar et al. 2012)(Ritonja, Rowan et al. 1989)
11.	Bromelain	BAA21849	351	100% O23791.1 (fruit bromelain, precursor) 98% OAY85857.1 (fruit bromelain)	(Ritonja, Rowan et al. 1989, Muntari, Amid et al. 2012)
12.	Cysteine proteinase precursor	CAA08861	357	97% AGS78388.1 (bromelain)	(George, Bhasker et al. 2014)
13.	bromelain, partial [Ananas comosus]	AGC54590	241	99% BAA22546 (FB1035 precursor)	NA

				98% BAA22546 (FB1035 precursor) 98% OAY68270 (Fruit bromelain) 98% AGC54590 (bromelain)	
28.	FB1035 precursor, partial [Ananas comosus]	BAA22546	324	99% OAY80102 (Fruit bromelain)	NA
29.	FB22 precursor, partial [Ananas comosus]	BAA22545	340	99% OAY85858 (Fruit bromelain)	NA
30.	FB31 precursor [Ananas comosus]	BAA22543	352	100% BAA21848 (bromelain) 100% BAA21929 (bromelain) 100% ADY68475 (stem bromelain)	NA
31.	Fruit bromelain [Ananas comosus]	OAY85857	721	100% OAY68854 (Fruit bromelain)	NA

*NA: Not Available

4.4.1 Amino acids and domain analysis of fruit bromelain and stem bromelain

Protein is made up of amino acids (Wahl and Holzgrabe 2016). The side chain (R group) of each 20 distinct amino acid marks the chemical properties of each protein. Various structural and computational methods have been developed to determine the properties of protein. The method includes using bioinformatics tools to establish knowledge regarding the protein of interest. The physicochemical characteristics of pineapple endopeptidases are summarized in Table 4.8. Stem bromelain and fruit bromelain is a mixture of different sulfhydryl proteases (thiol endo-peptidases) and several other composition (Ramalingam, Srinath et al. 2012). From the table, the molecular weight of stem bromelain is large, 23.40–35.73 kDa compared to fruit bromelain, 25-31.00 kDa, Besides that, the isoelectric point of stem and fruit bromelain is at 9.55 and 4.6, respectively (Yamada, Takahashi et al. 1976). Commonly most of the enzyme at higher temperature of up to 60°C are destroyed or denatured (Martins, Rescolino et al. 2014). Unlike the other enzyme, fruit bromelain and stem bromelain can retain its proteolytic activity at this range. The optimal temperature for the proteolysis of bromelain varies from 35°C to 60 °C on certain condition. However, when exposed to temperatures normally applied in pasteurization at 72°C, it becomes inactive and its thermal denaturation of the enzyme is irreversible (Novaes, Jozala et al. 2016). Furthermore, bromelain activity remains active in both acidic and alkaline environment. Biochemical studies indicate that bromelain has broad spectrum enzyme activity over an optimum pH range of 5.0 to 8.0 (Manzoor, Nawaz et al. 2016). Fruit bromelain is an acidic protein whereas stem bromelain is more alkaline and larger than papain. In contrast to fruit bromelain, the higher carbohydrate moiety of stem bromelain contribute towards its functional stability at alkaline pH (Kaur, Kaur et al. 2015).

Both enzyme of fruit and stem bromelain favourably cleaves glycyl, alanyl, and leucyl peptide bonds (Maurer 2001). Fruit bromelain displays certain kinetic characteristics that distinguish it from stem bromelain. From the kinetic data obtained with synthetic protease substrates of Bz-Phe-Val-Arg-pNA and Z-Arg-Arg-pNA, both bromelain displays a distinct substrate-specificity profile. The presence of p-nitroaline (pNA), a fluorescence biomarker after proteolysis can be detected either fluorescently or colorimetrically. Fruit bromelain prefer Bz-Phe-Val-Arg-pNA than Z-Arg-Arg-pNA whereas stem bromelain is vice versa.

and acidic amino acids, aspartate and glutamate, where the percentage of former is higher in the stem protein while the latter is more in fruit bromelain. This difference is reflected in the isoelectric points (pI value) of the two proteins. Protein pI is calculated using pKa values of amino acids and solely depends on its side chain. The stem enzyme is basic and has a pI value at about 9.5 (Murachi 1964) while the fruit enzyme is acidic with the pI value at about 4.6 (Ota, Moore et al. 1964). From Table 4.9 and Table 4.10, the theoretical pI value of fruit bromelain is within 4.6 to 5.6. Whereas, the theoretical isoelectric point of basic protein, stem bromelain is 8.32 and 8.60 for CAA08861 and P14518 respectively. Nevertheless, ADY68475 and BAA21849 has an isoelectric point of 4.41 and 5 close to the pI value of fruit bromelain (Table 4.10).

The presence of glucosamine in the stem enzyme and its absence in the fruit enzyme is another difference between those two enzymes as reported by Murachi (1964). The finding suggest stem bromelain as a glycoprotein (protein containing one or more covalently linked carbohydrates of various types) due to the present of four hexosamines, and 2.1 % carbohydrate in stem bromelain. Similarly, Ota, Moore et al. (1964) detected six glucosamines with 1.5 % carbohydrate in a purified stem bromelain. In an attempt to confirm the presence of a glycoprotein, gas chromatograpy (Murachi, Suzuki et al. 1967, Yasuda, Takahashi et al. 1970) and automated borate chromatography (Scocca and Lee 1969) examinations were performed. The studies reported that the present of mannose, fucose, xylose, and glucosamine in the ratio of 3:1:1:4 in stem bromelain using gas chromatography analysis, whereas using the second approach of chromatography, the same carbohydrate composition was obtained with a different ratio of 2:1:1:2. Yamada, Takahashi et al. (1976) reported that fruit bromelain is a simple protein with an acidic isoelectric point, while stem bromelain is a basic glycoprotein. Similarly, papain is basic protein, but is a simple protein. This data is contradicted to the finding of Ota et al that fruit bromelain was contained firmly bound carbohydrates (3% neutral sugars). Yamada et al have established, however, with their highly purified preparation that fruit bromelain (FA2) is not a glycoprotein but a simple protein having a molecular weight of approximately 31,000. In this study, the analysis of glycosylation site has been performed using NetNGlyc 1.0 server (Blom, Sicheritz-Pontén et al. 2004). Glycosylation refers to post translation modifications of proteins. It plays role in multiple protein function such as protein folding, interaction, stability, and mobility, as well as in signal transduction (Roth, Yehezkel et al. 2012). From the analysis it was revealed that all fruit and stem bromelain contain 1-2 sites of glycosylation except for AGS78388 (fruit),

Table 4.9: Amino acid composition of fruit bromelain

Accession number	AEH 26024	AGC 54590	AGS7 8388	BAA 22543	BAA 22545	BAA 22546	OAY 68270	BAA 22544	BAA 21848	BAA 21929	O237 91	OAY 71019	OAY 68894	OAY 68854	OAY 62650	OAY 68387	OAY 65848	OAY 85858	OAY 85826
Ala (A)	7.4%	8.3%	7.9%	8.2%	7.6%	6.8%	6.6%	9.3%	8.2%	8.0%	7.7%	8.3%	8.6%	7.5%	8.5%	9.3%	9.6%	8.3%	8.2%
Asn (N)	7.7%	7.5%	6.5%	7.7%	7.6%	8.3%	8.5%	7.0%	7.7%	8.7%	7.4%	5.5%	5.5%	7.0%	7.4%	7.8%	5.6%	7.7%	7.7%
Asp (D)	4.0%	3.7%	4.5%	6.0%	6.2%	4.0%	3.8%	4.2%	6.0%	6.1%	4.3%	5.5%	5.2%	4.7%	4.3%	5.0%	4.6%	6.0%	6.0%
Cys (C)	2.3%	2.9%	2.5%	2.3%	2.4%	2.2%	2.2%	2.2%	2.3%	2.2%	2.3%	1.8%	2.5%	1.9%	2.3%	2.3%	2.3%	2.3%	2.3%
Gln (Q)	3.7%	3.7%	4.2%	3.7%	3.5%	3.7%	3.8%	3.1%	3.7%	3.8%	3.4%	3.1%	4.0%	4.2%	3.7%	4.3%	3.6%	3.7%	3.7%
Glu (E)	5.7%	4.6%	4.5%	4.5%	4.7%	6.2%	6.0%	5.6%	4.5%	4.2%	6.0%	6.7%	7.4%	4.2%	3.1%	3.1%	5.0%	4.6%	4.5%
Gly (G)	8.5%	10.8%	7.6%	8.8%	8.8%	9.0%	9.1%	7.6%	8.8%	9.9%	8.0%	8.9%	8.3%	8.6%	8.8%	7.0%	9.6%	8.5%	8.8%
Ile (I)	6.0%	6.6%	6.5%	6.2%	5.3%	6.5%	6.3%	6.7%	6.2%	7.1%	5.7%	6.1%	4.9%	6.1%	6.8%	5.8%	4.6%	5.4%	6.2%
Leu (L)	3.7%	2.9%	3.9%	3.7%	4.1%	3.1%	3.4%	3.1%	3.7%	2.9%	4.3%	3.1%	5.5%	4.7%	5.1%	5.4%	4.3%	4.0%	3.7%
Met (M)	3.4%	2.1%	3.1%	3.1%	2.9%	3.1%	3.1%	3.1%	3.1%	2.2%	3.4%	2.8%	2.5%	3.6%	3.4%	3.5%	1.7%	3.1%	3.1%
Phe (F)	4.5%	2.9%	4.2%	4.3%	4.7%	4.0%	4.1%	4.5%	4.3%	3.5%	4.6%	4.9%	4.3%	4.5%	5.1%	5.4%	5.6%	4.6%	4.3%
Pro (P)	3.7%	3.7%	3.7%	3.4%	3.5%	3.7%	3.1%	3.9%	3.4%	2.9%	3.7%	3.7%	3.7%	3.3%	4.0%	4.3%	5.6%	3.4%	3.4%
Ser (S)	10.5%	11.6%	11.5%	10.2%	10.3%	9.9%	10.3%	9.3%	10.2%	9.9%	10.3%	8.0%	6.4%	10.0%	9.7%	9.7%	6.6%	10.3%	10.2%
Thr (T)	4.5%	4.6%	3.9%	4.0%	4.4%	4.9%	5.0%	3.9%	4.0%	4.5%	4.6%	5.2%	7.1%	5.8%	5.4%	5.4%	4.0%	4.0%	4.0%
Trp (W)	2.3%	2.5%	2.3%	2.6%	2.6%	2.2%	2.2%	2.5%	2.6%	2.2%	2.3%	3.1%	2.5%	2.2%	2.0%	1.6%	2.0%	2.6%	2.6%
Tyr (Y)	6.0%	7.1%	4.8%	6.0%	6.2%	6.5%	6.6%	5.1%	6.0%	6.7%	6.0%	5.5%	6.1%	5.8%	5.1%	5.8%	5.6%	6.0%	6.0%
Val (V)	7.7%	8.7%	8.2%	7.7%	7.6%	7.4%	7.5%	7.9%	7.7%	7.7%	7.7%	5.8%	6.4%	6.7%	6.3%	6.2%	7.6%	8.3%	7.7%
Lys (K)	3.7%	2.9%	4.2%	3.7%	3.2%	4.0%	3.8%	5.9%	3.7%	3.5%	4.0%	4.6%	5.2%	3.9%	4.3%	4.3%	6.3%	3.4%	3.7%
Arg (R)	4.0%	2.5%	5.1%	3.4%	3.5%	4.0%	4.1%	4.5%	3.4%	3.2%	4.0%	4.6%	3.1%	3.9%	4.0%	3.5%	4.0%	3.4%	3.4%
His (H)	0.9%	0.4%	0.8%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	2.8%	0.9%	1.1%	0.6%	0.4%	1.7%	0.6%	0.6%
(Asp + Glu)	34 (10%)	20 (8%)	32 (9%)	37 (11%)	37 (11%)	33 (10%)	31 (10%)	35 (10%)	37 (11%)	32 (10%)	36 (10%)	40 (12%)	41 (13%)	32 (9%)	26 (7%)	21 (8%)	29 (10%)	37 (11%)	37 (11%)
(Arg + Lys)	27 (8%)	13 (5%)	33 (9%)	25 (7%)	23 (7%)	26 (8%)	25 (8%)	37 (10%)	25 (7%)	21 (7%)	28 (8%)	30 (9%)	27 (8%)	28 (8%)	29 (8%)	20 (8%)	31 (10%)	24 (7%)	25 (7%)
Theoretical isoelectric point	5.15	4.66	7.48	4.67	4.56	5.05	5.08	8.00	4.67	4.64	5.00	5.41	4.74	5.65	8.32	5.86	8.06	4.61	4.67
Glycosylation site	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes

Proteins are often multi-domain of which it carried out different biological functions attributable to each different domain (Rawlings 2010). The NCBI Conserved Domain analysis revealed there are two domains of fruit and stem bromelain which is cathepsin propeptide inhibitor (I29) and peptidase C1A, papain C-terminal. Figure 4.9 shows that all fruit bromelain contain I29 domain at the N-terminal region followed by peptidase C1A domain at the C-terminal of polypeptide. In general, the length of a protein sequence is determined by its function and the wide variance in the lengths of an organism's proteins reflects the diversity of specific functional roles for these proteins. Despite of that, it is difficult to predict a short protein sequence on purely statistical grounds and is also less likely to have confirmatory homologies in other organisms. Stem bromelain also display the similar domain organization to fruit bromelain except for AGC 54590 and P14518 which lacking of I29 domain. Mostly, the I29 domain is located between amino acids number 1 to 100 of the N-terminal site.

(Berg, Tymoczko et al. 2002, Rawlings 2010).. According to Beers, Jones et al. (2004) such mechanism helps to control various activity that take place in protein such as its half-lives, subcellular trafficking and others. Despite of that, very little information was made on the origin and evolution of these protein families in plants (Martinez and Diaz 2008).

Proteases are synthesized as inactive or less active precursor molecules in order to prevent such inappropriate proteolysis. They are activated by limited intra- or intermolecular proteolysis cleaving off an inhibitory peptide. Precursor are usually being processed in the secretory pathway that consequently be secreted out into the cytoplasmic matrix or become lysosomal or vacuolar in animal and plant systems, respectively. This propeptide mostly similar to the papain propeptide that are predicted to bind substrate in the reverse orientation thus inactivate the active site similar to the proenzymes (Rawlings and Barrett 2004). The characteristic element of the propeptide which are highly conserved in evolution is the GXNXFXD, seven amino acid linked by peptide bond or heptapeptide which is located at the kink of the β–sheet. This motif element can be found in most of the cysteine propeptides (Wiederanders 2003). Examination of the I29 motif using an amino acid sequence alignment of all fruit and stem bromelain showed that the GXNXFXD motif residues are conserved between all of the examined bromelain except OAY65848 as shown in Figure 4.10. From the OAY65848 sequence alignment, the amino acid G was replaced by A. This mutation may take place due to a change in one of the DNA base pair that results in the substitution of one amino acid for another in the protein made by a gene. For instance, mutations to hydrophilic residue may disrupt the bond and thus decrease the probability of the protein reaching its active conformation.

thoroughly conserved in the sequence alignment. Some of the bromelain from fruit (OAY68894 and OAY71019) were found to have gap-containing segment that lack about 12 residues at the location where Gln reside. On the other hand, the residue Asn was conserved for all aligned bromelain sequences except for the fruit bromelain of OAY68854 that replaced the location with Tyr.

et al. 2016). In addition to nucleophiles a general base (histidine) is also required in order for catalysis to occur (Figure 4.12). This catalytic mechanism of cysteine peptidases is similar to the group of serine-type peptidases (Vernet, Tessier et al. 1995). Moreover, it has been identified that mostly histidine residues become the common proton donor for cysteine peptidases. In some peptidases family, only the dyad of cysteine and histidine seem to be essential for catalysis. However, some of other family exhibited the requirement of third residue to orientate the imidazolium ring of the histidine (Barrett, Woessner et al. 2012).

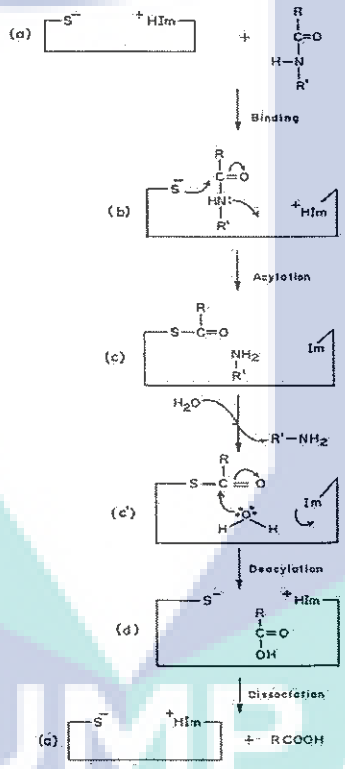

Figure 4.12 The catalytic mechanism of cysteine protease. Im and +HIm refer to the imidazole and protonated imidazole, respectively.

4.5 Structural comparison of fruit bromelain and stem bromelain

Procaricain (PDB ID: 1PCI) is an inactive form of peptidase found in caricain from the latex of *Carica papaya* (Groves, Taylor et al. 1996). An alignment search made using BLASTP (NCBI) against the PDB database, revealed that BAA21848 and CAA08861 sequence exhibits low similarity with all solved 3D structures. The highest match for BAA21848 showing only 43% identity and 64% similarity to chain A of the 1PCI. In the meantime, the highest match percentage of CAA08861 was 43% identity and 65% similarity based on the solved 3D structure of chain A 1PCI respectively. A suitable template is needed to generate an optimum target-template alignment. In order to do that, a template searches were performed using several servers such as HHPRED, Phyre2, BLAST-PDB, PSI-BLAST and Mod-link+ (Table 4.11). The analysis revealed various potential templates for molecular modelling purposes. Among them was1PCI, which had the highest sequence identity (41%) and query coverage (90%) with fruit bromelain (BAA21848), using the HHPRED server. Similar finding was found for stem bromelain (CAA08861) in which 1PCI showed the highest sequence identity (42%) and query coverage (89%). 1PCI was then selected as a template to build the 3D structure of fruit bromelain and stem bromelain. To further assess the reliability of the structural sequence alignment between BAA21848 and CAA08861 to 1PCI, consensus secondary structure prediction was used to confirm the alignment. This was achieved by comparing the aligned secondary structure of 1PCI and the consensus predicted secondary structure of BAA21848 and CAA08861. The structural alignment indicates a good alignment of 10 α-helices and 6 β-strands between BAA21848 and CAA08861 with the template structure of 1PCI. The rest of the protein regions are shown to consist of random coils and a few structural mismatches and gap-containing segments.

Table 4.11 Predicted templates for fruit and stem bromelain obtained from different servers along with their percentages of identity and query coverage. 1PCI appeared in the all of the results given and was chosen as the model template for BAA21848 and CAA08861.

Server	Template	Protein characteristics	Fold	Identity (%)	Query coverage (%)
Fruit bromelain (BAA21848)					
HHPRED	2COY	Procathepsin S	hydrolase, cysteine protease	37	90
	1PCI	Procaricain	hydrolase, cysteine	41	90

PSI-BLAST	3TNX	Papain	Hydrolase	43	85
	4QRG	Crystal Structure of I86l Mutant of Papain	Hydrolase	43	85
	1PCI	Procaricain	Hydrolase	43	86

Comparative protein modelling method was designed to find the most probable structure for a sequence given its alignment with related structures. The best alignment results from HHPRED program were used to build models with MODELLER program, which is an automated program of comparative modelling. This program basically performs by satisfying all spatial restraints derived from the structure-sequence alignment. Models that produced high violations of the restraints lead to higher objective functions (calculated by CHARM-22 force field) and will be considered as poor models. As a consequence, only the model with the lowest objective function will then be selected. The resulting model was subjected to energy minimization using the steepest descent algorithm as implemented in GROMOS from Deepview to avoid poor molecular contacts.

Preliminary model of fruit bromelain generated by the MODELLER has a high energy level of -1610.640 kcal/mol so do stem bromelain which has an energy level of -4110.637 kcal/mol respectively. The fruit bromelain and stem bromelain model was then subjected to side-chain and loop refinement which decreased the energy level to -13409.075 kcal/mol and -15172.673 kcal/mol, respectively. Normally this energy level is considered too high and due to this reason, a series of energy minimization steps are required for the model to reach a local or more preferential global minimum level of total energy (Crivelli, Eskow et al. 2002). In silico, protein structures are more stable in low energy level (Alberts, Johnson et al. 2002). After three rounds of minimization processes, the total energy of fruit bromelain and stem bromelain model was reduced to -16101.752 kcal/mol and -17900.148 kcal/mol, respectively.

For model validation and assessment, the quality of the refined model was assessed using the programs of ERRAT, RAMPAGE and VERIFY3D (Table 4.12). Generally, VERIFY3D works based on the compatibility of an atomic model (3D) with its own amino acid sequence (1D) (Jitendra and Vinay 2011). On the other hand, the overall quality factor for non-bonded

conformation, the residue interaction and the residue contacts of the structure were all well within the limits established for reliable structures. The resulting model after the third minimization step was used to elucidate the structure-function relationship of fruit bromelain and stem bromelain.

Table 4.12 Energy levels, ERRAT plot, VERIFY3D and RAMPAGE score for fruit bromelain and stem bromelain models at different stages.

Step	Structure energy level (kcal/mol)	Structure validation by ERRAT plot	Structure validation by VERIFY3D (%)	Structure validation by RAMPAGE (%)
Fruit bromelain (BAA21848)				
Model before energy minimization	-1610.640	69.578	82.10	Favoured: 91.7 Allowed: 6.0 Outlier: 2.3
Model after side-chain and loop refinement	-13409.075	76.923	81.25	Favoured: 91.4 Allowed: 5.1 Outlier: 3.4
Model after second minimization	-15287.221	80.062	80.40	Favoured: 91.4 Allowed: 5.7 Outlier: 2.9
Model after third minimization	-16101.752	81.818	81.25	Favoured: 91.4 Allowed: 6.0 Outlier: 2.6
Stem bromelain (CAA08861)				
Model before energy minimization	-4110.637	76.724	78.99	Favoured: 92.1 Allowed: 5.6 Outlier: 2.3
Model after side-chain and loop refinement	-15172.673	80.814	79.27	Favoured: 91.0 Allowed: 6.5 Outlier: 2.5
Model after second minimization	-17086.387	81.287	77.87	Favoured: 90.7 Allowed: 6.8 Outlier: 2.5
Model after third minimization	-17900.148	81.287	77.31	Favoured: 90.7 Allowed: 7.0 Outlier: 2.3

4.5.2 Structural analysis of fruit bromelain and stem bromelain

amino acid residues are in close proximity due to the folding structure as shown in Figure 4.14. The best characterised family of cysteine proteases, papain, is also having cysteine and histidine residues, Cys-25 and His-159, as the catalytic residues which are evolutionarily preserved in all cysteine proteases family. At first, Asp-158 was thought to play a role analogous to the role of aspartate in the serine protease catalytic triad, but that has since then been disproved (Menard, Khouri et al. 1990). However another one residue, Asn-175 was reported to aid in orientation of His-159 to allow the deprotonation of Cys-25 to occur. It is though these three amino acids working together in the active site that provides papain with its unique functions (Mamboya 2012).

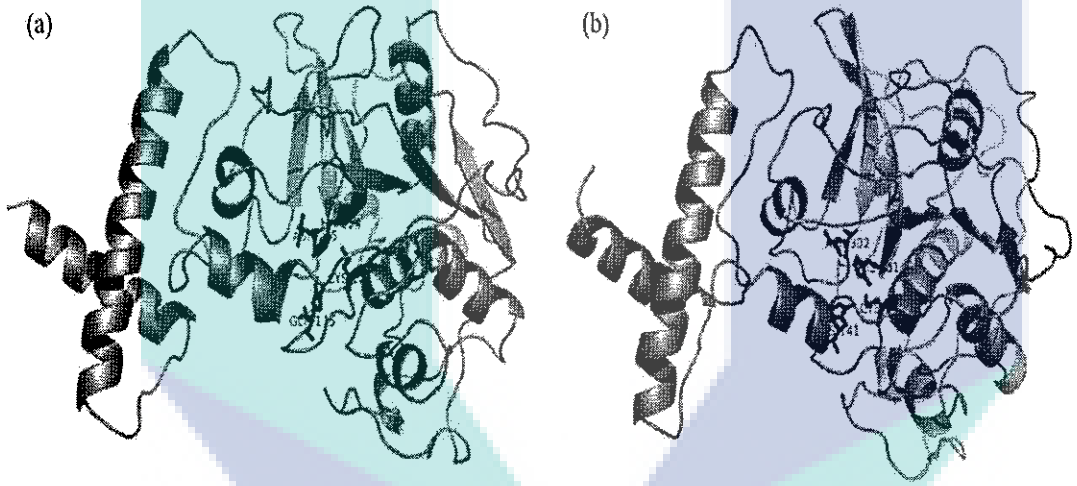

Figure 4.14 Substrate-binding site (a) fruit bromelain, BAA21848 (b) stem bromelain, CAA08861 using 1PCI as a template. Green region indicates domain I29 while orange region indicates domain peptidase C1. The catalytic amino acids of both models are represented as sticks.

Cysteine protease found in the latex of Carica papaya is known as caricain. Caricain is naturally expressed as an inactive zymogen called procaricain, 1PCI (PDB database). The 1PCI template consist of three non-crystallographically (Chain A, B and C) related molecules. This molecule forms a separate globular domain which binds to the C-terminal domain of a mature caricain. From the analysis, BAA21848 and CAA08861 is located in chain A of the 1PCI structure.

4.5.2.1 Structural characteristics comparison of BAA21848 and CAA08861

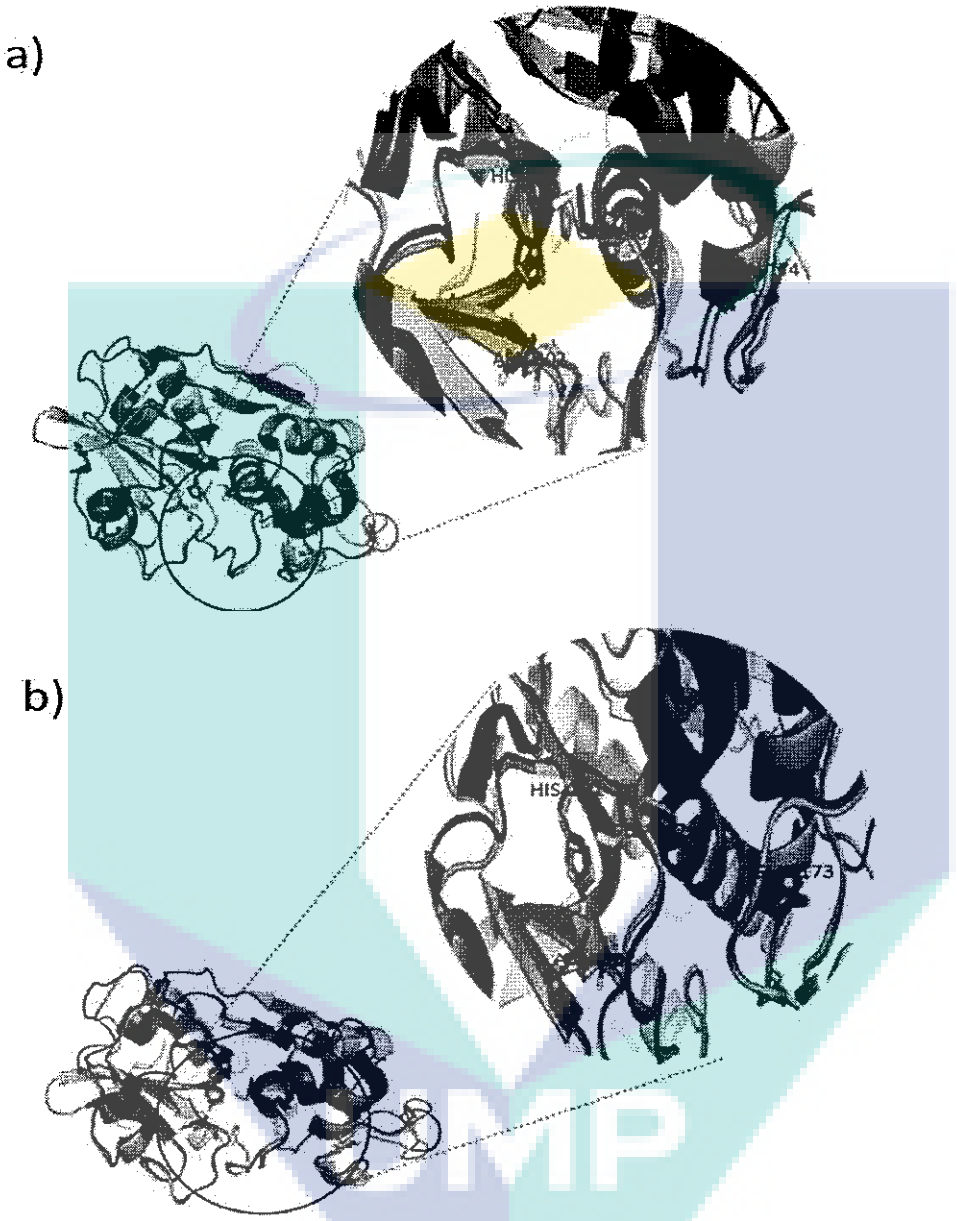

Figure 4.15 The 3D superimposition structure of BAA21848 and CAA08861 with 1CVZ. (a) The catalytic binding site (Cys-148, Gln-174, His-281 and Asn-302) of BAA21848 (green) with 1CVZ (purple). (b) The catalytic binding site (Cys-147, Gln-173, His-281 and Asn-302) of CAA08861 (green) with 1CVZ (purple).

The physiochemical properties of a single protein is determined by its side chain groups either it's hydrophobic (unfavourable with water), hydrophilic (favourable with water) or

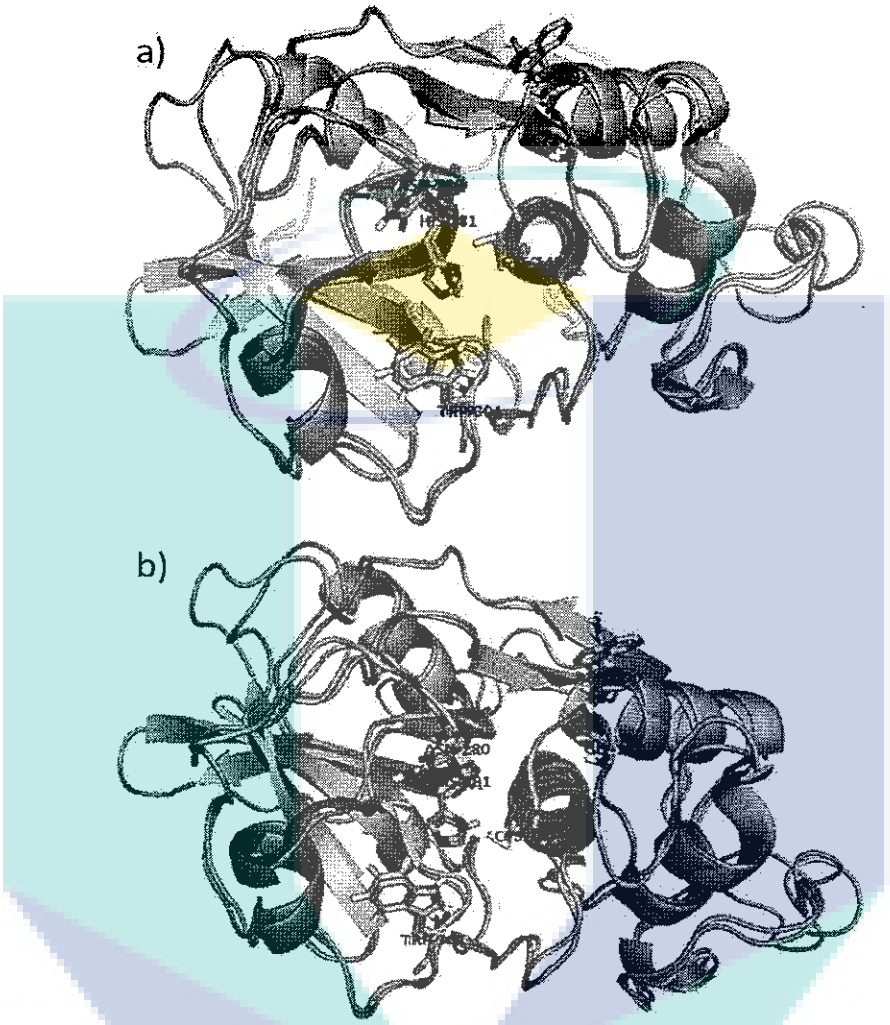

Figure 4.16 The 3D superimposition structure of BAA21848 and CAA08861 with the catalytic site of 1CVZ. (a) The catalytic binding site (Cys-25, Gly-66, Trp-69, Asp-158, His-159 and Trp-177) of 1CVZ (blue) after being superimposed with the catalytic site (Cys-148, Gly-188, Asp-191, His-281, Asn-280 and Trp-304) of BAA21848 (green) (b) The catalytic binding site (Cys-25, Gly-66, Trp-69, Asp-158, His-159 and Trp-177) of 1CVZ (blue) after being superimposed with the catalytic site (Cys-147, Gly-187, His-281, Asn-190, Asn-280 and Trp-304) of CAA08861 (green).

Generally, native proteins contain a hydrophobic core and a charged and/or polar group on the surface. The hydrophobic core aids in stabilizing the tertiary structure of the protein by providing hydrophobic interaction while the outer polar surfaces preferentially interact with the

is possible that the sequence differences that alter the overall hydrophobicity of those residues could affect the specificity and inhibitor-binding properties of stem bromelain, particularly compared with fruit bromelain.

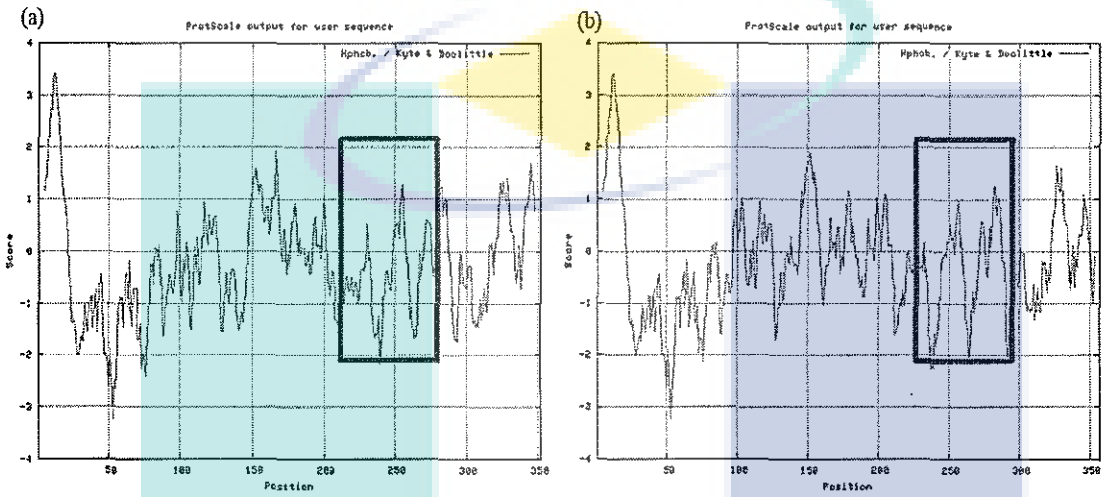

Figure 4.18 ProtScale output for hydropathy analysis of (a) Fruit bromelain (b) Stem bromelain.

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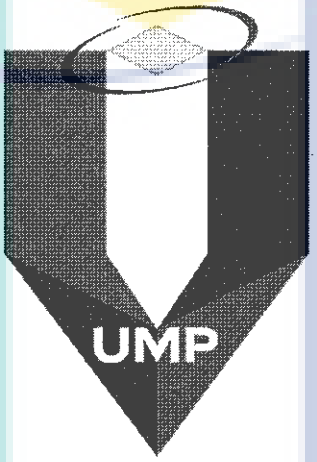
FINAL REPORT

INTERNAL GRANT UMP

RDU1403169



Molecular Cloning and Overexpression of Stem Bromelain from *Ananas Comosus* in *Kluyveromyces lactis*

By

Dr. Aizi Nor Mazila Ramli

ACKNOWLEDGEMENT

I would like to thanks the following people and organisations;

- The students working on this project:
  - Master student:
    - Tuan Norsyalieza Tuan Aznan – Waiting for Viva session (2017/2018)
  - Undergraduate student:
    - Siti Hajar Mohamad Nor – graduated November 2017
    - Nurul Hartini Ani – graduated November 2017
- The co-researcher
  - Dr Noormazlinah Ahmad
  - Dr Hazrulrizawati Abd Hamid

- Lempaga Perindustrian Nanas Malaysia cawangan Negeri Pahang for their assistance and full support
- Universiti Malaysia Pahang for the funding.
- Jabatan Penyelidikan & Inovasi UMP for their help and support.

REPORT SUMMARY

Nowadays, numerous therapeutic benefits have been claimed for bromelain. Bromelain, belonging to papain family, is a glycosylated single chain protein. Until recently, the three-dimensional (3D) structure of bromelain remained to be elucidated. The comprehensive information about the thorough structural organization of bromelain is vital for therapeutical application and in understanding their role in the cell and in other related molecular mechanisms. In addition, up to now, the available commercial bromelain in the market were partially produced by pineapple stem and fruit. Nevertheless, none of them are produced and formulated from recombinant forms other than *E. coli*. However, due to safety concerns of the potential bacteria endotoxin contamination, an alternative system needs to be applied. Therefore the purpose of this research work is to screen for bromelain from different variants of A. comosus followed by bromelain sequence analysis and accurate 3D structure determination using bioinformatics tools. Finally, recombinant bromelain construction in cloning and expression systems is performed. This research study will provide the information of the bromelain sequence importance for therapeutical application followed by the accurate predicted structure of bromelain from *A. comosus* that can be used to define the molecular catalysis mechanism of bromelain which is very important for protein engineering purpose in future. In addition, recombinant bromelain construction in GRAS system of *K. lactis* produced which will ensure simplicity, high quality of the enzyme, reduce contamination and also cost effective.

So far, we had one peer-reviewed article published in Journal of the science of food and agriculture, won 1 award and have one paper presented in International conference. Two undergraduate students and one MSc student were also graduated from work related to this project. In conclusion, this project has achieved its target for human capital development.

POSTGRADUATE AND UNDERGRADUATE STUDENTS

The following student works for this project

Master student

- Tuan Norsyalieza Tuan Aznan (MKT15004) – waiting for Viva session (expected 2017/2018)

Undergraduate student

- Siti Hajar Mohamad Nor (SB13014) -- graduated November 2017
- Nurul Hartini Ani (SB13019) – graduated November 2017

JOURNALS

This project produced ONE (1) peer-reviewed research article:

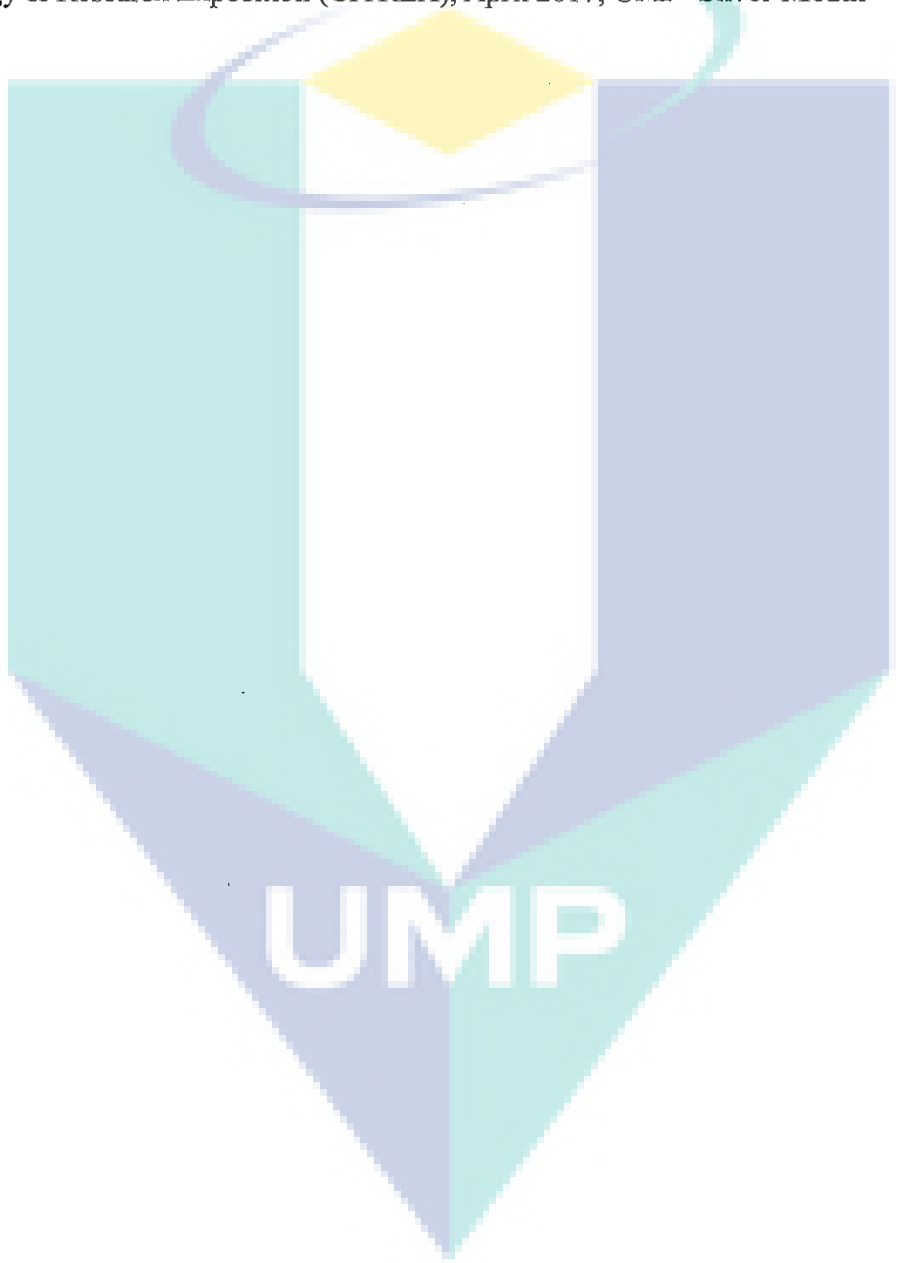
Aizi Nor Mazila Ramli, Tuan Norsyalieza, Tuan Aznan, Rosli Md. Illias, (2017). Bromelain: from production to commercialization. Journal of the Science of Food and Agriculture. 97(5): 1386–1395.

PRESENTATION IN CONFERENCE

Screening of bromelain from different variants of *Ananas comosus*, 2nd International Symposium on Phytochemicals in Medicine and Food, 7-10 April 2017, Organized by Institute of Chinese Medical Sciences State Key Lab of Quality Res in Chinese Med University of Macau. Fuzhou, China (Awarded as best oral presenter).

AWARD

Recombinant bromelain: potential source of pure bromelain product. Creation, Innovation, Technology & Research Exposition (CITREX), April 2017, UMP- **Silver Medal**



FINANCIAL REPORT

The total amount of budget approved for this project is RM 74500.00. At the end of this project RM 73977.12 was spent, representing 99.3% of funding utilisation as shown in Table 1.

Table 1: Budget and spending of the project

Project Leader	Approved	Spending	% spending
Dr Aizi Nor Mazila Ramli	**29,500.00**	**27,141.73**	**92.01**

ABSTRACT

There are many cultivar of pineapple, *A. comosus* available around the world. Morris, N36 and Sarawak cultivar use throughout this work was amongst cultivars grown in Malaysia. Proteolytic enzyme found in pineapple plant, is known as bromelain. The enzyme catalyzes the breakdown of peptide bond from the polypeptide chain by hydrolysis. Nowadays, numerous therapeutic benefits have been claimed for bromelain. Bromelain, belonging to papain family, is a glycosylated single chain protein consists of cysteine proteinases. A gene encoding bromelain from pineapple (*Ananas comosus*) was isolated from Morris cv. using the Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) techniques. The isolated gene was successfully expressed in the *E. coli*. Analysis of the nucleotide sequence revealed the presence of bromelain coding sequence, 1,056 base pair (bp) which encodes 302 amino acid. Up to now, the three-dimensional (3D) structure of bromelain remained to be elucidated. The comprehensive information about the thorough structural organization of bromelain is vital for therapeutical application and in understanding their role in the cell and in other related molecular mechanisms. Therefore the purpose of this research work is to screen for bromelain from different cultivars of *A. comosus* followed by bromelain sequence analysis and accurate 3D structure determination using bioinformatics tools. This research study will provide the information of the bromelain concentration in different cultivars of *A. comosus*. In addition, details of bromelain sequence importance for therapeutical application followed by the accurate predicted structure of bromelain from *A. comosus* that can be used to define the molecular catalysis mechanism of bromelain will be provided.

ABSTRAK

Banyak kultivar nanas, *A. comosus* yang terdapat di seluruh dunia. Penggunaan Morris, N36 dan kultivar Sarawak sepanjang kerja ini adalah antara kultivar yang ditanam di Malaysia. Enzim proteolitik yang terdapat dalam buah nanas, dikenali sebagai bromelain. Enzim ini memangkinkan pecahan ikatan peptida dari rantaian polipeptida oleh hidrolisis. Kini, banyak manfaat terapeutik telah dituntut untuk bromelain. Bromelain, yang berasal dari keluarga papain, adalah protein rantai tunggal glikosilasi yang terdiri daripada proteinase sistaina. Gen yang menghasilkan bromelain dari nanas (*Ananas comosus*) telah diasingkan dari Morris cv. menggunakan teknik Reverse Transcriptase (RT) - Polymerase Chain Reaction (PCR). Gen bromelain berjaya diasingkan dalam *E. coli*. Analisis urutan nukleotida menunjukkan kehadiran urutan gen bromelain, 1,056 pasangan tapak (bp) yang mengekod 302 asid amino. Sampai sekarang, struktur tiga dimensi (3D) bromelain masih belum dapat dijelaskan. Maklumat yang komprehensif tentang organisasi struktur bromelain yang menyeluruh adalah penting untuk aplikasi terapeutik dan memahami peranan mereka dalam sel dan dalam mekanisme molekul lain yang berkaitan. Oleh itu tujuan kerja penyelidikan ini adalah untuk menyaring bromelain dari pelbagai kultivar *A. comosus* diikuti dengan analisis urutan gen bromelain dan penentuan struktur 3D tepat menggunakan alat bioinformatik. Kajian penyelidikan ini juga akan memberikan maklumat kepekatan bromelain dalam kultivar *A. comosus* yang berbeza. Di samping itu, informasi lebih jelas mengenai bromelain akan disediakan bagi aplikasi terapeutik diikuti dengan struktur ramalan bromelain yang tepat dari *A. comosus* yang boleh digunakan untuk menentukan mekanisme pemangkinan molekul bromelain.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The necessary elements of the study were discussed in this chapter. The elements were the background of study, problem statement, objective and the research scope respectively.

1.2 Background of Study

Pineapple (*A. comosus*), the family member of Bromeliaceae is the third most produced plant in the world. The plant is grown in tropical and subtropical regions including Brazil, China, Indonesia, Malaysia, Thailand, the Philippines, Kenya and India. In Malaysia, pineapple is known to be one of the major domestic crops. According to the Malaysian Pineapple Industrial Board (MPIB) in 2006, small sector registered with the LPNM (Lembaga Perindustrian Nanas Malaysia) accounts the use of 8,731 hectares of lands for pineapple plantation.

There are nine pineapple cultivars including hybrid grown in Malaysia known as Gandul, Josapine (hybrid), Maspine (hybrid), MD2 (hybrid), Morris, Morris Gajah, N36 (hybrid), Sarawak and Yankee. These cultivars offer variation in characteristics such as the fruit size, color and taste. However, some of the cultivar receives a

selective interest in the market to fulfil the uniformity and consistency in taste, size and ripeness. According to the previous research of (Yuris and Siow 2014), the antioxidant activity of Morris cv., is higher compared to N36 cv. and Sarawak cv. In addition, these cultivar is only commercialized in Malaysia.

Bromelain is one of the proteolytic enzyme found in the pineapple plant, *A. comosus* (Bhattacharyya 2008). There are 351 amino acids residues estimated to be present in the bromelain of which seven cysteines were reported to be responsible in catalysis of the enzyme. The enzyme is used in therapeutic and industrial applications (Muntari, Ismail et al. 2012). Its therapeutic properties were first introduced in 1957 for various medical treatments. The properties of bromelain has shown to be effective in the platelet inhibition aggregation treatment, fibrinolysis, inflammation healers, regulation of body immunity, potentiation of other drugs, digestive assistance, wound healing and assisting with the improvement of cardiovascular circulatory (Kelly 1996). Apart from that, bromelain is also being used in the baking industries and to tenderize the meat (Arshad, Amid et al. 2014).

In general, the name of stem bromelain and fruit bromelain was used to accordingly distinguish the origin of the enzyme in the plant (Kelly 1996). Direct consumption of fruit bromelain provides various source of vitamins and minerals. Unlike stem bromelain, less studies has been reported on fruit bromelain. In the present work, stem bromelain are the most studied enzyme as an alternative source of bromelain compared to fruit bromelain which usually being eaten fresh. Therefore, the purpose of this research is to reveal an in-depth information regarding fruit bromelain.

1.3 Problem Statement

Pineapple helps to sustain some essential nutrient for wellbeing. Deficiency of these nutrient consumption can lead to several incorporated fatal diseases. There are several report being made on the evolutionary relationship of pineapple cultivar morphologically. However, less research study were reported to acknowledge the content of crude fruit bromelain in Morris cv., N36 cv. and Sarawak cv. available in Malaysia.

Unlike the stem bromelain gene, none of fruit bromelain gene was found to be cloned and characterized. Thus, it is a crucial to analyze and determine the characteristics of the fruit bromelain and its potential therapeutical properties. Furthermore, until recently, the 3D structure of fruit bromelain remained to be identified. Knowledge and information about the detailed structural organization of proteins is crucial in understanding their role in the cell and in other related molecular mechanisms. This task can be facilitated by determining the accurate three-dimensional (3D) structure of the studied protein and comparison with stem bromelain structure.

1.4 Research Objectives

The aims of this research are:

1. To analyse the enzymatic activity of fruit bromelain from the local pineapple (*A. comosus*) cultivars available in Malaysia.
2. To isolate and clone the fruit bromelain from pineapple flesh of a selected cultivar with the highest proteolytic activity for sequence analysis.
3. To compare the structural of fruit bromelain (primary, secondary and tertiary structure) with stem bromelain.

1.5 Scope of Study

This research was initiated with the screening of fruit bromelain from different cultivars of *A. comosus* (Morris cv, N36 cv. and Sarawak cv.) by enzyme assay and protein analysis. Next, the gene of fruit bromelain (BAA21848) was isolated using total RNA method. Cultivar with the highest enzymatic activity was selected and used as a template in the gene cloning approach. DNA sequencing was then performed. The gene sequence of fruit BAA21848 was eventually used for the structural analysis and comparison with stem bromelain.

CHAPTER 2

LITERATURE REVIEW

2.1 Bromeliaceae

A significant evolution shifts deliver a diverse features to plant diversity (Soltis and Soltis 2004). Such evolution also includes Bromeliaceae, the family member of pineapple (*A. comosus*) (Matallana, Godinho et al. 2010). The family are well known to be one of the epiphytic plants in the plant kingdom (SOUSA and COLPO 2017). Bromelioideae, Pitcairnioideae and Tillandsioideae are the subfamily of Bromeliaceae (Barfuss, Samuel et al. 2005). Amongst the fellow subfamily, Bromelioideae remains to be the most poorly understand due to the less information regarding its relationship within the subfamily (Evans, Jabaily et al. 2015). According to Carlier, d'Eeckenbrugge et al. (2007) the Ananas genus belongs to the Bromelioideae subfamily, the genus species includes the *Ananas comosus* with 6 variant. One of the variant is *Ananas comosus var. comosus* synonym to *Ananas comosus (L.) Merr.* which accordingly grouped to cultivar.

2.2 Pineapple (*A. comosus*)

Back to the early age, Christopher Columbus discovered pineapple (*A. comosus*) on his journey across the world. At that moment, the plant was domesticated by the native community of South America (Ming, Wai et al. 2016). The plant was later on being introduced to the European until it reaches worldwide nowadays (Hajar, Zainal et al. 2012). The pineapple was mainly cultivated vegetatively using the crown section (Fitchet and van de Venter 1988).

2.2.1 *A. comosus* Cultivar

According to Bhattacharya (2016), desirable characteristics of a plant can be identified as a cultivar. Many pineapple cultivars are grown with distinguishable characteristics such as size, flesh color and taste to meet the acceptable standards (Shoda, Urasaki et al. 2012). Gandul cv., Josapine cv. (hybrid), Maspine cv. (hybrid), MD2 cv. (hybrid), Morris cv., Morris Gajah cv., N36 cv. (hybrid), Sarawak cv. and Yankee cv. found in Malaysia are amongst cultivars that possess variation in its characteristic. Morris cv. (Queen) and Sarawak cv. (Smooth Cayenne) is the most notable cultivar used for commercialization around the world so do in Malaysia (Bartholomew, Paull et al. 2002, Lembaga Perindustrian Nanas Malaysia 2017). Whereas, the N36 pineapple is a hybrid selected from a cross between 'Gandul' (Spanish) and the Sarawak (Smooth Cayenne) cultivar.

2.3 Proteases

Proteases hydrolyze peptide bonds. They can act near the ends of the polypeptide chains (exopeptidases) or within them (endopeptidases). Both type of proteases cleave peptide bonds and remove the amino acids from either N (aminopeptidases) or C-terminus (carboxypeptidases). There are four major classes of proteases which is serine, cysteine, aspartic and metallo-proteases (González-Rábade, Badillo-Corona et al. 2011).

The numerous industrial and therapeutic applications of enzymes necessitated their production. Proteases are enzymes considered to be the most significant of all industrial enzymes with annual sale of about US$3 billion (Leary *et al.*, 2009). In fact, they represent about 60% of all commercial enzymes worldwide. They are widely used in food, pharmaceutical and detergent industries (Feijoo-Siota and Villa 2011). Plant proteases have been gaining unique attention in the field of biotechnology and medicine due to their exploitable properties. The most recognized plant proteases with greater commercial values are papain from *Carica papaya*, ficin from *Ficus spp.* and bromelain from pineapple plant (*Ananas comosus*) (Dubey, Pande et al. 2007).

2.4 Bromelain

Bromelain, a collective name for stem bromelain or fruit bromelain in pineapple plant, *A. comosus*, is a proteinase enzyme that hydrolyze protein into smaller polypeptides or amino acids (Bhattacharyya 2008). In addition, the sulfhydryl, peroxidase, acid phosphatase, several protease inhibitors and organically-bound calcium can also be found in bromelain (Gautam, Mishra et al. 2010). According to Muntari, Ismail et al. (2012) the stem and fruit part of pineapple stores bromelain. It was reported that despite of the level of amino acid composition differences, both stem and fruit bromelain retain similar catalytic action constantly (Mohapatra, Rao et al. 2013).

In order to produce a highly purified bromelain, several processes need to be carried out. The process covers the extraction, purification, drying and packing of bromelain. Above all, purification process was the most important process as it will affects the purity of the end product and the overall processing cost (Arshad, Amid et al. 2014). The extraction of bromelain from pineapple takes place when the desired part of purification was grinded without water using a domestic juicer. The juice extracted was the crude extract of the enzymes. Generally, bromelain is prepared from cooled pineapple juice by centrifugation, ultrafiltration, and lyophilisation (de Lencastre Novaes, Jozala et al. 2016). Consequently, the process yields a yellowish powder.

Bromelain, known since 1875 is mainly used as a phytomedical compound. Besides, bromelain also offers an abundant use for industrial and other applications (Ramli and Aznan 2017). Commercial bromelain is not cheap, the price can cost up to 2400 USD/kg (Muntari, Ismail et al. 2012). The process of bromelain production was very expensive thus making the bromelain value increased in the market.

2.4.1 Fruit bromelain

Fruit bromelain (EC 3.4.22.33) is a glucoprotein proteinase present in pineapple juice. Although fruit bromelain share the same component of element in stem bromelain, the amount of amino acids are different (Pavan, Jain et al. 2012). Apart from that, fruit bromelain apparently has a similar mechanism of action. In contrast to stem bromelain, fruit bromelain hydrolyzes proteins with a broad substrate specificity of the internal polypeptide bonds of proteins (Kaur, Kaur et al. 2015). These enzymes performs important role in proteolytic modulation at cellular matrix, in numerous physiologic process, including tissue morphogenesis, tissue repair, angiogenesis and tissue modulation, decreasing bruises, swelling, pain and healing time (Mohapatra, Rao et al. 2013). Casein, haemoglobin, gelatin and some synthetic substrates can be used as a substrate to measure the enzymatic activity of fruit bromelain. However, most the optimal condition of pH and temperature for each substrate are yet to be identified (Corzo, Waliszewski et al. 2012).

2.4.2 Stem bromelain

Stem bromelain (EC 3.4.22.32) is a glycosylated single-chain protein of cysteine proteinase (Ritonja, Rowan et al. 1989, Polaina and MacCabe 2007). This proteolytic enzyme cleaves peptides bonds within the protein molecule (Tap, Majid et al. 2016). According to Manzoor, Nawaz et al. (2016) the concentration of bromelain is high in the pineapple stem. However, the biochemical properties and composition of stem bromelain is different from fruit bromelain. The difference happen to be a frequent proteolytic activity reduction and a low specificity for peptide bonds (de Lencastre Novaes, Jozala et al. 2016). Being the most studied cysteine proteinase, stem bromelain helps to reduce substantial agriculture waste in the future by replacing the remaining source of bromelain (Pavan, Jain et al. 2012).

2.5 Application of Bromelain

Tones of industrial and therapeutic application using this enzyme were made nowadays including pharmaceutical, food industries and others. It was firstly introduced as a therapeutic compound on 1957. Inhibition of platelet aggregation, fibrinolytic activity, anti-inflammatory action, anti-tumor action, modulation of cytokines and immunity, skin debridement properties, enhanced absorption of other drugs, mucolytic properties, digestive assistance, enhanced wound healing, and cardiovascular circulatory improvement was the action of bromelain.

Bromelain was categorized as a food additive and generally accepted as safe by The Food and Drug Administration, USA (Muntari, Ismail et al. 2012). Food industries also using this enzyme for baking, meat tenderization as well as a daily supplement for healthy lifestyle (Muntari, Salleh et al. 2011). At present, bromelain application was divided into three categories: clinically, pharmaceutically and industrially (Muntari, Ismail et al. 2012).

2.5.1 Clinical Use

According to the National Institute of Health (2012) clinical involves individual people or the use of materials from human origin, such as observed behaviour, answers to questions or tissue samples, obtained through direct contact with a particular living person that volunteers and agrees to participate in a research study. Bromelain was clinically being used to treat several inflammatory disorders of the musculoskeletal system. With respect to this matter, a number of clinical studies were made as evidence to support the findings.

2.5.1.1 Treatment of Osteoarthritis

Osteoarthritis is a common musculoskeletal disorder caused by pain and disability in joint (Klein, Kullich et al. 2006). Based on recent statistics of

osteoarthritis case, the most common form of osteoarthritis in Western countries was set in USA (Buckwalter, Saltzman et al. 2004). Normally, osteoarthritis occurs more often in women at age of 65 above rather than men (March and Bagga 2004). Several attempts was done to cure osteoarthritis and a large body of scientific research shows that bromelain becomes a potential product for treatment of osteoarthritis (Buckwalter and Martin 2006, Yuan, Wahlqvist et al. 2006). Afterwards, with the properties of relieving pain, bromelain was thought to be the best natural treatment of inflammation. Initial study of bromelain was first reported in 1964 for an anti-inflammatory use in both rheumatoid arthritis and osteoarthritic patients. The study was carried out to assess the effectiveness of bromelain most frequently using preparations containing differing complexes of proteolytic enzymes and differing concentrations of bromelain. At the end of the studies, there is a reduction of inflammation in osteoarthritis patients (Brien, Lewith et al. 2004).

In addition, bromelain may also provide an alternative treatment to nonsteroidal anti-inflammatory drug (NSAID). The alternative treatment was investigated by administrating a combination of bromelain, trypsin and rutosid on 51 patients. At the same time, one of the most commonly use NSAID, diclofenac, was used as a control in 52 patients (Kaur, Abmwani et al. 2014). From the studies, both treatments resulted in significant and similar reduction in the pain and inflammation within the sixth week of consumption (Akhtar, Naseer et al. 2004). However, prolonged use of diclofenac was often associated with an increased risk of peptic ulcer complications (Langman, Weil et al. 1994). Daily consumption of bromelain reduced the potential risk of osteoarthritis rather than taking the nonsteroidal anti-inflammatory drug (NSAID) (Pavan, Jain et al. 2012).

2.5.1.2 Immunogenicity

Bromelain was used as an adjuvant to stimulate immune response in the treatment of chronic inflammatory, malignant, and autoimmune diseases of immunogenicity (Pavan, Jain et al. 2012). Earlier work on this particular matter has

been done by Engwerda, Andrew et al. (2001). In the study, bromelain can enhance IFN-γ-mediated nitric oxide and TNFα production by macrophages thus, enhancing the innate immune response against unknown threats (Engwerda, Andrew et al. 2001). In another study of immunogenicity, bromelain can also inhibit the T cell signal transduction to block the Raf-1/extracellular-regulated-kinase- (ERK-) 2 pathways to participate in mitogenesis, apoptosis, and cytokine production (Mynott, Ladhams et al. 1999). Moreover, with the treatment of cells using bromelain, the activation of CD4 (+) can be lowered down thus reducing the expression of CD25 (Secor, Singh et al. 2009). An increased levels of CD25 have been shown to correlate with disease severity in individuals with allergic asthma which commonly take place in children (Hoeger, Niggemann et al. 1994, Sujata Pandit Sharma and Brajbhushan 2015).

2.5.1.3 Fibrinolysis

The excessive synthesis of fibrin which causing the blood to clot tremendously was reported by Lotz-Winter (1990). By applying bromelain at higher concentration, the period of inhibition of prothrombin was prolonged (Pavan, Jain et al. 2012). The inhibition caused the prothrombin to be inactive thus lessen the time for the blood to coagulate. Besides that, bromelain is an effective fibrinolytic agent. It stimulates the conversion of plasminogen to plasmin, resulting in increased fibrinolysis by degrading fibrin (Taussig and Batkin 1988).

2.5.1.4 Diarrhoea

Several evidence proposed that bromelain able to prevent some of the effects of *Vibrio cholera* and *E. coli*, an intestinal pathogens (Bitange Nipa Tochi, Zhang Wang et al. 2008). This pathogen produces enterotoxin and causes diarrhoea in animals (Pavan, Jain et al. 2012). To counteract with this pathogen, bromelain interact with the intestinal secretory signalling pathways, that includes several component of

biomolecules which is adenosine 3:5- cyclic monophosphatase, guanosine 3:5-cyclic monophosphatase, and calcium-dependent signalling cascades (Mynott, Guandalini et al. 1997). There are also studies that suggest different mechanism of action. For instance, an active supplementation of bromelain leads to some anti-adhesion effects. The anti-adhesion effects works by preventing the bacteria from attaching to specific glycoprotein receptors located on the intestinal mucosa by proteolytically modifying the receptor attachment sites in *E. coli* infection (Mynott, Luke et al. 1996).

2.5.1.5 Cancer Cells

Apparently, cancerous cell happen as a result of abnormal growth of cell (GM). Several studies made claims that bromelain possesses anti-cancerous properties (Chobotova, Vernallis et al. 2010). According to Chakraborty (2015) the anticancer activity of bromelain studies was mostly focused on rat and human cells. A research study carried out by Báez, Lopes et al. (2007) treat the mouse skin tumour with bromelain. Later on, it was observed that bromelain reduced the tumour formation, tumour volume and caused apoptotic cell death. A similar study was carried out by Grabowska, Eckert et al. (1997). The study was using bromelain F9 and papain on B16F10 mouse melanoma cell lung colonization to inhibit the growth of the melanoma cells in a dose dependent manner. In another research work, the effect of bromelain against breast cancer was studied. From the research finding, the population of GI101A (breast cancer cell line) was reduced (Paroulek, Jaffe et al. 2010). Moreover, a significant reduction of the cell growth of gastric carcinoma Kato III cell lines was observed after being treated with bromelain (Amini, Ehteda et al. 2013).

2.5.2 Pharmaceutical care

The pharmaceutical care has been introduced in the early 1990s which focus on the delivery of patient care throughout hospital and professionals community

(Ahmed, Hasan et al. 2010). The effectiveness of bromelain most frequently being used in the preparations containing differing complexes of proteolytic enzymes and differing concentrations of bromelain (Brien, Lewith et al. 2004).

2.5.2.1 Surgery

The average number of days for pain and post-surgery inflammation recovery can be reduced by the administration of bromelain before a surgery (Unknown 2010). Besides that, several trials state that the use of bromelain might be effective in reducing swelling, bruising, and pain in women having episiotomy. In 1993, a German government commission approved the use of bromelain to treat swelling and inflammation following surgery, especially sinus surgery (Dighe, Pattan et al. 2010). Bromelain was today used for treating acute inflammation and sports injuries (Brien, Lewith et al. 2004).

2.5.2.2 Potentiation of Antibiotics

The demand for novel effective antimicrobial or antibiotic drugs is high nowadays (Pieren and Tigges 2012). According to Kelly (1996) antibiotic potentiation is one of the primary uses of bromelain. Bromelain works by modifying the permeability of organs and tissues to different drugs (Bhattacharyya 2008). Neubauer (1961) evaluate the administration of combined bromelain together with antibiotic therapy. As a result, no treatment responses take place in 22 patients which only consuming antibiotics whereas 23 patients give out a positive result with the addition of bromelain.

2.5.2.3 Debridement Burns

Debridement is known as the removal of damaged tissue from wounds or second/third degree burns (Pavan, Jain et al. 2012). Bromelain accelerates healing

when it is applied as a cream (35% bromelain in a lipid base). Non-proteolytic compound escharase found in bromelain is responsible for this effect as it has no hydrolytic enzyme activity against normal protein substrate or various glycosaminoglycan substrates (Houck, Chang et al. 1982). The activity of escharase varies due to different preparations of bromelain. An effective enzymatic debriding agent can provide the basis for first-line minimally invasive treatment. The use of enzymatic debridement was reported by Rosenberg, Krieger et al. (2012) and Singer et al., (Singer, McClain et al. 2010) in two studies carried out in porcine model. The study was using different types of bromelain-based agents, namely, Debriding Gel Dressing (DGD) and Debrase Gel Dressing. It is observed that this agent was capable of removing the necrotic layer of the dermis within four hours of application without harming non-burned tissues. In another study made by Wu, Hu et al. (2012) on enzymatic debridement of topical bromelain in incised wound tracks accelerate the recovery of blood perfusion, pO2 in wound tissue, controlled the expression of TNF-α, and raised the expression of TGT- β in Chinese landrace pigs. The use of bromelain as an enzymatic debridement possess better solutions rather than the surgical debridement which is painful, nonselective and exposes the patients to the risk of repeated anaesthesia and significant bleeding during surgical incision (Strohal, Dissemond et al. 2013).

2.5.2.4 Dermatological disorders

Dermatological disorders was a type of health problem on skin which frequently affecting people from the infant to the elderly (Tabassum and Hamdani 2014). The disorder occurred was lack of adequate skin firmness, wrinkles, and dry skin. In order to treat the disorder formulations such as gels, creams, lotion and ointment was used. The composition of the formulation was one alpha hydroxyl acid, salicylic acid, and at least one digestive enzyme derived from fruit (Ozlen 1996). Usually the digestive enzyme used originates from a mixture of bromelain and papain (Rosli 2012). In a research study conducted by Massimiliano, Pietro et al. (2007), the

effectiveness of bromelain was proven to treat eight patients with *Pityriasis lichenoides chronica* a skin disease of unknown etiology.

2.5.3 Industrial use

Proteolytic enzymes, bromelain was widely used industrial enzyme (Kirk, Borchert et al. 2002). It have been used extensively in industrial fields such as in tenderization, baking industry, textile, tooth whitening and cosmetics (Polaina and MacCabe 2007).

2.5.3.1 Tenderization

Meat tenderness refers to the characteristic of meat texture. Basically, meat tenderness is an important factor that affects the consumers' need. However, it is not easy to make the meat tenders due to the complex structure of muscle (Calkins and Sullivan 2007). In order to do this, many approaches have been taken to improve the tenderness of meat such as blade tenderization, moisture enhancement technology and enzymatic treatment (Pietrasik, Aalhus et al. 2010). Previously, meat is keep cool for up to 10 days to allow cathepsins and calphins (proteolytic enzyme) break the toughness of meat (Arshad, Amid et al. 2014). Nevertheless bromelain, papain, and ficin has been use to tenderize meat as these exogenous enzyme has been classified as Generally Regarded as Safe (GRAS) by USDA's Food Safety Inspection Service (ISIS) (Calkins and Sullivan 2007). Compared to other exogenous enzyme, bromelain is the most effective enzyme that improve the meat texture (Sullivan and Calkins 2010). As a matter of fact, bromelain is reported to efficiently hydrolyse a few meat tissue fibre by degrading the connections between the sarcolemma and the myofibrils as observed on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Sunantha and Saroat 2011). In the meantime, bromelain possess a clear proteolytic effect on myosin and other myofibrillar proteins when it was added to a dry chopped sausage (Melendo, Beltrán et al. 1996).

2.5.3.2 Baking industry

For hundreds of year, the baking industry use yeast and enzyme to manufacture baked product (Hamer 1995). According to Polaina and MacCabe (2007) among the enzyme used was bromelain. Bromelain was used in baking to lower down the protein level of flour and promotes dough relaxation by preventing the dough to shrink back due to the presence of gluten which is a functional component of wheat product (Al Tamim 2014). This process will allow the dough to rise evenly during the baking process to produce a better bread volume, crumbliness and browning uniformity.

2.5.3.3 Textile industry

Bromelain improves the dyeing qualities of protein fibre in silk and wool (Muntari, Maizirwan et al. 2013). The fibre on silk was produced by cooking the cocoon to soften it. While cooking, strong alkaline agents and chemicals are added that usually gave bad influence to the quality of the silk thread produced. Therefore, an advance in the cocoon cooking process using enzyme treatment is important as this treatment will reduce softening time and at the same time will increasing production and saving energy. For example a recent research study by Anwar, Ahmad et al. (2007) reported that when the protease stem bromelain was cross-linked with 0.25 and 1.25% of glutaraldehyde (GTA) the properties of fabric was improved. Other similar studies were reported by Koh, Kang et al. (2006) and Rupachandra Singh, Ranjana Devi et al. (2003). The study of Koh, Kang et al. (2006) reported that bromelain was used to improve the dyeing properties of protein fibres such as silk and wools. In this study it was revealed that bromelain can removed the impurities and scales from the wool and silk that led to the improvement of dye accessibility and at the same time conserved their tensile properties. On the other hand, Rupachandra Singh, Ranjana Devi et al. (2003) described in their study that the usage of pineapple extract having bromelain activity in addition with 9.8 mM of sodium carbonate at

60°C during silk cocoon process can reduced the softening time from 20 hours to only 30 minutes.

2.5.3.4 Tooth whitening

A large number of people seek for dentist regarding their teeth appearance and colour. The colour of the teeth is determined by the coupling effects of intrinsic and extrinsic colorations on the surface of teeth. The main cause of extrinsic discoloration is due to the intake of coloured foods, caffeinated drinks and smoking (Joiner 2004). According to Chakravarthy and Acharya (2012) dentifrices containing papain and bromelain extracts exerts a significant lightening effect compared to the control dentifrice (Colgate Regular). Discoloured teeth have raised the demand for tooth-whitening products such as toothpaste with the ability to remove the extrinsic stains. The stain can be removed using papain and bromelain based on the current evidence discovered by Kalyana, Shashidhar et al. (2011) which reported that higher lightening value after brushing compared to the control dentifrice.

2.5.3.5 Cosmetic industry

With gentle peeling effect to skin, bromelain has been used as an active ingredient in cosmetics product (Ketnawa, Chaiwut et al. 2011). For instance, bromelain can be used in the treatment of skin problems such as wrinkles, acne and dry skin. The action of bromelain works by gently digesting the protein layer of dead cells and replace it with a younger skin cells (Ozlen 1995).

2.6 Structural Studies of Bromelain

Bioinformatics involves the use of computational approach to analyze a biological data (Fenstermacher 2005). According to Luscombe, Greenbaum et al. (2001), there are numerous amounts of biological data were deposited in the Gen

Bank database every year. The biological data can be stored, mined, retrieved, and analyzed from the database. On average, this database manage to assist researchers around the world in the molecular level (Ding, Wang et al. 2014). Associated programs such as the homology and similarity tools, protein functional analysis tools, sequence analysis tools and modelling tools are commonly use tools to analyze the unknown biomolecule in the database (Fulekar 2009).

The use of bioinformatics approach in structural study of protein is crucial to predict the possible protein structure and function (Mills, Beuning et al. 2015). There are many software can be used to predict the structure of protein such as protein threading, comparative modelling, secondary structure prediction, ab initio methods and signal peptide prediction (Jones 1999, Martí-Renom, Stuart et al. 2000, Zhang and Henzel 2004, Lee, Wu et al. 2009, Wang, Peng et al. 2016). The prediction take place based on the conserved site of the evolutionary patterns (Dutt, Singh et al. 2010).

The catalytic mechanism of protein can be identified using the three dimensional structure of a protein (Facchiano, Stiuso et al. 2001). Besides that, the 3D protein structures generates an efficient design of experiments (Söding, Biegert et al. 2005). X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) are some of the experimental method used to solve protein structure (Ramli 2012).

Up to now, the exact three dimensional (3D) structure of bromelain remains unclear. The structural ambiguity leads to several predictable model using available bioinformatics tools (Tap, Majid et al. 2016). An attempt was made in 1980 to predict the structure of stem bromelain by comparative modelling. In the study, papain is as the modelling template (GOTO, TAKAHASHI et al. 1980). Later, Tap, Majid et al. (2016) use 1PCI (procaricain) as a template to build the predicted 3D structure model of stem bromelain.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample Preparation

The fruit and stem used throughout this research were obtained from the local pineapple (*A. comosus*) cultivars around Pahang, Malaysia. The pineapple cultivars were Morris, N36 and Sarawak. Initially, the pineapple fruit and stem were cut into small pieces and the pieces were weighed using the analytical balance (Appendix 1) and grinded in the industrial blender separately. Next, each of the extract obtained was filtered using the whatman filter paper and were centrifuged at 10,000 rpm in 4°C for 20 min to remove left residue. The crude supernatant was collected and stored at -20°C until further use.

3.2 Buffer and reagent preparation

3.2.1 Enzymatic Assay Buffers and Reagents

The pH of reagent A (50mM Potassium Phosphate Buffer) was adjusted to 7.5, at 37°C. In the preparation of reagent B (Casein), 0.65% (w/v) was dissolved in 125ml of reagent A. Dilution of reagent C, tricholoroacetic acid (TCA), D (F-C) Folin

& Ciocalteu's Phenol and E, Sodium Carbonate (Na2CO3) solution was done using the
deionized water. For reagent F (1.1mM L-tyrosine), L-tyrosine was dissolved in 100mL of deionized water under a low heat and cooled to a room temperature.

3.3 Growth media preparation

3.3.1 Bacteria growth media

Luria Bertani (LB) agar is used to grow the competent cell of *Escherichia coli*, *E.coli* after the transformation reaction. The media are rich in nutrient which provide the propagation of bacteria with peptides and peptones, vitamins, and trace elements. The media was prepared in distilled water at 500mL in volume which consist of 5g of sodium chloride (NaCL, 5g of tryptone, 2.5g of yeast extract and 7.5g of agar. When necessary, ampicillin (50 or 100 μg/ml) was added to the medium as a selectable marker.

3.4 Enzyme Assay of Crude Enzyme

Reagent B (casein) is used as a substrate to evaluate the proteolytic activity of crude extract of fruit bromelain. At first, the reagent B was incubated at 37°C for 5 minutes in the water bath. Then, the crude pineapple enzymes were inserted into each of the test tube after the incubation completed. Later on, a secondary incubation then take place for 10 minutes. In order to prevent continuous proteolytic activity, 5ml of reagent C (TCA) were added in each of the test tube. Last but not least, the mixture

was incubated again at 37°C for 30 minutes. The mixtures were filtered out after 30 minutes using the 0.45μm syringe filter.

Reagent D (F-C) and E (Na_2CO_3) were used to detect the presence of L-tyrosine content in the test sample. Therefore, 5ml of reagent E was added to the sample mixture followed by the addition of reagent D. The mixture were incubated in the water bath at 37°C in 30 minutes. After 30 minutes, the color development was observed. The enzymatic activity of protease activity was calculated using the tyrosine standard curve. The standard curve is prepared by using different concentration of L-tyrosine at range of 0.05 ml to 0.50 ml. At the end, the test sample and standard were measured using the spectrophotometer to obtain the absorbance reading.

3.5 Isolation of DNA sequence from pineapple fruit

The pineapple fruit was grounded in liquid nitrogen to obtain extracts in the form of a fine powder (Figure 3.1). The procedure was done under the free-RNAse environment and stored at -80°C for the total RNA extraction of fruit and stem bromelain.

Figure 3.1 The sample of grounded fruit bromelain in powder form

3.6 Fruit bromelain (BAA21848) isolation and cloning

3.6.1 Full length amplification of *A. comosus* fruit bromelain via reverse-transcription polymerase chain reaction (RT-PCR)

The total RNA of fruit bromelain were extracted using the innuPREP Plant Extraction Kit from Analytik Jena. The total RNA of *A. comosus* with the highest concentration of RNA was used as a template to perform RT-PCR using the Tetro cDNA Synthesis Kit from Bioline, as recommended by the manufacturers. About 1-3 µg of total RNA was used for first-strand full length cDNA synthesis in combination with 2.5 µl of 10 mM dNTP, 0.5 µl of 10 µM primer and 2.5 µl of DEPC-treated water to give a total volume of 10 µl of priming premix. Then, the premix was incubated at 70°C for 5 minutes prior to chilling on ice for at least 1 minutes. In another tube, the reaction premix was prepared by the addition of 4 µl of 5X RT buffer, 1 µl of Ribosafe RNase inhibitor, 1 µl of Tetro reverse transcriptase (200 u/µl) and DEPC-treated water up to 10 µl of the premix. Next, 10 µl of the reaction premix was added into the priming premix and incubated for 30 minutes at 45°C. The reaction was terminated at 85°C for 5 minutes and cooled on ice for 2 minutes. The first strand cDNA obtained was eventually used for the PCR reaction.

The mRNA sequence of fruit bromelain, D14058.1 was selected using the BLAST method in the National Centre of Biotechnology Information (NCBI). The complete amplification of the full-length gene of fruit bromelain by RT-PCR were obtained using the primer of forward primer, AFBro-F9 (5'-ATG GCT TCC AAA GTT CAA CTC GTG-3') and reverse primer, AFBro-R1039 (5'- TCA AGT TTT AGA AAC CAT CTT AAT AAC TGC-3') respectively. These primers were designed using the ExPASy tool available online (http://web.expasy.org/translate/). The primers were prepared by dissolving the lyophilized primer product in nuclease free water or double distilled water to a stock solution, 100µM and eventually to a working stock concentration of 10µM.

3.6.2 Fruit bromelain amplification gene via Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify the nucleic acid respectively. An amount of 5 ng of cDNA was used for this PCR amplification. The volume of each component was stated in the Table 3.1. The reaction was carried out in a 0.2 ml PCR tube. A 20 µl of reaction mixture contained: 5 µl genomic DNA of cDNA from fruit bromelain, 2 µl of 10X PCR buffer, 1.2 µl of 3.0 mM $MgCl_2$, 1 Unit of Taq polymerase, 0.5 µM of each forward and reverse primer (FBroF and FBroR) and 1.6 µl of 0.2 mM dNTPs. A PCR Mastercyler (Eppendorf Scientific Inc.) was used to perform the amplification under specific condition (Table 3.2). The reaction was carried out for 34 cycles under the following conditions: 3 minutes initial denaturation at 94°C followed by the PCR cycles consisting of denaturation at 94°C for 30 seconds, annealing temperature at 58°C for 30 seconds and extension at 72°C for 1 minutes 30 seconds. The PCR cycles were followed by an additional final extension at 72°C for another 10 minutes.

Table 3.1 Reagents for the PCR amplification reactions of fruit bromelain

Reagents	Concentration	Volume (µl)
10x PCR Buffer	1x	2
dNTP	0.2mM	1.6
Primer Forward	0.5µM	0.5
Primer Reverse	0.5µM	0.5
dH_2O		8.7
$MgCl_2$	3.0mM	1.2
cDNA template	5-20 ng	5

Taq DNA Polymerase	1.0-2.5	0.5
Total volume		20µl

Table 3.2 The PCR amplification reaction conditions of fruit bromelain

Cycle step	Temperature	Time	Number of cycle
Pre-denaturation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	34
Anealling	58°C	30 seconds	
Extension	72°C	1 minutes 30 seconds	
Final Extension	72°C	10 minutes	1

The PCR reaction products were purified using the innuPrep gel extraction kit to eliminate traces of the remaining reagents that can interfere with subsequent downstream applications. Samples were stored at --20 °C.

3.6.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to separate and identify any nucleic acid based on their size under the influence of electric field. Prior to any band detection, GelRed™ was added to fluoresce the nucleic acid and visualized under the Gel Imager™. The agarose gel used throughout this study was 0.8% and 1% in concentration.

A mix of TAE buffer and agarose powder were made using the following volume and weight to produce a 0.8% and 1% of agarose gel; 0.24g of Agarose

powder mix with 30mL of TAE buffer and 0.30g of Agarose powder mix with 30mL of TAE buffer. The mixture was heated in the microwave for at least 30s until the agar melt and boiled. The prepared agar was left to cool down at 70°C before it was being added with 0.5µl of GelRed™. The gel were poured down inside the gel casting tray with the casting comb attached to it and was left to solidify for approximately 20 minutes. After the gel had solidified, the casting comb was removed. The gel was then ready for sample loading when it was placed in the 1X TAE buffer tank.

Prior to running the gel, the DNA ladder was loaded into the first well. This ladder was used as ruler to predict the size of the separated nucleic acid. An amount of 2µl of loading dye and 5µl of distilled water, dH_2O was mixed together with the sample to produce a volume of 10µl. The samples were added accordingly into the well. The buffer tank was closed after sample loading and 80V of were applied up to 35 minutes. The visualization of gel was carried out using the Gel Imager™.

3.6.4 Insertion of the target gene into the cloning vector

Cloning of PCR product was done using the pGEM®-T Easy Vector Systems. Initially, TA cloning was carried out using the Taq DNA Polymerase assisted by PCR. The cloning produceed 3'-adenine overhang to each end of the PCR product. The use of pGEM®-T Easy Vector Systems relatively joins the vector and fragment together as the vector possess a 3' T overhangs end. Hence, an efficient ligation can be achieved when the fragment of interest was introduced into the prepared vector.

The pGEM®-T Easy Vector and Control Insert was centrifuged together to collect the contents to the bottom of the tube. The ligation reaction was performed (Table 3.3). Prior to each use, the 2X Rapid Ligation Buffer was vigorously vortexed. The reaction was mixed evenly. The reaction was incubated either at 1 hour in the room temperature or overnight in 4°C to achieve maximum number of transformants.

Table 3.3 Reagents for the ligation reactions of fruit bromelain

Reagents	Standard reaction	Positive control	Background control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T Easy Vector	1µl	1µl	1µl
PCR product (Purified)	2µl	-	-
Control Insert DNA	-	2µl	-
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
Deionized water	1µl	1µl	1µl
Total volume	10µl	10µl	10µl

3.6.5 Transformation of recombinant plasmid in cloning host

3.6.5.1 Competence cell preparation

The Dh5-Alpha competent cell was initially grown in the LB agar plate overnight at 37°C. Then a single grown colony was selected and aseptically transferred into 50 mL Falcon tube containing 10 mL LB broth. The inoculums broth was grown overnight at 37°C on an orbital shaker for 250 rpm prior to another subculture under the same condition.

3.6.5.2 Transformation in competence cell

The transformation of Dh5-Alpha competent cell was carried out by inserting 2µl of the ligation reaction inside 50µl of the competent cell. Then, the mixture was subjected to heat-shock for 45-50 seconds at 42°C in the PCR block machine. The ligation reaction was cooled on ice for 2 minutes. LB medium of 950µl was added

into the ligation reaction and the other 900 µl was added to the uncut DNA control tube. The inoculums were incubated in the orbital shaker (~150rpm) for 1.5 hours at 37°C. After the incubation, 100 µl of each transformation culture was spread and grown overnight at 37°C onto the LB ampicillin agar spread with IPTG and X-Gal. Successful transformant grown in white was selected. The presence of the fruit bromelain gene were identified by colony PCR method.

3.6.6 Colony PCR of transformed colonies

Colony PCR is a commonly used method to quickly screen for plasmids containing a desired insert directly from bacterial colonies. This method eliminates the need to culture individual colonies and prepare plasmid DNA before analysis. All transformed colony were subjected to colony PCR. The reagents used were shown in Table 3.4. A slight modification of PCR program was done by changing the period of initial denaturation from 3 minutes to 10 minutes to allow effective cell wall breakdown (Table 3.5).

The PCR reaction products were purified using the innuPrep gel extraction kit to eliminate traces of the remaining reagents that can interfere with subsequent downstream applications. Samples were stored at –20 °C.

Table 3.4 Reagents for Colony PCR amplification reactions of selected fruit bromelain colony

Reagents	Concentration	Volume
10x PCR Buffer	1x	2
dNTP	0.2mM	1.6
Primer		
Forward	0.5µM	0.5
Reverse	0.5µM	0.5
dH_2O		9.7
$MgCl_2$	3.0mM	1.2
Colony sample	2-5ng	4
Taq Polymerase	1.0-2.5	0.5
Total volume		20µl

Table 3.5 Colony PCR amplification reaction condition

Cycle step	Temperature	Time	Number of cycle
Pre-denaturation	94°C	10 minutes	1
Denaturation	94°C	30 seconds	34
Anealling	58°C	30 seconds	
Extension	72°C	1 minutes 30 seconds	
Final Extension	72°C	10 minutes	1

3.7 Sequencing of DNA

Recombinant plasmid obtained was further subjected to DNA sequencing using two universal primers: T7 polymerase and SP6 polymerase. The plasmid was sent for sequencing service provided by the 1st Base Laboratories, Malaysia.

3.8 Bioinformatics analysis of fruit bromelain

3.8.1 Data mining

The NCBI GenBank and Protein Data Bank (PDB) databases were used to retrieve all the informations regarding fruit bromelain.

3.8.2 Primary sequence analysis

Basic local alignment search tool (BLAST) server is used to perform a homology search corresponding to fruit bromelain (Altschul et al., 1990). The sequence alignment of fruit bromelain with the sequence obtained from NCBI Database was done using the Clustal Omega programme. The catalytic domain of the fruit bromelain protein was identified by InterProScan (Zdobnov and Apweiler, 2001); while the theoretical molecular weight, isoelectric point and amino acid composition of the protein were calculated using the ProtParam tool (reference).

3.8.3 Secondary sequence analysis

Fruit bromelain amino acid sequence (352 residues) was subjected to various sequence prediction analyses, including BLAST-PDB, HHPred, Mod-link+, Phyre2, and PSI-BLAST to identify possible families or conserved domains in the protein.

3.8.4 Tertiary sequence analysis

Comparative protein modelling method was performed by submitting fruit bromelain amino acid sequence to the library of known folds using BLAST-PDB, HHPred, Mod-link+, Phyre2, and PSI-BLAST. The sequence-structure alignment was optimised and the resulting alignment was used as the input for the development of 3D models using MODELLER. Out from one hundred models generated using MODELLER, a model with the lowest objective function was selected and evaluated based on its root-mean-square deviation (RMSD). Optimisation and energy minimisation of the resulting model was performed using SPDVB from Deepview (Guex and Peitsch, 1997), respectively. The protein structure assessment tools used were PROCHECK, VERIFY3D and ERRAT.

3.8.5 Structural comparison of fruit bromelain with stem bromelain

A solved crystal structure of papain (1CVZ) from *Carica papaya*, obtained from the PDB database, was used as a reference for structural comparison against fruit bromelain and stem bromelain developed using the homology template caricain (1PCI). All of the graphic presentations of the 3D model were prepared using Deepview and PyMOL (Delano, 2002).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

Bromelain enzyme can be obtained from the pineapple, *A. comosus*. This enzyme is widely used to assist in various industrial applications. Despite of that, the enzymatic mechanism remains unclear due to less information on its three dimensional structure. All data related to the screening of fruit bromelain from different *A. comosus* cultivars and the molecular work on bromelain with the highest proteolytic content was collected and discussed in this chapter. In addition, the data also includes the structural comparison of fruit bromelain sequence, BAA21848 with other available fruit and stem bromelain retrieved from the NCBI database,

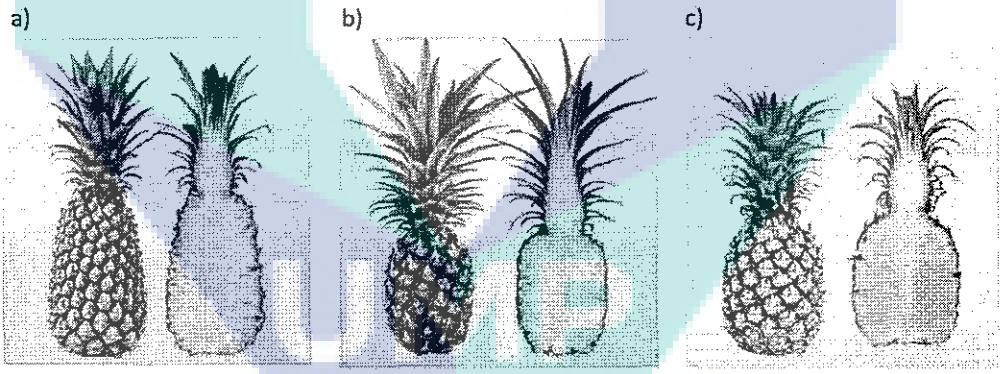
4.2 Bromelain analysis from different cultivars

4.2.1 Sample collection and analysis

The enzymatic activity of bromelain has been reported in several research studies. However, some of the enzymatic activity of bromelain in different *A. comosus* cultivars (cv.) including Morris cv., N36 cv. and Sarawak cv. has yet to be reported through fully. Morris, N36 and Sarawak cultivar as shown in Figure 4.1 and

Table 4.1 was relatively different in crown size, fruit shape and stem size. According to Fassinou Hotegni, Lommen et al. (2014), crown (leafy part on top) are part of vegetative organ use in plant propagation. The fruiting stage of pineapple relies on the size of planting material; shorter crown leads to a longer propagation period. From the observation, it's appeared that Morris cv. and Sarawak cv. crown is shorter compared to N36 cv.

Upon reaches maturity, the color of pineapple fruit turns from greenish to orange-yellow (Lobo and Yahia 2017). Besides that, a mature pineapple fruit shape also varies differently to each cultivar. The fruit shape of N36 cv. and Sarawak cv. is cylindrical except Morris cv. which is tapered in shape. According to Hidayat, Abdullah et al. (2012) the N36 cv. was the product of hybrid between Gandul and Sarawak. The size of Morris cv. and Sarawak cv. (also known as the Smooth Cayenne) is bigger than N36 cv..



Source: Lembaga Perindustrian Nanas Malaysia (LPNM)

Figure 4.1: Cultivars of *A. comosus* (a) Morris (b) N36 (c) Sarawak

Table 4.1 Morphological features of *A. comosus* cultivars

Cultivars	Crown size	Fruit shape	Stem size
Morris	Medium	Tapered	Small (2 cm)
N36	Long	Cylindrical	Large (2.0-2.8 cm)
Sarawak	Short	Cylindrical	Large (2.0-2.8 cm)

Regardless on the pineapple physical appearance, different volume of pineapple juice extract is obtained from each of the cultivar. These differences may contribute to different protein content. The highest volume of juice extracts from pineapple fruit, 230.0 mL is obtained from Sarawak cv. (Table 4.2). On the other hand, the volume of juice extracts from pineapple fruit of Morris cv. was 187.5 mL. In contrast to Morris cv., the volume of juice extract in N36 cv. is higher in fruit, 200.0 mL respectively. Based on the observation, the pineapple peel content in Sarawak cv. is less than the other cultivar. Less juice is produced when more pineapple peels present in the fruit, thus causing the space of water content to be reduced. It's difficult to commercialize small fruits which are under marketable value. Sarawak cv. is large in size and weighs approximately around 1.5 to 4.0 kilograms (maximum). Either consumed as fresh fruit, desserts or canned products, Sarawak cv. is known to its sweet taste and crunchy texture (Soloman George, Razali et al. 2016). Despite of that, the N36 cv. is far more resistance to spoilage. Therefore, N36 cv. is suitable to be exported using a reefer container (Hajar, Zainal et al. 2012).

Besides that, the pineapple juice content is also affected by the ripening fruit. The fruit ripening produce more juice than the unripe fruit. This statement can be supported by the other journal that study on the moisture content of the unripe and ripe of mango fruit and the result shown that the moisture content of the fruits increased significantly during ripening from 79.75% to 83.11% (Appiah, Kumah et al. 2011). Thus, high moisture percentage will produced more juice.

Table 4.2 Weight and volume of fruit from each pineapple cultivar; Morris, N36 and Sarawak

Cultivar	Total mass (g)	Final mass used to get the concentrated juice (g)	Volume in 300 g (mL)
Fruit Bromelain			
Morris	556.55		187.5
N36	64.26	300.0	200.0
Sarawak	475.4		230.0

4.2.2 Enzymatic Analysis of Crude Bromelain from Different Cultivars

The proteolytic activity of protease are compared to a standard curve by reacting known quantities of L-tyrosine with the F-C reagent to correlate changes in absorbance with the amount of L-tyrosine in micromoles. From the standard curve the activity of protease can be determined in terms of Units, which is the amount in micromoles of L-tyrosine equivalents released from casein per minute.

The absorbance value increase when more L-tyrosine is added (Table 4.3). From the standard curve of L-tyrosine in Figure 4.2, the concentration of L-tyrosine is directly proportional to the absorbance value. According to Cupp-Enyard (2008) the breakdown of casein using protease liberates L-tyrosine with other amino acids and peptide fragments. A blue coloured chromophore is formed when an amount of free L-tyrosine reacts with Folin and Ciocalteus (F-C) reagent. The coloured chromophore is quantifiable and measured as an absorbance value on the spectrophotometer.

The equation obtained from the generated graph is $y=1.2653x-0.0001$ and regression value (R^2) shown is 0.9996. R^2 represents the portion of the variation in y that is associated with the variation of the predictor, x. When R^2 is 1, all data points lie perfectly on a straight line (Chu, Feng et al. 2011). Therefore, this is means that, the graph plotted shown the close data are fitted to the regression line. The standard curve plotted is in the best fit line since the R^2 is near 1.

Table 4.3 The absorbance value of L-Tyrosine at different L-Tyrosine concentration for Standard Curve

Concentration of L-Tyrosine (µmol)	Absorbance (A)

0.05	0.063
0.1	0.121
0.2	0.261
0.4	0.504
0.5	0.632

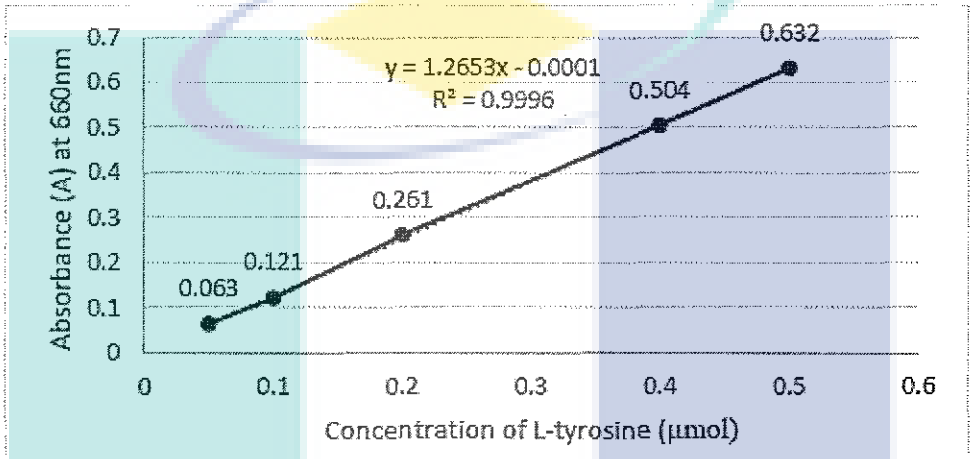


Figure 4.2 The standard curve of enzyme assay using different concentration of L-Tyrosine

The enzymatic activity of fruit bromelain in Morris cv., 1.891 A (average value) is high compared to the other cultivar; the N36 cv. is 1.772 A and Sarawak cv. is 1.593 A (Table 4.4). Besides that, the amount of fruit bromelain (Appendix 2) in Morris cv. is also higher, 0.8220 units/ml than N36 cv. (0.7703 units/ml) and Sarawak cv. (0.6925 units/ml).

The breakdown of casein by fruit bromelain and stem bromelain (generally determined as substrate converted into product) in Morris cv., N36 cv. and Sarawak cv. release L-tyrosine. On the other hand, the enzyme units serve to quantify the amount of an enzyme. The volume of juice content doesn't affect the amount of fruit bromelain and stem bromelain. Hence, it can be presume that there is no relationship between the amounts of enzyme present in the cultivar with the volume of extracts obtained previously.

Table 4.4 The absorbance and units/ml enzyme value of fruit bromelain in different cultivar; Morris, N36 and Sarawak

Cultivar	Absorbance (A)		Average Absorbance (A)	Units/ml Enzyme
	Replicate 1	Replicate 2		
Morris cv.	1.880	1.902	1.891	0.8220
N36 cv.	1.740	1.804	1.772	0.7703
Sarawak cv.	1.591	1.595	1.593	0.6925

4.3 Isolation and Cloning of Fruit Bromelain

4.3.1 Total fruit RNA extraction of *A. comosus*

RNA is an active molecule that works in regulating the transcription of protein and stores some of the biological data of an organism similar to the DNA. However, the production of a new protein cannot be accomplished if the previous RNA was present. Consequently, the previous RNA needs to be removed by RNase enzyme produced by the organisms to prevent any possible transcriptional event take place. The enzyme action degrades the RNA (Deutscher 2006). Nevertheless, the presence of the enzyme caused the RNA sample collection becomes difficult. RNA is a fragile and unstable biomolecule. It can be easily degraded. Therefore, the extraction of RNA needs to be performed under special care to prevent total loss of the RNA sample. RNA is extremely susceptible to degradation by RNases in the environment. A proper quantification is also needed for a successful RNA analysis after careful handling during purification (Tan and Yiap 2009).

The concentration of the RNA samples; fruit bromelain was recorded below in Table 4.5. The yields for fruit bromelain RNA was relatively increase accordingly based on the concentration of each samples extracted. The extraction took place in a well maintained environment with all of the apparatus used were properly sterilized to remove the RNase enzymes. The sterilization also includes the use of 15% of SDS to wash the apparatus before it was autoclaved. The

samples were prepared by cutting the pineapple flesh into pieces. The fruit part was taken out and ground into a fine powder to increase the surface area of samples when the extraction of RNA was performed. From the result, the purity of RNA can be assessed with a wavelength of 260/280 nm. Apparently all sample are nearly pure based on the accepted ratio of RNA purity which falls approximately to 1.8. An ideal 260/280 absorbance reading also strongly implies the RNA is free from any substances which could interfere with its stability (Fleige and Pfaffl 2006).

Table 4.5 The yield and purity of fruit bromelain, total RNA

Sample	Conc.	Wavelength					
		A230	A260	A280	A320	A260/ 280	A260/ 320
F1	108.8	3.91	2.47	1.08	-0.252	2.045	0.654
F2	117.6	3.19	2.67	1.16	-0.269	2.056	0.850
F3	127.6	1.27	2.83	1.22	-0.360	2.019	1.957
F4	145.6	1.76	3.39	1.59	-0.252	1.978	1.811

4.3.2 Total RNA detection using gel electrophoresis

Gel electrophoresis makes use of the electrical field to separate mixture of biomolecules such as DNA, RNA and proteins according to its molecular size. The migration of biomolecule takes place when electrical charges were applied, the samples will move from negative electrode towards positive electrode. The movement of the biomolecule across the gel was measured using 1kb DNA marker. In general, shorter bands migrate faster than the larger bands.

Two out from four samples of fruit total RNA were selected; F3 and F4. The selection was done as both of the sample gives out an optimal absorbance reading of approximately to 1.8 at wavelength of A260/280nm. Agarose gel analysis (Figure 4.3) was then carried out using sample F3 (Lane 1) and F4 (Lane 2) to visualize the RNA bands. The total RNA extract was identified through the

formation of two RNA crisp fragment bands on the agarose gel. There is also less smearing effect forms on the RNA bands.

The formation of a visible RNA fragment F3 and F4 in the agarose gel strongly suggest that the RNA also degrades regardless of their high RNA concentration. In general, the a pure total RNA was usually made up of 80 to 95% of ribosomes (Rodriguez-Oquendo 2015). The RNA can be assessed using the RNA integrity (RIN) test formed on the denaturing agarose gels. In figure 4.2, two fractions of ribosomal RNA (rRNA), 28s on the top, and 18s on the bottom can be observed. The rRNA is a molecular component of a ribosome, the cell's essential protein factory. rRNA does not make proteins. It makes polypeptides (assemblies of amino acids) that go to make up proteins (Lodish, Berk et al. 2000). The intensity of 28S rRNA band should be at ratio of 2:1 against the 18S rRNA band for an intact RNA. However, it appears that total RNA of both sample is lower than expected. According to Imbeaud, Graudens et al. (2005), degradation affects the quality of RNA thus lower down its rRNA ratios.

Degradation happens when the rRNA bands are of equal intensity which much likely can be observed in both samples. RNA contaminated with DNA possesses a higher molecular weight bands. The mRNA runs between the 2 ribosomal bands might be seen as a smear. The mRNA is much lower abundance, and the size of mRNAs varies enormously. Hence, the presence of mRNA cannot be detected on agarose as mRNA possesses a low molecular weight.

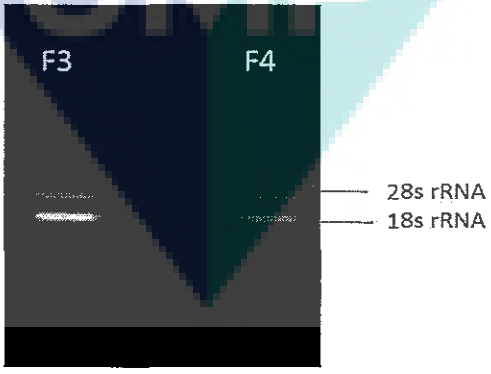


Figure 4.3 Agarose gel 0.8% (w/v) of fruit bromelain, RNA fragment; F3 (Lane 1) and F4 (Lane 2)

4.3.3 Complementary DNA (cDNA) synthesis of fruit bromelain

A reverse transcription of RNA was carried out to produce a complementary cDNA library. The cDNA is a double stranded DNA synthesized by the messenger RNA (mRNA) template. The fruit bromelain cDNA of *A.comosus* was successfully obtained from the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using a specific primer from the fruit bromelain gene, BAA21848. Up to now, no studies has described about the isolation and cloning of this fruit bromelain gene.

Later, the cDNA was amplified using PCR. To check whether the PCR generated the anticipated cDNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products. Amplification of the cDNA product using a routine PCR manage to obtain desired biomolecules size (Figure 4.4). The size of successfully amplified fruit bromelain is 1056kb. These PCR fragment was then purified to remove primer dimer and other contaminant that can interfere during the ligation of the target gene into the first cloning vector.

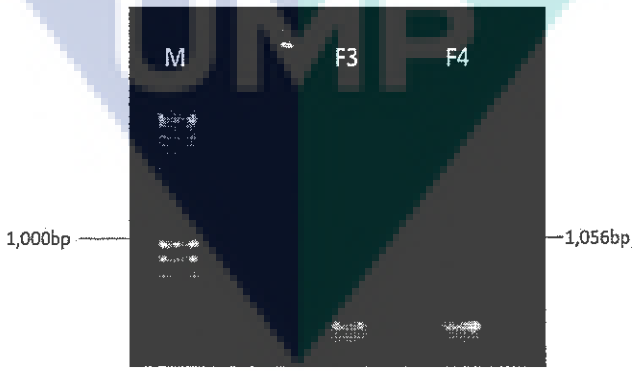


Figure 4.4 Determination of the cDNA amplified fragment of fruit bromelain; M: 1kb DNA Hyperladder (Bioline), Lane 2: F3 and Lane 3: F4

4.3.4 Insertion of the target gene into the cloning vector

The construction of a recombinant vector was initially begins with a ligation. Nevertheless, the target gene must be compatible to the vector backbone. The overhangs, called "sticky ends", allow the vector and insert to bind to each other. When the sticky ends are compatible, the overhanging base pairs on the vector and insert fused together by the ligation reaction. The pGEM-T Easy vector is used for cloning of PCR products by Taq polymerase. According to Mulhardt (2010), Taq polymerase produces no fragments with blunt ends through the construction of a vector with a thymine overhang. Thus, the PCR fragment can be cloned easily rather than vector with the blunt ends. Taq polymerase adds a single adenosine (A) to the end of every 3' PCR product. The action take place as the Taq polymerase possesses a non-template dependent terminal transferase activity. Generally, the method is known as TA cloning. An incubation of the ligation mixture multiplies the number of transformants by cloning.

4.3.5 The transformation of competent cell, *E.coli*

The vectors containing the target gene were introduced into the competent cell by transformation. In comparison to the available bacterial strains, competent cell is used to increase the transformation efficiency by keeping the rearrangement of the vector DNA from occurred. Nearly, all vectors carry the bacterial origin of replication and an antibiotic resistance gene for use as a selectable marker in the bacteria (Lodish, Berk et al. 2000).

The LB agar plate was treated with the ampicillin antibiotic to allow only the growth of bacterial colony that is resistance to the antibiotic treatment. The pGEM-T Easy vector contain ampicillin resistance gene that inactivates ampicillin. A blue-white screening test was carried out by applying the IPTG (100mM) and a chromogenic substrate, X-Gal (20mg/mL) at the top of the LB ampicillin agar. IPTG induces the expression of lac Z gene which produce β-galactosidase. Whenever the transformations take place, the lac Z gene will be disrupted and no β-galactosidase is produced. Unlike the untransformed colonies, the X-gal hydrolyze β-galactosidase to form 5-bromo-4-chloro-indoxyl, which

later on oxidized into a blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo (Karasova, Spiwok et al. 2002, Padmanabhan, Banerjee et al. 2011). The growth of the transformed colonies will appear to be white in color whereas, the untransformed ones appear blue. Six white transformed single colonies are selected and cultured from the blue-white screening plate as shown in Figure 4.5.

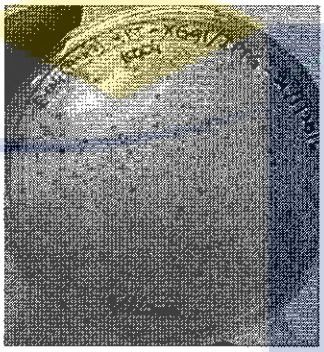


Figure 4.5 Blue-white screening of the fruit bromelain transformed colonies

4.3.6 Screening of the recombinant plasmid DNA using the colony PCR

The presence of the insert DNA in the vector constructs can be identified using the colony PCR method. Initially, the individual transformants were grown for 16 to 24 hours in LB medium and harvested. Plasmid from the cell were released by a short heating step with a small pick of colony lysed in sterile ddH_2O (Ramli 2012). Other than that, the colony also can be added directly to the PCR reaction and lysed during the initial denaturation step. The released plasmid can serve as template for the amplification reaction. The amplification goes specifically when specific primer was used. In all experimental designs, presence or absence of a PCR amplicon and size of the product are determined by electrophoresis alongside a DNA size marker on an agarose gel. From the agarose gel electrophoresis (Figure 4.6), all colonies (Appendix 3) selected integrates with the gene of interest except for colony F1. The upper band represents the pGEM vector with insert (4,107 bp) while the lower band is fruit bromelain gene (1,056 bp). The size of parent plasmid was 3,051 bp. The PCR product should involve parent plasmid (3,051 bp) and fruit bromelain gene (1,056 bp).

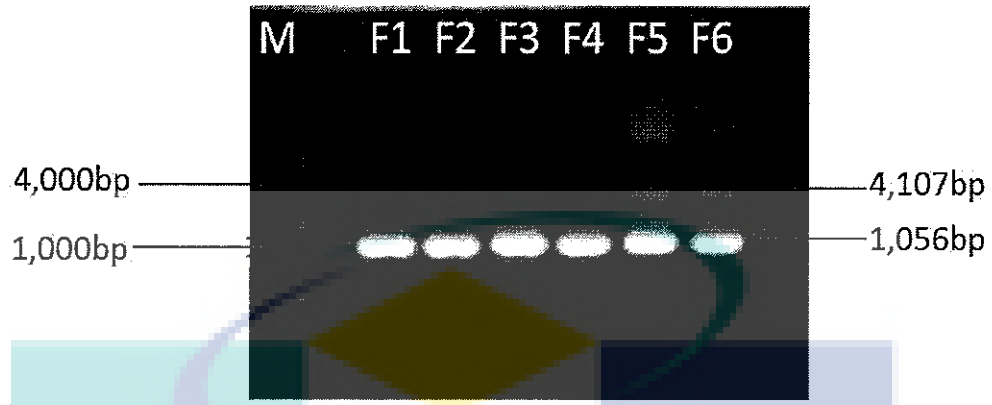


Figure 4.6 Determination of the plasmid integration via Colony PCR in pGEM-T Easy vector. M: 1kb DNA ladder (Fermentas), Lane 1: F1, Lane 2: F2, Lane 3: F3, Lane 4: F4, Lane 5: F5 and Lane 6: F6

4.3.7 Plasmid extraction of the recombinant vector

The extraction of recombinant plasmid is carried out by growing the transformed bacterial colony in LB broth culture. The overnight grown culture was harvested to collect the plasmid. The transformed colonies grown on the LB agar plate can only be stored at 4°C for a few weeks. Hence, bacterial glycerol stocks can be prepared to preserve the transformed cell. The glycerol prevents cell membranes damages by stabilizing the frozen bacteria and keeping the cells alive at the dormant state (Morrison 1979). The bacterial stock can be stored at -80°C for many years. The plasmid collected was purified to remove any contaminants that would interfere sequencing.

4.3.8 Sequencing of plasmid

Samples of fruit bromelain PCR fragment; F2, F3, F5 and F6 was subjected for sequencing using the T7 and SP6 Polymerase universal primers. From the order of nucleotides of fruit bromelain gene results, a new search was made to identify all known fruit bromelain gene sequences in the NCBI database. The NCBI BLASTX analysis indicated that the fruit bromelain gene from the sequencing is homologous to bromelain (*Ananas comosus*) at a close identity (99%) and the highest query coverage (99%).

Figure 4.7 The NCBI BLASTX analysis from the purified plasmid of fruit bromelain from the order of nucleotide generated by sequencing

Therefore, a sequence comparison analysis was carried out to identify which sequence is entirely opposite to each other. From the analysis, the coded region translates 352aa of fruit bromelain which is located at 9 to 1038 in bromelain gene. Based on the multiple sequence alignment result the two sequences shared almost a similar protein encoded region. However, there is a minor alteration in the coding sequence (Figure 4.8).

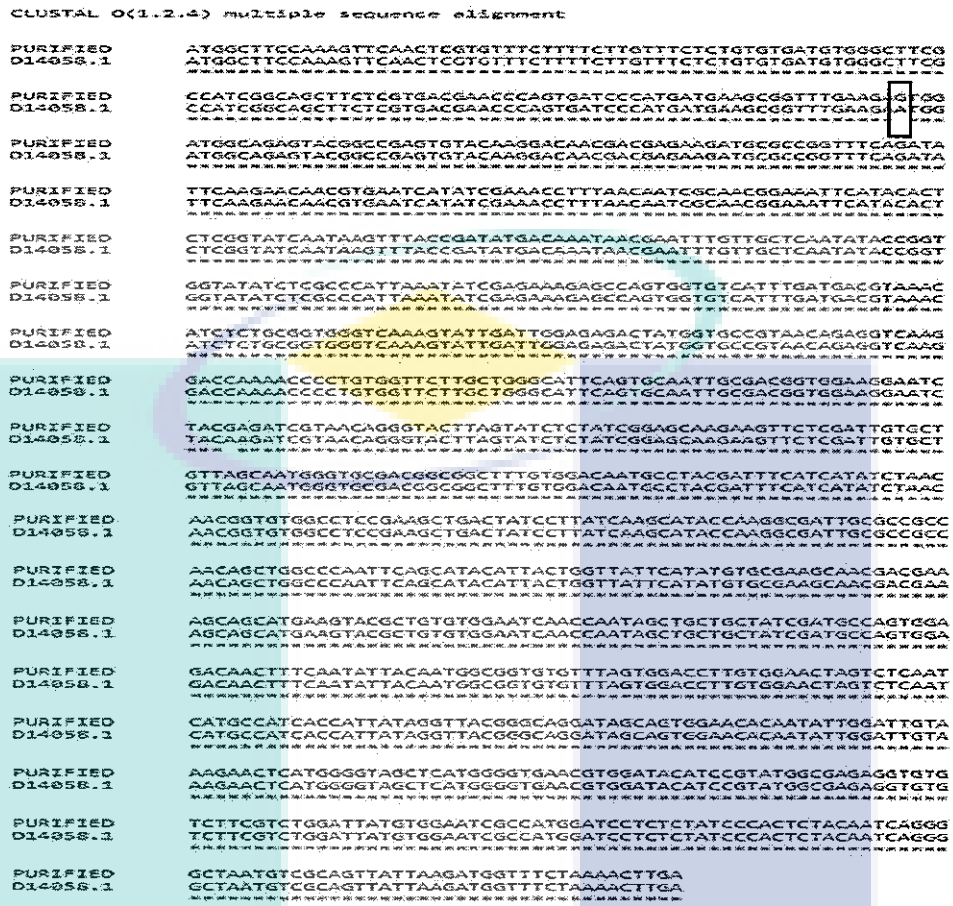


Figure 4.8 The multiple sequence alignment of purified fruit bromelain with the nucleotide sequence obtained from the NCBI BLASTX analysis. The coding sequence alteration is indicated in the box.

4.4 Data mining of fruit bromelain and stem bromelain using bioinformatics tools

GenBank is a database system responsible to store and assign the submission of nucleotide and amino acid sequence with no cost over the internet prior to any publication. Accessible through the NCBI (www.ncbi.nlm.nih.gov/) server, it provides and encourage access within the scientific community to the most up to date and comprehensive DNA sequence information by data exchange with European Nucleotide Archive (ENA) and the DNA Data Bank of Japan (DDBJ) (Benson, Cavanaugh et al. 2017). Over the pass decades, the database manage to receive thousands of sequence data submission leading to an extensive growth in the database (Pina-Martins and Paulo 2016).

Bromelain is a proteolytic enzyme made up from endopeptidases, glycoproteins, and carbohydrates that catalyse peptide bond from the polypeptide chain by hydrolysis. It was reported that the read length of bromelain starts from 250 to 360 amino acids. The sequence retrieval of bromelain from the NCBI database found out there are 31 amino acid sequences of bromelain from *A. comosus (Table 4.6)*. Regardless of that, 8 out from 31 amino acid sequence has been discard away due to the read length sequence which is less than 200 or more than 360 of amino acid respectively. The amino acid sequence accession number are OAY85828, OAY80102, OAY83410, OAY80099, OAY76881, OAY67114, OAY85856 and OAY85857. Thus, only 23 amino sequences has been selected to be further evaluated with 4 of them is known as stem bromelain and the rest is considered as fruit bromelain. Yet, most of the bromelain amino acid sequences retrieved in the database remains unpublished in any publication. Table 4.6 shows the summary of the bromelain sequences available in the NCBI database.

There are 19 amino acid sequences of fruit bromelain was found, the sequences accession number are AEH26024, AGC54590, AGS78388, BAA21929, BAA21848, BAA22544, BAA22546, BAA22545, BAA22543, O23791, OAY85858, OAY85826, OAY71019, OAY68894, OAY68270, OAY68854, OAY68387, OAY65848 and OAY62650 respectively. Commonly, a

library of newly determined sequences is compared to an existing protein sequence database using a tool such as BLAST. Each sequence in the library is compared to the database, and the annotation for the closest homologue found is transferred to that of the gene for the query sequence. From the NCBI BLASTP analysis, every amino acid was likely related to each other based on their percentage identity. Most of the fruit bromelain percentage identities is in the range of 90% to 100% to other bromelain as shown in Table 1. For example bromelain with the accession number of BAA21849.1 show 100% sequence identity to O23791. Other than that amino acid sequence of OAY68854 is 100% similar to OAY85857. Fruit bromelain of BAA21848 showed 100% sequence identity with OAY85826, BAA21929 and ADY68475. However for BAA21929 and ADY68475 the coverage of the sequence alignment is only 88% and 82%, respectively.

For stem bromelain, 4 sequences have been identified that are ADY68475, P14518, BAA21849 and CAA08861. However, due to the 100% similarity of ADY68475 to fruit bromelain, BAA21848, the sequence under this accession number was excluded from stem bromelain group. Although BAA21849 has been identified as fruit bromelain in NCBI GenBank database, this sequence has been reported to be isolated from stem bromelain and produced in recombinant form (Amid, Ismail et al. 2011). Therefore, in this study the sequence under accession number of BAA21849 has been classified as stem bromelain.

Table 4.6 Summary of bromelain sequence from Genbank NCBI

No.	Type of bromelain	Accession No.	Read length	% identity	Reference
1.	Fruit bromelain	OAY71019	326	95% OAY76881.1 (fruit bromelain)	NA
2.	Fruit bromelain	OAY68894	326	75% OAY68891.1 (fruit bromelain)	NA
3.	Fruit bromelain	OAY68854	359	100% OAY85857.1 (fruit bromelain)	NA
4.	Fruit bromelain	OAY85858	351	98% BAA21848.1 (bromelain)	NA
5.	Cysteine peptidase	AEH26024	352	97% OAY85857.1 (fruit bromelain)	(Wang, Zhang et al. 2014)
6.	Fruit bromelain	OAY68270	319	99% OAY85857.1 (fruit bromelain)	NA
7.	Bromelain	BAA21929	312	100% BAA21848.1 (bromelain)	NA
8.	Bromelain	BAA21848	352	100% OAY85826.1 (100% recovery) 100% ADY68475.1 (82% coverage) 100% BAA21929.1 (88% recovery) 98% OAY85858.1 (fruit bromelain)	NA
9.	Stem bromelain, partial	ADY68475	291	100% BAA21848.1 (bromelain)	(Tap, Majid et al. 2016)
10.	Stem bromelain	P14518	212	94% OAY80104.1 (Ananain)	(Sekhar et al. 2012)(Ritonja, Rowan et al. 1989)
11.	Bromelain	BAA21849	351	100% O23791.1 (fruit bromelain, precursor) 98% OAY85857.1 (fruit bromelain)	(Ritonja, Rowan et al. 1989, Muntari, Amid et al. 2012)
12.	Cysteine proteinase precursor	CAA08861	357	97% AGS78388.1 (bromelain)	(George, Bhasker et al. 2014)
13.	bromelain, partial [Ananas comosus]	AGC54590	241	99% BAA22546 (FB1035 precursor)	NA

				99% AEH26024 (cysteine peptidase) 99% OAY80102 (Fruit bromelain)	
14.	Bromelain [Ananas comosus]	AGS78388	355	97% CAA08861 (cysteine proteinase precursor, AN11)	NA
15.	Fruit bromelain [Ananas comosus]	OAY85856	1924	93% OAY68854 (Fruit bromelain)	NA
16.	Fruit bromelain [Ananas comosus]	OAY85828	170	99% OAY80099 (Fruit bromelain)	NA
17.	Fruit bromelain [Ananas comosus]	OAY85826	352	100% BAA21848 (bromelain) 100% BAA21929 (bromelain) 100% ADY68475 (stem bromelain)	NA
18.	Fruit bromelain [Ananas comosus]	OAY83410	1530	79% OAY85856 (Fruit bromelain)	NA
19.	Fruit bromelain, partial [Ananas comosus]	OAY80102	714	99% BAA22546 (FB1035 precursor) 99% OAY68270 (Fruit bromelain)	NA
20.	Fruit bromelain [Ananas comosus]	OAY80099	170	99% OAY85828 (Fruit bromelain)	NA
21.	Fruit bromelain [Ananas comosus]	OAY76881	666	95% OAY71019 (Fruit bromelain)	NA
22.	Fruit bromelain, partial [Ananas comosus]	OAY68387	258	97% OAY62650 (Fruit bromelain)	NA
23.	Fruit bromelain, partial [Ananas comosus]	OAY67114	150	-	NA
24.	Fruit bromelain [Ananas comosus]	OAY65848	302	-	NA
25.	Fruit bromelain [Ananas comosus]	OAY62650	351	97% OAY68387 (Fruit bromelain)	NA
26.	FBSB precursor [Ananas comosus]	BAA22544	356	99% OAY80104 (Ananain)	NA
27.	RecName: Full=Fruit bromelain; AltName: Allergen=Ana c 2; Flags: Precursor	O23791	351	98% OAY85857 (Fruit bromelain) 98% OAY80102 (Fruit bromelain)	NA

				98% BAA22546 (FB1035 precursor) 98% OAY68270 (Fruit bromelain) 98% AGC54590 (bromelain)	
28.	FB1035 precursor, partial [Ananas comosus]	BAA22546	324	99% OAY80102 (Fruit bromelain)	NA
29.	FB22 precursor, partial [Ananas comosus]	BAA22545	340	99% OAY85858 (Fruit bromelain)	NA
30.	FB31 precursor [Ananas comosus]	BAA22543	352	100% BAA21848 (bromelain) 100% BAA21929 (bromelain) 100% ADY68475 (stem bromelain)	NA
31.	Fruit bromelain [Ananas comosus]	OAY85857	721	100% OAY68854 (Fruit bromelain)	NA

*NA: Not Available

The MEROPS (https://www.ebi.ac.uk/merops/) database assign proteinase of common ancestry into clans, the highest level of evolutionary ancestor, and families (Barrett, Rawlings et al. 2001). In each clan, proteases are grouped together according to similar tertiary structure (protein fold), specific mechanism and catalytic residue order despite of limited sequence similarity (Rawlings 2010). Subsequently, within each clan, proteases are divided into families that are homologous. In this database, 68 families of cysteine proteinases (EC 3.4.22) were registered in the MEROPS database. They are grouped into ten clans where papain family become the most studied cysteine proteinase which classified in the clan CA and family C1 (peptidase family C1).

The peptidase family C1 contain many endopeptidases and several exopeptidases which are relatively small proteins (Mr values in the range of 20,000–35,000) comprising other related plant proteinases, for example both stem and fruit bromelain including bromelain inhibitor (bromein), chymopapain, caricain, actinidin, arcain, ananain, comosain, and many more (Turk, Turk et al. 1996, Rawlings, Barrett et al. 2010). Ever since 1996, a collection of cleavages in substrate of related proteinase has been deposited in the MEROPS database. The action of peptidase towards amino acid, peptides and synthetic substrate can be elucidated accordingly based on their biochemical interaction (Rawlings 2016). Exopeptidases were found to cleave the amino or carboxy terminal of the peptide bond, whereas endopeptidases prefer peptide bonds distant from the N- or C-terminal. Basically, cysteine protease can be found in most organisms. The evolutionary tree in the MEROPS database showed that family C1 is divided into two subfamilies of C1A and C1B that is related to papain and bleomycin hydrolase subfamily, respectively. The catalytic mechanism of proteases is classified into the following six types: aspartic, cysteine, glutamic, metallo, serine, and threonine. Therefore, there will be different types of proteases with different action mechanisms and biological processes. Most of the catalytic residue from family C1 belongs to cysteine. Table 4.7 shows examples of catalytic residues from peptidase family C1 in the MEROPS database.

Table 4.7 The clans and family of cysteine protease

Clan	Family	Subfamilies	Peptidase	Catalytic residue
CA	C1	C1A	Papain	Cysteine
			Peptidyl-dipeptidase	
			Aminopeptidase	
			Carboxypeptidase	
		C1B	Bleomycin hydrolase	

4.4.1 Amino acids and domain analysis of fruit bromelain and stem bromelain

Protein is made up of amino acids (Wahl and Holzgrabe 2016). The side chain (R group) of each 20 distinct amino acid marks the chemical properties of each protein. Various structural and computational methods have been developed to determine the properties of protein. The method includes using bioinformatics tools to establish knowledge regarding the protein of interest. The physicochemical characteristics of pineapple endopeptidases are summarized in Table 4.8. Stem bromelain and fruit bromelain is a mixture of different sulfhydryl proteases (thiol endo-peptidases) and several other composition (Ramalingam, Srinath et al. 2012). From the table, the molecular weight of stem bromelain is large, 23.40–35.73 kDa compared to fruit bromelain, 25-31.00 kDa, Besides that, the isoelectric point of stem and fruit bromelain is at 9.55 and 4.6, respectively (Yamada, Takahashi et al. 1976). Commonly most of the enzyme at higher temperature of up to 60°C are destroyed or denatured (Martins, Rescolino et al. 2014). Unlike the other enzyme, fruit bromelain and stem bromelain can retain its proteolytic activity at this range. The optimal temperature for the proteolysis of bromelain varies from 35°C to 60 °C on certain condition. However, when exposed to temperatures normally applied in pasteurization at 72°C, it becomes inactive and its thermal denaturation of the enzyme is irreversible (Novaes, Jozala et al. 2016). Furthermore, bromelain activity remains active in both acidic and alkaline environment. Biochemical studies indicate that bromelain has broad spectrum enzyme activity over an optimum pH range of 5.0 to 8.0 (Manzoor, Nawaz et al. 2016). Fruit bromelain is an acidic protein whereas stem bromelain is more alkaline and larger than papain. In contrast to fruit bromelain, the higher carbohydrate moiety of stem bromelain contribute towards its functional stability at alkaline pH (Kaur, Kaur et al. 2015).

Both enzyme of fruit and stem bromelain favourably cleaves glycyl, alanyl, and leucyl peptide bonds (Maurer 2001). Fruit bromelain displays certain kinetic characteristics that distinguish it from stem bromelain. From the kinetic data obtained with synthetic protease substrates of Bz-Phe-Val-Arg-pNA and Z-Arg-Arg-pNA, both bromelain displays a distinct substrate-specificity profile. The presence of p-nitroaline (pNA), a fluorescence biomarker after proteolysis can be detected either fluorescently or colorimetrically. Fruit bromelain prefer Bz-Phe-Val-Arg-pNA than Z-Arg-Arg-pNA whereas stem bromelain is vice versa.

According to Arroyo-Reyna, Hernandez-Arana et al. (1994), the specificity profiles of stem bromelain are closely matched comosain which also be found in the pineapple respectively. Nevertheless, there are slight differences in amino acid composition and kinetic specificity towards the epoxide inhibitor E-64 which was used in enzyme inactivation.

Table 4.8 Physicochemical properties of bromelain from pineapple, *A. comosus*

Bromelain type	Molar Mass (kDa)	pI	Presence of Glycoproteins	Protein type	Optimum pH	Substrate specificity
Stem bromelain	23.8–27	9.5	Yes	Basic protein	8	Z-Arg-Arg-pNA
Fruit bromelain	25–31	4.6	No	Acidic protein	5-6	Bz-Phe-Val-Arg-pNA

Further analysis regarding amino acid composition was performed to identify the relationship between stem and fruit bromelain. The amino acid composition data shown in Table 4.9 and Table 4.10 revealed minimal differences between fruit and stem bromelain obtained from ExPASy Protpram tool (Gasteiger, Hoogland et al. 2005). The most abundant amino acids constituent in both stem bromelain and fruit bromelain are alanine, glycine and serine while histidine is present in the lowest amounts. Alanine, glycine and serine are flavour amino acid, this amino acid constituent gives the sweet taste of stem bromelain and fruit bromelain (Nadzirah, Zainal et al. 2016). Despite of that, stem bromelain markedly contain more alanine than glycine while fruit bromelain contains less alanine than glycine. This observation was corroborated by the findings of Yamada, Takahashi et al. (1976), which reported the amino acid composition of alanine in fruit bromelain is smaller than glycine in contrast to stem bromelain. A similar finding was reported by Murachi (1964) using stem bromelain where the most abundant amino acids are alanine and glycine, while histidine and methionine are present in the lowest amounts. In addition, the composition of stem bromelain was also studied by Napper, Bennett et al. (1994) when they compare stem bromelain with ananain, and comosain. Stem bromelain appeared to differ somewhat from the fruit bromelain, particularly in the number of the polar amino acids, arginine and lysine,

and acidic amino acids, aspartate and glutamate, where the percentage of former is higher in the stem protein while the latter is more in fruit bromelain. This difference is reflected in the isoelectric points (pI value) of the two proteins. Protein pI is calculated using pKa values of amino acids and solely depends on its side chain. The stem enzyme is basic and has a pI value at about 9.5 (Murachi 1964) while the fruit enzyme is acidic with the pI value at about 4.6 (Ota, Moore et al. 1964). From Table 4.9 and Table 4.10, the theoretical pI value of fruit bromelain is within 4.6 to 5.6. Whereas, the theoretical isoelectric point of basic protein, stem bromelain is 8.32 and 8.60 for CAA08861 and P14518 respectively. Nevertheless, ADY68475 and BAA21849 has an isoelectric point of 4.41 and 5 close to the pI value of fruit bromelain (Table 4.10).

The presence of glucosamine in the stem enzyme and its absence in the fruit enzyme is another difference between those two enzymes as reported by Murachi (1964). The finding suggest stem bromelain as a glycoprotein (protein containing one or more covalently linked carbohydrates of various types) due to the present of four hexosamines, and 2.1 % carbohydrate in stem bromelain. Similarly, Ota, Moore et al. (1964) detected six glucosamines with 1.5 % carbohydrate in a purified stem bromelain. In an attempt to confirm the presence of a glycoprotein, gas chromatograpy (Murachi, Suzuki et al. 1967, Yasuda, Takahashi et al. 1970) and automated borate chromatography (Scocca and Lee 1969) examinations were performed. The studies reported that the present of mannose, fucose, xylose, and glucosamine in the ratio of 3:1:1:4 in stem bromelain using gas chromatography analysis, whereas using the second approach of chromatography, the same carbohydrate composition was obtained with a different ratio of 2:1:1:2. Yamada, Takahashi et al. (1976) reported that fruit bromelain is a simple protein with an acidic isoelectric point, while stem bromelain is a basic glycoprotein. Similarly, papain is basic protein, but is a simple protein. This data is contradicted to the finding of Ota et al that fruit bromelain was contained firmly bound carbohydrates (3% neutral sugars). Yamada et al have established, however, with their highly purified preparation that fruit bromelain (FA2) is not a glycoprotein but a simple protein having a molecular weight of approximately 31,000. In this study, the analysis of glycosylation site has been performed using NetNGlyc 1.0 server (Blom, Sicheritz-Pontén et al. 2004). Glycosylation refers to post translation modifications of proteins. It plays role in multiple protein function such as protein folding, interaction, stability, and mobility, as well as in signal transduction (Roth, Yehezkel et al. 2012). From the analysis it was revealed that all fruit and stem bromelain contain 1-2 sites of glycosylation except for AGS78388 (fruit),

OAY68894 (fruit) and CAA08861 (stem). This apparent discrepancy seemed to require further investigation.

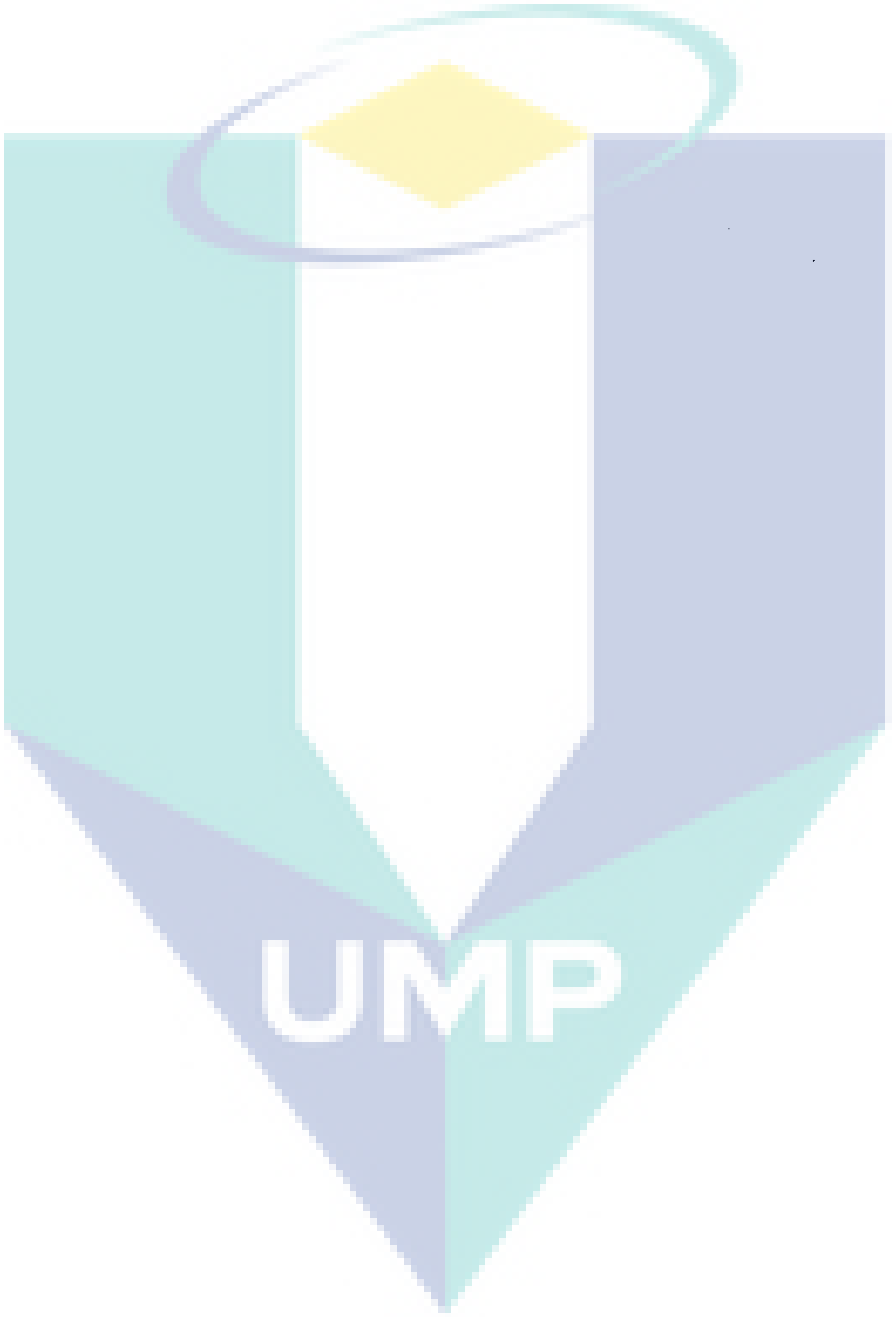


Table 4.9: Amino acid composition of fruit bromelain

Accession number	AEH 26024	AGC 54590	AGS7 8388	BAA 22543	BAA 22545	BAA 22546	OAY 68270	BAA 22544	BAA 21848	BAA 21929	O237 91	OAY 71019	OAY 68894	OAY 68854	OAY 62650	OAY 68387	OAY 65848	OAY 85858	OAY 85826
Ala (A)	7.4%	8.3%	7.9%	8.2%	7.6%	6.8%	6.6%	9.3%	8.2%	8.0%	7.7%	8.3%	8.6%	7.5%	8.5%	9.3%	9.6%	8.3%	8.2%
Asn (N)	7.7%	7.5%	6.5%	7.7%	7.6%	8.3%	8.5%	7.0%	7.7%	8.7%	7.4%	5.5%	5.5%	7.0%	7.4%	7.8%	5.6%	7.7%	7.7%
Asp (D)	4.0%	3.7%	4.5%	6.0%	6.2%	4.0%	3.8%	4.2%	6.0%	6.1%	4.3%	5.5%	5.2%	4.7%	4.3%	5.0%	4.6%	6.0%	6.0%
Cys (C)	2.3%	2.9%	2.5%	2.3%	2.4%	2.2%	2.2%	2.2%	2.3%	2.2%	2.3%	1.8%	2.5%	1.9%	2.3%	2.3%	2.3%	2.3%	2.3%
Gln (Q)	3.7%	3.7%	4.2%	3.7%	3.5%	3.7%	3.8%	3.1%	3.7%	3.8%	3.4%	3.1%	4.0%	4.2%	3.7%	4.3%	3.6%	3.7%	3.7%
Glu (E)	5.7%	4.6%	4.5%	4.5%	4.7%	6.2%	6.0%	5.6%	4.5%	4.2%	6.0%	6.7%	7.4%	4.2%	3.1%	3.1%	5.0%	4.6%	4.5%
Gly (G)	8.5%	10.8%	7.6%	8.8%	8.8%	9.0%	9.1%	7.6%	8.8%	9.9%	8.0%	8.9%	8.3%	8.6%	8.8%	7.0%	9.6%	8.5%	8.8%
Ile (I)	6.0%	6.6%	6.5%	6.2%	5.3%	6.5%	6.3%	6.7%	6.2%	7.1%	5.7%	6.1%	4.9%	6.1%	6.8%	5.8%	4.6%	5.4%	6.2%
Leu (L)	3.7%	2.9%	3.9%	3.7%	4.1%	3.1%	3.4%	3.1%	3.7%	2.9%	4.3%	3.1%	5.5%	4.7%	5.1%	5.4%	4.3%	4.0%	3.7%
Met (M)	3.4%	2.1%	3.1%	3.1%	2.9%	3.1%	3.1%	3.1%	3.1%	2.2%	3.4%	2.8%	2.5%	3.6%	3.4%	3.5%	1.7%	3.1%	3.1%
Phe (F)	4.5%	2.9%	4.2%	4.3%	4.7%	4.0%	4.1%	4.5%	4.3%	3.5%	4.6%	4.9%	4.3%	4.5%	5.1%	5.4%	5.6%	4.6%	4.3%
Pro (P)	3.7%	3.7%	3.7%	3.4%	3.5%	3.7%	3.1%	3.9%	3.4%	2.9%	3.7%	3.7%	3.7%	3.3%	4.0%	4.3%	5.6%	3.4%	3.4%
Ser (S)	10.5%	11.6%	11.5%	10.2%	10.3%	9.9%	10.3%	9.3%	10.2%	9.9%	10.3%	8.0%	6.4%	10.0%	9.7%	9.7%	6.6%	10.3%	10.2%
Thr (T)	4.5%	4.6%	3.9%	4.0%	4.4%	4.9%	5.0%	3.9%	4.0%	4.5%	4.6%	5.2%	7.1%	5.8%	5.4%	5.4%	4.0%	4.0%	4.0%
Trp (W)	2.3%	2.5%	2.3%	2.6%	2.6%	2.2%	2.2%	2.5%	2.6%	2.2%	2.3%	3.1%	2.5%	2.2%	2.0%	1.6%	2.0%	2.6%	2.6%
Tyr (Y)	6.0%	7.1%	4.8%	6.0%	6.2%	6.5%	6.6%	5.1%	6.0%	6.7%	6.0%	5.5%	6.1%	5.8%	5.1%	5.8%	5.6%	6.0%	6.0%
Val (V)	7.7%	8.7%	8.2%	7.7%	7.6%	7.4%	7.5%	7.9%	7.7%	7.7%	7.7%	5.8%	6.4%	6.7%	6.3%	6.2%	7.6%	8.3%	7.7%
Lys (K)	3.7%	2.9%	4.2%	3.7%	3.2%	4.0%	3.8%	5.9%	3.7%	3.5%	4.0%	4.6%	5.2%	3.9%	4.3%	4.3%	6.3%	3.4%	3.7%
Arg (R)	4.0%	2.5%	5.1%	3.4%	3.5%	4.0%	4.1%	4.5%	3.4%	3.2%	4.0%	4.6%	3.1%	3.9%	4.0%	3.5%	4.0%	3.4%	3.4%
His (H)	0.9%	0.4%	0.8%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	2.8%	0.9%	1.1%	0.6%	0.4%	1.7%	0.6%	0.6%
(Asp + Glu)	34 (10%)	20 (8%)	32 (9%)	37 (11%)	37 (11%)	33 (10%)	31 (10%)	35 (10%)	37 (11%)	32 (10%)	36 (10%)	40 (12%)	41 (13%)	32 (9%)	26 (7%)	21 (8%)	29 (10%)	37 (11%)	37 (11%)
(Arg + Lys)	27 (8%)	13 (5%)	33 (9%)	25 (7%)	23 (7%)	26 (8%)	25 (8%)	37 (10%)	25 (7%)	21 (7%)	28 (8%)	30 (9%)	27 (8%)	28 (8%)	29 (8%)	20 (8%)	31 (10%)	24 (7%)	25 (7%)
Theoretical isoelectric point	5.15	4.66	7.48	4.67	4.56	5.05	5.08	8.00	4.67	4.64	5.00	5.41	4.74	5.65	8.32	5.86	8.06	4.61	4.67
Glycosylation site	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes

Table 4.10 Amino acid composition of stem bromelain

Accession number	ADY68475	BAA21849	CAA08861	P14518
Ala (A)	8.6%	7.7%	7.8%	11.8%
Asn (N)	8.6%	7.4%	6.4%	4.7%
Asp (D)	6.9%	4.3%	4.2%	3.8%
Cys (C)	2.4%	2.3%	2.2%	3.3%
Gln (Q)	3.8%	3.4%	3.9%	3.3%
Glu (E)	5.2%	6.0%	4.5%	4.2%
Gly (G)	7.9%	8.0%	7.8%	10.4%
Ile (I)	6.2%	5.7%	7.3%	8.0%
Leu (L)	3.4%	4.3%	3.9%	2.8%
Met (M)	2.7%	3.4%	3.1%	1.4%
Phe (F)	5.2%	4.6%	4.2%	2.8%
Pro (P)	3.4%	3.7%	3.6%	5.2%
Ser (S)	8.9%	10.3%	11.5%	8.0%
Thr (T)	3.8%	4.6%	3.6%	4.2%
Trp (W)	2.1%	2.3%	2.2%	2.4%
Tyr (Y)	6.2%	6.0%	5.0%	6.6%
Val (V)	7.6%	7.7%	7.8%	6.6%
Lys (K)	3.4%	4.0%	4.2%	7.1%
Arg (R)	3.1%	4.0%	5.3%	2.8%
His (H)	0.7%	0.6%	1.1%	0.5%
(Asp + Glu)	35 (12%)	36 (10%)	31 (9%)	17 (8%)
(Arg + Lys)	19 (7%)	28 (8%)	34 (12%)	21 (10%)
Theoretical isoelectric point	4.41	5.00	8.32	8.60
Glycosylation site	Yes	Yes	No	Yes

Proteins are often multi-domain of which it carried out different biological functions attributable to each different domain (Rawlings 2010). The NCBI Conserved Domain analysis revealed there are two domains of fruit and stem bromelain which is cathepsin propeptide inhibitor (I29) and peptidase C1A, papain C-terminal. Figure 4.9 shows that all fruit bromelain contain I29 domain at the N-terminal region followed by peptidase C1A domain at the C-terminal of polypeptide. In general, the length of a protein sequence is determined by its function and the wide variance in the lengths of an organism's proteins reflects the diversity of specific functional roles for these proteins. Despite of that, it is difficult to predict a short protein sequence on purely statistical grounds and is also less likely to have confirmatory homologies in other organisms. Stem bromelain also display the similar domain organization to fruit bromelain except for AGC 54590 and P14518 which lacking of I29 domain. Mostly, the I29 domain is located between amino acids number 1 to 100 of the N-terminal site.

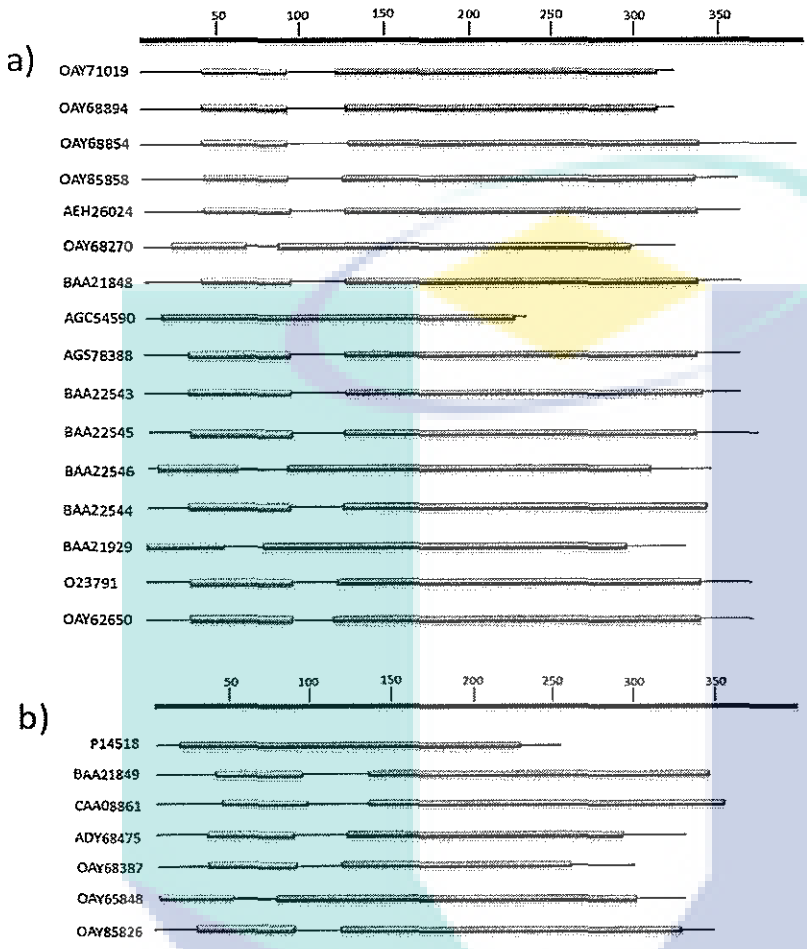


Figure 4.9 Domain organisation of (a) fruit bromelain and (b) stem bromelain.

Domain I29 (as classified by MEROPS) can be observed at the N-terminus of a protein precursor. This single or multiple domains can be found within protein as part of a large precursor protein. They can either become as a propeptide or as an N-terminal domain associated with an inactive peptidase or zymogen. The elimination of the N-terminal inhibitor domain either by interaction with a second peptidase or by autocatalytic cleavage will activates the inactive peptidase (Trejo, López et al. 2009). According to Rawlings, Tolle et al. (2004), the peptidase inhibitor reactive-site loop remains stable even after being cleavage by enzyme due to the presence of disulphide bonds. An uncontrolled proteolysis of proteins can be very damaging. In some case, the accumulation of product possibly leads to tissue necrosis. Hence, the action of inhibitor need to be taken to prevent the unwanted proteolysis at a wrong time and location

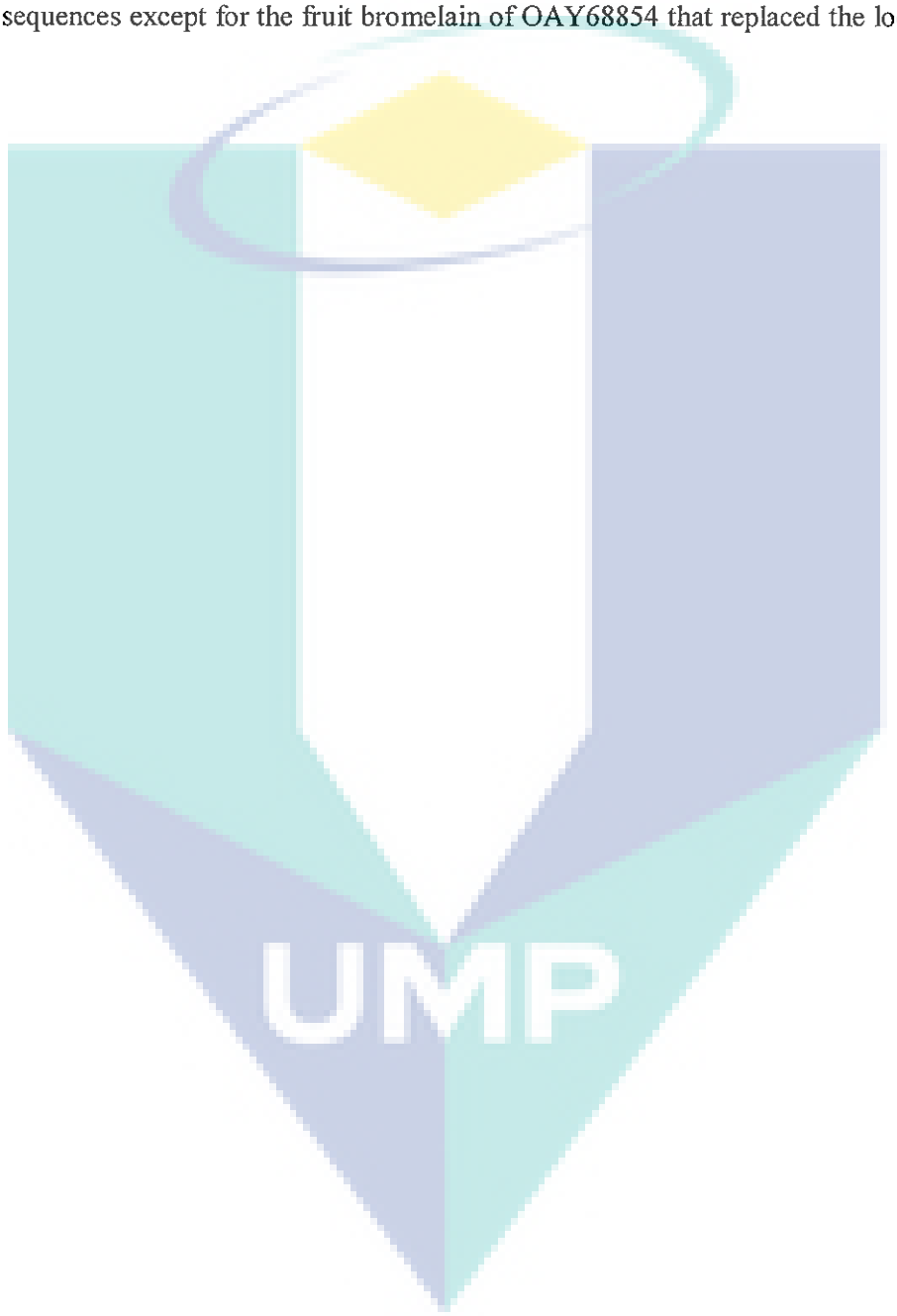
(Berg, Tymoczko et al. 2002, Rawlings 2010).. According to Beers, Jones et al. (2004) such mechanism helps to control various activity that take place in protein such as its half-lives, subcellular trafficking and others. Despite of that, very little information was made on the origin and evolution of these protein families in plants (Martinez and Diaz 2008).

Proteases are synthesized as inactive or less active precursor molecules in order to prevent such inappropriate proteolysis. They are activated by limited intra- or intermolecular proteolysis cleaving off an inhibitory peptide. Precursor are usually being processed in the secretory pathway that consequently be secreted out into the cytoplasmic matrix or become lysosomal or vacuolar in animal and plant systems, respectively. This propeptide mostly similar to the papain propeptide that are predicted to bind substrate in the reverse orientation thus inactivate the active site similar to the proenzymes (Rawlings and Barrett 2004). The characteristic element of the propeptide which are highly conserved in evolution is the GXNXFXD, seven amino acid linked by peptide bond or heptapeptide which is located at the kink of the β–sheet. This motif element can be found in most of the cysteine propeptides (Wiederanders 2003). Examination of the I29 motif using an amino acid sequence alignment of all fruit and stem bromelain showed that the GXNXFXD motif residues are conserved between all of the examined bromelain except OAY65848 as shown in Figure 4.10. From the OAY65848 sequence alignment, the amino acid G was replaced by A. This mutation may take place due to a change in one of the DNA base pair that results in the substitution of one amino acid for another in the protein made by a gene. For instance, mutations to hydrophilic residue may disrupt the bond and thus decrease the probability of the protein reaching its active conformation.

Figure 4.10 Alignments of amino acid sequences of fruit and stem bromelain. The conserved motif residues of GXNXFXD motif are indicated with box.

C1 Peptidase family (MEROPS database nomenclature), also referred to as the papain family; composed of two subfamilies of cysteine peptidases: C1A (papain) and C1B (bleomycin hydrolase). NCBI Conserved Domain analysis revealed that all the stem and fruit bromelain contains the signature domain of C1 Peptidase with the read length between 150 to 200 amino acids. All C1A proteins contain several disulphide bonds and share three conserved catalytic residues. The catalytic residues of C1 family peptidases are known as Asn, Cys and His, forming a catalytic triad (Cambra, Hernandez et al. 2012). The catalytic site is already preformed in the precursor. It is localized at the bottom of the active site cleft and involves the three residues mentioned above. The data of multiple sequence alignment of both stem and fruit bromelain showed that the catalytic residues of Cys and His are conserved between all of the examined bromelains as shown in Figure 4.11. In addition to Cys and His, the other two residues, Gln and Asn, were assumed to contribute a significant role in catalysis as well as maintaining an active enzyme conformation (Cambra, Hernandez et al. 2012). A Gln preceding the catalytic Cys, believed to aid in the formation of the oxyanion hole while an Asn residue orients the imidazolium ring of the catalytic His (Boudreaux, Chaney et al. 2012). Compare to Cys and His which were existed in all sequence of bromelain, the residues of Gln and Asn were not

thoroughly conserved in the sequence alignment. Some of the bromelain from fruit (OAY68894 and OAY71019) were found to have gap-containing segment that lack about 12 residues at the location where Gln reside. On the other hand, the residue Asn was conserved for all aligned bromelain sequences except for the fruit bromelain of OAY68854 that replaced the location with Tyr.



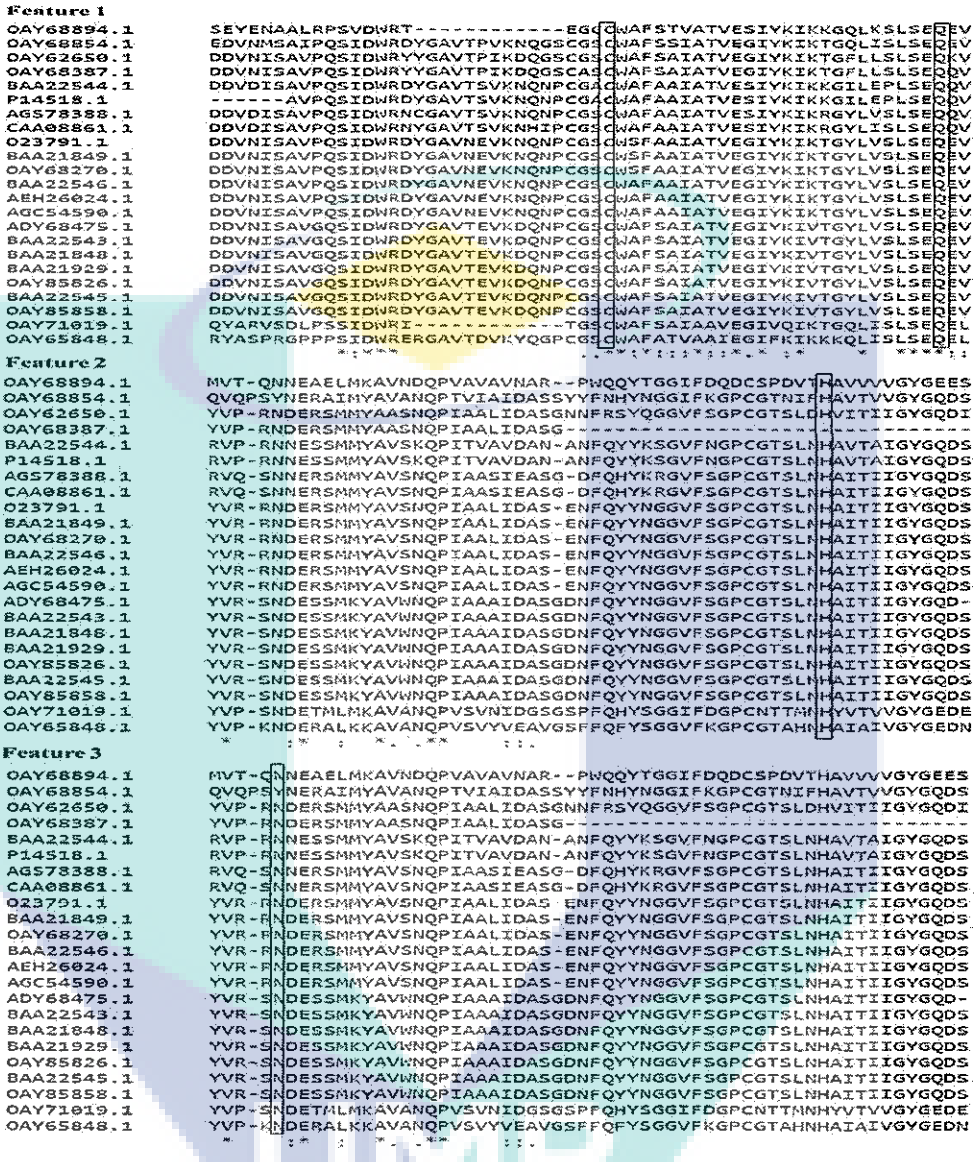


Figure 4.11 The sequence alignment of stem (ADY68475.1, P14518.1, BAA21849.1 and CAA08861.1) and fruit bromelain (OAY62650, OAY65848, OAY71019, OAY68894, OAY68854, OAY85858, AEH26024, AGS78388, OAY68270, BAA21929 and BAA21848) sequences from the NCBI Genbank Database. The catalytic residues of Asn (N), Cys (C), His (H) and Gln (Q) were boxed.

In general, cysteine-type peptidase is the peptidases in which the functional group of Cys residues, sulfhydryl group, acts as nucleophiles to attack the scissile peptide bonds (Verma, Dixit

et al. 2016). In addition to nucleophiles a general base (histidine) is also required in order for catalysis to occur (Figure 4.12). This catalytic mechanism of cysteine peptidases is similar to the group of serine-type peptidases (Vernet, Tessier et al. 1995). Moreover, it has been identified that mostly histidine residues become the common proton donor for cysteine peptidases. In some peptidases family, only the dyad of cysteine and histidine seem to be essential for catalysis. However, some of other family exhibited the requirement of third residue to orientate the imidazolium ring of the histidine (Barrett, Woessner et al. 2012).

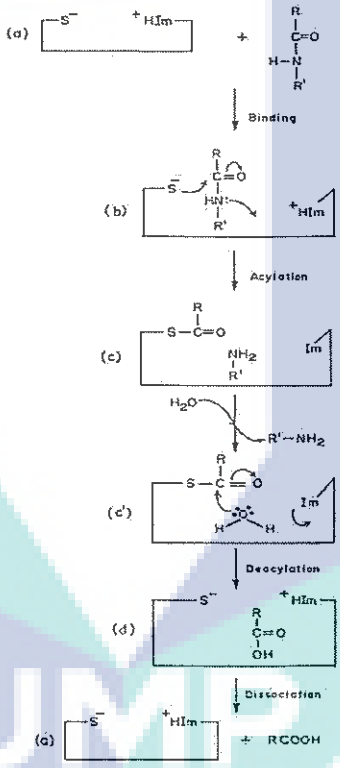


Figure 4.12 The catalytic mechanism of cysteine protease. Im and +HIm refer to the imidazole and protonated imidazole, respectively.

4.5 Structural comparison of fruit bromelain and stem bromelain

The similarities and differences in fruit and stem bromelain can be determined using the structural comparison method. There are various method can be used to analyse the structural similarities and differences between fruit and stem bromelain (Bourne and Shindyalov 2003).

4.5.1 Secondary and tertiary structure analysis

There are two general techniques used to predict protein. The techniques include experimental and computational methods. In general, protein structure can be obtained by X-ray crystallography and multi-dimensional magnetic resonance in laboratory, and these experimental methods can get accurate protein structure information with high precision; however, these method have the disadvantages of extremely difficult, cost prohibitive, time consuming and limited molecular weight. Consequently, the experimental methods apparently do not cope with the challenge of the rapidly growing protein sequences data. Computational methods for protein structure prediction is one of the most important and effective technologies in the emerging interdisciplinary field of bioinformatics due to the rapid advances of protein sciences, and it has the characteristics of simple, low cost and fast speed, which can overcome the disadvantage of experimental methods (Venkatesan, Gopal et al. 2013).

Primary sequence is used to predict the secondary structure of protein by predicting the formation of protein structure in alpha helix, beta strands and turns (Tuckwell, Humphries et al. 1995). Predictions were performed on a single sequences rather than families of homologous sequences, and there were relatively few known 3D structures from which to derive parameters. To elucidate and understand the characteristics of the bromelain, 3D structure models of bromelains from *A. comosus* were constructed. Up to now no solved structure were reported for both fruit and stem bromelain. For the structural analysis of fruit bromelain of BAA21848, the 3D model was built using the homology modelling approach, MODELLER9.17. Comparative modelling or homology modelling can help develop a useful 3D model for a protein's structure in the absence of an experimentally determined structure. The application of comparative modelling in cases with high sequence identities (more than 35 %) typically results in accurate models. Furthermore, for structural comparison between fruit and stem bromelain, a sequence representative of stem bromelain (CAA08861) was also selected for 3D model building.

Procaricain (PDB ID: 1PCI) is an inactive form of peptidase found in caricain from the latex of *Carica papaya* (Groves, Taylor et al. 1996). An alignment search made using BLASTP (NCBI) against the PDB database, revealed that BAA21848 and CAA08861 sequence exhibits low similarity with all solved 3D structures. The highest match for BAA21848 showing only 43% identity and 64% similarity to chain A of the 1PCI. In the meantime, the highest match percentage of CAA08861 was 43% identity and 65% similarity based on the solved 3D structure of chain A 1PCI respectively. A suitable template is needed to generate an optimum target-template alignment. In order to do that, a template searches were performed using several servers such as HHPRED, Phyre2, BLAST-PDB, PSI-BLAST and Mod-link+ (Table 4.11). The analysis revealed various potential templates for molecular modelling purposes. Among them was1PCI, which had the highest sequence identity (41%) and query coverage (90%) with fruit bromelain (BAA21848), using the HHPRED server. Similar finding was found for stem bromelain (CAA08861) in which 1PCI showed the highest sequence identity (42%) and query coverage (89%). 1PCI was then selected as a template to build the 3D structure of fruit bromelain and stem bromelain. To further assess the reliability of the structural sequence alignment between BAA21848 and CAA08861 to 1PCI, consensus secondary structure prediction was used to confirm the alignment. This was achieved by comparing the aligned secondary structure of 1PCI and the consensus predicted secondary structure of BAA21848 and CAA08861. The structural alignment indicates a good alignment of 10 α-helices and 6 β-strands between BAA21848 and CAA08861 with the template structure of 1PCI. The rest of the protein regions are shown to consist of random coils and a few structural mismatches and gap-containing segments.

Table 4.11 Predicted templates for fruit and stem bromelain obtained from different servers along with their percentages of identity and query coverage. 1PCI appeared in the all of the results given and was chosen as the model template for BAA21848 and CAA08861.

Server	Template	Protein characteristics	Fold	Identity (%)	Query coverage (%)
Fruit bromelain (BAA21848)					
HHPRED	2COY	Procathepsin S	hydrolase, cysteine protease	37	90
	1PCI	Procaricain	hydrolase, cysteine	41	90

			protease		
	1BY8	Procathepsin K	hydrolase, sulfhydryl proteinase	36	89
Phyre2	5EGW	Cysteine protease	hydrolase, cysteine protease	39	91
	5EF4	Cysteine protease	hydrolase, cysteine protease	39	88
	1PCI	Procaricain	hydrolase, cysteine protease	43	88
BLAST PDB	5EF4	Cysteine protease	hydrolase, cysteine protease	41	85
	3TNX	Thermostable papain	hydrolase, cysteine protease	42	86
	1PCI	Procaricain	hydrolase, cysteine protease	43	87
PSI-BLAST	4QRG	Crystal Structure of I86l Mutant of Papain	Hydrolase	42	86
	3TNX	Structure of The Precursor of A Thermostable Variant of Papain at 2.6 Angstroem Resolution	Hydrolase	42	86
	1PCI	Procaricain	Hydrolase	43	87
Mod-link+	1S4V	Vignain	Cysteine proteinases	53	68
	1O0E	Ervatamin C	Cysteine proteinases	53	66
	1PCI	Caricain	Cysteine proteinases	41	62
Stem bromelain (CAA08861)					
HHPRED	1PCI	Procaricain	hydrolase, cysteine protease	42	89
	2COY	Procathepsin S	hydrolase, cysteine protease	36	87
	1BY8	procathepsin K	hydrolase(sulfhydryl proteinase)	35	87
Phyre2	1PCI	Procaricain	hydrolase, cysteine protease	42	86
	5EGW	Cysteine protease	hydrolase, cysteine protease	39	88
	3TNX	Papain	hydrolase, cysteine protease	41	87
BLAST PDB	1PCI	Procaricain	hydrolase, cysteine protease	43	86
	4QRG	Papain	hydrolase, cysteine protease	43	85
	3TNX	Papain	hydrolase, cysteine protease	43	85
Mod-link+	1PCI	Caricain	Cysteine proteinases	40	62
	1IWD	Ervatamin B	Cysteine proteinases	52	69
	1O0E	Ervatamin C	Cysteine proteinases	50	62

PSI-BLAST	3TNX	Papain	Hydrolase	43	85
	4QRG	Crystal Structure of I86l Mutant of Papain	Hydrolase	43	85
	1PCI	Procaricain	Hydrolase	43	86

Comparative protein modelling method was designed to find the most probable structure for a sequence given its alignment with related structures. The best alignment results from HHPRED program were used to build models with MODELLER program, which is an automated program of comparative modelling. This program basically performs by satisfying all spatial restraints derived from the structure-sequence alignment. Models that produced high violations of the restraints lead to higher objective functions (calculated by CHARM-22 force field) and will be considered as poor models. As a consequence, only the model with the lowest objective function will then be selected. The resulting model was subjected to energy minimization using the steepest descent algorithm as implemented in GROMOS from Deepview to avoid poor molecular contacts.

Preliminary model of fruit bromelain generated by the MODELLER has a high energy level of -1610.640 kcal/mol so do stem bromelain which has an energy level of -4110.637 kcal/mol respectively. The fruit bromelain and stem bromelain model was then subjected to side-chain and loop refinement which decreased the energy level to -13409.075 kcal/mol and -15172.673 kcal/mol, respectively. Normally this energy level is considered too high and due to this reason, a series of energy minimization steps are required for the model to reach a local or more preferential global minimum level of total energy (Crivelli, Eskow et al. 2002). In silico, protein structures are more stable in low energy level (Alberts, Johnson et al. 2002). After three rounds of minimization processes, the total energy of fruit bromelain and stem bromelain model was reduced to -16101.752 kcal/mol and -17900.148 kcal/mol, respectively.

For model validation and assessment, the quality of the refined model was assessed using the programs of ERRAT, RAMPAGE and VERIFY3D (Table 4.12). Generally, VERIFY3D works based on the compatibility of an atomic model (3D) with its own amino acid sequence (1D) (Jitendra and Vinay 2011). On the other hand, the overall quality factor for non-bonded

atomic interactions will be examined by the ERRAT program. For model validation and assessment of BAA21848, the resulted Ramachandran plot derived from RAMPAGE analysis before energy minimization shows that 91.7% of the residues were located in the favoured region and the remaining residues resided in the allowed (6.0%) and outlier (2.3%) regions. The VERIFY3D analysis indicates that 82.1% of the residues have an average 3D–1D score greater than 0.2, indicating that only 17.9% of the residues did not complement the 3D–1D profile. A VERIFY3D score of greater than 80 % indicates that the predicted model is of satisfactory quality. In addition, the model assessment using ERRAT program provides a score of 69.578%. The ERRAT program is used to inspect the total quality factor for non-bonded atomic interactions of the structure. A higher score of ERRAT means a higher model quality and the normally accepted range is >50% (Chaitanya, Babajan et al. 2010). The ERRAT score of the BAA21848 model indicates that the backbone conformation and non-bonded interactions of the model are all reasonable within a normal range.

On the other hand, the model validation and assessment of CAA08861, Ramachandran plot predicted that 91.0% of the residues are located in the favoured region and the remaining residues reside in the allowed (6.5%) and outlier (2.5%) regions. ERRAT and VERIFY3D program gave the score of 80.814% and 79.27%, respectively which indicate the acceptable quality for the predicted model. It can be observed from the validation programs, the score for fruit bromelain and stem bromelain models was slightly refined in the third minimizations process. No significant improvement in the VERIFY3D score was observed after the minimization steps. The minimization score of 81.25% suggests that the total amino acids in the fruit bromelain model were located in the theoretically correct positions and had an average 3D-1D score greater than 0.2. Only 18.75% of the structure was inaccurately predicted. Same goes to stem bromelain, the score value of VERIFY3D was 77.31%, with 22.69% prediction inaccuracy. The ERRAT plot detect geometrical errors in a protein structure using a nine residue sliding window approach and could suggest the overall quality factor for non-bonded atomic interactions, calculated by a comparison with statistics from highly refined structures. From the analysis, the ERRAT score for fruit bromelain and stem bromelain was 81.818 and 81.287, respectively. Based on the validation and assessment analysis, a reasonable predicted model of BAA21848 and CAA08861 were constructed. The geometric quality of the backbone

conformation, the residue interaction and the residue contacts of the structure were all well within the limits established for reliable structures. The resulting model after the third minimization step was used to elucidate the structure-function relationship of fruit bromelain and stem bromelain.

Table 4.12 Energy levels, ERRAT plot, VERIFY3D and RAMPAGE score for fruit bromelain and stem bromelain models at different stages.

Step	Structure energy level (kcal/mol)	Structure validation by ERRAT plot	Structure validation by VERIFY3D (%)	Structure validation by RAMPAGE (%)
Fruit bromelain (BAA21848)				
Model before energy minimization	-1610.640	69.578	82.10	Favoured: 91.7 Allowed: 6.0 Outlier: 2.3
Model after side-chain and loop refinement	-13409.075	76.923	81.25	Favoured: 91.4 Allowed: 5.1 Outlier: 3.4
Model after second minimization	-15287.221	80.062	80.40	Favoured: 91.4 Allowed: 5.7 Outlier: 2.9
Model after third minimization	-16101.752	81.818	81.25	Favoured: 91.4 Allowed: 6.0 Outlier: 2.6
Stem bromelain (CAA08861)				
Model before energy minimization	-4110.637	76.724	78.99	Favoured: 92.1 Allowed: 5.6 Outlier: 2.3
Model after side-chain and loop refinement	-15172.673	80.814	79.27	Favoured: 91.0 Allowed: 6.5 Outlier: 2.5
Model after second minimization	-17086.387	81.287	77.87	Favoured: 90.7 Allowed: 6.8 Outlier: 2.5
Model after third minimization	-17900.148	81.287	77.31	Favoured: 90.7 Allowed: 7.0 Outlier: 2.3

4.5.2 Structural analysis of fruit bromelain and stem bromelain

The validated models of BAA21848 and CAA08861 was finalised and shown in Figure 5. The general structures of the models show a typical papain family protein fold (Sekhar, Rajkeshwar et al. 2013). Similarly, the model of BAA21848 and CAA08861 consist secondary protein structure of 10 α-helices and 6 β-strands. From the observation, α-helices is rod in shaped and coiled forming a spring-like structure held by the hydrogen bonds while β-strands is connected laterally by two or more hydrogen bonds forming a backbone. In addition, like papain, the fruit and stem bromelain models of BAA21848 and CAA08861 are characterised by having two domain structures with a cleft between the two domains harbours the active site (catalytic pocket) where the substrate is bound. The predicted fruit bromelain and stem bromelain structures have an all-α-helix domain (domain I29) and an antiparallel β-sheet domain (domain peptidase C1) as shown in Figure 4.13.

a) b)

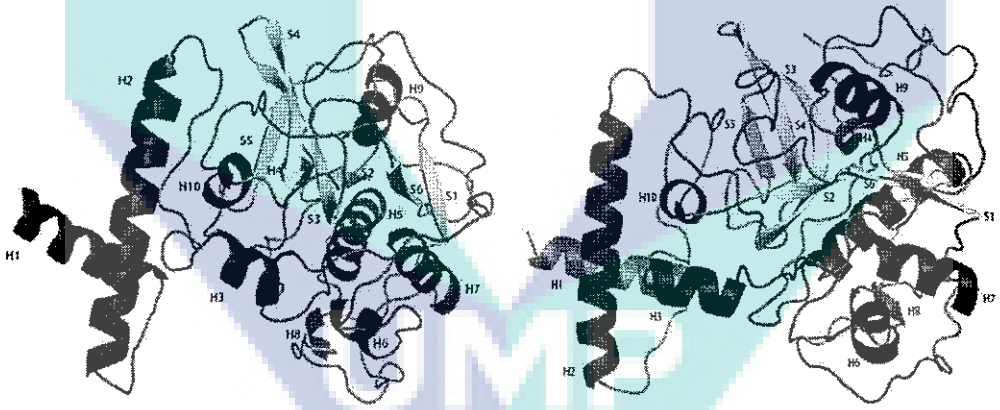


Figure 4.13 Three dimensional model of fruit bromelain, BAA21848 (a) and stem bromelain, CAA08861 (b) with the β-strands, S1 to S6 (yellow) and the α-helices, H1 to H10 (red).

In this study, Cys-148, His-281, Gln-174 as well as Asn-275 are the catalytic residues of BAA21848 which are anticipated to subsidise a significant role in catalysis. Meanwhile, CAA08861 model also possesses all the important residues at the active site, Cys-147 and His-281, which play crucial roles in the chemical catalysis as general acid/base catalysts together with the aid of His-141 and Asn-302. Although located far apart within the chain, these four

amino acid residues are in close proximity due to the folding structure as shown in Figure 4.14. The best characterised family of cysteine proteases, papain, is also having cysteine and histidine residues, Cys-25 and His-159, as the catalytic residues which are evolutionarily preserved in all cysteine proteases family. At first, Asp-158 was thought to play a role analogous to the role of aspartate in the serine protease catalytic triad, but that has since then been disproved (Menard, Khouri et al. 1990). However another one residue, Asn-175 was reported to aid in orientation of His-159 to allow the deprotonation of Cys-25 to occur. It is though these three amino acids working together in the active site that provides papain with its unique functions (Mamboya 2012).

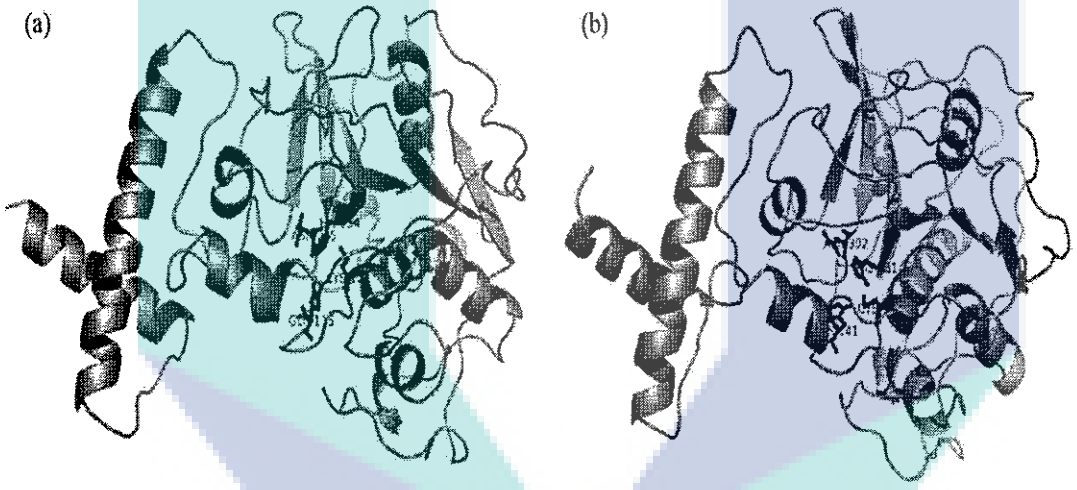


Figure 4.14 Substrate-binding site (a) fruit bromelain, BAA21848 (b) stem bromelain, CAA08861 using 1PCI as a template. Green region indicates domain I29 while orange region indicates domain peptidase C1. The catalytic amino acids of both models are represented as sticks.

Cysteine protease found in the latex of Carica papaya is known as caricain. Caricain is naturally expressed as an inactive zymogen called procaricain, 1PCI (PDB database). The 1PCI template consist of three non-crystallographically (Chain A, B and C) related molecules. This molecule forms a separate globular domain which binds to the C-terminal domain of a mature caricain. From the analysis, BAA21848 and CAA08861 is located in chain A of the 1PCI structure.

4.5.2.1 Structural characteristics comparison of BAA21848 and CAA08861

A protein comparison of unknown function with a set of well-characterized structures (known function) is made to check whether there are local similarities involving the known functional patches. Previously, the similar structure organization was reported for the structure of papain refined at 1.65 Å resolution (1CVZ) (Kamphuis, Kalk et al. 1984). The conformational behaviour of this solved structure has been investigated to show a high α-helical content and substantial secondary structure of ß-sheet in the molten globule state (pH 2.0) (Huet, Looze et al. 2006). The superimposition of the 3D BAA21848 model with the structure of 1CVZ (Figure 4.15 (a)) yielded a root mean square deviation (RMSD) of 0.437 Å that covered 66% of the backbone atoms, indicating a good overall structural alignment. The proposed catalytic residues for the modelled BAA21848 structures (Cys-148, Gln-174, His-281 and Asn-302) are represented as sticks and fall into approximately the same locations as in papain (1CVZ) catalytic residues (Cys-25, Gln-51, His-159 and Asn-175). In the meantime, the superimposition of the 3D CAA08861 model with the structure of 1CVZ (Figure 4.15 (b)) yielded an RMSD of 0.410 Å that covered 67% of the backbone atoms. The proposed catalytic residues for the modelled CAA08861 structures (Cys-147, Gln-173, His-281 and Asn-302) fall into approximately the same locations as in 1CVZ catalytic residues (Cys-25, Gln-51, His-159 and Asn-175). The catalytic residue location of Cys and Gln in BAA21848 and CAA08861 was nearly close to each other whereas His and Asn is similar. The conservation of the catalytic residues and the identical structural assignments in the modelling suggest that the modelled BAA21848 and CAA08861 of fruit and stem bromelain, respectively, are an accurate representation of the actual cysteine proteases protein structure.

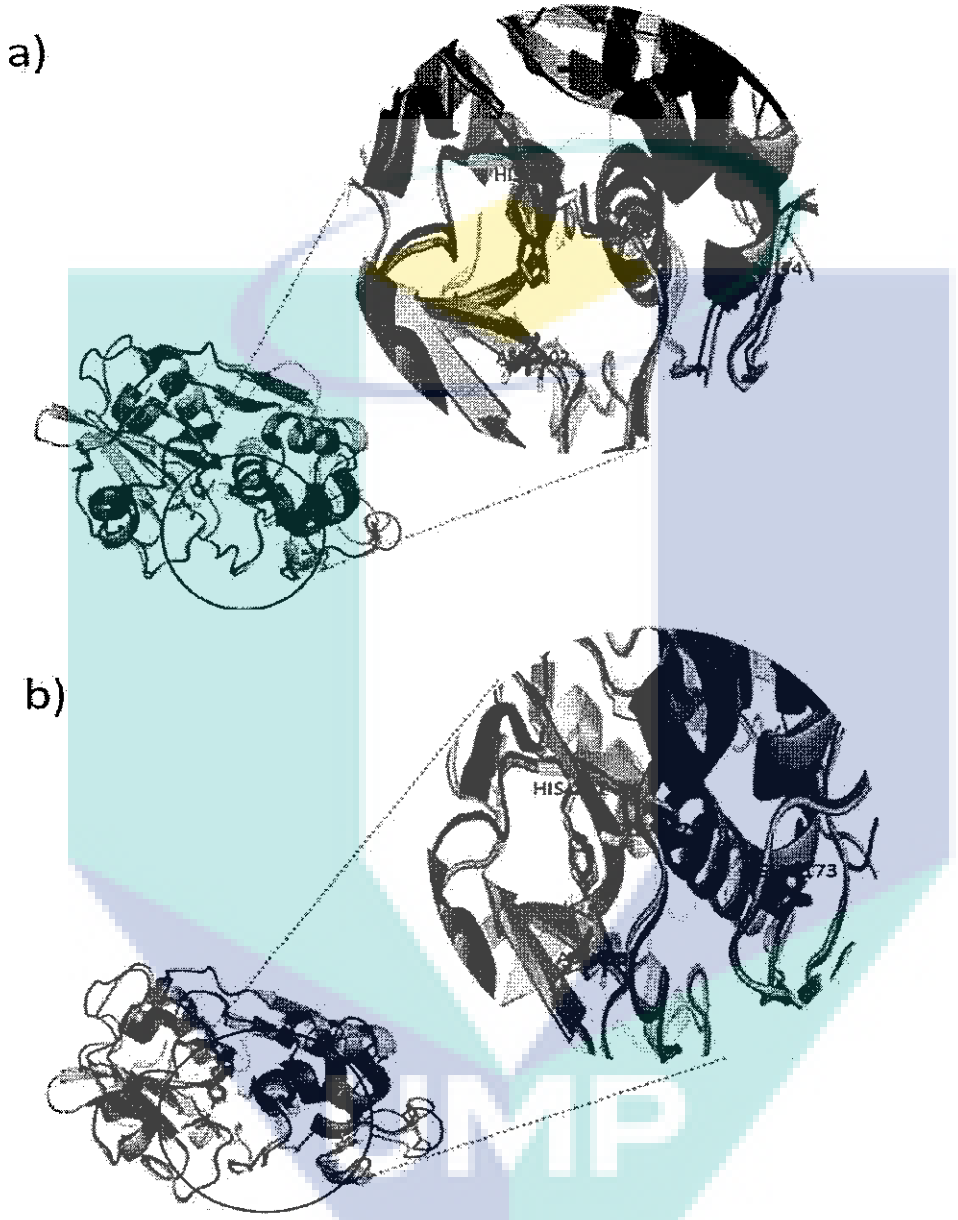


Figure 4.15 The 3D superimposition structure of BAA21848 and CAA08861 with 1CVZ. (a) The catalytic binding site (Cys-148, Gln-174, His-281 and Asn-302) of BAA21848 (green) with 1CVZ (purple). (b) The catalytic binding site (Cys-147, Gln-173, His-281 and Asn-302) of CAA08861 (green) with 1CVZ (purple).

The physiochemical properties of a single protein is determined by its side chain groups either it's hydrophobic (unfavourable with water), hydrophilic (favourable with water) or

charged. In general most peptidases of subfamily C1A, S2 subsite is a dominant subsite which demonstrates a preference for a bulky hydrophobic site chain binding rather than a charged one. Remarkably, the S2 subsite of stem bromelain has been found to efficiently catalyse only on Arg-Arg-containing synthetic substrate (Rowan, Buttle et al. 1988, Gosalia, Salisbury et al. 2005). The similar mechanism was found in several cysteine peptidases including cathepsin B protein and comasain (Napper, Bennett et al. 1994). This distinguishing specificity phenomenon can be elucidated by the residue lying at the bottom of the S2 pocket which has been replaced by charged residue. For example in stem bromelain the residue lying at the bottom pocket of S2 is aspartate while in cathepsin B is glutamate where at the same location in papain is occupied by serine. Therefore the convenient synthethic substrate for stem bromelain is a Z-Arg-Arg| NH-pNA which is scarcely affected by ananain or fruit bromelain. In addition, the relatively weak kinetics of E-64 inactivation and lack of inhibition by chicken cystatin distinguishes stem bromelain from fruit bromelain and most other peptidases of family C1. However, the significance of such differences will not become apparent until the complete structures are known.

Using the information from the papain crystal structure, residues important in substrate binding and specificity have been identified in papain including Cys-25, Gly-66, Trp-69, Asp-158, His-159 and Trp-177 (Drenth, Kalk et al. 1976). The equivalent residues in fruit bromelain are Cys-148, Gly-188, Asp-191, His-281, Asn-280 and Trp-304 and stem bromelain are Cys-147, Gly-187, His-281, Asn-190, Asn-280 and Trp-304 are shown in Figure 4.16. One notable difference observed as a residue of the S2 subsite in papain, Trp-69, is replaced by an aspartate, Asp-191 in fruit bromelain and an asparagine, Asn-190 residue in stem bromelain. The difference in charged side chain between aspartate and asparagine could contribute to the distinctive substrate specificities displayed by fruit bromelain and stem bromelain towards synthetic peptide substrates. Similar study of Lee et al (Lee, Albee et al. 1997), exhibited that ananain also replaced the hydrophobic residues at S2 subsite by asparagine which could indicate the similar action of ananain substrate specificities with stem bromelain of CAA08861.

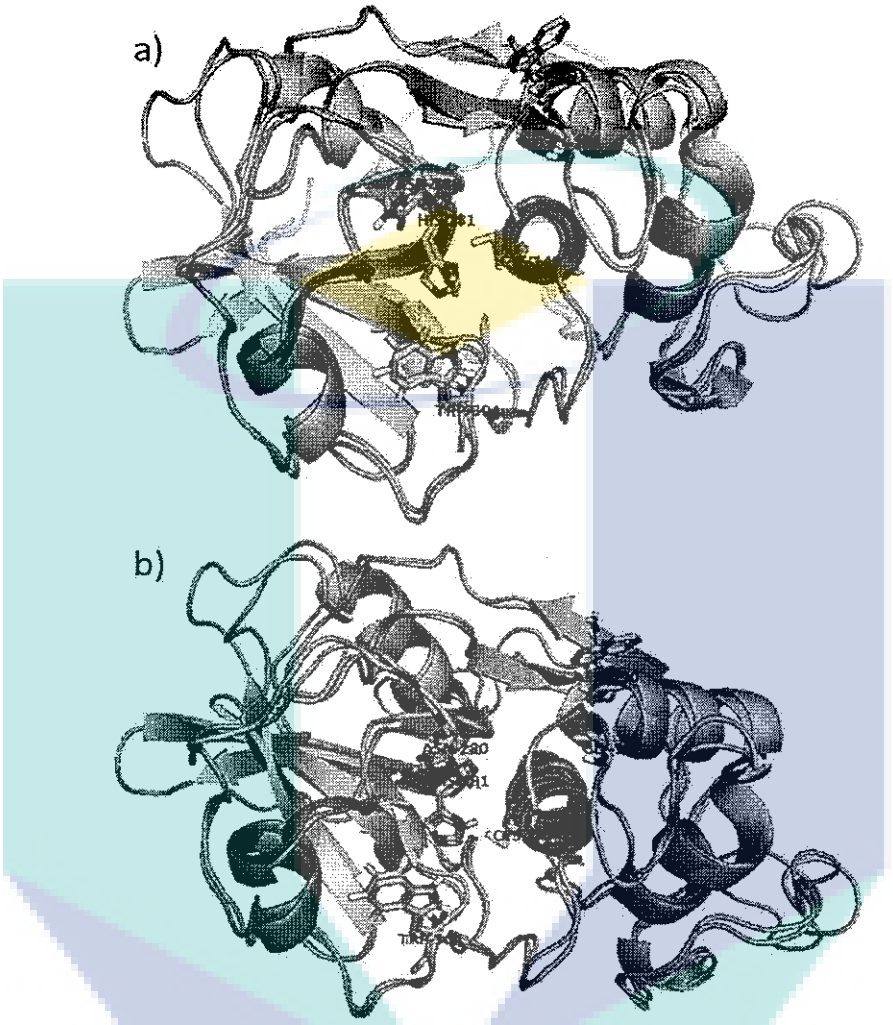


Figure 4.16 The 3D superimposition structure of BAA21848 and CAA08861 with the catalytic site of 1CVZ. (a) The catalytic binding site (Cys-25, Gly-66, Trp-69, Asp-158, His-159 and Trp-177) of 1CVZ (blue) after being superimposed with the catalytic site (Cys-148, Gly-188, Asp-191, His-281, Asn-280 and Trp-304) of BAA21848 (green) (b) The catalytic binding site (Cys-25, Gly-66, Trp-69, Asp-158, His-159 and Trp-177) of 1CVZ (blue) after being superimposed with the catalytic site (Cys-147, Gly-187, His-281, Asn-190, Asn-280 and Trp-304) of CAA08861 (green).

Generally, native proteins contain a hydrophobic core and a charged and/or polar group on the surface. The hydrophobic core aids in stabilizing the tertiary structure of the protein by providing hydrophobic interaction while the outer polar surfaces preferentially interact with the

exterior aqueous medium (Wang, Sun et al. 2006). Hydrophobic amino acid of papain in space fill model is shown in Figure 4.17.

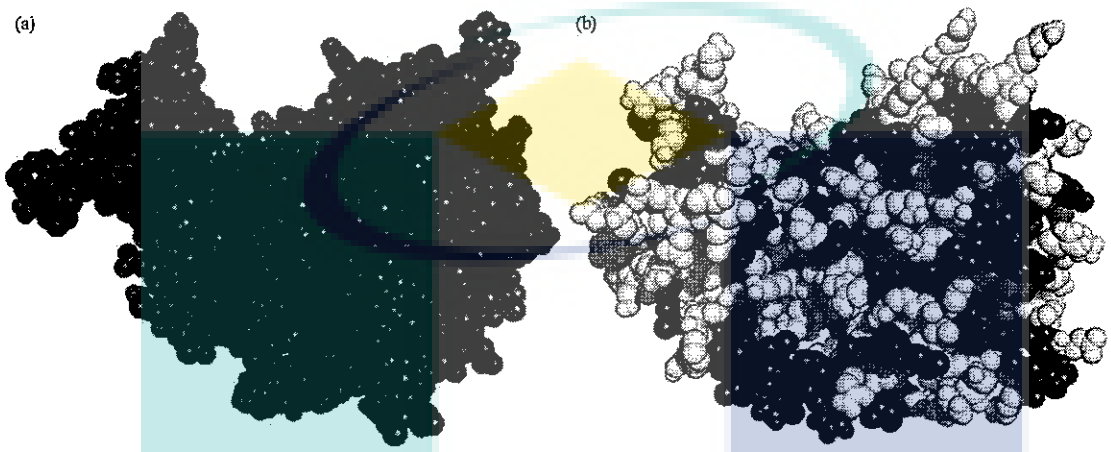


Figure 4.17 Hydrophobic amino acid of papain in space fill model (a) Fruit bromelain (b) Stem bromelain. Colored grey in a space fill model are the backbone oxygen and nitrogen of the residues with the hydrophobic side chain.

In addition, the hydrophobic-hydrophilic interactions of amino acids in the side chain contribute to the thermodynamic forces which drive protein folding. Besides, the hydrophobic effect is considered to be the major driving force for the folding of globular proteins. Therefore, it is often beneficial to investigate the relative hydrophobicity or hydrophilicity values of the amino acids in a protein sequence. Since hydrophobic residues tend to be buried in the interior of the protein away from the solvent while hydrophilic residues are more exposed to the solvent, a profile of these values can indicate the overall folding pattern. Hydrophobic amino acids have essentially nonpolar side chains, for example, valine, leucine, isoleucine, phenylalanine, and methionine (Malleshappa Gowder, Chatterjee et al. 2014). Previously, study of the intermediate state formation of papain via inducing n-alkyl sulfates compounds such as sodium octyl sulfate, sodium decyl sulfate, and sodium dodecyl sulphate using diverse concentrations has revealed that hydrophobic interactions contribute a vital part in inducing the two different intermediates along the two various thermodynamic pathways (Chamani, Heshmati et al. 2009). The hydropathy index analysis, presented in Figure 4.18, indicated that the stem bromelain structure between residues 150 and 200 (red box) was more hydrophobic than the same residues in fruit. It

is possible that the sequence differences that alter the overall hydrophobicity of those residues could affect the specificity and inhibitor-binding properties of stem bromelain, particularly compared with fruit bromelain.

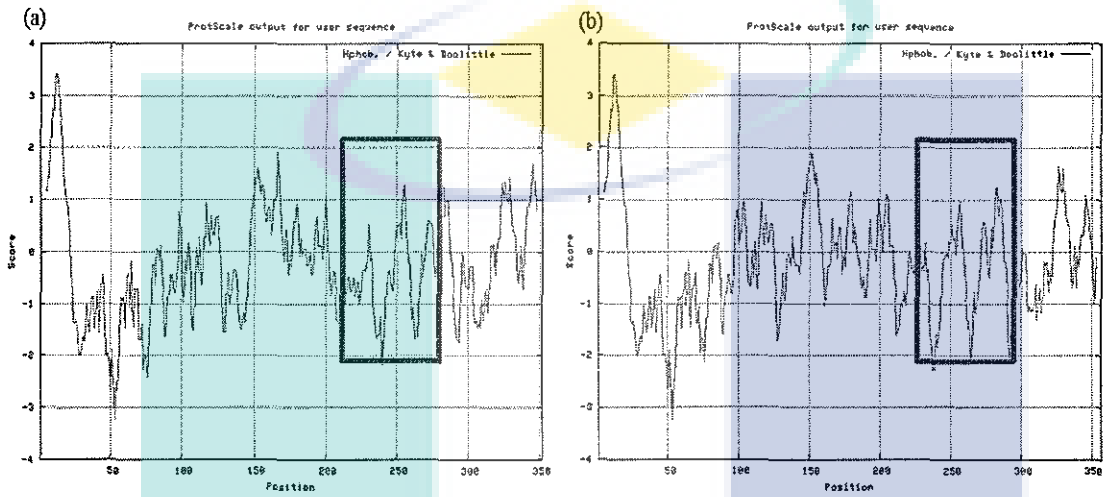


Figure 4.18 ProtScale output for hydropathy analysis of (a) Fruit bromelain (b) Stem bromelain.

CHAPTER 5

CONCLUSION

As a conclusion, the enzymatic activity of fruit bromelain from the local pineapple (*A. comosus*) cultivars; Morris cv., N36 cv and Sarawak cv. available in Malaysia was successfully quantified. Cultivar with the highest proteolytic activity is Morris cv.

The isolation and cloning of fruit bromelain from pineapple cultivar Morris is successful through the amplification of fruit bromelain using the RT-PCR approach. The sequence analysis of bromelain gene obtained from Morris cv. also reveals that the sequence are partially homologous.

The 3D model successfully proved and identified how protein structure conformation contributed to the catalytic action of bromelain. With this breakthrough an efficient and cost effective processes can be established and thus contributes to the development of highly purified and low cost bromelain which is beneficial to industrial and therapeutic applications worldwide.

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