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ENZYMATIC, ANTIOXIDANT ACTIVITIES AND STABILITY OF BROMELAIN-POLYPHENOL PRECIPITATE IN PINEAPPLE JUICE

POH SHIANG SHIEW

A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
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FEBRUARY 2012

I declare that this thesis entitled "*ENZYMATIC, ANTIOXIDANT ACTIVITIES AND STABILITY OF BROMELAIN-POLYPHENOL PRECIPITATE IN PINEAPPLE JUICE*" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Specially dedicated to my beloved mother, Wong Swee Lan
and father, Poh Sing Hua

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ABSTRACT

Bromelain, a cysteine protease derived from pineapple, is highly susceptible to denaturation. To stabilize its enzymatic activity, polyphenols from cashew leaf extract with antioxidant activity and protein-binding characteristic was used to precipitate bromelain in pineapple juice and formed bromelain-polyphenol precipitate subsequently. Nutraceutical properties (bromelain and antioxidant activities) and effect of temperature and pH on free and precipitated bromelain were evaluated and compared. Bromelain from pineapple juice was successfully precipitated with cashew polyphenols. The bromelain activity of bromelain-polyphenol precipitate was retained at about 65 %, 63 % and 40 % of its original activities by precipitating with 1.5 %, 1.0 % and 0.5 % cashew polyphenols respectively. 1.5 % and 1.0 % bromelain-polyphenol precipitates were found to have 15 % greater antioxidant activity than pineapple juice. 1.5 % and 1.0 % precipitates were shown to have good heat resistance by displaying 7 and 3.5 times lower denaturation rate constants respectively as compared to free bromelain. All bromelain-polyphenol precipitates exhibited stable bromelain activity and antioxidant activity at temperature range of 25 °C – 85 °C, whereas free bromelain showed an activity declination. Kinetics of thermal inactivation (E, ΔH^*, ΔG^* and ΔS^*) showed that the precipitate was more thermostable than free bromelain. Bromelain-polyphenol precipitate exhibited an increased bromelain activity over alkaline pH whereas free bromelain was stable at acidic and neutral pH. Besides, the precipitate displayed a high and stable antioxidant activity at acidic region. The study showed that precipitating of bromelain with cashew polyphenols could stabilize bromelain activity, increase antioxidant capacity and improve stability in response to temperature and pH. These results suggested that precipitating of bromelain with polyphenols could be a new method for enhancing the nutraceutical properties of enzyme.

ABSTRAK

Bromelain, protease sisteina yang diperolehi daripada nanas, adalah sangat terdedah kepada penyahaslian. Bagi menstabilkan aktiviti enzim, polifenol daripada ekstrak daun gajus dengan aktiviti antioksidan dan ciri-ciri ikatan protein digunakan untuk membentuk mendakan dengan bromelain dalam jus nanas dan seterusnya membentuk mendakan bromelain-polifenol. Sifat-sifat nutraseutikal (aktiviti bromelain dan antioksidan), kesan suhu dan pH pada bromelain bebas dan bromelain-polifenol telah dinilai dan dibandingkan. Bromelain daripada jus nanas telah berjaya membentuk mendakan dengan polifenol gajus. Aktiviti bromelain bagi mendakan bromelain-polifenol telah dikekalkan sebanyak 65 %, 63 % dan 40 % daripada aktiviti asalnya dengan polifenol gajus masing-masing 1.5 %, 1.0 % dan 0.5 %. 1.5 % dan 1.0 % mendakan bromelain-polifenol didapati mempunyai aktiviti antioksidan 15 % lebih besar daripada jus nanas. 1.5 % dan 1.0 % mendakan bromelain-polifenol dapati mempunyai rintangan haba yang baik dengan pemalar kadar penyahaslian masing-masing menunjukan 7 dan 3.5 kali ganda lebih rendah berbanding dengan bromelain bebas. Semua mendakan bromelain-polifenol mempamerkan aktiviti bromelain dan aktiviti antioksidan yang stabil pada julat suhu 25 °C – 85 °C, manakala bromelain bebas menunjukkan kemerosotan aktiviti. Kinetik pentakaktifan haba (E, ΔH^*, ΔG^* dan ΔS^*) menunjukkan bahawa mendakan lebih termostabil daripada bromelain bebas. Mendakan bromelain-polifenol mempamerkan peningkatan aktiviti bromelain pada julat pH beralkali manakala bromelain bebas stabil pada julat pH berasid dan neutral. Di samping itu, mendakan bromelain-polifenol memaparkan aktiviti antioksidan yang tinggi dan stabil pada julat pH berasid. Kajian ini menunjukkan bahawa pembentukan mendakan bromelain dengan polifenol gajus boleh menstabilkan aktiviti bromelain, meningkatkan keupayaan antioksidan dan memperbaiki kestabilan atas tindak balas suhu dan pH. Keputusan ini mencadangkan bahawa pembentukan mendakan bromelain-polifenol boleh menjadi satu kaedah yang baru bagi mempertingkatkan sifat nutraseutikal enzim.

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LIST OF ABBREVIATIONS

ANOVA	One Way Analysis of Variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DPPH	2, 2-diphenyl-1-picrylhydrazyl free radical
E	Activation energy
EC50	Half maximal effective concentration
GAE	Gallic acid equivalents
k	Rate constant
SD	Standard deviation
$t_{1/2}$	Half life
U	Units
UV/ VIS	Ultraviolet-visible spectroscopy
w/v	Weight per volume
$\Delta G^{\bullet}$	Free energy of inactivation
$\Delta H^{\bullet}$	Activation enthalpy
$\Delta S^{\bullet}$	Activation entropy

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Bromelain (EC 3.4.22.32) is a natural mixture of proteases and non proteases derived from pineapple plant (*Ananas comosus*), Bromeliaceae family. Takahashi *et al.* (1973) showed that the reactive sites of bromelain are one free sulfhydryl group and two disulphide bonds. Bromelain is a cysteine protease and the catalytic activity of bromelain and cysteine protease, in general, is attributed to its catalytic nucleophile – sulfhydryl side chain of cysteine reactive group located at active site. However, sulfhydryl group of cysteine is readily to be oxidized to form disulphide bond in the presence of oxidizing agents. Oxidation on cysteine groups will alter their important properties in substrate binding, catalysis, structure stabilization and thus cause change in structure of active site and loss in catalytic activity (Manning *et al.*, 1989; Rawlings *et al.*, 2007). Conformation of bromelain is stabilized by disulphide bonds situated between two domains of polypeptide chain (subunits). Change in environmental conditions (pH, temperature and addition of denaturing agents) will result in protein deformation and protein inactivation (Daniel *et al.*, 1996, Belitz *et al.*, 2009; Yon-Kahn and Herve, 2010).

Bromelain extracted from pineapple stem has been regarded as therapeutic component since 1957. Bromelain has been clinically proven to possess several pharmacological benefits for example anti-inflammation, inhibition of platelet aggregation and thrombus formation, anti-edematous, fibrinolytic activity, promotion of antibiotic absorption, burn debridement and anticancer activity (Kelly, 1996;

Maurer, 2001; Hale *et al.*, 2005a; Tochi *et al.*, 2008; Chobotova *et al.*, 2010). Proteolytic activity appears to be the major mechanism of therapeutic and protective action of bromelain especially on cysteine proteases (Hale *et al.*, 2002; Hale, 2004; Hale *et al.*, 2006). Since several pharmacological activities of bromelain are accounted for its proteolytic activity, it is essential to maintain bromelain in conformational intact and proteolytic/ catalytic active, in order to preserve its therapeutic value.

Pineapple is a natural and popular fruit consumed by Malaysian. This fruit provides health beneficial effects due to the presence of high bromelain concentration. However, bromelain in pineapple is highly susceptible to denaturation under conditions of high temperature or even room temperature, acidic/ alkaline conditions, solvent and chemical denaturants (Khan *et al.*, 2003; Hale *et al.*, 2005b; Jutamongkon and Charoenrein, 2010; Xue *et al.*, 2010). Pineapple fruit is generally consumed fresh and also processed as canned pineapple juice and cubes, to meet the demands from food market. The effect of temperature, heat, and mechanical pressure applied in fruit postharvest handling and processing would cause inactivation of enzyme and the processed product are likely proteolytic inactive (Bhattacharya and Bhattacharyya, 2007). Because the therapeutic properties of bromelain are associated with its proteolytic activity, therefore it is crucial to preserve the bromelain's activity in pineapple. Information on the influence of external factors on protein's structure and activity is required for improving and preserving the beneficial properties of protein by manipulating some operating conditions in food processing.

Enzyme activity could be altered and redesigned through covalent modification for enhanced functionality. This is a method to confer the enzyme some new and useful functional properties. Enzyme with improved catalytic activity and conformational stability will have an increased potential application in industrial, food processing, pharmaceutical and biotechnological areas. Xue *et al.* (2010) reported that stem bromelain modified with anhydride groups exhibited good heat resistance. The greater thermal stability may be due to decreased positive charge repulsion between lysine residues within polypeptide exerted by anhydride groups. Modified bromelain maintained high activity over a wider pH range towards alkaline

pH and it suggests the linking of anhydride to lysine gives bromelain overall negative charge and favors alkaline condition.

Apart from using chemical methods as mentioned above, there was a study by Liang *et al.* (1999) demonstrated complexion of bromelain in pineapple juice with tea polyphenols. Tea-polyphenol complexed bromelain had an improved thermal stability by displaying longer activity half life and greater enzyme activity at same temperature as compared with free bromelain. The authors hypothesized that the improved thermal stability of complexed bromelain might be attributed to antioxidant properties and protein binding characteristics of polyphenols.

Complexing of bromelain with polyphenols provides a useful method and strategy for conferring bromelain with enhanced enzymatic activity and stability. Hence, in the present study, this method of precipitation and complexing of bromelain in pineapple juice with polyphenols is employed to stabilize and retain the bromelain's enzyme activity and reduce its extent of denaturation. Polyphenols is the potential candidate used for bromelain precipitation and complexing owing to its antioxidant activity and protein-binding affinity.

Polyphenols with strong antioxidant properties could provide protection on catalytic active site (sulfhydryl group) of bromelain and prevent this enzyme from being oxidized, and thus stabilize its enzymatic activity. In this study, cashew leave is chosen as a source of polyphenols to precipitate with bromelain. Cashew leave could be available in large amount, cost effective and the reproduction period of leave is shorter than that of cashew fruit and cashew nut. Cashew leave has been shown to possess antibacterial, antiviral, antifungal, antioxidant and antiulcer properties (References are stated in section 2.5.2). These pharmacological activities are mainly attributed to phenolic compounds which are abundant in cashew plant.

Antioxidant activity of some cashew plant extracts has been shown to be correlated with total phenol content. Cashew nut shell liquid which contains the highest amount of alkyl phenol (anacardic acid, cardonols and cardols) was reported to display the most significant antioxidant activity among tested cashew products.

Anacardic acids exhibit great antioxidant activity and suggested that it is the major polyphenols contributing to high antioxidant activity of nut shell liquid (Trevisan *et al.*, 2006). Cashew leave was previously reported to have antioxidant activity (Runnie *et al.*, 2004; Abas *et al.*, 2006) and it is believed that the phenolic content of cashew leave extract is the contributors to its antioxidant capacity.

In addition to antioxidant activity of polyphenols as discussed above, polyphenols has a significant protein binding characteristics, especially for extended proline rich polypeptide. Proline residues appear to be essential in protein/ polyphenol binding and precipitation (Asano *et al.*, 1982). From the view of polyphenols in protein-polyphenol complex, proanthocyanidins is found to be the common polyphenols participating in haze formation in beverage such as beer and fruit juice (McMurrough *et al.*, 1992; Spanos and Wrolstad, 1992). In a model of interaction proposed by Hagerman and Butler (1981), polyphenols interacts reversibly with open extended polypeptide at proline binding sites by noncovalent bonding. Further multivalent interaction leads to the precipitation of protein-polyphenol complex (Charlton *et al.*, 2002). Protein/ polyphenol binding and precipitation is predominantly driven by hydrophobic interaction and hydrogen bonding (Hagerman and Butler, 1981; Murray *et al.*, 1994; Haslam, 1996; Siebert *et al.*, 1996; Baxter *et al.*, 1997; Hagerman *et al.*, 1998; Charlton *et al.*, 2002).

Bromelain in pineapple juice and polyphenols in cashew leaf extract might have a tendency in bromelain-polyphenol precipitate formation since both bromelain and polyphenols have been proven to possess precipitate active components which are essentially involved in binding and precipitation. Bromelain is a single polypeptide with 212 residues and possesses 11 proline residues per molecule (Takahashi *et al.*, 1973). Proline residues in extended polypeptides are essentially involved in interaction with polyphenols. Cashew leave has been found to contain flavonoids, proanthocyanidin, tannic acid (Arya *et al.*, 1989; Konan and Bacchi, 2007) and these phenolic compounds are active in protein-polyphenol precipitate formation.

Both proteins (bromelain) and polyphenols (cashew leaf extract) have been clinically evidenced to have significant health benefit pharmacological properties. Apart from the current findings related to activity and stability, more intensive studies should be carried out to evaluate the medicinal potential of bromelain-polyphenol precipitate.

In the present study, cashew leave is ethanolic extracted for polyphenolic compounds and the resultant ethanolic cashew leaf extract is mixed with pineapple juice. Bromelain in pineapple juice could be separated and precipitated by polyphenols of cashew leaf extract through protein-polyphenol binding and interaction and the precipitate formed of bromelain-polyphenols is obtained. It is followed by evaluation and comparison of nutraceutical properties (enzymatic and antioxidant activity) and stability (thermal and pH stability) between bromelain-cashew polyphenol precipitated bromelain and free bromelain.

1.2 Problem Statement

Bromelain in pineapple juice is highly susceptible to denaturation and oxidation under the conditions of elevated temperature and acidic/ alkaline reagent. Effect of these environmental factors would cause enzyme inactivation and result in loss of bromelain's health beneficial proteolytic activity and functionality. Polyphenols from cashew leaf extract with antioxidant activity and protein-binding characteristic is used to separate and precipitate bromelain in pineapple juice, to stabilize its enzymatic activity and increase antioxidant capacity by formation of bromelain-polyphenol precipitate. Bromelain contains proline residues and cashew leaf extract has been detected with flavonoids, proanthocyanidins and phenolic acids as polyphenolic compounds. Both bromelain and cashew polyphenols have tendency to form bromelain-polyphenol precipitate since they possess precipitate active components. Bromelain-polyphenol precipitate is hypothesized to exhibit high resistance against heat or pH-caused denaturation effect, whereby retaining its nutraceutical properties (enzymatic and antioxidant activity), which is not achievable by free bromelain.

1.3 Hypotheses

Precipitation of bromelain with polyphenols is hypothesized to stabilize bromelain's enzymatic activity, increase antioxidant activity and improve stability in terms of temperature and pH.

Catalytic nucleophile of bromelain (sulfhydryl group of cysteine) is readily to be oxidized and this change of side chain of reactive residues would inactivate its catalytic and structural functions (Manning *et al.*, 1989). If bromelain is precipitated with polyphenols, the strong antioxidant properties of polyphenols will protect the sulfhydryl groups of cysteine from being oxidized and stabilize and retain the bromelain's enzymatic activity.

Cross linking of polyphenols with enzyme might become one of the techniques to stabilize enzyme. The native form of enzyme is stabilized by putting hydrogen bonds, hydrophobic interactions, Van der Waal interactions, salt bridges and electrostatic interactions. This intramolecular cross linking will increase protein conformational stability and make the enzyme resistant to protein denaturation and degradation (Matthews, 1993; Daniel *et al.*, 1996; Bhatti *et al.*, 2006). It has been shown that protein deactivation is found to be slow in conformational rigid protein (Daniel *et al.*, 1996; Naidu and Panda, 2003; Quintanilla-Guerrero *et al.*, 2008). Precipitated bromelain is hypothesized to be more thermostable as compared to native form of bromelain owing to the stability effect exerted by precipitation with polyphenols.

pH change will alter ionization states of amino acids in enzyme's active site and charge properties of substrate, this will affect reactivity of catalytic groups and substrate binding and thus cause reduction of enzyme's catalytic activity. In addition, high and low pH change the total overall charge of protein, leading to charge repulsion and protein unfolding (Matthews, 1993). Unfolded protein is highly susceptible to degradative process. Bromelain in the precipitate with polyphenols is hypothesized to have a rigid conformation and an increase in enzyme stability against pH-caused protein denaturation.

1.4 Objectives

The objectives of this study are stated below:

1. To determine the enzymatic activity and antioxidant activity of bromelain-polyphenol precipitate.
2. To determine the stability of bromelain-polyphenol precipitate in terms of temperature and pH.

1.5 Scopes

The scopes of this study are stated below:

1. Screening of three ethanolic plant extracts and selection of plant extract with high antioxidant activity and phenolic content.
2. Evaluation of nutraceutical properties (enzymatic and antioxidant activities) and protein and phenolic contents of bromelain-polyphenol precipitate.
3. Study the effect of temperature on thermal stability of bromelain-polyphenol precipitate.
4. Evaluation of effect of pH on bromelain-polyphenol precipitate.

CHAPTER 2

LITERATURE REVIEW

As mentioned in Chapter 1, to have an improved enzymatic, antioxidant activities and stability, bromelain in pineapple is precipitated with polyphenols from cashew leaf extract to form bromelain-polyphenol precipitate. Bromelain will be firstly introduced with its catalytic activity, therapeutic properties and followed by a brief description on the source of bromelain – pineapple. Polyphenols, another character in this study is described with its chemical structure and antioxidant activity. Cashew leave, as a source of polyphenols will be discussed with its phytochemical substances, pharmacological properties and toxicological analysis. Details on the mechanism of protein-polyphenol interaction, the structural factors of both proteins and polyphenols involved in binding and precipitation and also the interaction bonding will be clearly elaborated in the following sections. How do the external conditions exert advese effect on conformational stability of protein is also described.

2.1 Bromelain

2.1.1 Introduction to Bromelain

Bromelain is aqueous extracted from pineapple stem and it is purified and characterized into stem bromelain (EC 3.4.22.32), fruit bromelain (EC 3.4.22.33), ananain (EC 3.4.22.31) and comosain depending on their source, biochemical properties and proteolytic specificity (Rowan *et al.*, 1990; Hale *et al.*, 2005b). In an

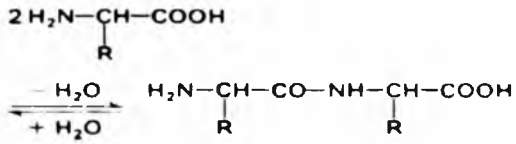
earlier study (Rowan *et al.*, 1988), stem bromelain and ananain were found in crude pineapple stem by FPLC purification followed by affinity chromatography and cation exchange chromatography. Later, comasain was purified from ananain preparation by active site affinity chromatography and cation exchange chromatography and the resultant comasain fraction has different substrate specificity as compared with ananain (Rowan *et al.*, 1990). Stem bromelain is the most abundant proteases amongst cysteine proteases present in pineapple stem. Stem bromelain is commercially available, predominant in nutraceutical preparation and widely used in industry, laboratory and pharmaceutical field (Hale *et al.*, 2005b). The molecular weight of stem bromelain is between 23000 and 25000 (Harrach *et al.*, 1995). Fruit bromelain is a major cysteine protease in pineapple fruit (Rowan *et al.*, 1990) with molecular mass of 23000 (Maurer, 2001).

In a later study (Harrach *et al.*, 1995), nine basic cysteine protease components are detected on fractionation of crude pineapple stem extract by FPLC cation exchange chromatography. The main protein fractions F4 and F5 and highest proteolytic active component F9 are purified and characterized. Bromelain fraction F9 modulates the metastasis, migration of cancer cells and human peripheral blood lymphocytes by removing CD44 surface molecules (Harrach *et al.*, 1994; Munzig *et al.*, 1994).

In addition to proteases, bromelain also contains peroxidases, phosphatases, glucosidases, cellulases, protease inhibitors, carbohydrates, glycoproteins and organic bound calcium (Kelly, 1996; Maurer, 2001). In some physiological conditions, the beneficial effects of bromelain are attributed to its non proteases, not to single protease fraction.

2.1.2 Molecular Structure, Catalytic Active Site and Catalytic Activity of Bromelain

Proteases, also known as peptidases, proteinases and proteolytic enzymes, hydrolyze the peptide into free amino acids.



(Belitz *et al.*, 2009)

Bromelain is a thiol proteases which its cysteine group is responsible for catalyzing peptide bond hydrolysis. Bromelain is originated from *Ananas comosus* (pineapple) and all thiol proteases exist in plant are endopeptidases which cleave internal, α peptide bond (Yon-Kahn and Herve, 2010).

Complete sequence and three-dimensional structure of papain have been determined and well known (Kamphuis *et al.*, 1985). Since bromelain is a member of papain family (Ritonja *et al.*, 1989), the molecular structure and catalytic centre of bromelain may be speculated from papain's structural and catalytic points of view. Polypeptide chain of papain with 212 residues is folded into two domains which are N-terminal domain (β sheets) and C-terminal domain (α helix). There are three α helix and one of the helix is situated at interface between two domains. The structure of papain is stabilized by three disulphide bonds and numerous hydrogen bonds. Cys-25 (in N-terminal domain) and His-159 (in C-terminal domain) are catalytic groups localized at active centre between two structural domains. Amino acid sequence situated around residues Cys-25 and His-159 of stem bromelain and fruit bromelain are compared and it was found that their sequences are highly conserved with small insertions and deletions (Yon-Kahn and Herve, 2010).

In 1967, Schechter and Berger proposed a model to describe the substrate specificity of peptidase in which each substrate binding subsite of enzyme (S1, S2, ..., S1', S2') accommodates one amino acid side chain of substrate (P1, P2, ..., P1', P2'). * represents enzyme catalytic site and + is substrate scissile bond.

N terminus – Substrate: P3-P2-P1 + P1'-P2'-P3' – C terminus

Enzyme: S3-S2-S1 * S1'-S2'-S3'

Catalytic binding groups arranged on surface molecules in groove of catalytic site in peptidases must be in favorable position relative to hydrolysis of scissile bond in substrate (Rawlings *et al.*, 2007).

In cysteine proteases, sulfhydryl group (SH) of reactive group (cysteine, Cys) is catalytic nucleophile in which the charged form (S^-) is excellent proton donor. Histidine (His) is usually a general base because the basic nitrogen atom in imidazole ring of histidine abstracts proton from cysteine and becomes protonated form (H^+Im). The enzyme is active when the catalytic groups are ion paired between S^- (deprotonated thiol group of cysteine) and H^+Im (protonated imidazole ring of histidine) (Yon-Kahn and Herve, 2010) (Figure 2.1). It is followed by transfer of proton group from active enzyme to nitrogen of peptide bond in substrate, which would break the peptide bond. In papain, the third residue is asparagine (Asn) which orientates the imidazole ring of histindine and interacts with nitrogen atom in the ring by hydrogen bond (Figure 2.1). Cysteine, histidine and asparagine form catalytic triad in papain. Bromelain lacks Asn-175, therefore it only has Cys/His catalytic dyad (Yon-Kahn and Herve, 2010).

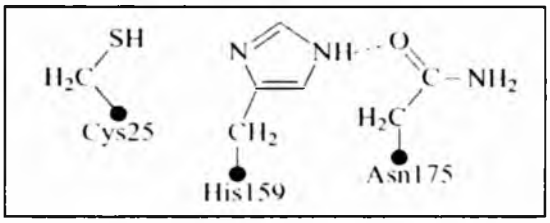


Figure 2.1 Catalytic triad of cysteine protease (Yon-Kahn and Herve, 2010).

2.1.3 Safety of Oral Bromelain

Oral bromelain is considered to be safe and nontoxic. LD50 of bromelain is greater than 10 g/kg in mice. Bromelain of 1.5 g/kg administrated daily to rats shows no carcinogenic effects (Kelly, 1996). In human, oral exposure to bromelain up to 12 g daily produces no major side effect (Castell *et al.*, 1997). No effect on blood pressure and heart rate in hypertensive patients was observed after administration of bromelain up to 460 mg. There is no major side effect with daily oral doses from

200 to 2000 mg and great therapeutic benefits of bromelain start at dose of 750 mg per day (Kelly, 1996).

2.2 Proteolytic Activity of Bromelain in Pharmacological Actions

Since 1957, Heinecke and Gortner found that bromelain, which is extracted from pineapple stem, could be used as therapeutic components (Kelly, 1996). In several native tropics, pineapple has been grown and used as traditional medical plant. From numerous traditional observations, control and uncontrolled clinical studies, *in vitro* and *in vivo* animal based or cell based experiments, bromelain is evidenced to exhibit several therapeutic efficacies for example antithrombotic, antiplatelets, fibrinolytic, anti-inflammatory effects, anti-edematous activity, promotion of antibiotic absorption, debridement properties and anticancer activity.

Some therapeutic properties of bromelain are attributed to proteolytic activity of bromelain. The following examples demonstrate the dependency of bromelain's pharmacological actions on its proteolytic activity.

2.2.1 Fibrinolytic, Antiplatelets and Antithrombotic Activity of Bromelain

Some studies have indicated that bromelain inhibits platelet aggregation by demonstrating prolonged prothrombin and reduced fibrinogen level, dose dependently and proteolytically (Heinicke *et al.*, 1972; Morita *et al.*, 1979; Maurer, 2001). *In vitro*, bromelain decreases conversion of prothrombin to thrombin which is needed for activation of fibrinogen to yield fibrin (Kelly, 1996). Fibrin could aggregate into threads around injured area and inhibit drainage and blood circulation. Besides, bromelain increases fibrinolytic activity by activating fibrinolytic factor to convert plasminogen to plasmin which could then degrade fibrin (Cirelli and Smyth, 1963; Kelly, 1996). It has been shown that fibrinolytic property of bromelain is dependent on its proteolytical activity since proteinase inhibitors in plasma could inhibit fibrinolysis action of bromelain (Morita *et al.*, 1979).

Binding of platelet to tumor cells initiates production of platelet-based factors (thrombospondin, thrombin, transforming growth factor-β, prostaglandin, etc), causes the deregulation of blood flowing and coagulation system, facilitates thrombus formation and platelet-tumor cells aggregation. This aggregation enhances tumor cell's endothelial adhesion and also its invasiveness and metastases (Mehta, 1984; Belloc *et al.*, 1995; Maurer, 2001). Bromelain could proteolytically reduce cell surface molecules (CD44) on platelet coated metastalized tumor cells to prevent tumor cells from adhesion to endothelial cells, migration through vessel and metastatic potential (Grabowska *et al.*, 1997).

Fibrin around tumor cells polymerizes with human serum albumin into matrix coat which could protect tumor cells against proteolysis and immune recognition (Lipinski and Egyud, 2000; Chobotova *et al.*, 2010). Fibrinolytic activity of bromelain could degrade the fibrin coat around tumor cells and reduce blood plasma fibrin level, increasing cytotoxicity of lymphocytes and exposing tumor cells to immune system (Lipinski and Egyud, 2000; Biggerstaff *et al.*, 2008; Chobotova *et al.*, 2010).

2.2.2 Anti-inflammatory Effect of Bromelain

Bromelain exerts anti-inflammatory effect by proteolytically cleavage of CD4 on T lymphocytes to reduce production of proinflammatory effectors (Manhart et al, 2002). *In vitro*, bromelain proteolytically alters 14/59 cell surface molecules on leukocyte in whole blood and results in modulation of leukocyte adhesion and activation and alteration of pathogenesis of inflammation (Hale *et al.*, 2002). Oral bromelain remains proteolytically active within gastrointestinal tract, removes bromelain sensitive cell surface molecules from leukocytes and colon endothelium, stimulating immune response for decreasing colon inflammation in mice in dose-dependent manner (Hale, 2004).

In immunological response to inflammation and neoplastic disease, bromelain could proteolytically stimulate cytokine synthesis including tumor necrosis factor-α

(TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in human peripheral blood mononuclear cells (Desser *et al.*, 1993; Chobotova *et al.*, 2010). The cytokine secretion increases synergistically with interferon-α and γ (IFN- α and IFN- γ) production when bromelain is applied (Desser and Rehberger, 1990; Kelly, 1996). All these regulators could activate immune system and inhibit tumor progression.

2.2.3 Anticancer Effect of Bromelain

Bromelain reduces CD44 expression on metastasizing tumor cells for example leukemia and melanoma cells (Harrach *et al.*, 1994). It is associated with diminished cancer cell aggressiveness and metastasis (Subramaniam *et al.*, 2007). Decreased aggressiveness of tumor cells may attribute to suppression of CD44-mediated lymphocyte adhesion to vessel endothelium and blocking the migration of lymphocytes to inflammation sites, thereby reducing the binding with tumor cells (Chobotova *et al.*, 2010).

2.2.4 Anti-edematous Activity of Bromelain

Bromelain reduces edema formation by fibrinolysis of blood clots which block vessel permeability, thus promotes the absorption of edema into blood circulation. Increased tissue permeability by fibrinolysis also enhances antibiotic absorption (Kelly, 1996). Since fibrinolytic activity of bromelain is dependent on its proteolytic activity, it could say that the proteolytic activity is the relevant feature of bromelain's anti-edematous potential and promotion of antibiotic absorption.

2.3 Pineapple

Pineapple is a primary source of bromelain. Pineapple belongs to family Bromeliaceae, is a tropical fruit and well cultivated on large scale plantations in

many tropical countries including Brazil, India, Thailand, the Philippines, Indonesia, China and Malaysia. Pineapple fruit makes up 60% of the weight and contains water, sugar (sucrose), protein, fibre, fat, citric acid, vitamins A, B and C (Rangan, 1984; Davey *et al.*, 2007). Besides, pineapple is a source of proteolytic enzyme (bromelain) which has therapeutic effect (Kelly, 1996). Pineapple has been grown as medicinal plant in many native tropics and its clinical applications include reducing inflammation, anticoagulation, antioxidant activity, improving digestion and prevention of cardiovascular disease (Taussig and Batkin, 1988). Parts of pineapple plant are shown in Figure 2.2 and classification of pineapple is stated in Table 2.1.

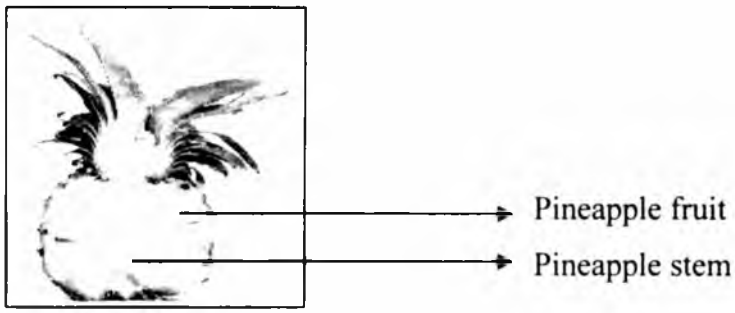


Figure 2.2 Pineapple fruit and fruit stem.

Table 2.1 Scientific classification of pineapple plant (Coppens d' Eeckenbrugge and Leal, 2003; Davey *et al.*, 2007).

Classification	Name
Kingdom	*Plantae*
Order	*Poales*
Family	*Bromeliaceae*
Subfamily	*Bromelioideae*
Genus	*Ananas*
Species	*macrodontes, comosus*
Varieties	*ananassoides, bracteatus, comosus, erectifolius, paraguazensis*
Groups/ Cultivars	Cayenne, Queen, Spanish, Pernambuco (Abacaxis), Perolera

Pineapple of cultivation is *Ananas comosus* var. *comosus*. Pineapple cultivars are grouped into Cayenne, Queen, Spanish and Abacaxis in international trade with several variations within each major group. The following table (Table 2.2) will discuss several major pineapple cultivars planted in Malaysia including Maspine, Moris, Sarawak and Josapine.

Table 2.2 Features of some Malaysia's pineapple cultivars (MARDI, 1996; MPIB, 2008).

Cultivars / Features	Maspine	Moris	Sarawak	Josapine
Originated cultivar	Spanish	Queen	Cayenne	Cross breeding between Spanish and Cayenne
Shape	Cylindrical	Sharp pointed	Cylindrical	Cylindrical
Weight (kg)	1.8	0.8-1.5	2.0-4.0	1.3
Flesh colour	Golden yellowish	Yellowish	Dull yellowish	Golden yellow
Flesh taste	Crispy with sweetness (16°-17° Brix), dry taste	Crispy with sweetness (15°-17° Brix)	High sugar and acid content	Dry, crispy with sweetness (16°-17° Brix)
Leaves	Without spine	Sharp thorns	Without spine	Without spine
Peel	Smooth	Small pith but deeper eyes	Big pith	Dark purple when immature, reddish when ripe

Advantages in commercialization	Good keeping period, well adapted to shipping (no black stripe, maintain freshness), fresh consumption	Fresh consumption, keep well after ripening	Fresh consumption, well adapted to canning and processing	Resistance to black heart disorder, good shelf life, well adaptation for exporting

Josapine (Figure 2.3) selected in this study is a hybrid pineapple fruit between Spanish and Smooth Cayenne and released officially by Malaysia Agriculture Research and Development Institute (MARDI) on August 5th,1996 (MARDI, 1996).

Figure 2.3 Josapine.

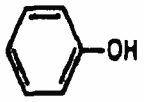
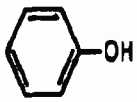
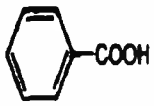
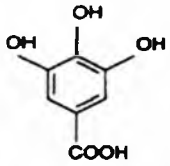
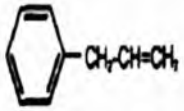
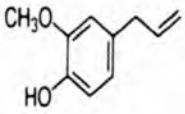
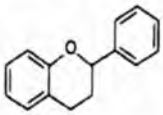
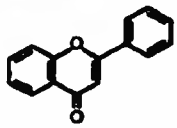
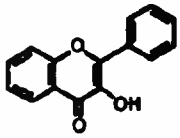
2.4 Polyphenols

2.4.1 Chemistry of Polyphenols

Polyphenols, which is secondary metabolites, constitutes one of the most common and widely distributed substances in plant kingdom. Polyphenols is characterized by the presence of one or more sugars (typically glucose) attached with

hydroxyl groups. Polyphenolic compounds are divided into many classes range from simple phenols with a C6, phenolic acids with C6-C1 and flavonoids with C6-C3-C6 skeleton (Table 2.3). Tannins are highly polymerized compounds (Bravo, 1998; Bennick, 2002). Flavonoids and tannins will be discussed in detail.

Table 2.3 Classes of polyphenols (Bravo, 1998).

Basic Skeleton	Class	Basic Structure	Example	Structure of example
C6	Simple phenols		Phenol	
C6-C1	Phenolic acids		Gallic acid	
C6-C3	Phenylpropenes		Eugenol	
C6-C3-C6	Flavonoids		Flavones	
			Flavonols	

Flavonoids is the largest and diverse group of plant polyphenols. The basic unit consists of two aromatic rings separated by propane in which usually forms an oxygenated heterocyclic ring. Ring A is derived from acetate pathway whereas ring B is from Shikimate pathway (Bravo, 1998) (Figure 2.4). Some classes of flavonoids are shown in Table 2.4.

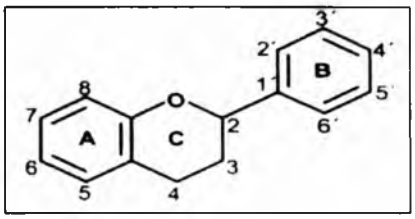
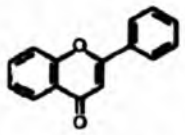
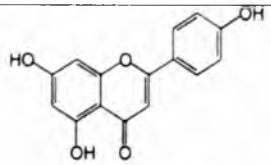
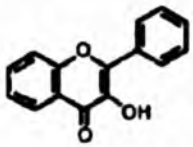
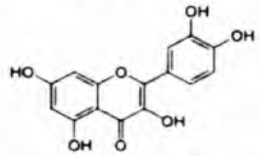
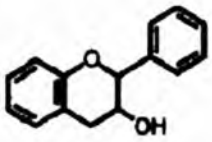
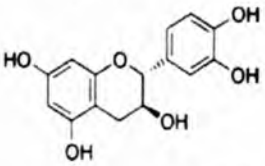
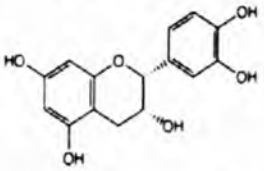
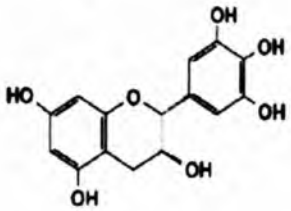
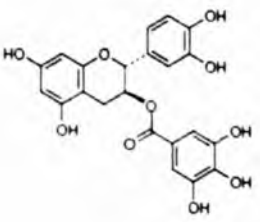
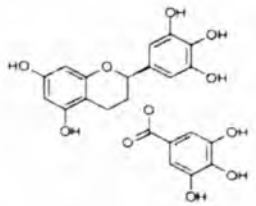
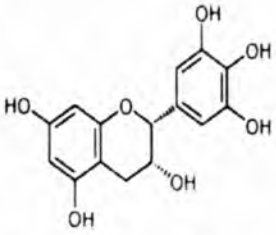
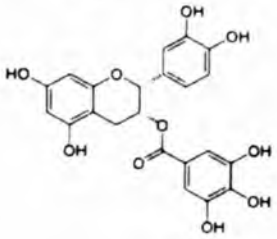
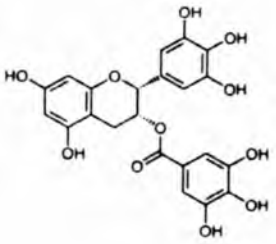
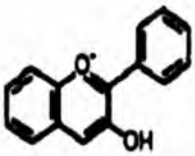
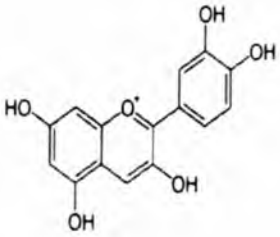


Figure 2.4 Basic structure of flavonoids (Bravo, 1998).

Table 2.4 Classes of flavonoids (Bravo, 1998).

Flavonoids	Basic Structure	Example	Structure of example
Flavones		Apigenin	
Flavonols		Quercetin	
Flavanols		Catechin	
		Epicatechin	
		Gallocatechin	
		Catechin gallate	

		Gallocatechin gallate	
		Epigallocatechin	
		Epicatechin gallate	
		Epigallocatechin gallate	
Anthocyanidin		Cyanidin	

Tannins is high polymerized polyphenols and able to form insoluble precipitates with protein, gelatin and carbohydrate (Mehansho *et al.*, 1987). Tannins is divided into hydrolyzable tannins and condensed tannins based on structural characteristics.

Hydrolyzable tannins consists of gallic acid or hexahydroxydiphenic acid (ellagic acid) esterified to a polyhydric alcohol, typically glucose (Figure 2.5). These tannins can be hydrolyzable in acid, alkali and enzymatic reaction to give polyhydric alcohol and phenylcarboxylic acid (Bravo, 1998; Bacon and Rhodes, 2000; Bennick, 2002).

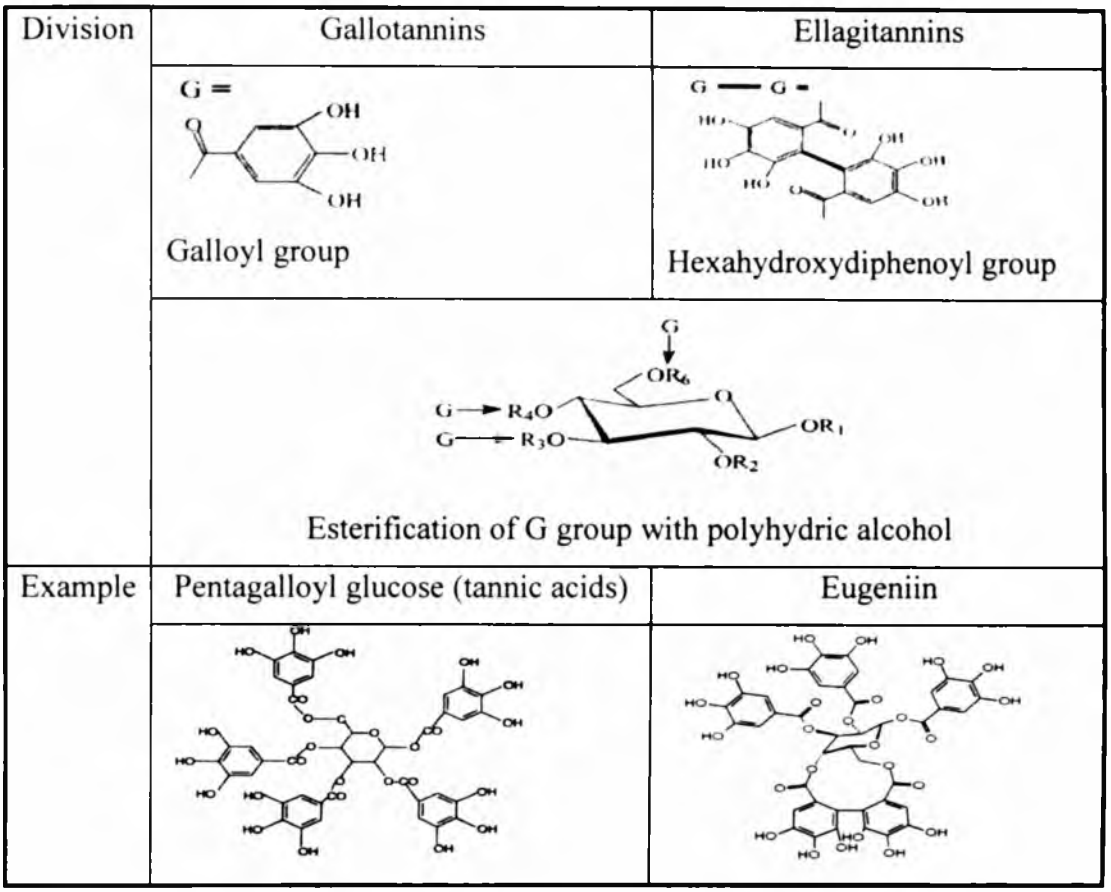


Figure 2.5 Structure of hydrolyzable tannins (Bacon and Rhodes, 2000).

Condensed tannins or proanthocyanidins is polymer of monomer flavan-3-ols (catechin/ epicatechin) (Figure 2.6), which is formed by oxidative condensation. Tannins is depolymerized in strong acid and alkaline solution to yield anthocyanidins (Haslam, 1996; Bravo, 1998; Bennick, 2002).

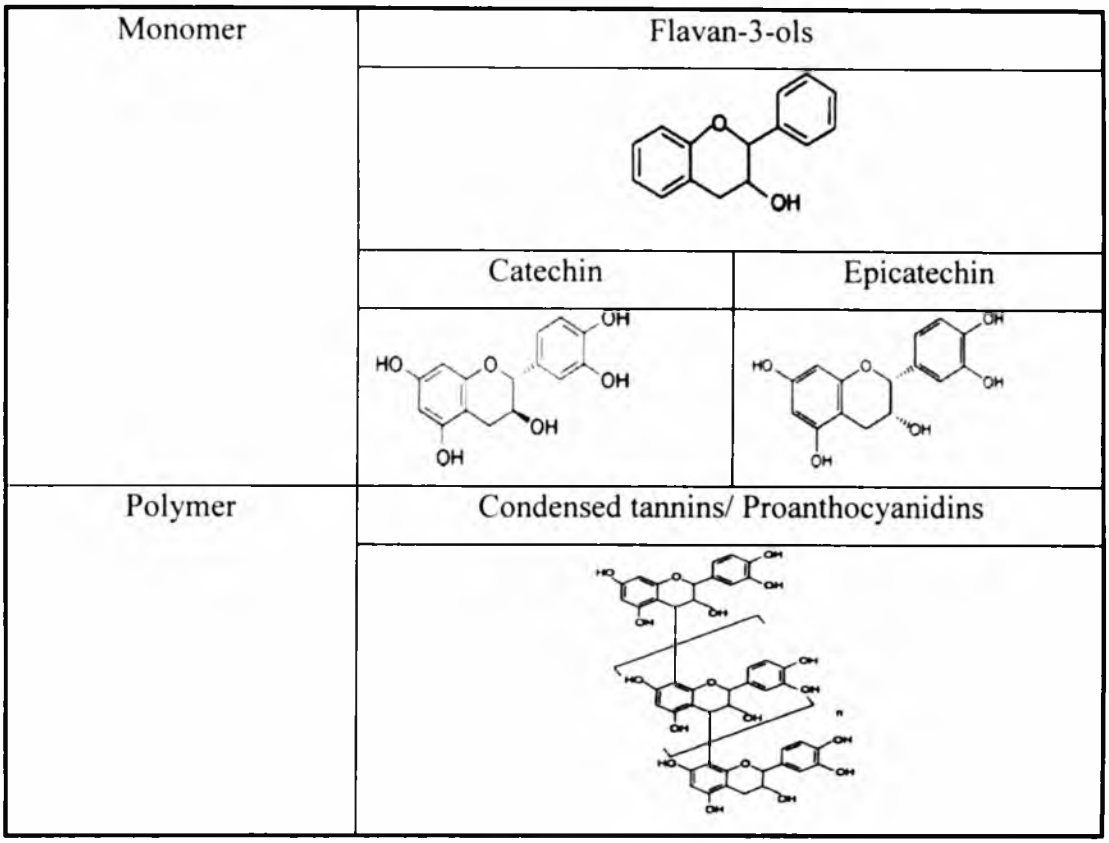


Figure 2.6 Structure of condensed tannins.

2.4.2 Antioxidant Activity of Plant Polyphenols

Damage from oxygen species and radicals is associated with increased incidence of aging, inflammation, arthritis, neurodegenerative disease, cardiovascular disease and cancer (Ames *et al.*, 1993; Frankel *et al.*, 1993; Goodwin and Brodwick, 1995; Floyd, 1999; Narayana *et al.*, 2001). Polyphenols, mainly flavonoids exerts protective effect against the diseases related with prooxidant states through its several antioxidant actions such as free radicals and reactive oxygen species scavenging, metal ion chelation, inhibition of lipid peroxidation (Cook and Samman, 1996; Haslam, 1996; Bravo, 1998). The anticarcinogenic effects of polyphenols might be due to its protective action on DNA from oxidative damage, inhibition of expression of mutant gene and related enzyme systems (Noroozi *et al.*, 1998).

Numerous studies are performed on antioxidant activity and total phenolic content of various plant extracts, to determine the relationship between antioxidant

activity, phenolic content and the main phenolic compounds. Tea extract has been known for its rich phenolic compounds (flavonoids) such as catechin, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate and these polyphenols exhibit strong antioxidant activity (Salah *et al.*, 1995). Tea polyphenols exerts protection action against carcinogenic, cardiovascular and mutagenic disease (Dufresne and Farnworth, 2001; Hodgson, 2008). Xu *et al.* (2010) reported that total content of phenolic compounds (phenol, flavonoids, flavan-3-ols) correlates significantly with antioxidant activity in grape seeds and flavan-3-ols is the main phenolic compound. In a study on selected herbs (Zheng and Wang, 2001), a positive linear relationship is observed between antioxidant capacity and total phenolic contents of medicinal herbs and culinary herbs. The identified phenolic compounds in the selected herbs are rosmarinic acid, quercetin and kaempferol.

Plant polyphenols is important as natural antioxidant found in food (Haslam, 1996) and it is capable of scavenging radicals such as hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), reactive oxygen species such as superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), inhibition of lipid peroxidation and metal ions chelation (Salah *et al.*, 1995). Polyphenols with polyhydroxyl groups has low activation energy to donate its hydrogen atoms to radicals. The resulting antioxidant free radical is stable due to delocalization of spare electron as a whole, therefore it does not initiate another oxidation. Besides, phenoxy antioxidant radical intermediates react with other free radicals and terminate propagation of free radical chain (Bravo, 1998). The mechanism of antioxidants with radicals is shown below (P represent polyphenols):

$ROO^{\bullet} + PH \rightarrow ROOH + P^{\bullet}$

$P^{\bullet} + ROO^{\bullet} \rightarrow ROOP$

$RO^{\bullet} + PH \rightarrow ROH + P^{\bullet}$

$P^{\bullet} + RO^{\bullet} \rightarrow ROP$

2.5 Cashew

Cashew leave is the source of polyphenols which is used to precipitate bromelain in this study. In the below section, cashew plant will be firstly introduced and followed by description of its polyphenolic compounds, pharmacological properties and the relationship of polyphenols with antioxidant activity. Biosafety of cashew leaf extract will also be investigated by acute toxicity.

2.5.1 Introduction to Cashew Plant

The species *Anacardium occidentale* L. or typically known as cashew, is a member of family Anacardiaceae. It is a plant indigenous to Brazil and it is now extensively cultivated in India, East Africa and well grown in tropical countries including Cambodia, Indonesia, Kenya, the Philippines, Thailand, Vietnam and Malaysia (Orwa *et al.*, 2009). The scientific classification of cashew plant is stated in Table 2.5.

Table 2.5 Scientific classification of cashew plant.

Classification	Name
Kingdom	*Plantae*
Order	*Sapindales*
Family	*Anacardiaceae*
Genes	*Anacardium*
Species	*Anacardium occidentale*

Figure 2.7 shows a cashew fruit consists of pseudofruit (cashew apple) and true fruit (cashew nut).

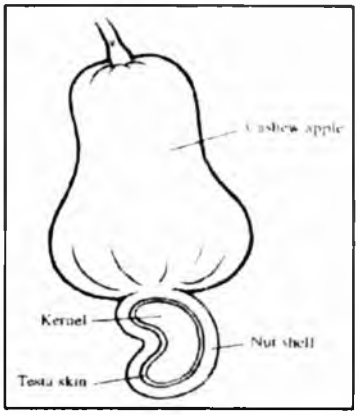


Figure 2.7 Cashew fruit consists of pseudofruit (cashew apple) and true fruit (cashew nut).

Cashew has been consumed as folk medicine to treat toothache, sore gums, dysentery, piles, pellagra, mouth ulcer (Kudi *et al.*, 1999; Akinpelu, 2001; Luiz-Ferreira *et al.*, 2008), diarrhea, gastric disease (Mota *et al.*, 1985; Kudi *et al.*, 1999; Goncalves *et al.*, 2005; Luiz-Ferreira *et al.*, 2008), wound, boil, dyspepsia (Kudi *et al.*, 1999; Luiz-Ferreira *et al.*, 2008), skin ulcer (Franca *et al.*, 1996) and anti-inflammation (Mota *et al.*, 1985; Luiz-Ferreira *et al.*, 2008).

Cashew is a common source of antioxidant phenolic compounds including flavonoids, tannins (Arya *et al.*, 1989), anacardic acid (Paramashivappa *et al.*, 2001), resorcinolic lipids (Kozubek and Tyman, 1999) and also vitamin C, vitamin A, carotenoid (Cecchi and Rodriguez-Amaya, 1981; Maciel *et al.*, 1986; Assuncao and Mercadante, 2003), fatty acid, sugar and proteins (Venkatachalam and Sathe, 2006).

2.5.2 Polyphenols, Pharmacological Properties and Antioxidant Activity of Cashew Plant

Various parts of cashew plant have been subjected to phytochemical substances screening, pharmacological screening, antioxidant activity testing and toxicological test. The following tables provide some experimental data on polyphenolic compounds (Table 2.6) and pharmacological properties (Table 2.7) obtained from chemical screening and clinical studies on different parts of cashew

plant. Phytochemical substances in plant extract could be screened using high performance liquid chromatography coupled with mass spectrophotometer.

Table 2.6 Polyphenolic compounds of various parts of cashew plant.

Parts of cashew	Polyphenolic compounds	References
Cashew leave	• Flavonoids [1a, 1b]: glycosylated quercetin [1a, 1c], glycosylated kaempferol [1a], kaempferol-3-O [1a], methyl ether [1a], glycosylated myricetin [1a], amentoflavone [1a, 1c] • Condensed tannins: proanthocyanidin [1b, 1c] • Phenolic acids [1d]: gallic acid, protocatechuic acid, ρ-hydroxybenzoic acid, cinnamic acid, ρ-coumaric acid, ferulic acid	1a. Arya *et al.*, 1989 1b. Goncalves *et al.*, 2005 1c. Konan and Bacchi, 2007 1d. Kogel and Zech, 1985
Cashew apple	• Anacardic acid [2a, 2b] • Anthocyanin: glycosylated cyanidin [2c] • Glycosylated flavonols [2c]: glycosylated quercetin, myricetin, kaempferol	2a. Trevisan *et al.*, 2006 2b. Kubo *et al.*, 2006 2c. Brito *et al.*, 2007
Cashew nut shell liquid	• Resorcinolic lipids cardol [3a, 3b, 3c, 3d] • Phenolic lipid cardanol [3a, 3b, 3c, 3d] • Phenolic acid anacardic acid [3b, 3c, 3d, 3e, 3f, 3g]	3a. Kozubek *et al.*, 2001 3b. Kozubek and Tyman, 1999 3c. Trevisan *et al.*, 2006 3d. Paramashivappa *et al.*, 2001 3e. Himejima and Kubo, 1991 3f. Toyomizu *et al.*, 1993 3g. Kubo *et al.*, 2008

Cashew nut	Tannins [4a], anacardic acid [4b, 4c], alkyl salicylic acids [4b]	4a. Venkatachalam and Sathe, 2006 4b. Kubo *et al.*. 2006 4c. Trevisan *et al.*, 2006

Table 2.7 Pharmacological activities of various parts of cashew plant.

Cashew leave	
Pharmacological properties	**References**
1. Antivirus activity • Aqueous leaf extract inhibits 82.2% activity against *Simian* rotavirus.	1. Goncalves *et al.*, 2005
2. Antibacterial activity • Ethanolic leaf extract inhibits gram positive bacteria (*Staphylococcus aureus*, *Enterobacter* species, *Streptococcus pneumoniae*) and gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*).	2. Kudi *et al.*, 1999
3. Antifungal activity • After ethanol precipitation of macromolecules of leaf extract, supernatant and precipitates exhibit antifungal activity against *Cryptococcus neoformans*.	3. Schmourio *et al.*, 2005
4. Antiulcerogenic effect • Ethanolic leaf extract inhibits gastric mucosal lesions [4a] and it may be due to presence of quercetin [4b].	4a. Konan and Bacchi, 2007 4b. Martin *et al.*, 1993
5. Protection against diabetes • Aqueous leaf extract reduces *Streptozotocin*-induced blood glucose level in rats and no glycosuria presents in pretreated rats.	5. Kamtchouing *et al.*, 1998
6. Vasorelaxation effect • Leaf extract exhibits vasorelaxation effect on rat aortic ring and reduces arterial blood	6. Runnie *et al.*, 2004

pressure. 7. Antioxidant activity [7a, 7b] • Leaf extract exhibits antioxidant activity in lipopolysaccharide activated macrophage [7b].	7a. Runnie *et al.*, 2004 7b. Abas *et al.*, 2006
Cashew apple	
Pharmacological properties	**References**
1. Antioxidant and antimutagenic activity • Cashew apple juice extract prevents hydrogen peroxide-induced mutation of *Salmonella typhrimurium* strain TA102 and the antimutagenic effect is due to free radical scavenging and extracellular mutagenic compounds complexing activity of cashew apple juice. Besides, the extract stimulates repair of DNA damage.	1. Cavalcante *et al.*, 2003
2. Enzyme inhibition • Anacardic acid exhibits xanthine oxidase [2a], lipoxygenase [2b], urease [2c] inhibitory effect. 3. Antibacterial activity • Anacardic acid inhibits gram negative bacteria (*Helicobacter pylori* [3a], *Staphylococcus aureus* [3b]) through interaction of alkyl side chain of anacardic acid [3c].	2a. Kubo *et al.*, 2006 2b. Ha and Kubo, 2005 2c. Kubo *et al.*, 1999 3a. Kubo *et al.*, 1999 3b. Kubo *et al.*, 2003 3c. Kubo *et al.*, 1993
Cashew nut shell liquid	
Pharmacological properties	**References**
1. Antioxidant activity • Anacardic acids inhibits superoxide anion generation in xanthine oxidase system [1a, 1b]. Anacardic acids exhibits lipoxygenase inhibitory effect by inhibiting linoleic acid peroxidation [1c, 1d]. 2. Antibacterial activity • Anacardic acids exhibits antibacterial activity	1a. Trevisan *et al.*, 2006 1b. Masuoka and Kubo, 2004 1c. Kubo *et al.*, 2008 1d. Ha and Kubo, 2005 2a. Kubo *et al.*, 2003 2b. Kubo *et al.*, 1993

against gram negative bacteria (*Staphylococcus aureus*) which is resistant to Methicillin. The antibacterial activity is enhanced by the presence of unsaturated alkyl side chain of anacardic acids which can effectively disrupt the membrane of bacteria [2a, 2b]. 3. Enzyme inhibition • Anacardic acids inhibits glycealdehyde-3-phosphate dehydrogenase in *Trypanosoma cruzi* (flagellate protozoa) which is the cause of parasitic infection, Chagas' disease.	3. Pereira *et al.*, 2008

Cashew nut	
Pharmacological properties	**References**
1. Antioxidant activity • Tocopherols [1a] and anacardic acids [1b] inhibit xanthine oxidase. 2. Antitumor activity • Kernel oil enhances activity of antioxidant enzyme (superoxidase dismutase SOD, catalase etc.), decreases lipid peroxidation in mice liver, inhibits tumor growth in mice skin papillomagenesis.	1a. Kornsteiner *et al.*, 2006 1b. Kubo *et al.*, 2006 2. Singh *et al.*, 2004

Cashew bark	
Pharmacological properties	**References**
1. Antibacterial activity • Ethanolic bark extract is active in inhibiting gram positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Corynebacterium pyogenes*, *Enterobacter* species) and gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). 2. Ant- inflammatory activity • Bark extract decreases inflammatory mediators-	1. Kudi *et al.*, 1999; Akinpelu, 2001 2. Mota *et al.*, 1985

induced permeability effect and prevents leucocytes from migration to inflammation site. Therefore, the extract exerts anti-inflammatory activity in edemas polyarthritis in rats. 3. Anti-parasitic activity • Hydroalcoholic bark extract treats skin ulcer which is infected by *Leishmania* (*Viannia*) *braziliensis*.	3. Franca *et al.*, 1996

Antioxidant activity of cashew plant extract measured in various *in vitro* antioxidant systems and analysis of its relationship with phenolic content will be discussed in the following paragraphs.

In a study by Trevisan *et al.* (2006), cashew nut shell liquid contains much higher amount of anacardic acids (major alkyl phenols), followed by cashew fibre and low amount is detected in other cashew products (cashew apple, raw and coasted nut). Apparent no amount of cardanols and cardols is detected in cashew fibre, apple, nut while these alkyl phenols are abundant in cashew nut shell liquid. In addition, cashew nut shell liquid displays significant antioxidant activity followed by hexane extract of cashew fibre in hypoxanthine/ xanthine oxidase assay. The result exhibits a correlation between antioxidant capacity and concentration of alkyl phenols in cashew product extract. The alkyl phenols in cashew nut shell liquid are fractionated by HPLC and major fraction of anacardic acids, cardanols and cardols are tested with antioxidant activity. Anacardic acid fractions display far greater antioxidant activity than cardanols and cardols in hypoxanthine/ xanthine oxidase assay which is related to inhibition of superoxide anion generation by xanthine oxidase. This could be explained that anacardic acids is the major contributor to the high antioxidant activity of cashew nut shell liquid and this antioxidant activity correlates significantly with concentration of anacardic acids. Anacardic acids contains phytyl side chain along with three double bonds in alkyl side chain, conferring strong antioxidant activity. Antioxidant activity of plant extract correlates with concentration of phenolic compounds, major constituents of phenolic content and also its structural properties related to antioxidant activity.

Kamath and Rajini (2007) worked on determination of antioxidant activity of cashew nut skin extract in various antioxidant assay systems. Ethanolic cashew nut skin extract is evaluated as a potential antioxidant with high total phenolic content. The antioxidant activity of this extract appears to be more related to free radical scavenging rather than inhibition of oxidation and metal chelation since its effectiveness in antioxidant assay is in order of ABTS radical scavenging > superoxide radical scavenging > deoxyribose oxidation > inhibition of lipid peroxidation > metal chelation. In addition, the study also reported that epicatechin may be the major polyphenols contributing to antioxidant activity of cashew nut skin extract.

Cashew shoot extract obtained from three different solvent extractions has been tested for antioxidant activity and total phenolics (Razali *et al.*, 2008). Methanolic cashew shoot extract displays significant antioxidant activity in scavenging $ABTS^{+}$, DPPH, superoxide anion, nitric oxide radicals and ferric ions as compared to ethyl acetate and hexane extract. Methanol extracts high polar antioxidants from shoot sample and these antioxidants appear to be the contributors to observed antioxidant activity. In this study, antioxidant activity of methanol extract correlates significantly with total phenolic content, which is 7 fold higher as compared to total phenolics of the other shoot extracts. This result indicates that high level of phenolic compounds appears to be contributor to observed antioxidant activity.

2.5.3 Toxicological Analysis of Cashew Plant

Hydroethanolic cashew leave extract produced 50% fatal dose only of >2000 mg/kg body weight in rats and no toxic symptoms (Konan *et al.*, 2007; Konan and Bacchi, 2007). A study by Konan *et al.* (2007) showed that administration of ethanolic cashew leaf extract did not significant affect renal and hepatic functions in rats and liver and kidney morphology. These protective effects might be attributed to antioxidant activity of cashew leaf extract.

2.6 Mangosteen Fruit Rind and Cengal Wood Chipping Extract

In this study, mangosteen fruit rind and cengal wood chipping were also ethanolic extracted and tested for antioxidant activity and phenolic content.

Mangosteen fruit rind extract has been consumed as herbal medicines for anti-acne treatment because this plant is active in free radical scavenging and inhibits the growth of acne bacteria including *P. acnes* and *S. epidermidis*. It contains high content of tannins, flavonoids and phenolic acids such as protocatechuic acid, m-Hydroxybenzoic acid and 3,4-dihydroxymandelic acid (Pothitirat *et al.*, 2009; Zadernowski *et al.*, 2009).

Cengal is a medicinal plant which is available in Sabah, Malaysia for cancer therapy and this plant has been shown to have antioxidant activity (Gansau *et al.*, 2009). It is believed that the polyphenolic compounds contained in this plant extract are the contributors to their antioxidant activity and therapeutic properties.

2.7 Protein-polyphenol Interaction

Polyphenolic compounds have a significant characteristic of possessing capacity to bind proteins and form precipitates subsequently. Considerable number of studies have been performed which involved especially the binding between salivary proline rich proteins and tannins (Murray *et al.*, 1994; Charlton *et al.*, 1996; Baxter *et al.*, 1997; Hagerman *et al.*, 1998; Bacon and Rhodes, 2000; Freitas and Mateus, 2001).

In the paper of Charlton *et al.* (2002), they proposed the processes occurring in binding and precipitation of salivary proline rich proteins and polyphenols in detailed molecular aspect. The model of the interaction has in the past been generated by Hagerman and Butler (1981). The interaction processes are divided into three stages (Figure 2.8). Initially, polyphenols interact reversibly with extended and random coiled polypeptide at proline binding sites by hydrophobic bonding and

give soluble complexes. As more polyphenols are added, polyphenols link two peptides and the peptides dimer coated by polyphenols becomes insoluble and starts to precipitate. Further intermolecular interactions lead to precipitation of complexes and the complexes aggregate into large and small particles.

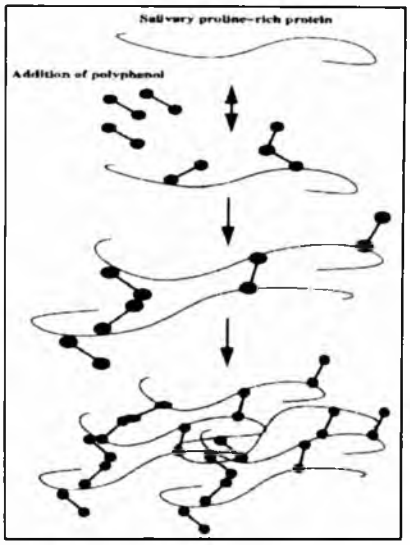


Figure 2.8 Model of interaction between salivary proline rich proteins and polyphenols (Charlton *et al.*, 2002).

Studies in the past have indicated that tannins interacts with proteins in a specific and selective mode. Several structural features including molecular size, conformation, composition, stereochemistry, ratio of elements and bonding which are involved in protein-polyphenol interaction will be elaborated in the following sections regarding nature of proteins and polyphenols in protein-polyphenol complex.

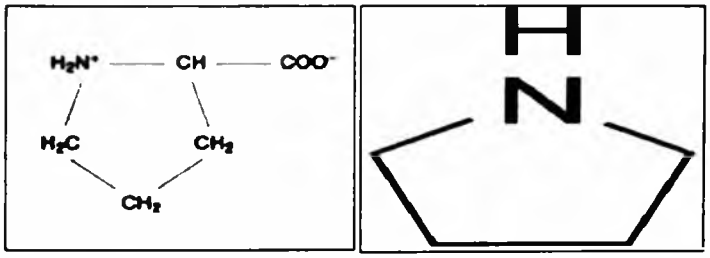
2.7.1 Nature of Proteins in Protein-polyphenol Complex

2.7.1.1 Proline Rich Proteins

Polyphenols has a significant affinity for extended proline rich proteins (Hagerman and Butler, 1981). Proline residues in polypeptides are essentially involved in binding with polyphenols. Polypeptides with proline content form haze in various degree roughly according to the mole percent of proline. A study by

Asano *et al.* (1982) has shown that the ability of proteins to form haze with catechin (polyphenols) is proportional to proline content. Gelatin which contains 18% proline and hydroxyproline (Hagerman and Butler, 1981) and gliadin of 15% proline (Asano *et al.*, 1982) exhibit strong haze forming activity (Asano *et al.*, 1982; Makkar *et al.*, 1988) whereas low proline lysozyme and papain (1% and 5% respectively) (Asano *et al.*, 1982) have low activity (Siebert *et al.*, 1996). Homopolymeric polypeptides lack of proline such as polyglutamic acid, polyasparagine, polyphenylalanine, polyleucine and polylysine form no haze (Asano *et al.*, 1982).

Proline belongs to hydrophobic amino acid with nonpolar side chain. Its side chain is cyclized back and binds with backbone amide. The cyclized side chain is called pyrrolidine ring (Figure 2.9). This backbone conformation makes the proline polypeptide highly restricted in mobility (Balasubramanian *et al.*, 1971) and disfavors α-helix conformation (MacArthur and Thornton, 1991). Proline is not a hydrogen bond donor because its protein amide is replaced by a methyl group (Fernandez and Lilley, 1992).



(a) (b)

Figure 2.9 (a) Proline amino acid and (b) pyrrolidine ring.

Proline rich regions in protein existing as repetitive short proline rich sequence $(XP)_n$ and tandemly repeated sequence exhibit binding function in many examples. Proline rich tandemly repeated sequence has a protein function to bind with polyphenols preferably. It has been discussed above that polyphenols bind well with long open proline rich sequence (Hagerman and Butler, 1981; Murray *et al.*, 1994). Salivary proline rich protein, which is frequently used to complex with polyphenols in numerous experiments of interaction, is an example of tandemly repeated sequence $(PQGPPQQGG)_n$ (Bennick, 1982).

Some proline structure features are the devices for increasing binding affinity. The restricted mobility of proline rich polypeptide has an entropy advantage for binding. A rigid peptide has low entropy and therefore its binding is strengthened by a smaller entropy loss, giving a greater overall binding energy (Page and Jencks, 1971). Besides, multiple tandem repeats on proline rich proteins allow multidentate cross linking between polyphenols and peptides and thereby increase the overall binding affinity (Baron *et al.*, 1991; Williamson, 1994). Repeated sequence of proline, which is not highly specific for substrate binding, can bind with a wide range of ligands at high speed and remarkably strongly (Corden, 1990; Williamson, 1994).

From the consideration of interaction binding, proline with flat and hydrophobic side chain can interact well with polyphenols because of the same hydrophobic surface of aromatic ring in phenolic compound, promoting hydrophobically stacking of phenol ring in face-face manner against proline pyrrolidine ring (Oh *et al.*, 1980; Murray *et al.*, 1994; Hagerman *et al.*, 1998) (Figure 2.10). In addition to hydrophobic binding, hydrogen bonding in tannin-protein complex has an important role in secondary interaction stabilization (Hagerman and Butler, 1980; Hagerman and Butler, 1981) (Figure 2.11). The stabilized complex and enhanced binding affinity for tannins are attributed to proline which acts as a hydrogen bond acceptor. It is because methylene group substituted tertiary amide nitrogen donates electrons to peptide bond and causes the adjacent carbonyl group to be electron rich for forming hydrogen bond with other ligands/ polyphenols (Hagerman and Butler, 1981; Hagerman and Klucher, 1986).

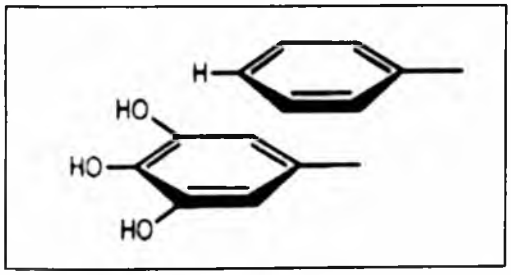


Figure 2.10 Hydrophobic face to face stacking of proline pyrrolidine ring (above ring) with phenolic aromatic ring (below ring) (Haslam, 1996).

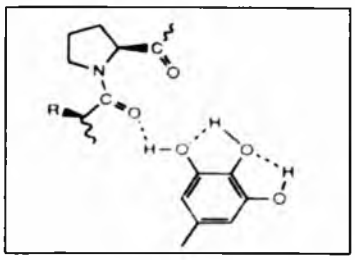


Figure 2.11 Hydrogen bonding of tertiary amide carbonyl group of proline residues (above group) with a phenolic group (below group) (Haslam, 1996).

2.7.1.2 Studies of Interaction between Proline Rich Proteins and Polyphenols

Investigation of interaction between proteins and polyphenols is usually performed on H-NMR studies. Chemical shift changes ($\Delta\delta$), dissociation constants and intermolecular nuclear Overhauser effects (NOEs/ROEs) are derived experimentally to indicate specificity and strength of binding interaction (Murray *et al.*, 1994; Baxter *et al.*, 1997).

A study by Murray *et al.* (1994) demonstrated the precipitation of pentagalloyl glucose by mouse salivary proline rich proteins MP5. The binding interaction between proteins and polyphenols is NMR evidenced by chemical shift change seen on peptide resonance and presence of NOEs on the addition of polyphenols. Large chemical changes are observed on proline residues, proceeding amide bond and proceeding proline residues and these observations indicate the galloyl rings of polyphenols bind beyond the proline to proceeding bond and amino acids. Besides, large changes in chemical shift are observed on CαH, $C\beta^3H$ and CδH which are the proline protons located on the pyrrolidine ring surface containing CαH. It suggests that polyphenols binds preferentially onto this face of pyrrolidine ring which is less structurally hindered.

In the study by Baxter *el al.* (1997), the observation on intermolecular ROEs and upfield chemical shift change of peptide proton resonance on the titration of polyphenols serves to propose that galloyl rings of polyphenols are stacking onto the pyrrolidine ring of proline together with proceeding residues (Figure 2.12). In

addition, this study also demonstrated that there is no change in the structure of peptide on the titration of polyphenols and it is believed that proline residues in proline rich protein keep the conformation extended and provide maximum binding surface (Williamson, 1994).

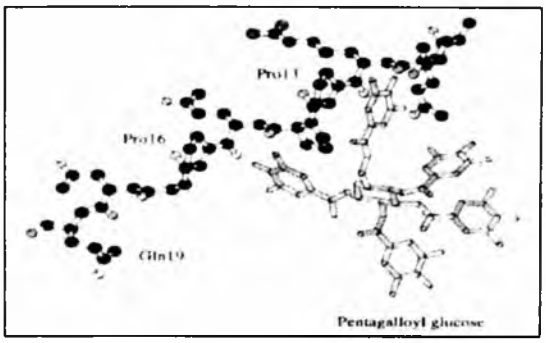


Figure 2.12 Hydrophobic stacking of galloyl rings of pentagalloyl glucose with pyrrolidine rings of proline and proceeding proline residue (Baxter *et al.*, 1997).

All the studies stated above are to present some structural features of proline rich proteins such as hydrophobic planar ring, multiple tandem repeat, long open extended, rigid conformation and electron rich carbonyl group, make it a good ligand for effective and cooperative association with polyphenols.

2.7.2 Nature of Polyphenols in Protein-polyphenol Complex

Numerous studies on the binding between tannins and protein have been reported. Tannins is classified into hydrolyzable tannins and condensed tannins. Owing to the structural difference between hydrolyzable tannins and condensed tannins, the structural considerations of these two tannins on binding capacity and precipitation will be discussed separately.

2.7.2.1 Structural Factors of Hydrolyzable Tannins Involved in Binding and Precipitation

For hydrolyzable tannins (galloylglucose), its specificity in binding and precipitation will be elaborated in view of molecular size (galloylation) and multidentate binding.

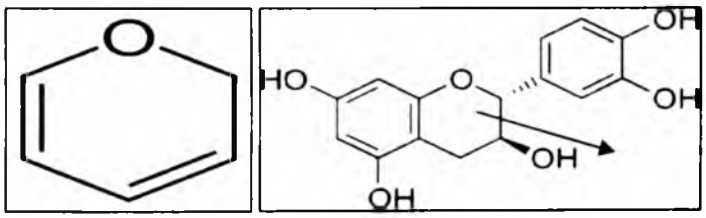
Baxter *et al.* (1997) indicated that larger polyphenols (pentagalloyl glucose) binds more strongly with proline rich proteins than the smaller one (trigalloyl glucose). This proportional relationship between the level of galloylation and enzyme binding affinity is also demonstrated in the studies of inhibition of β-glucosidase by hydrolyzable tannins (Ozawa *et al.*, 1987), precipitation of galloylglucose by bovine serum albumin (Kawamoto *et al.*, 1995) and hydrolyzable tannins/ salivary proline rich proteins binding and precipitation (Charlton *et al.*, 2002). However, a study by McManus *et al.* (1985) indicated that the binding affinity with bovine serum albumin by pentagalloyl glucose would not be enhanced with addition of further galloyl rings when optimum affinity is achieved.

The effectiveness in association between proteins (proline rich proteins and bovine serum albumin) and large, complex hydrolyzable tannins might be due to multidentate binding (Hagerman and Butler, 1981; McManus *et al.*, 1985; Baxter *et al.*, 1997; Haslam, 1998; Bacon and Rhodes, 2000; Charlton *et al.*, 2002). Highly galloyl substituted polyphenols enhances the cross linking with another peptide by extra free galloyl rings, to allow further cooperative interaction and strengthen the association. This could explain less concentration of pentagalloyl glucose (with five galloyl rings) is required in precipitation of tannins/ peptide complex as compared to epigallocatechin gallate which has only two 1,2-dihydroxyphenyl rings (Charlton *et al.*, 2002). In addition, multidentate binding of large polyphenols encourages cooperative binding of several galloyl rings present on same polyphenols and stacking of galloyl groups between two adjacent polyphenols on same peptide (Murray *et al.*, 1994; Baxter *et al.*, 1997).

2.7.2.2 Structural Factors of Condensed Tannins Involved in Binding and Precipitation

Proanthocyanidins is polymers of catechin/ epicatechin. Proanthocyanidins is the most common condensed tannins participating in haze formation in beverage such as beer (McMurrough *et al.*, 1992) and fruit juice (Spanos and Wrolstad, 1992). Several structural factors such as molecular size, degree of polymerization and stereochemistry of monomeric units would affect the binding of condensed tannins to proteins.

It has been shown that only high polymer of proanthocyanidins (dimers and above) is active in haze formation (Mulkay and Jerumanis, 1983; Asano *et al.*, 1984). Freitas and Mateus (2001) indicated that (+) catechin – (+) catechin dimer has two fold higher binding affinity as compared to its monomer. Large molecular size and increased degree of polymerization of tannins have an effective binding and precipitation. It might be due to high density of hydroxyl groups attached on phenolic pyranic ring (Figure 2.13) and these well exposed hydroxyl groups enhance noncovalent binding with peptides without conformation restriction.



(a) (b)

Figure 2.13 Pyranic ring of catechin.

Small polyphenols is ineffective in activity because it could not cross link with peptides strongly and fail to simultaneously bind at several binding sites on same or different peptides, due to lack of adequate composition and complexity (Siebert and Lynn, 1998; Freitas and Mateus, 2001). However, this positive relationship between binding affinity and degree of polymerization is not very obvious in the reaction with (-) epicatechin. This agrees with another observation that (+) catechin has a greater tannins specific activity in reaction with proline rich

proteins as compared to (-) epicatechin (Siebert and Lynn, 1998; Freitas and Mateus, 2001) and demonstrates the effect of stereochemistry of monomeric unit of proanthocyanidins on the interaction with proline rich proteins.

2.7.3 Nature of Protein-polyphenol Interaction

2.7.3.1 Relative Ratio of Proteins and Polyphenols in Protein-polyphenol Complex

In a study of Siebert *et al.* (1996), gelatin (protein with 12-14% hydroxyproline and proline) and tannic acid (polyphenols) are combined in various proportions and the resultant haze formed is measured by light scattering using turbidimeter. The relationship between concentration of proteins, polyphenols and precipitates obtained in the study is generated as a model for protein-polyphenol interaction (Figure 2.14). In this interaction model, polyphenol molecule is depicted to have two binding ends and protein is depicted to have fixed number of binding sites that can bind to polyphenols. Only roughly equal number of binding sites in both proteins and polyphenols would produce the largest aggregates and maximum light scattering.

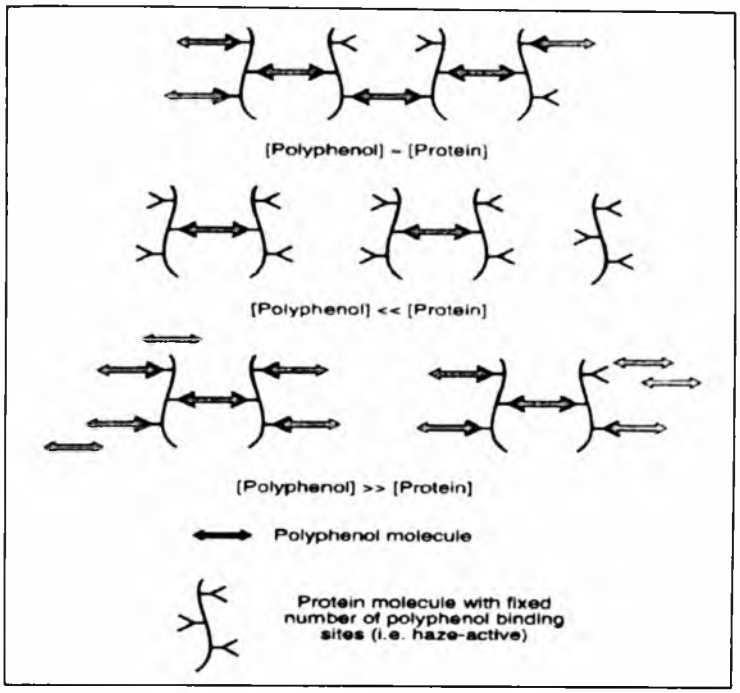


Figure 2.14 Model for protein-polyphenol interaction (Siebert *et al.*, 1996).

The amount and size of precipitates would decrease if large access of proteins relative to polyphenols or polyphenols relative to proteins occurs during protein-polyphenol interaction (Table 2.8). If the ratio of polyphenols to proteins is less than 1, polyphenols of fewer amount are insufficient to bridge many peptide together. If the ratio is more than 1, most of the protein binding sites are occupied and free polyphenols find difficultly available protein binding sites to bridge to (Siebert *et al.*, 1996). These studies showed that the ratio of polyphenols and proteins is essential in estimation of size of complex/ precipitates formed.

Table 2.8 Relative ratio of polyphenols to proteins and size of precipitates.

Relative ratio of polyphenols to proteins, [polyphenols]/[proteins]	**Size of precipitates formed**
=1	Large
>1	Small
<1	Small

2.7.3.2 Driving Forces towards Association of Proteins and Polyphenols

Hydrophobic effect and hydrogen bonding are primary driving forces towards protein-polyphenol interaction and these two modes of interaction will be elaborated in detail in the following paragraphs.

Hydrophobic interaction of protein-polyphenol interaction will be discussed firstly. Both of the two main elements – polyphenols and proteins in protein-polyphenol complex have multiple hydrophobic sites. Polyphenols possesses aromatic galloyl rings and pyranic rings of carbon-hydrogen skeleton. Proline amino acid contains pyrrolidine ring and arginine has a flexible, hydrophobic side chain. These hydrophobic sites provide potential driving forces in participation of hydrophobic interaction (Haslam, 1996).

In a study of interaction between pentagalloyl glucose and mouse salivary proline rich proteins, gallic groups of tannins are hydrophobically stacked against planar pyrrolidine rings of proline (Murray *et al.*, 1994). Hydrophobic interaction is also evidenced in the precipitation of pentagalloyl glucose with bovine serum albumin by surrounding proteins with hydrophobic bond (Oh *et al.*, 1980; Hagerman *et al.*, 1998). However, the efficacy of hydrophobic interaction will be influenced by structural complexity of peptide. Proline rich proteins interacts strongly and effectively with tannins due to its random coiled structure with well exposed binding sites and this allows face to face stacking of phenolic groups onto proline planar surface (Murray *et al.*, 1994). Small globular proteins (e.g. bovine serum albumin) bind but not cross link with phenolic groups of tannins (Murray *et al.*, 1994; Baxter *et al.*, 1997; Siebert and Lynn, 1998).

Examples of study (Oh *et al.*, 1980; Murray *et al.*, 1994; Hagerman *et al.*, 1998) given above suggest the interaction between hydrolyzable tannins and proteins is dominantly hydrophobic interaction. However, a different mechanism which involves hydrogen bonding is observed with the condensed tannins – proteins interaction. In a study of Hagerman *et al.* (1998), precipitation of bovine serum albumin by pentagalloyl glucose (hydrolyzable tannins) is sensitive to temperature

and suppressed by the presence of organic solvent (alcohol), indicating the precipitation involves mainly hydrophobic force and very weak hydrogen bonding, whereas precipitation by proanthocyanidins (condensed tannins) gives an opposite trend which is insensitive to temperature and reaction solution containing alcohol and this suggests hydrogen bonding dominates the reaction (Hagerman and Butler, 1981).

Several studies have demonstrated protein-polyphenol interaction is primarily driven by hydrophobic force and hydrogen bonding is a secondary complex stabilizing force (Hagerman and Butler, 1980; Hagerman and Butler, 1981). Hydrogen bonding is formed between phenolic hydroxyl groups and peptide residues' carbonyl groups. However, it has been stated that these two substrate groups are surrounded with water molecules, therefore solvation around the substrate groups needs to be broken upon complexion of proteins and polyphenols (Haslam, 1996). Carbonyl group of tertiary amide in prolyl peptide is a good participant in hydrogen bonding due to poor solvation of tertiary amide (Figure 2.11). Less energy is required to break solvating hydrogen bond on tertiary amide groups in comparison to that of secondary amide. In addition, the strength of binding is enhanced because methylene substitution on tertiary amide nitrogen next to carbonyl group donates electrons into peptide bond and thus the carbonyl group becomes strong hydrogen acceptor (Hagerman and Butler, 1981; Hagerman and Klucher, 1986; Haslam, 1996; Murray *et al.*, 1994).

In summary, the interaction of proteins and polyphenols is dominantly driven by hydrophobic stacking of polyphenolic rings against hydrophobic side chain on peptide and subsequently hydrogen bonding between phenolic hydroxyl groups and carbonyl groups of peptide as secondary forces. Different mechanisms involving hydrophobic interaction and hydrogen bonding are observed for precipitation of hydrolyzable tannins and condensed tannins respectively.

2.8 Effect of Temperature and pH on Conformational Stability of Protein

Studies on temperature and pH are essential. These factors will alter the conformational structure of protein and in turn affect its functionality. In this study, free and complexed bromelain were exposed to elevated temperature and acidic/ alkaline conditions to determine the effect of these factors on enzymatic acitivty and stability. Therefore, the mechanism on disruption of protein's structure by temperature and pH is elaborated.

Conformational stability of protein is attributed to the sum of stabilizing interactions/ various noncovalent bondings for folded structure maintenance and destabilizing forces arising from entropy of unfolding. Hydrophobic interaction, hydrogen bonding and electrostatic interaction are the major forces in stabilizing the folded states of protein (Matthews, 1993; Baldwin and Matthews, 1994). Decrease the conformation freedom of unfolded form will increase the difference in free energy between folded and unfolded state and thus enhance protein stability (Matthews, 1993; Daniel *et al.*, 1996). Any changes in the strength of stabilizing and destabilizing interactions caused by environmental conditions will have an effect on integrity and stability of protein.

Increased temperature facilitates disruption of conformational integrity of folded protein by thermal unfolding and the loss of conformation can proceed to degradation and thus leads to thermo-inactivation in protein. Irreversible degradative reactions include deamidation, oxidation of amino acid, intramolecular succinimide formation, peptide bond hydrolysis and aggregation of denatured protein and all these processes strongly damage the covalent structure of protein and cause activity loss (Clarke, 1987; Manning *et al.*, 1989; Tomizawa *et al.*, 1995; Daniel, 1996; Daniel *et al.*, 1996).

Isoelectric point is the characteristic pH of a protein at which the net charge is zero and activity is optimum. At this pH, acidic and basic amino acids are equally charged and the electrostatic interactions provide benefit in protein stability. Large changes in pH alter the state of ionization of amino acids, increase charge repulsion,

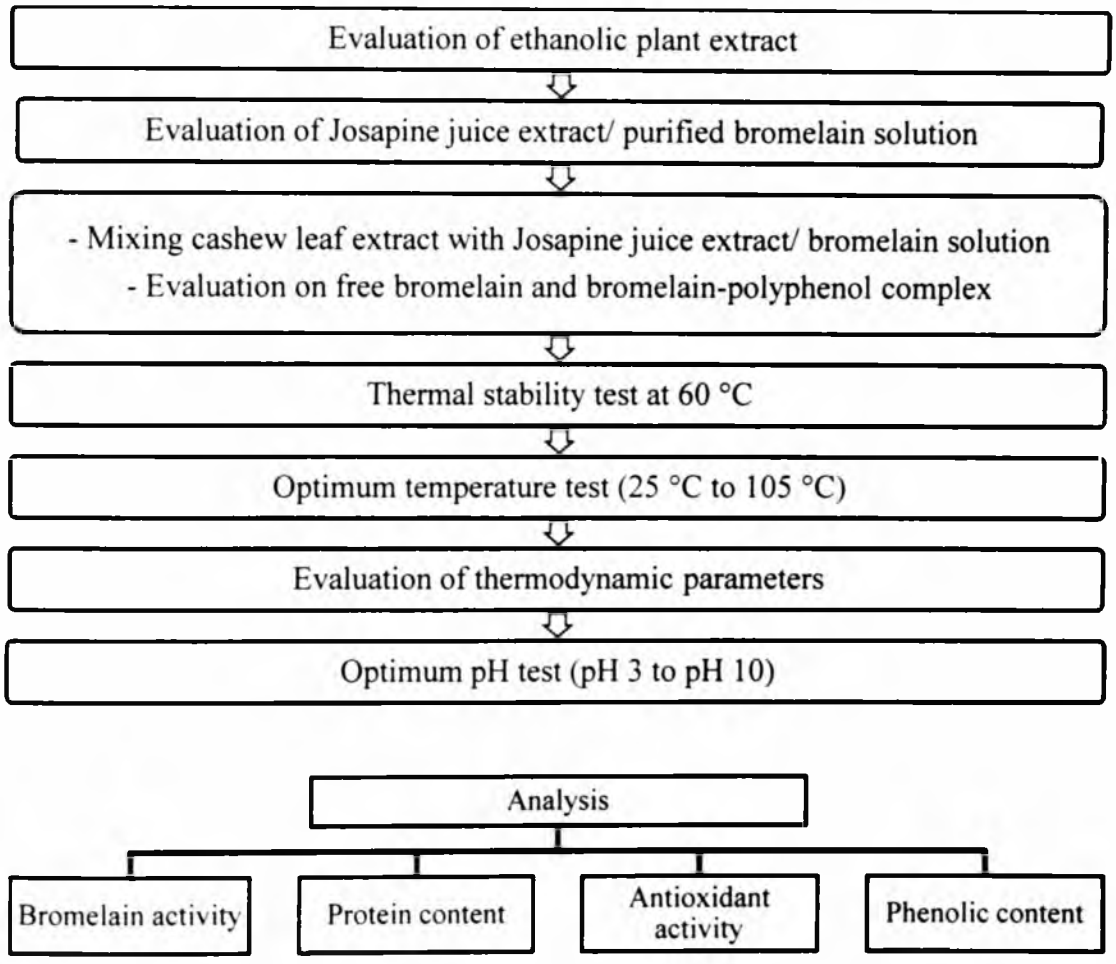
change electrostatic interactions/ ionic bond which stabilize the conformation. It would cause protonation or deprotonation of residues and decrease substrate binding to catalytic residues. Extreme pH further increases intramolecular charge repulsion which counteracts the stabilizing interaction maintaining the folded form and results in protein unfolding (Matthews, 1993; Fink *et al.*, 1994; Ahmad *et al.*, 2004). pH effect will cause protein denaturation and irreversible deactivation. Under alkaline condition, some amino acids (eg. cysteine, serine, lysine, etc) will be degraded by β-elimination. Cysteine residues are involved in disulphide exchange. Peptide bond hydrolysis will occur in acidic solution (Manning *et al.*, 1989; Munch and Tritsch, 1990).

The literatures have clearly indicated the medicinal potential of proteins (bromelain) and polyphenols (cashew leaf extract) and the tendency of these two molecules to form protein-polyphenol complex. Current studies are focused on physical characteristics of the complex by evaluating its nutraceutical (enzymatic and antioxidant activity) and also physiochemical properties (thermal and pH stability). These two characteristics could be beneficial in designing high nutraceutical product. The study will be conducted by methodology described in Chapter 3.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design



3.2 Chemicals

Bromelain from pineapple stem, L-Tyrosine, 2N Folin-Ciocalteau's phenol reagent, gallic acid, sodium phosphate dibasic, sodium phosphate monobasic and L-Cysteine hydrochloride monohydrate were obtained from Sigma Aldrich (St Louis, MO, USA). 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) was purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Ascorbic acid was purchased from GCE Laboratory Chemical. Casein was obtained from Biochemical BDH (England). Pierce BCA protein assay reagent kit was purchased from Thermo Scientific (Rockford, USA). Sodium carbonate, pH 4 and pH 10 buffer solutions were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid (TCA), sodium hydroxide pellet and pH 6 buffer solution were obtained from QReC. EDTA di-sodium salt was obtained from Univar (N.S.W., Australia). pH 7 buffer solution was obtained from Eutech Instruments (Malaysia). Hydrochloric acid was purchased from Mallinckrodt (Xalostoc, Mexico). 95% (purity) ethanol was purchased from HmbG Chemicals.

3.3 Preparation and Evaluation of Plant Extracts

3.3.1 Plant Materials

Cashew leaves were collected from a cashew tree planted at Universiti Teknologi Malaysia campus, Malaysia. The leaves were air dried at room temperature for 24 hours. Mangosteen fruit rind was the residue collected after mangosteen fruit processing using belt pressing machine (Voran) at downstream processing laboratory, Faculty of Chemical Engineering, UTM. The mangosteen residue was dried in drying oven (Memmert) at 50 °C for 48 hours (Pothitirat *et al.*, 2009). Cengal wood chipping was purchased from a Malay traditional medicine shop. These samples were cut, ground to powder in grinder (IKA Labortechnik, Staufen, Germany) and stored in tight container until used.

3.3.2 Ethanolic Plant Extraction

Dried powder of cashew leave, mangosteen fruit rind and cengal wood chipping were separately extracted with 95% (purity) ethanol at room temperature for 24 hours, in mass to volume ratio of 2:10 (g/ml). The extracts were filtered through cotton cloth to remove the residues. The filtrate was concentrated to dryness to remove ethanolic phase with rotary evaporator (Heidolph Laboratory, Laborota 4000) at 70 °C, 30 rpm, under low pressure. The ethanolic extract was kept at -18 °C until used (Konan and Bacchi, 2007; Razali *et al.*, 2008).

3.3.3 Evaluation of Antioxidant Activity and Phenolic Content of Plant Extracts

20% ethanolic plant extract was defined since the ratio of dried plant powder (g) to ethanol (ml) was 2:10 (w/v). The plant extract was resuspended in ethanol and the plant extract solution was tested for antioxidant activity and phenolic content. In details, hyperbolic saturation curve of DPPH scavenging activity (antioxidant activity) was attained by varying the extract solution concentration. Antioxidant capacity could also be estimated based on EC50 value. Plant extract with high antioxidant activity and phenolic content was selected to form precipitate with bromelain.

3.4 Preparation and Evaluation of Josapine Juice Extract and Purified Bromelain Solution

3.4.1 Plant Material

Josapine pineapples were collected at pineapple farm (managed by Malaysian Pineapple Industry Board, MPIB) located at Alor Bukit, Pontian, Johor, Malaysia. Josapine pineapples with mature index of three were harvested. For pineapple with this index number, one or two layers of pineapple eyes from the lowest part become

yellowish while the rest is still in green colour. This fruit begins to be ripe at this stage. The harvested fruits were stored at cool room at 4 °C and it was processed within 1-2 days.

3.4.2 Preparation of Josapine Juice Extract

Josapine pineapples were peeled, cut, sliced and juiced in a laboratory juicer (National, MJ-68M). The juice was then centrifuged (Hettich Zentrifugen, Rotina 420R) at 10000 g, 4 °C for 30 minutes and fibrous material was removed. The supernatant was subjected to two stages of vacuum filtration using filter paper of pore size 20 μm and 2.7 μm (Advantec, Japan) with Buchner funnel (Gast pump, U.S.A). It was followed by sterile filtration (media filtration, Masterflex L/S pump, Cole Parmer Inc.) using 0.2 μm cellulose acetate membrane (Sartorius Stedium, Biotech GmbH, Germany). The clarified juice (Josapine juice extract) was stored at 4 °C for further analysis (Devakate *et al.*, 2009; Mirdawati *et al.*, 2010).

3.4.3 Evaluation of Properties of Josapine Juice Extract

The volume of juice remained after each processing step (centrifugation, 20 μm filtration, 2.7 μm filtration and 0.2 μm filtration) was measured to determine the percentage loss in each progress. Juice extract was analyzed with bromelain activity, protein content, antioxidant activity and phenolic content.

3.4.4 Preparation and Evaluation of Properties of Purified Bromelain Solution

Purified commercial bromelain from pineapple stem was purchased from Sigma-Alrich. Purified bromelain solution in concentration of 4 mg/ml or 0.4% (w/v) which was prepared in solution of cysteine hydrochloride (30 mM) and EDTA

disodium salt (6 mM) (Devakate *et al*., 2009) was used as a positive control free bromelain solution.

3.5 Precipitation of Bromelain with Cashew Leaf Extract Polyphenols

3.5.1 Mixing of Cashew Leaf Extract with Josapine Juice Extract/ Purified Bromelain Solution

Ethanolic cashew leaf extract was found to have high antioxidant activity and phenolic content and therefore selected to be precipitate bromelain. Cashew extract over the range 0.1% to 1.5% (w/v) was added to Josapine juice extract and the mixture was constant stirred using hotplate stirrer (Agimatic-N, 40 rpm) at 25 °C for 30 minutes. The mixture was subsequently cooled overnight at 4 °C to allow precipitation. The sample was centrifuged at 10000 g for 15 minutes and the precipitate was separated from the supernatant (Liang *et al.*, 1999; Devakate *et al.*, 2009).

This precipitate of bromelain-polyphenol was named cashew-josapine precipitated bromelain. 1.5% cashew-josapine precipitated bromelain was named if the concentration of cashew extract in Josapine juice was 1.5% (w/v). A 0.4% (w/v) bromelain solution precipitated with same range of cashew leaf extract was used as positive control and the resultant precipitate was named cashew-bromelain precipitated bromelain/ positive control precipitate.

3.5.2 Evaluation of Properties of Free Bromelain and Bromelain-polyphenol Precipitate

The supernatant of cashew-josapine and cashew-bromelain precipitates was separated from the precipitate that formed in each precipitate and the residual bromelain activity in supernatant solution was measured. Percentage recovery of bromelain activity from free bromelain solution (Josapine juice extract/ 0.4%

bromelain solution) after addition of cashew leaf extract was calculated by the following equation:

% recovery = [1- (supernatant activity of precipitate formation / bromelain activity of free bromelain solution)] x 100%

where the bromelain activity in supernatant after precitation with cashew leaf extract was calculated in relative to bromelain activity of free bromelain solution before addition of cashew extract.

1.5%, 1.0% and 0.5% cashew-josapine/ cashew-bromelain precipitates were resuspended separately with same amount of water and the bromelain activities in precipitate solutions were measured. Actual precipitate activity in each sample could be compared with estimated value which was calculated by multiplication of its corresponding % recovery with bromelain activity of free bromelain solution. In addition, total bromelain activity of supernatant and precipitate in each sample was compared with that of free bromelain solution to access percentage loss in precipitation process. Correlation between supernatant and precipitate activity was also determined.

Bromelain activity, protein content, antioxidant activity and phenolic content derived from bromelain-polyphenol precipitate were determined on the above stated precipitate solution. These precipitate properties were evaluated in comparison with Josapine juice extract, 0.4% bromelain solution and cashew-water precipitate (negative control).

3.6 Effect of Temperature on Properties of Free Bromelain and Bromelain-polyphenol Precipitate

3.6.1 Evaluation of Thermal Stability of Free and Precipitated Bromelain at Temperature of 60 °C

Bromelain-polyphenol precipitate was dissolved in 0.1 M sodium phosphate buffer (Na_2HPO_4/ NaH_2PO_4) with pH of 4.5. Josapine juice extract/ 0.4% bromelain solution is used for free bromelain test. Cashew-water precipitate is used as negative control sample. Thermal stability was evaluated by incubating these solutions at 60 °C for 180 minutes, using a water bath (Edelstahl Rostfrei). Aliquot of heated sample was withdrawn onto ice bath at 30 minute interval (Liang *et al.*, 1999). Samples were analyzed for bromelain activity, protein content, antioxidant activity and phenolic content and the activities were expressed as percentage (relative activity), that was the ratio of activity to maximum activity in each sample.

Thermal denaturation of enzyme is assumed to follow first order reaction which the equation is described as

$$\ln [v] = -kt + \ln [v_o]$$

where v and v_o are enzymatic activities at treatment time, t and initial time, t = 0 respectively and k is denaturation rate constant. From the logarithm of residual enzyme activity versus incubation time, denaturation rate constant (k) could be determined by linear regression analysis. Half life, $t_{1/2}$, is defined as time required to loss half of the initial activity, which could be described as

$$t_{1/2} = 1/k \ (\ln 2)$$

The obtained kinetics of thermal inactivation (denaturation rate constant and half life) were analyzed and compared among the tested samples.

For protein content, the slope of logarithm of relative content versus treatment time gave a value of decrease rate constant at defined incubation time range.

3.6.2 Evaluation of Optimum Temperature of Free and Precipitated Bromelain at Various Temperatures

Free bromelain and precipitated bromelain solutions were incubated at temperatures from 25 °C to 105 °C, 30 minutes at each temperature, using standard procedure as described above. Heat treated sample was analyzed with standard assays of bromelain activity, protein content, antioxidant activity and phenolic content, and optimum temperature for these properties was determined.

For bromelain activity, logarithm of residual activity versus incubation temperature was plotted and gradient of the slope at defined temperature range was determined for the effect of increasing temperature on enzymatic activity.

3.6.3 Estimation of Activation Energy and Thermodynamic Parameters

Thermal inactivation of enzyme could be evaluated by denaturation rate constant as a function of temperature. Josapine juice extract and 1.0% cashew-josapine precipitated bromelain were incubated at temperatures of 80 °C, 82 °C, 85 °C, 87 °C and 90 °C, at each temperature the inactivation was carried out at 30, 60, 90, 120 and 150 minutes. Denaturation rate constant and half life were determined from the plot of ln [relative bromelain activity] versus t for both samples at each incubation temperature.

Arrhenius equation describes temperature dependency of denaturation rate constant

$$\ln k = \ln A - (E/R)\ 1/T$$

where k is denaturation rate constant, A is Arrhenius constant, R is universal gas constant (8.314 $JK^{-1}mol^{-1}$) and T is absolute temperature. Activation energy, E could be calculated by linear regression analysis of plot of ln k versus 1/T.

Thermodynamic parameters such as activation enthalphy (ΔH^*), free energy of inactivation (ΔG^*) and activation entropy (ΔS^*) could be calculated based on the following absolute reaction rates

$$\Delta H^* = E - RT$$
$$\Delta G^* = -RT \ln (kh/ k_bT)$$
$$\Delta S^* = (\Delta H^* - \Delta G^*) / T$$

where h is Planck constant and k_b is Boltzmann constant (Naidu and Panda, 2003; Bhatti *et al.*, 2006; Xue *et al.*, 2010).

3.7 Effect of pH on Properties of Free Bromelain and Bromelain-polyphenol Precipitate

pH optimum and stability were accessed by incubating free bromelain and precipitated bromelain in 0.1 M sodium phosphate buffer (Na_2HPO_4/ NaH_2PO_4) which were prepared with pH range of 3.0-10.0 (Orion SA720 pH meter), for 4 hours at 25 °C. Residual activity was measured using the standard assays to determine the effect of pH on samples (Liang *et al.*, 1999; Quintanilla-Guerrero *et al.*, 2008).

3.8 Methods of Analysis

3.8.1 Determination of Antioxidant Activity

DPPH free radical scavenging activity was the antioxidant activity assay used in this study to access the potential of plant extract to quench free radical species. The scavenging activity was assayed according to method of Liyana-Pathiranan and Shahidi (2005). An aliquot of sample/ standard was mixed with same volume of 0.135 mM DPPH solution in ethanol. The mixture was shaken vigorously on vortex mixer (Asasi VM-20) and stand for 30 minutes at room temperature in dark. The reduction in absorption of DPPH solution was measured at 517 nm using a

spectrophotometer (Secomam, PRIM). All the determinations were carried out in triplicate.

DPPH radical scavenging effect was accessed by measuring percentage of DPPH radical scavenged. DPPH scavenging activity could be calculated as

DPPH scavenging activity (%) = [1- (Absorbance at 517 nm of sample/ absorbance at 517 nm of contol)] x 100%

where control was DPPH solution in absence of sample. Sample with antioxidant capacity will scavenge DPPH free radical and results in reduction in absorbance of DPPH solution.

Ascorbic acid was used as a standard. A series of concentrations ranging from 0.001 to 0.100 mg/ml were tested. The DPPH scavenging activity of ascorbic acid was shown in Appendix A, Figure A.1.

EC50 was an alternative parameter used for result interpretation of scavenging activity. It could be defined as the concentration of substrate in reaction mixture which causes a 50% reduce of initial DPPH concentration. The higher the antioxidant capacity exhibited by substrate, the lower is the EC50 value. EC50, expressed as concentration in mg/ml, could be determined from the plot of DPPH scavenging activity versus concentration of sample.

3.8.2 Determination of Phenolic Content

Phenolic content was determined by Folin-Ciocalteau colorimetry method. Determination of phenolic content was carried out using method of Wolfe *et al.* (2003). 0.1 ml sample or standard was mixed with 0.5 ml Folin-Ciocalteau reagent (diluted to 1:10 with deionized water) and left for 5 minutes in dark at room temperature. It was followed by addition of 0.4 ml sodium carbonate, Na_2CO_3 solution (7.5% w/v), mixing and the mixture was allowed to incubate for a further 1

hour. The absorbance at 765 nm was measured using Secoman. PRIM spectrophotometer

A gallic acid calibration curve was constructed by measuring the absorbance at 765 nm in the presence of various concentrations of gallic acid solution (0.005-0.060 mg ml). The linearity of the plot gave an equation of $y = 43.105x$, $R^2 = 0.9982$ where y was absorbance at 765 nm, x was gallic acid concentration (mg/ml) (Appendix A, Figure A.2). Phenolic content of sample was expressed as mg gallic acid equivalents (GAE) per g dried weight plant extract (mg GAE g) or mg GAE per ml sample solution (mg GAE ml).

3.8.3 Determination of Bromelain Activity

Bromelain activity enzymatic activity was determined based on spectrophotometrically estimation of the amount of tyrosine dissolved in Trichloroacetic acid (TCA) which is formed from casein substrate due to proteolytic activity of bromelain. According to definition of bromelain activity assay, one unit of bromelain is taken as 1 µg tyrosine produced per minute from 1 ml of sample when casein is hydrolyzed under the standard conditions of 37 °C, pH 7 for 10 minutes (Takahashi *et al.*, 1973).

Bromelain activity was measured based on the method described by Devakate *et al* (2009). Casein was the substrate used in this assay. Casein of 0.75% (w/v) was prepared in 50 mM dibasic sodium phosphate buffer (Na_2HPO_4), which the buffer solution was previously adjusted to pH 7 by hydrochloric acid. The substrate solution was heated until boiling point to allow the casein dissolved in phosphate buffer. Casein solution of 0.68 ml was preincubated at 37 °C for 10 minutes. To this substrate, 0.14 ml sample or standard was added and incubated further for 10 minutes. The proteolytic reaction was stopped by mixing with 0.68 ml 30% TCA (w/v) and allowed to stand for 30 minutes at 37 °C. The mixture was then cooled to room temperature and centrifuged at 10000 g for 5 minutes, using a microcentrifuge (Sigma H4). The supernatant of the centrifuged sample was separated and measured

with bromelain activity at 280 nm on UV/VIS spectrophotometer (Perkin Elmer Precisely, Lambda 25) using UV Winlab software (Perkin Elmer, Inc.).

Tyrosine standard curve was generated by using tyrosine at concentration range between 0.01 to 4.00 mg/ml. Bromelain activity was determined based on the equation obtained from the linearity of plot of absorbance 280 nm versus tyrosine concentration ($y = 2.5157x$, $R^2 = 0.9630$), where y was absorbance 280nm and x was tyrosine concentration (mg/ml) (Appendix A, Figure A.3). The bromelain activity was expressed as units per ml (U/ml).

3.8.4 Determination of Protein Content

Bicinchoninic acid (BCA) protein assay was used for colorimetric measurement of protein content. Pierce BCA Protein Assay kit was used (Walker, 2002). Sample/ standard of 25 µl was added into 200 µl BCA reagent in microplate and mixed thoroughly. The mixture was incubated at 37 °C in CO_2 incubator (Labline) for 30 minutes. The sample was cooled to room temperature prior to measurement of absorbance at 570 nm on microplate reader (BioTek Elx808 spectrophotometer) using KC Junior software (BioTek Instruments).

Bovine serum albumin (BSA) was used as protein standard. A set of diluted standards in concentration range 20-2000 µg/ml was prepared by diluting 2.0 mg/ml albumin standard by deionized water. A standard curve was made by plotting 570 nm for each standard versus its concentration (µg/ml). Protein content of sample could be determined according to the standard curve of $y = 0.0009x$, $R^2 = 0.9962$ (Appendix A, Figure A.4).

3.9 Data Expression

Most of the data are presented as 2D line and scatter plot, column and bar chart, in which x axis represents independent variables, while y axis represents

residuals of dependent variables, using Microsoft Office Excel 2007 software. Each scatter and column plot graphs the value as point with error bar indicating mean and standard deviation. The calculated standard deviation is used as positive and negative values in each error bar. Data is expressed as mean ± standard deviation (SD) of three replicated determinations (n=3). Significant differences ($p<0.05$) which are detected by ANOVA multiple comparison test between experimental groups are indicated with different letters near the means of each tested group.

Tested properties of tested sample are expressed as following units. Antioxidant activity is indicated as DPPH scavenging activity and EC50 (mg/ml). Pheolic content is presented as gallic acid equivalent (GAE) per dried weight or volume of sample (mg GAE/g or mg GAE/ml). Bromelain activity is expressed as U (Units) ml and units for protein content is μg/ml. Data is also expressed as relative activity (%) which is ratio of the activity to maximum activity for each tested sample, as percentage.

Some results in the studies on effect of temperature and pH are expressed as logarithm. The points represent logarithm of data plotted against tested parameters (incubation time, temperature and pH) based on linear regression analysis on Microsoft Excel 2007. Linear straight line of scatter plot is generated with equation ($y=mx+c$). The derived gradient (m) could describe the trend and strength of association between variables and the effect of tested parameter on dependent variables.

Four tested properties of each sample are combined and presented in 3D category bar char (XYY bar chart) and the graph is exported from Origin 8 software (The data analysis and graphing workspace). The 3D XYY bar chart graphs the two factors (tested properties and parameters) from the independent data columns along X and Y axes against dependent variable along Z axis.

Data correlation between tested properties (variables) is analyzed with Pearson correlation. Correlation coefficient (r), P value for correlation coefficient

and scatter matrix for Pearson correlation between combinations of variables are presented.

3.10 One Way Analysis of Variance (ANOVA)

ANOVA is used to determine the differences between the means of several groups. The data is analyzed with ANOVA by using 'Statistics' option in Sigma Plot 11 software (Systat software, Inc.). Tukey test is used for pairwise comparison between experimental groups and isolation of the differences that is statistically significant. The significant level for multiple comparisons is set at 0.05 and this concludes there is a significant difference when the likelihood of being incorrect is less than this level. Power of a test is set at α=0.05. p value of <0.05 indicates a significant difference between means of tested groups.

3.11 Pearson Product Moment Correlation

Pearson correlation is carried out in Sigma Plot 11 software. It could measure the strength of association or whether the relationship between the variables is a straight line. The association is quantified with correlation coefficient (r) which varies from -1 to +1. r near to +1 indicates a strong positive relationship, -1 indicates a strong negative relationship and 0 indicates no relationship between variables. P value is the probability of being wrong in concluding a true association between variables. The smaller the P value is, the greater the probability that variables are correlated.

Scatter matrix for Pearson correlation graphs correlation between all possible combinations of variables as scatter plots. In each small scatter plot, the point represent one variable stated in corresponding Y axis data against another variable stated in corresponding X axis data. The confidence level to conclude the coefficient is different from zero is set at 95%.

antioxidant activity could prevent oxidation of catalytic nucleophile at reactive group of bromelain. Several plant extracts were screened with antioxidant activity and phenolic content. Plant extract with high content of these two properties was used to precipitate bromelain in pineapple juice. Free bromelain solutions (pineapple juice/ purified bromelain solution) and bromelain-polyphenol precipitate were analyzed with nutraceutical properties (bromelain activity and antioxidant activity) together with protein content and phenolic content. Evaluation of these properties on bromelain-polyphenol precipitate could demonstrate the ability of polyphenolic compounds of selected plant extract to separate bromelain from pineapple juice and also the amount of tested properties retained on the precipitate.

4.1.1 Selection of Plant Extract with High Antioxidant Activity and Phenolic Content

In this section, cashew leave, mangosteen fruit rind and cengal wood chipping were extracted with ethanol and their resultant ethanolic extracts were determined for antioxidant activity and phenolic content. Plant extract that exhibited the highest antioxidant activity and phenolic content was selected and used as a source of polyphenols to be precipitated with bromelain. These plants were extracted with ethanol in mass to volume ratio of 2:10 (w/v) and the plant extracts were tested for antioxidant activity. Ethanolic cashew leaf extract exhibited marked antioxidant activity. As depicted in Figure 4.1, there was a rapid increase in free radical scavenging activity as the concentration of cashew extract increased up to 0.060 mg/ml at which concentration it reached the highest DPPH inhibition and followed by a plateau with a small increase or decrease in inhibition. Ascorbic acid was used as a standard. It exhibited a steady increase in DPPH scavenging activity up to 0.030 mg/ml after which it reached a plateau at 92.39%, with an EC50 of 0.016 mg/ml (Figure 4.1). The pattern of DPPH inhibition observed with cashew extract was similar with that of ascorbic acid. Potential ability to scavenge DPPH free radical was presented as EC50 value.

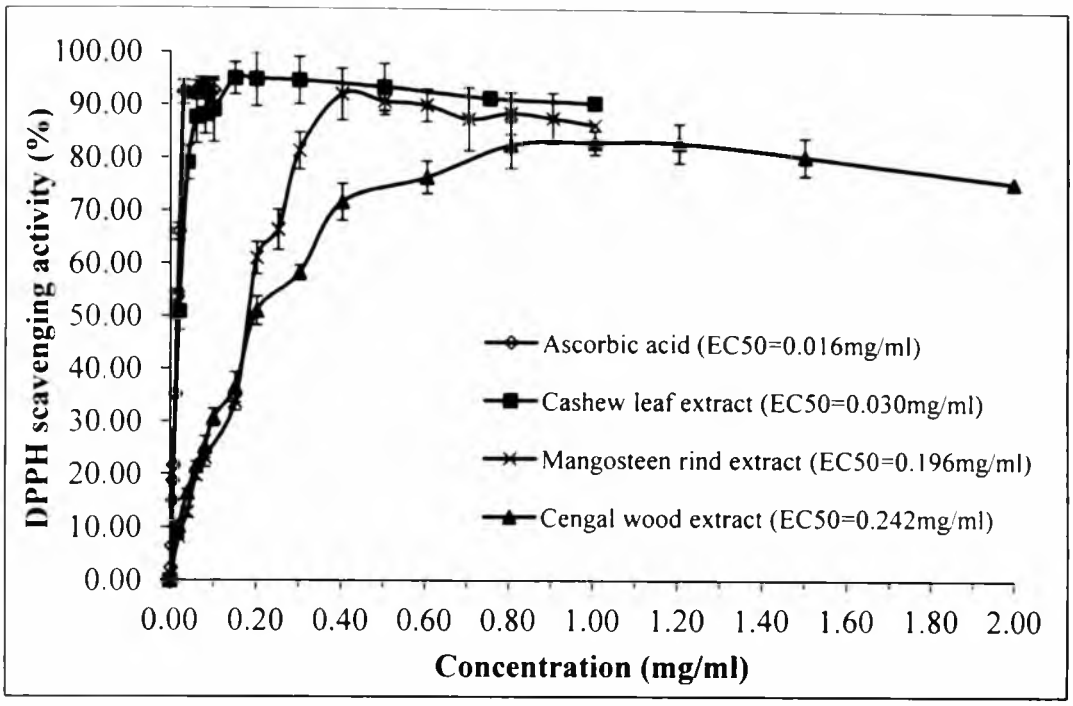


Figure 4.1 DPPH scavenging activity of ethanolic cashew leaf extract, mangosteen fruit rind extract, cengal wood extract and ascorbic acid. Indicated values are expressed as mean ± SD (n=3).

Mangosteen fruit rind extract was able to scavenge DPPH with percentage inhibition of approximately 90% at 0.40 mg/ml and its EC50 was found to be 0.196 mg/ml. Cengal wood extract displayed a relatively low scavenging effect (EC=0.242 mg/ml) compared to cashew leaf and mangosteen fruit rind extracts. Cengal extract reached highest percentage of DPPH inhibition at around 70% (Figure 4.1).

The antioxidant activity assay used in this study was DPPH free radical scavenging activity, therefore the results only indicated that plant extract has a potential to quench DPPH free radical species. It is essential to use different antioxidant assay systems to reveal the effectiveness of plant extract in scavenging against hydroxyl radicals and superoxide radicals scavenging, metal chelation or inhibition of lipid peroxidation and deoxyribose oxidation, instead of relying on one assay. For example, cashew nut skin extract was tested with various antioxidant assays and it was found that this extract displayed a much stronger activity in free radical scavenging rather than inhibition of enzyme/ lipid oxidation and metal chelation (Kamath and Rajini, 2007).

All these plant extracts were able to scavenge DPPH free radical to various extents. Cashew leaf extract was found to have the lowest EC50 among these plant extracts and its DPPH scavenging activity was about 1.86 times lower than positive control (ascorbic acid). This plant extract could be considered as a potent scavenger of free radical. It is hypothesized that this antioxidant capacity of cashew leaf extract is attributed to polyphenolic compounds.

It is important to evaluate the phenolic content of a plant extract due to its correlation with antioxidant capacity and protein binding characteristic which is essential in protein-polyphenol interaction (Details on antioxidant activity of plant polyphenols and protein-polyphenol interaction are elaborated in section 2.4.2 and section 2.7 respectively). Phenolic contents of ethanolic cashew leaf, mangosteen rind and cengal wood extracts were depicted in Table 4.1. The order of three ethanolic extracts with highest value of phenolic content was as follow: cashew leave > mangosteen rind > cengal wood. Ethanolic cashew leaf extract contained the greatest level of phenolic content and it could be a potent source of natural antioxidant.

The antioxidant activity and phenolic content of ethanolic plant extracts were summarized in Table 4.1. ANOVA indicated that means of EC50 and GAE (mg/ml) were significant different ($p<0.05$) among the plant extracts/ standard. Cashew extract displayed the strongest antioxidant capacity and highest phenolic content while cengal extract was the least effective antioxidant with lowest polyphenol content. Results clearly showed that an increase in antioxidant capacity (lower the EC50 value) was obtained with higher content of phenolic content. Pearson correlation indicated that coefficient for correlation between DPPH scavenging activity and phenolic content was 0.92624. A great positive correlation existed between these two parameters suggested that phenolic compounds were likely to be contributing to antioxidant activity of these extracts. Polyphenolic compounds in ethanolic plant extracts were capable of scavenging DPPH free radicals. Figure 4.2 showed that antioxidant activity of plant extracts decreased with the decrease of phenolic content.

Table 4.1 Antioxidant activity and phenolic content of ethanolic plant extracts and ascorbic acid. Indicated values are expressed as mean ± SD (n=3). Means with different letters within same column indicate a significant difference at $p<0.05$.

Sample	Antioxidant activity, EC 50 (mg/ml)	Phenolic content (mg GAE/g)
Ascorbic acid (standard)	0.0160 ± 0.0003 a	-
Ethanolic cashew leaf extract	0.0297 ± 0.0001 b	628.70 ± 30.63 a
Ethanolic mangosteen fruit rind extract	0.1958 ± 0.0020 c	462.82 ± 33.20 b
Ethanolic cengal wood extract	0.2422 ± 0.0079 d	249.78 ± 18.44 c

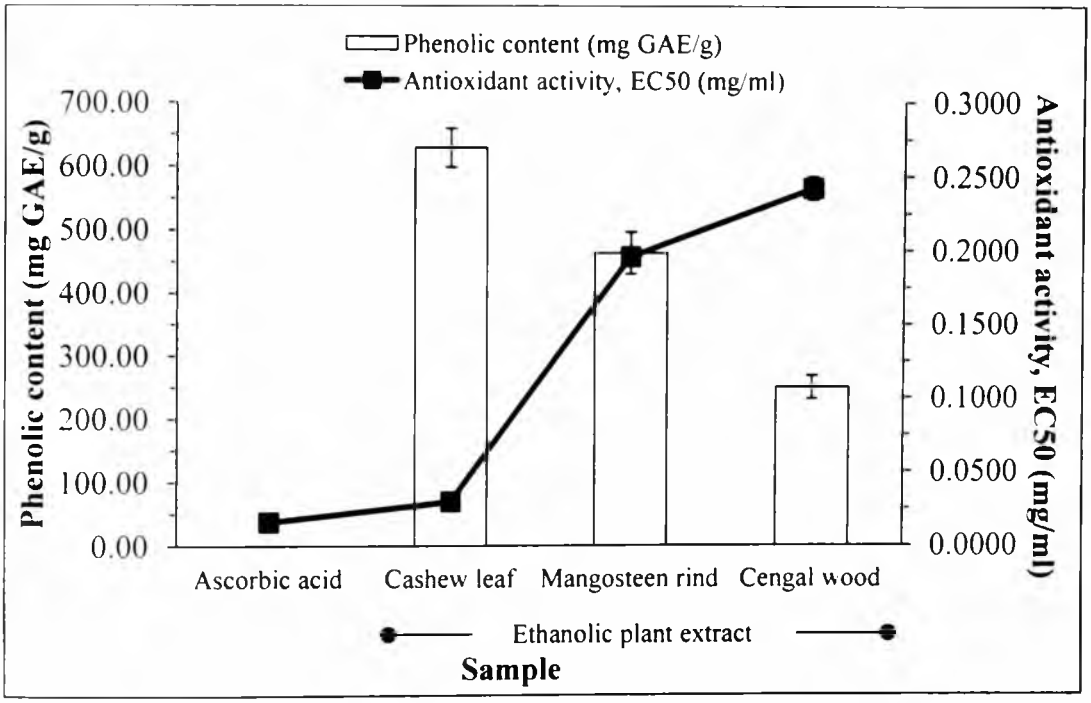


Figure 4.2 Antioxidant activity and phenolic content of ethanolic plant extracts and ascorbic acid. Indicated values are expressed as mean ± SD (n=3).

Trevisan *et al.* (2006) reported that the antioxidant activity of cashew nut shell liquid, cashew nut and cashew apple correlated significantly with concentration of alkyl phenols, and anacardic acid was found to be the major alkyl phenols contributing to the strong antioxidant capacity of cashew extract. Similar finding on positive correlation between free radical scavenging activity and total phenolic

content was observed on cashew nut skin extract in a study carried out by Kamath and Rajini (2007). In the study, epicatechin was identified to be major constituent of phenolic content related to antioxidant activity. Since our study indicated that total phenols exhibited a correlation with antioxidant activity in cashew leaf extract, therefore a further study on phytochemical substance analysis of the extract needs to be carried out in order to identify and characterize individual phenolic compounds. It is because high antioxidant response depends remarkably on chemical structure of major phenolic constituents, instead of rely on total phenolic content (Atoui *et al.*, 2005; Ikram *et al.*, 2009). For example, anacardic acid (alkyl phenols) displayed far greater antioxidant activity than cardanols and cardols in cashew nut shell liquid extract (Trevisan *et al.*, 2006).

Properties of extracting solvent such as polarity and concentration have great influence on phenolic yield and antioxidant efficiency of plant extract. In this study, cashew leave was incubated with ethanol (95% purity) and the extraction process was conducted at room temperature for 24 hours. Ethanol is a safe extracting solvent which could promote permeation of endocellular material by degrading plant cell wall of extract (Pothitirat *et al.*, 2009). The phenolic content in crude ethanolic cashew leaf extract was evaluated at 628.70 mg GAE/g. Results indicated that extraction of cashew leave with ethanol generated a considerably high phenolic yield. Ethanol selectively dissolves high polar polyphenol antioxidants present in the extract and these antioxidants are believed to be the contributors to the observed antioxidant capacity. A study on cashew shoot extract conducted by Razali *et al.* (2008) showed that methanolic extract displayed significant free radical scavenging activity compared to ethyl acetate and hexane extracts. In a study conducted by Turkmen *et al.* (2006) reported that black and mate tea extracts using high polar extracting solvent had a high level of phenolic compounds with effective antioxidant activity. This result agrees with another study on peanut skin (Yu *et al.*, 2005) which reported that 80% (purity) ethanol and methanol were the more effective polyphenols extracting solvent as compared to water.

Overall, the ethanolic extract of cashew leave, mangosteen fruit rind and cengal wood contained polyphenols. The antioxidant activity of these plant extracts was correlated with phenolic content. Ethanolic extract of cashew leave had

significant DPPH radical scavenging activity and polyphenols present in this extract appeared to have a major contribution to antioxidant activity. Ethanolic cashew leaf extract with phenolic content of 628.70 mg GAE/g and DPPH radical scavenging of EC50 equal to 0.030 mg/ml was selected to be the source of polyphenols for bromelain-polyphenol precipitation.

4.1.2 Properties of Josapine Juice Extract and Purified Bromelain Solution

In this study, Josapine was chosen for analysis among varieties of pineapple. The fruit was processed, centrifuged, filtered and clarified pineapple juice (Josapine juice extract) was obtained. Purified bromelain solution was used as a positive control. Josapine juice extract and purified bromelain solution were subjected to analysis of bromelain activity, protein content, antioxidant activity and phenolic content.

As shown in Table 4.2, Josapine juice extract was found to have bromelain activity of 621.92 U/ml and protein content of 14.26 mg/ml. The specific activity was calculated to be 43.90 U/mg. Devakate *et al.* (2009) reported that the specific activity of clarified crude pineapple juice was 58.8 U/mg. Purified bromelain solution in concentration of 4.0 mg/ml or 0.4% (w/v) contained 519.07 U/ml activity and protein content of only 3.32 mg/ml. Higher specific activity of 157.09 U/mg was obtained as compared to that of Josapine juice extract due to the presence of low protein content in bromelain solution.

Table 4.2 Bromelain activity and protein content of Josapine juice extract and purified bromelain solution. Indicated values are expressed as mean ± SD (n=3).

Sample	Bromelain activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
Josapine juice extract	621.92 ± 3.49	14.26 ± 1.46	43.90 ± 4.44
4 mg/ml or 0.4% purified bromelain solution	519.07 ± 17.99	3.32 ± 0.19	157.09 ± 14.29

For antioxidant activity, DPPH scavenging activity of Josapine juice extract increased with the percentage/ concentration of juice up to 5% (Figure 4.3). Apparent no increase in activity was observed from 5% to 6%. In consideration of both activity and concentration, 5% juice with antioxidant activity of 70.04% was appropriate to be used for analysis in the following sections. There was no phenolic compound observed in Josapine juice. Therefore, the antioxidant activity might be derived from vitamins (ascorbic acid, β-carotene) and free amino acid (histidine, glycine, alanine) other than polyphenols (flavonoids) (Steinberg, 1991; Cao *et al.*, 1996; Wu *et al.*, 2003; Meda *et al.*, 2005).

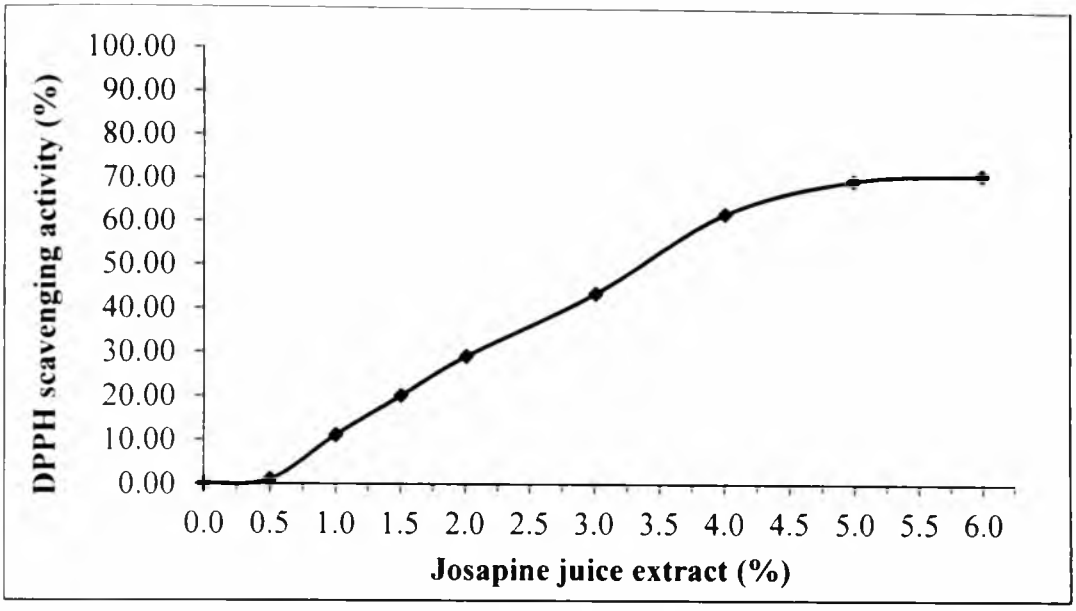


Figure 4.3 DPPH scavenging activity of Josapine juice extract in various percentages. Indicated values are expressed as mean (n=3).

Josapine juice extract with 621.92 U/ml bromelain activity, 14.26 mg/ml protein content and 70.04% DPPH scavenging activity and 0.4% bromelain solution with 519.07 U/ml bromelain activity and 3.32 mg/ml protein content were used as sources of free bromelain for the following analysis.

4.1.3 Properties of Free Bromelain and Bromelain-polyphenol Precipitate

One of the main objectives in this study is the formation of precipitate which involved the interaction between bromelain and polyphenols. Polyphenols would have significant characteristics in protein binding and precipitation. In this study, ethanolic cashew leaf extract was mixed with Josapine juice extract or 0.4% purified bromelain solution (positive control), the precipitate formed was regarded as bromelain-polyphenol precipitate. It is hypothesized that the concentration of bromelain in Josapine juice extract will decrease since bromelain is precipitated by the added cashew leaf extract, and the decrease in supernatant activity is correlated with an increase in bromelain activity on precipitate.

After mixing various concentrations (%) of cashew leaf extract with Josapine juice extract, supernatant was separated from precipitate formed. For every increase in % added cashew extract, the supernatant was analyzed for bromelain activity. Figure 4.4 showed the residual bromelain activity in supernatant. Value for 0% meant the bromelain activity of original Josapine juice extract before adding cashew leaf extract (666.41 U/ml). Bromelain activity in the supernatant declined as the percentage of cashew extract added to juice increased. This indicated that greater amount of bromelain in Josapine juice extract was precipitated by mixing with higher level of cashew polyphenols. However, it could be observed that the efficiency of cashew extract in precipitating bromelain decreased as the concentration of bromelain in the supernatant decreased, so eventually bromelain activity in the supernatant reached a low but relatively constant plateau. Figure 4.4 showed that means of supernatant bromelain activity of Josapine juice extract, 0.1%, 0.3%, 0.5%, 0.7% and 1.0% cashew-josapine sample exhibited significant difference ($p<0.05$), while 1.0% and 1.5% sample did not show any significant difference.

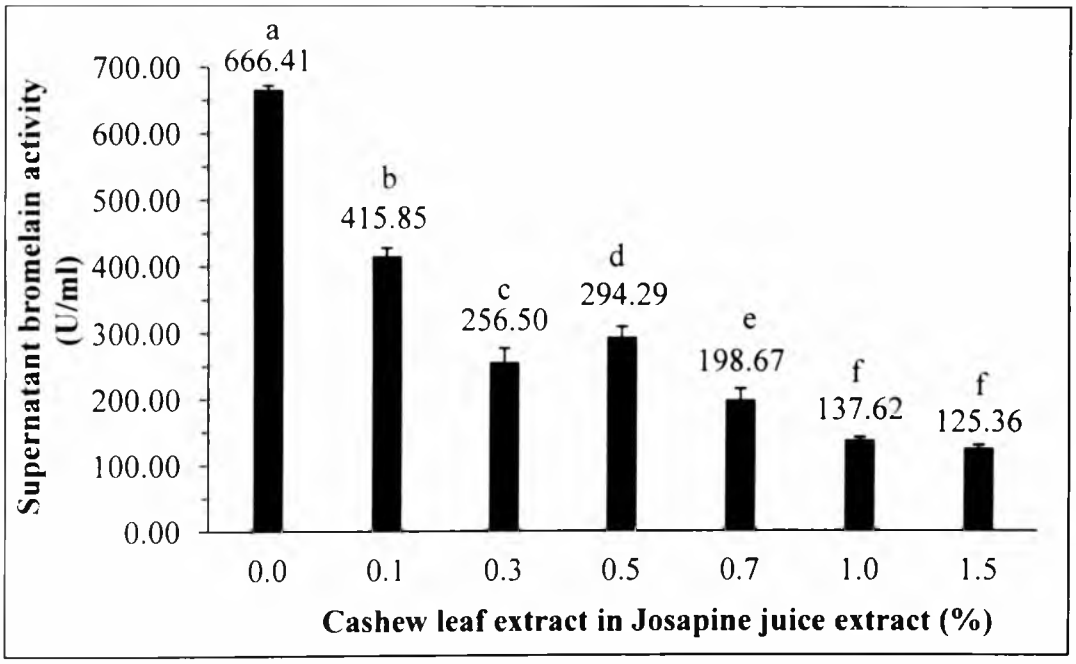


Figure 4.4 Bromelain activity in supernatant after mixing cashew leaf extract with Josapine juice extract. Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

A saturation of protein-polyphenol precipitation will be reached when most of the protein is recovered on precipitate by adding a certain amount of polyphenols.

The data obtained from Figure 4.4 was further described as percentage recovery of bromelain activity on bromelain-polyphenol precipitate which was shown in Figure 4.5. The percentage recovery was hyperbolic and leveled off after 1.0% cashew extract has been added. This hyperbolic pattern indicated that the reduction of bromelain activity in Josapine juice extract was greater when small amount of polyphenols was added (0.1% and 0.3%). Maximum recovery of bromelain activity was attained at 1.0% cashew extract in juice extract (79.35%). Further increase in % cashew extract beyond 1.0% did not vary the value significantly. 1.5% cashew extract gave % recovery of 81.19% which was only 1.84% greater than that of 1.0% extract. Overall, bromelain could be recovered from Josapine juice extract on bromelain-polyphenol precipitate after adding cashew leaf extract.

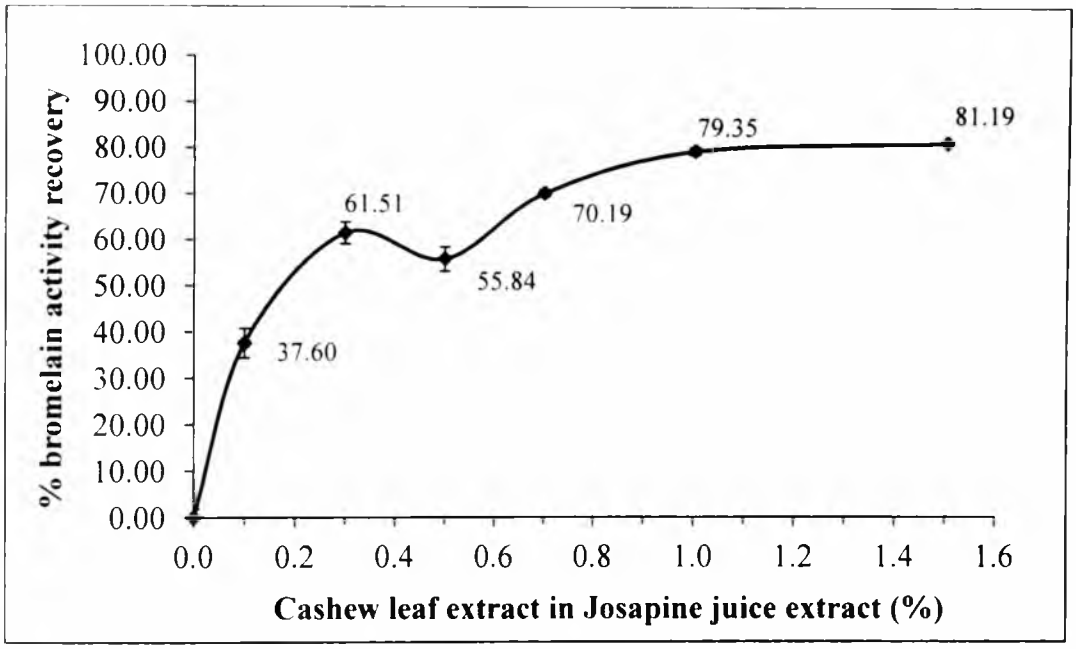


Figure 4.5 Percentage recovery of bromelain activity on precipitate after mixing cashew leaf extract with Josapine juice extract. Indicated values are expressed as mean ± SD (n=3).

Analysis was also performed on positive control sample which was a mixture of cashew leaf extract with 0.4% bromelain solution and the resultant supernatant bromelain activity and % recovery were depicted in Figure 4.6 and Figure 4.7 respectively. Result of supernatant activity and % recovery observed with positive control sample was in accordance with those of cashew-josapine sample. In Figure 4.6, supernatant bromelain activity in 0.4% bromelain solution decreased with an

increase of % cashew extract added, supernatant using higher % extract showed lower activity. 485.81 U/ml at 0% was the bromelain activity of original 0.4% bromelain solution without addition of cashew extract. As could be seen in Figure 4.7, % recovery reached a plateau after addition of 0.7% cashew extract in bromelain solution. No significant difference was found between 0.7%, 1.0% and 1.5% samples. Maximum recovery was attained by adding 0.7g cashew extract in 100ml 0.4% bromelain solution. It was found that the maximum recovery attained by positive control precipitate reached about 90%, which was about 10% higher as compared to cashew-josapine precipitate.

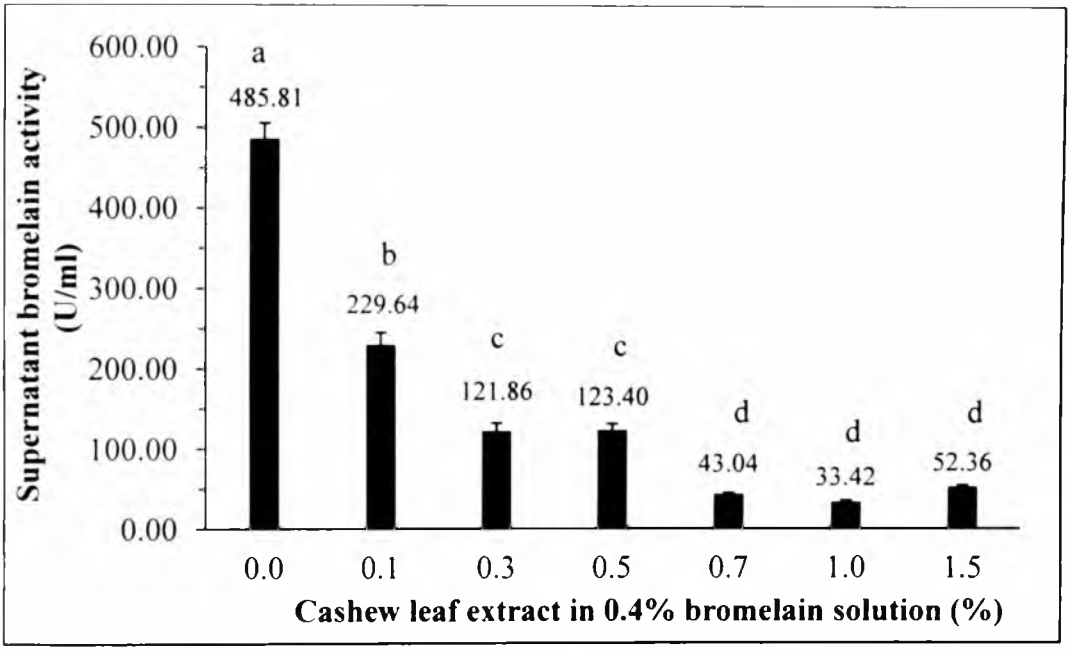


Figure 4.6 Bromelain activity in supernatant after mixing cashew leaf extract with 0.4% bromelain solution. Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

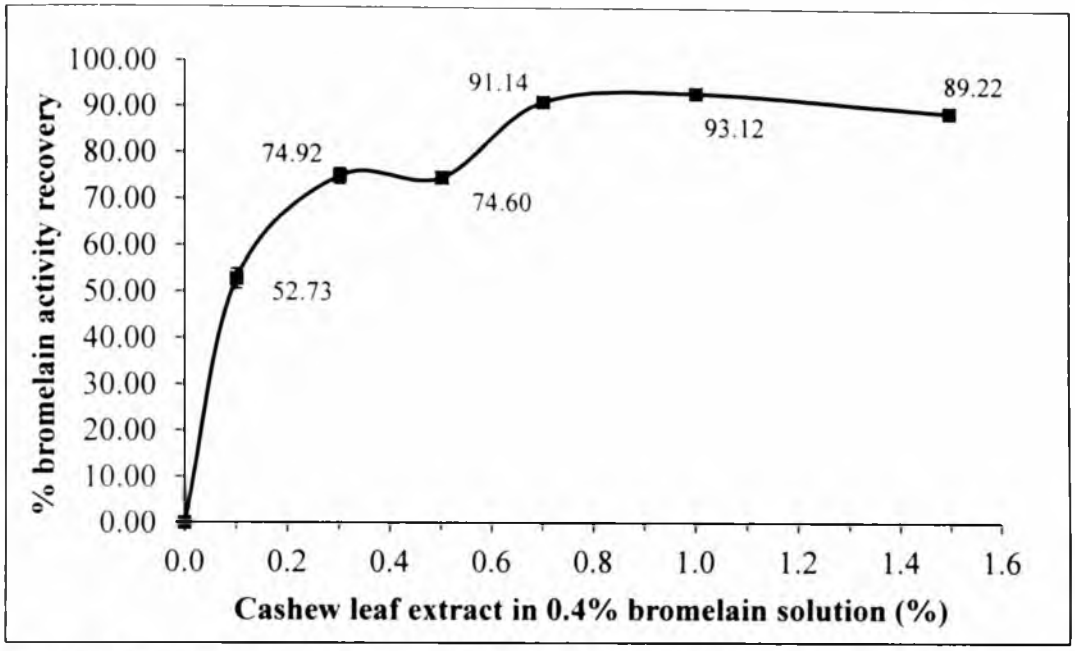


Figure 4.7 Percentage recovery of bromelain activity on precipitate after mixing cashew leaf extract with 0.4% bromelain solution. Indicated values are expressed as mean ± SD (n=3).

Overall, ethanolic cashew leaf extract possesses polyphenolic compounds which are able to precipitate and separate bromelain from Josapine juice extract and form bromelain-polyphenol precipitate subsequently.

Bromelain-polyphenol precipitate activities in terms of bromelain activity, protein content, antioxidant activity and phenolic content are analyzed in the following section. Bromelain activity on precipitate is assumed to be correlated with the decrease in supernatant activity. 1.0% cashew leaf extract in Jospaine juice extract and 0.7% extract in 0.4% bromelain solution were found to have maximum recovery in bromelain activity. In the next section, activity of precipitate was investigated on 1.5%, 1.0% and 0.5% cashew leaf extract in Josapine juice extract and bromelain solution to study the effect of different concentration of cashew leaf extract on precipitate activity.

4.1.3.1 Bromelain Activity of Bromelain-polyphenol Precipitate

Precipitated bromelain was named as 1.5% cashew-josapine precipitate (1.5% C-J), which was for 1.5% cashew leaf extract in Josapine juice extract or precipitate obtained from the mixing of 1.5 g cashew extract with 100 ml juice. While 1.5% cashew-bromelain precipitate (1.5% C-B) was named for 1.5% cashew extract in 0.4% bromelain solution. Cashew-bromelain precipitate was positive control precipitate. Cashew-water precipitate (C-Water) was named for cashew leaf extract in deionized water (negative control). The % value in front of C-J or C-B represented the concentration of cashew leaf extract in its corresponding free bromelain solution.

Figure 4.8 presented bromelain activity of cashew-josapine/ cashew-bromelain precipitate in comparison with Josapine juice extract, 0.4% bromelain solution and negative control (cashew leaf extract). Black colour bar represented actual bromelain activity measured in the sample. In the case of cashew-josapine precipitate, 1.5% and 1.0% cashew extract in juice had similar activities which were 434.09 U/ml and 422.45 U/ml respectively, whereas 0.5% precipitate retained only 262.06 U/ml. Likewise, the behaviour of 1.5%, 1.0% and 0.5% positive control precipitates (cashew-bromelain precipitate) in bromelain activity was very similar as compared to the corresponding cashew-josapine precipitate. No significant difference was observed among 1.5% and 1.0% cashew-josapine/ cashew-bromelain precipitates. 0.5% cashew-josapine precipitate and its corresponding positive control precipitate did not show significant difference as well. Lower activity was measured in cashew-josapine, cashew bromelain precipitates as compared to Josapine juice extract and 0.4% bromelain solution respectively. It was because bromelain in juice and bromelain solution was not fully recovered by precipitation with cashew polyphenols. For example, about 80% of bromelain in Josapine juice was recovered on 1.5% and 1.0% precipitates, whereas 0.5% precipitate showed only 55.84% recovery (Figure 4.5). There was apparent no bromelain activity was observed with negative control precipitate.

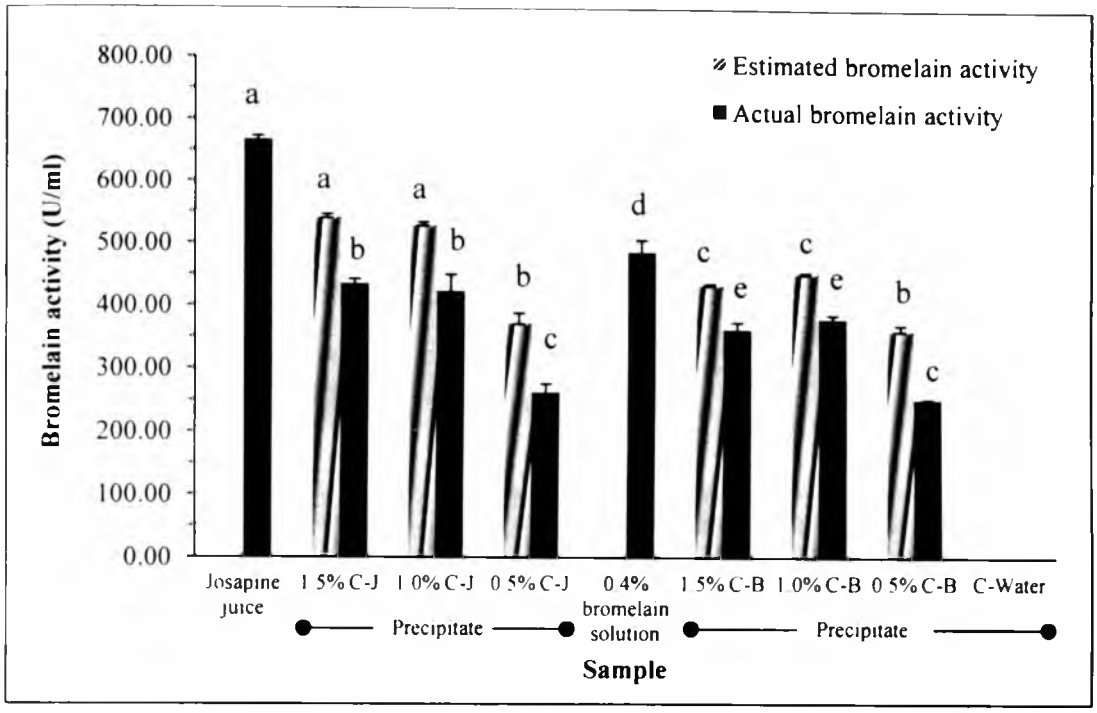


Figure 4.8 Bromelain activity of cashew-josapine precipitate, cashew-bromelain precipitate, Josapine juice extract, 0.4% bromelain solution and cashew-water precipitate (negative control). Indicated values are expressed as mean ± SD (n=3). Means with different letters on bars of actual or estimated bromelain activity indicate a significant difference at $p<0.05$.

Grey lining bar in Figure 4.8 was estimated bromelain activity which was calculated by using % recovery. The % recovery could be obtained from Figure 4.10 and Figure 4.7. For example, Josapine juice extract had activity of 666.41 U/ml and the % recovery on 1.5% cashew-josapine preciptiate was 81.19%, therefore the estimated activity present in this precipitate was 541.06 U/ml. It was observed that actual bromelain activity measured in precipitate in all cases was lower than their corresponding estimated activity. The difference between estimated and actual bromelain activity on precipitate was presented in Table 4.3. Activity loss might be due to incomplete precipitation between bromelain and cashew polyphenols or denaturation of bromelain occurred during precipitation process. Therefore bromelain activity which was estimated by the decrease in supernatant activity was not fully recovered on precipitate. The bromelain activity measured on precipitate was not correlated with those of being estimated.

Supernatant and precipitate bromelain activity of cashew-josapine precipitate and cashew-bromelain precipitate. Ind
pressed as mean ± SD (n=3). Means with different letters within same column indicate a significant difference at $p<0.05$.

Sample		Bromelain activity (U/ml)					
		Supernatant activity	**Estimated precipitate activity**	**Actual precipitate activity**	**% difference between estimated and actual precipitate activity**	**Total activity (Supernatant + precipitate activity)**	**% total act relative t Josapine ju bromelai solution**
	Josapine juice	666.41 ± 6.02 a					
	1.5% C-J	125.36 ± 5.31 b	541.06 ± 5.30 a	434.09 ± 7.61 a	19.77% ± 0.75 a	559.45 ± 3.41 a	83.95% ± 0.5
	1.0% C-J	137.62 ± 4.86 b	528.80 ± 4.86 a	422.45 ± 26.97 a	20.11% ± 5.83 a	560.07 ± 31.82 a	84.04% ± 4.7
	0.5% C-J	294.29 ± 15.90 c	372.12 ± 15.90 b	262.06 ± 13.96 b	29.58% ± 2.67 b	556.35 ± 11.76 a	83.49% ± 1.7
trol e	0.4% bromelain solution	485.81 ± 19.66 d					
	1.5% C-B	52.36 ± 1.90 e	433.44 ± 1.89 c	362.44 ± 11.83 c	16.38% ± 3.09 a	414.80 ± 13.73 b	85.38% ± 2.8
	1.0% C-B	33.42 ± 1.99 e	452.40 ± 1.98 c	379.70 ± 6.52 c	16.07% ± 1.54 a	413.12 ± 7.07 b	85.04% ± 1.4
	0.5% C-B	123.40 ± 8.07 b	362.41 ± 8.08 b	252.31 ± 0.90 b	30.38% ± 1.54 b	375.71 ± 8.07 b	77.34% ± 1.6

Bromelain activity in supernatant and precipitate were combined and presented in Figure 4.9. It is interesting to observe that for all cashew-josapine precipitates, the total bromelain activity was similar, which was about 560 U/ml, 84% relative to Josapine juice. Whereas with 1.5% and 1.0% positive control precipitates, both of them attained approximately 85% relative to 0.4% bromelain solution. 0.5% cashew-bromelain precipitate had a moderate lower activity which was 77.34%. No significant difference was observed among cashew-josapine and cashew-bromelain precipitates. Figure 4.10 showed that low value of supernatant activity could be correlated with high precipitate activity.

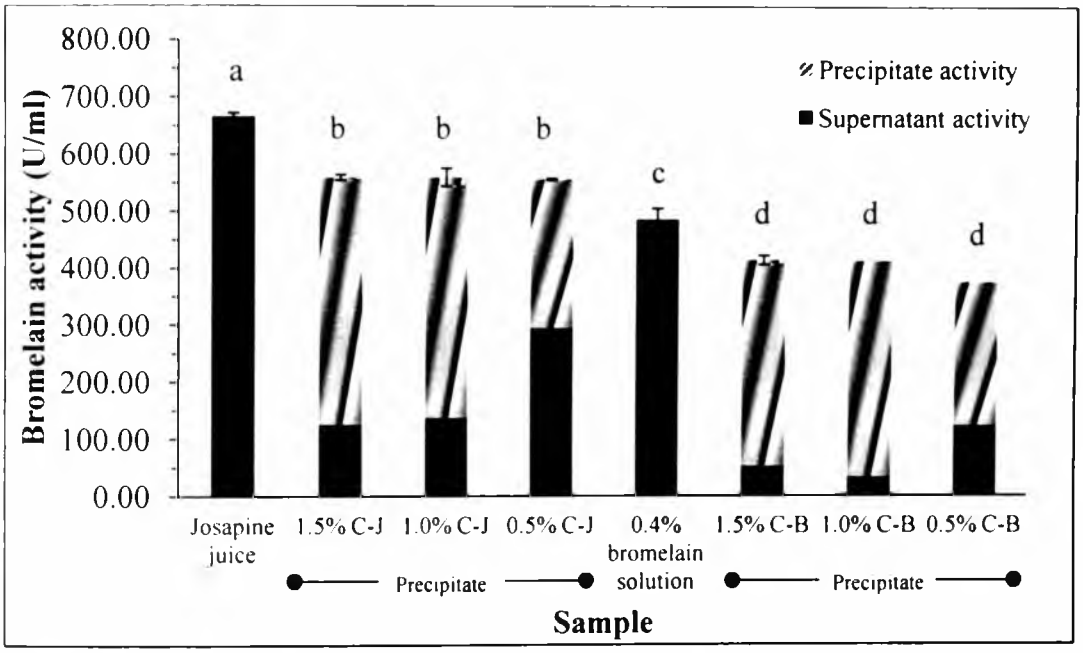


Figure 4.9 Supernatant and precipitate bromelain activity of cashew-josapine precipitate and cashew-bromelain precipitate. Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

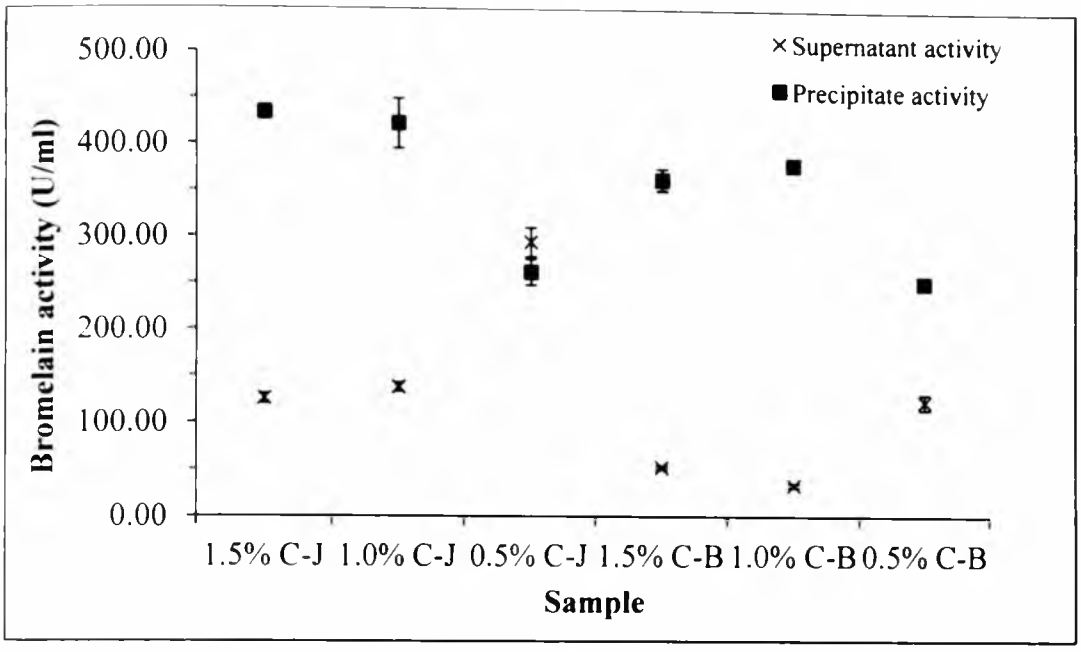


Figure 4.10 Correlation between supernatant and precipitate bromelain activity of cashew-josapine precipitate and cashew-bromelain precipitate. Indicated values are expressed as mean ± SD (n=3).

Overall, bromelain from Jospaine juice extract was recovered on precipitate by mixing with cashew leaf polyphenols. 65.14%, 63.39% and 39.32% of total bromelain in juice extract were recovered on 1.5%, 1.0% and 0.5% cashew-josapine precipitates respectively.

4.1.3.2 Protein Content of Bromelain-polyphenol Precipitate

Polyphenols has a significant ability to form complex with proteins especially proline rich polypeptides. Bromelain which consists of proline residues might occupy the protein content on precipitate to a certain extent. Protein content is measured by BCA protein assay, a type of analysis which is different from the method used for bromelain activity measurement. This provides additional information regarding protein concentration of precipitate.

In Figure 4.11, it could be seen that 1.5% and 1.0% cashew-josapine precipitates had high and similar protein content whereas relative lower protein

content was observed with 0.5% precipitated bromelain ($p<0.05$). Means of protein content of 1.5% and 1.0% precipitated bromelain were not significant different. Same behaviour was observed in the case of positive control precipitate. This phenomenon on protein content appeared to be similar to that of precipitate bromelain activity (Figure 4.8). It could suggest that protein content which was recovered from juice/ bromelain solution on precipitate was somehow related to % recovery in bromelain activity. This observation further supports the hypothesis that bromelain might be major constituent of protein content in the precipitate.

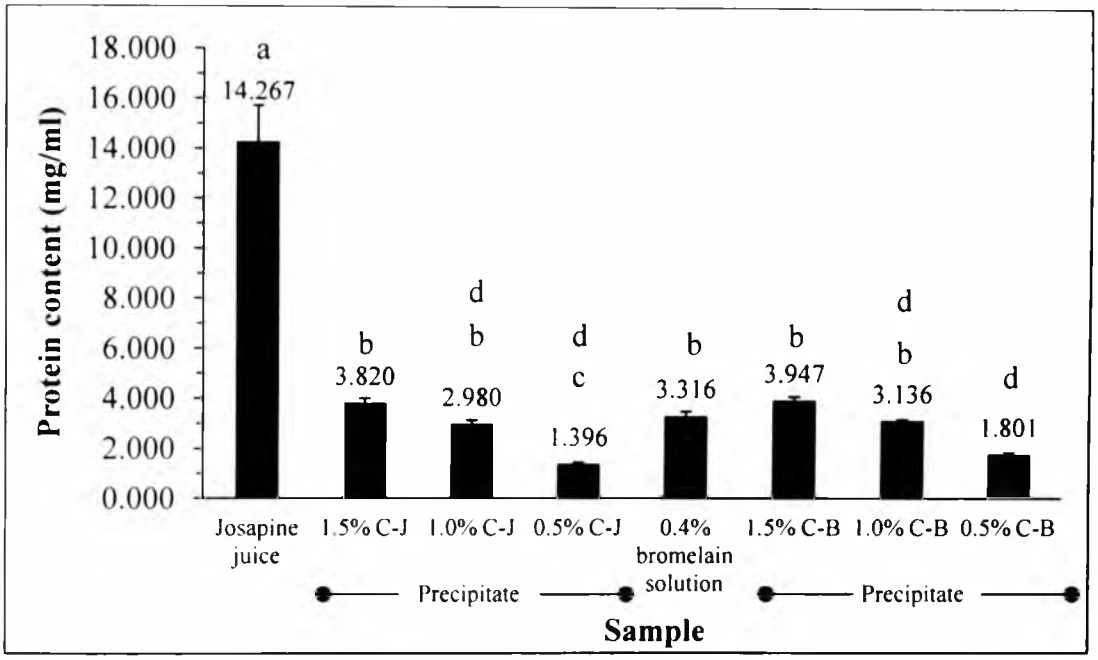


Figure 4.11 Protein content of cashew-josapine precipitate, cashew-bromelain precipitate, Josapine juice extract and 0.4% bromelain solution. Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

High protein content was seen in Josapine juice extract due to the presence of enzymes/ proteins and various amino acids other than bromelain. 0.4% bromelain solution consists of purified bromelain and its protein content could represent bromelain concentration in the solution, amongst various amino acids. Results indicated that protein content in 0.4% bromelain solution was fully recovered on 1.5% and 1.0% positive control precipitates (Figure 4.11). No significant difference was observed between 0.4% bromelain solution, 1.5% and 1.0% positive control precipitate.

4.1.3.3 Antioxidant Activity of Bromelain-polyphenol Precipitate

Both Josapine juice and cashew leaf extract had been previously proven to possess antioxidant activity (Figure 4.1 and Figure 4.3). In this section, the effect of interaction of proteins and polyphenols on total antioxidant activity was studied. It was observed in Figure 4.12, based on DPPH scavenging activity, the precipitate of cashew-josapine had higher antioxidant potential than did Josapine juice extract, the exception being the low activity for 0.5% cashew-josapine precipitate. It was hypothesized that bromelain-polyphenol precipitate would have increased antioxidant capacity compared to free bromelain and that proved to be the case. 1.5% and 1.0% cashew-josapine precipitated bromelain had 15% greater activity as compared to Josapine juice extract ($p<0.05$). Means of antioxidant activity were not significant different between 1.5% and 1.0% cashew-josapine precipitates. Low activity reported for 0.5% cashew-josapine precipitate might be due to the presence of low cashew polyphenols concentration. The relative contribution to the resultant antioxidant activity by Josapine juice or cashew polyphenols is not known. However, it was hypothesized that polyphenols from cashew leaf extract are the major contributors, since the precipitated bromelain with higher content of cashew extract (1.5% and 1.0%) were found to have greater antioxidant capacity than that with lower content (0.5%). Free bromelain in Josapine juice extract had weaker antioxidant activity therefore it is readily oxidized and denatured.

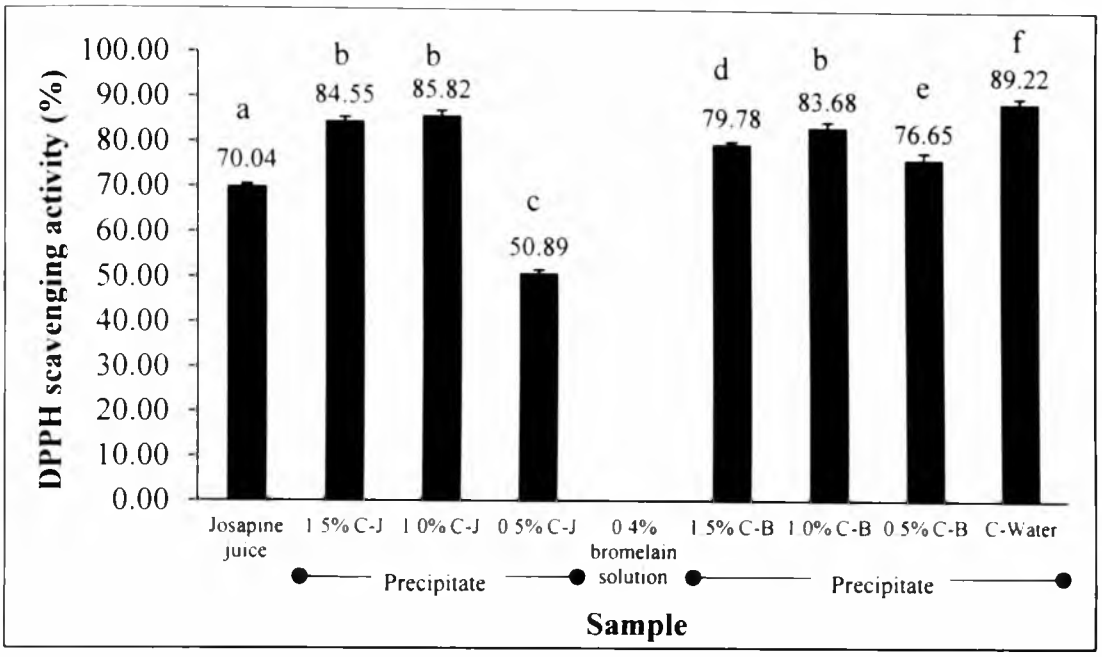


Figure 4.12 DPPH scavenging activity of cashew-josapine precipitate, cashew-bromelain precipitate, Jospaine juice extract, 0.4% bromelain solution and cashew-water precipitate (negative control). Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

0.4% bromelain solution had no effect on precipitates antioxidant capacity. Since no activity was observed with this solution, therefore the reported DPPH scavenging activity for cashew-bromelain precipitate (positive control precipitate) was attributed by polyphenols of cashew leaf extract (Figure 4.12). Apparent moderate similar activity was attained at 1.5% and 1.0% positive control precipitates whereas activity with 0.5% complex was slightly less.

Addition of cashew leaf extract into Josapine juice extract increased antioxidant activity of the juice by forming bromelain-polyphenol precipitate, however the increase was smaller than the activity of cashew extract itself (Figure 4.12). Likewise in cashew-josapine precipitate, antioxidant capacity of mixture of cashew polyphenols and purified bromelain (cashew-bromelain precipitate) was less than that of cashew leaf extract. Polyphenolic antioxidants did not reach their maximum antioxidant activity in precipitate.

DPPH scavenging activity by cashew polyphenols was decreased after precipitation with bromelain indicating the interaction between proteins and

polyphenols reduced the ability of polyphenol to scavenge DPPH radicals. Riedl and Hagerman (2001) had indicated that decreased ABTS scavenging of procyanidin might be due to the restricted collision between procyanidin and ABTS radicals which is caused by the proteins bound to procyanidin. Proline ratio of proteins, which is in precipitate with polyphenols, and also flavonoids component in plant extract are the factors affecting the masking of antioxidant activity of phenolic compounds (Arts *et al.*, 2002). Albumin and β casein, which contain large amount proline, mask the antioxidant activity of catechin. It speculated that proline residues in bromelain might involve in masking and interrupting the scavenging ability of cashew polyphenols. It is also essential to determine the composition of cashew leaf extract since the phenolic compounds exhibit various degree of masking.

Polyphenols/ protein complexing and precipitation affects antioxidant capacity of polyphenols. Riedl and Hagerman (2001) reported that the antioxidant activity decreases depends on the conditions (such as relative ratio of polyphenols to proteins, pH and temperature) where large precipitation is occurred (Details on nature of protein-polyphenol interaction are elaborated in section 2.7.3). In addition, structural factors and nature of proteins and polyphenols (stereochemistry of monomeric unit, degree of polymerization, esterification of gallate group, interaction bonds and ionic strength), which is involved in binding and precipitation, could be the determinants for efficacy of polyphenols (Details on nature of protein and polyphenols in protein-polyphenol complex are elaborated in section 2.7.1 and 2.7.2).

4.1.3.4 Phenolic Content of Bromelain-polyphenol Precipitate

In Figure 4.13, phenolic content of precipitate either cashew-josapine precipitate or positive control precipitate was increased with the amount of cashew polyphenols present in precipitate. However, the phenolic content in precipitate could not determine the magnitude of scavenging activity. Both 1.5% and 1.0% precipitates exhibited high, similar DPPH scavenging activity even though they possessed different phenolic content. Therefore, it is likely that cashew polyphenols with concentration of about 5.600 mg/ml already reach their optimum antioxidant

capacity. There were no phenolic compounds observed with Josapine juice extract and 0.4% bromelain solution. Negative control, cashew leaf extract had similar or slightly less phenolic content as compared to 1.5% cashew-josapine and 1.5% positive control precipitate respectively. However, its scavenging activity was about 5% higher than 1.5% and 1.0% precipitates (Figure 4.12).

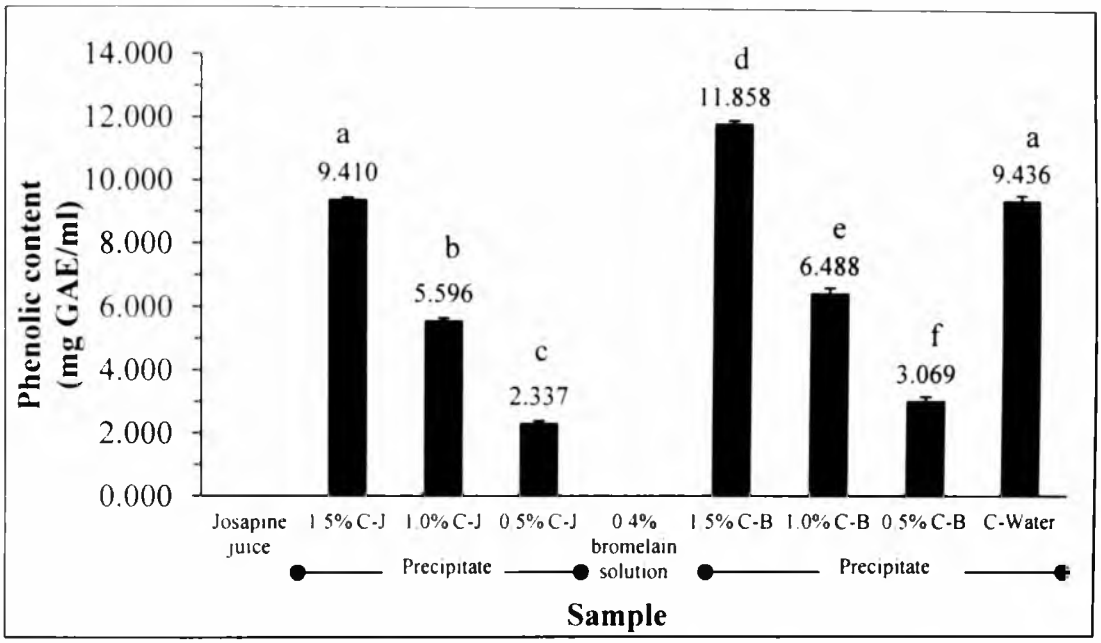


Figure 4.13 Phenolic content of cashew-josapine precipitate, cashew-bromelain precipitate, Josapine juice extract, 0.4% bromelain solution and cashew-water precipitate (negative control). Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

Combination of DPPH scavenging activity and phenolic content of bromelain-polyphenol precipitate, Josapine juice extract, 0.4% bromelain solution and cashew leaf extract (negative control) was presented in Figure 4.14.

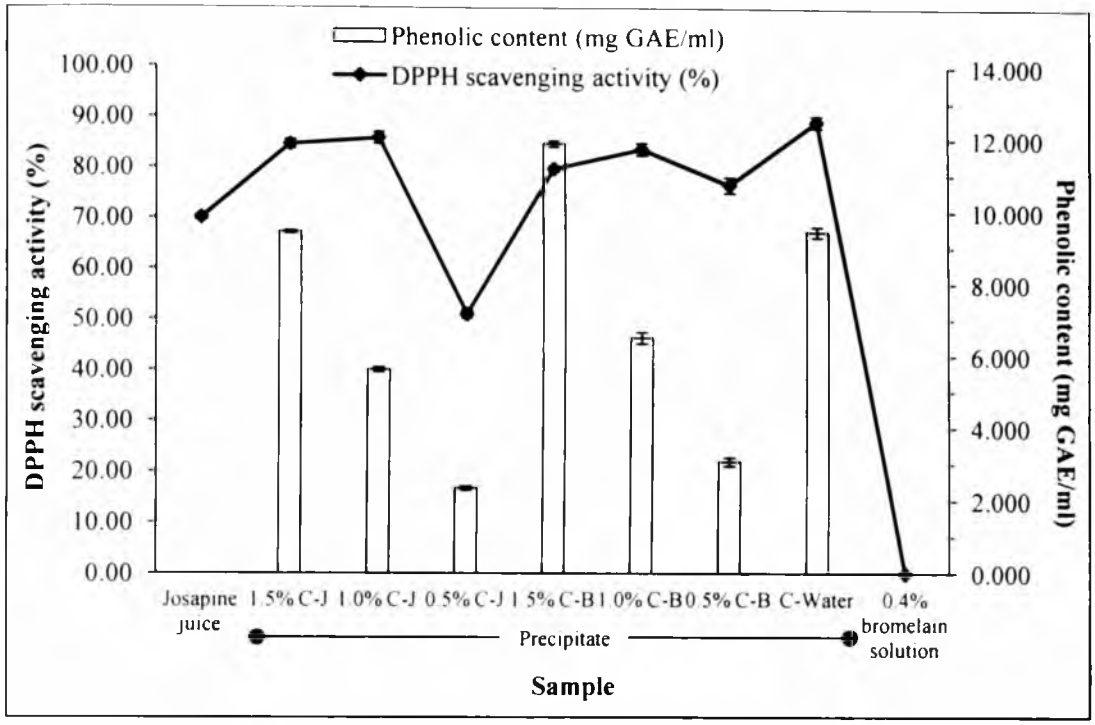


Figure 4.14 DPPH scavenging activity and phenolic content of cashew-josapine precipitate, cashew-bromelain precipitate, Josapine juice extract, 0.4% bromelain solution and cashew-water precipitate (negative control). Indicated values are expressed as mean ± SD (n=3).

Combination of bromelain activity, protein content, antioxidant activity and phenolic content of free bromelain and bromelain-polyphenol precipitate (precipitated bromelain) was shown in Figure 4.15.

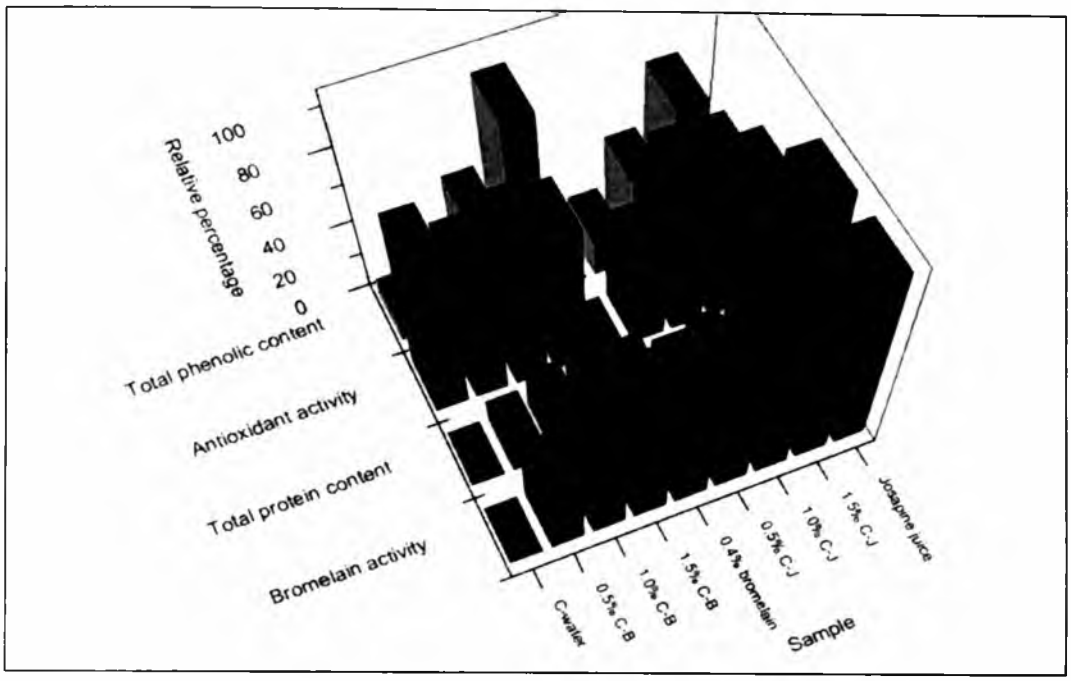


Figure 4.15 Bromelain activity, protein content, antioxidant activity and phenolic content of Josapine juice extract, cashew-josapine precipitate, 0.4% bromelain solution, cashew-bromelain precipitate and cashew-water precipitate (negative control). Indicated values are expressed as mean (n=3).

Results obtained from precipitate activity indicated that bromelain in Josapine juice extract could be recovered by precipitation with cashew leaf extract polyphenols and bromelain's enzymatic activity was retained on bromelain-polyphenol precipitate. Bromelain recovered on 1.5%, 1.0% and 0.5% cashew-josapine precipitates was found to be 65.14%, 63.39% and 39.32% of total bromelain respectively. Devakate *et al.* (2009) reported that purification of bromelain using precipitation at 40-80% ammonium sulfate saturation could obtain about 80% enzyme recovery. Although % recovery found in our study was lower than that observed with ammonium sulfate precipitation method, complexing of bromelain with polyphenols could be employed as an alternative way for recovery of bromelain from pinepapple juice on bromelain-polyphenol precipitate, and at the same time, its enzymatic activity is preserved and stabilized.

It was shown that bromelain-polyphenol precipitate had a greater antioxidant capacity as compared to Josapine juice extract. Polyphenolic compounds of cashew leaf extract are believed to be the contributors. Bromelain's catalytic nucleophile,

sulfhydryl group is readily to be oxidized and the alternation of side chain of catalytic group will lead to inactivation of structural and catalytic function of enzyme. The enzyme could have been protected and stabilized by complexing with polyphenols, which would prevent it from being oxidized and complexed with metal ions. Thus, it could say that antioxidant properties of polyphenols could stabilize and retain bromelain's enzymatic activity. In addition, resultant antioxidant activity of precipitated bromelain was greater than that of free bromelain due to the presence of polyphenols in precipitate.

Bromelain-cashew polyphenol precipitated bromelain is hypothesized to have stabilized bromelain activity and increased antioxidant activity and it is proven to be the case.

4.2 Effect of Temperature on Enzymatic and Antioxidant Activities of Free Bromelain and Bromelain-polyphenol Precipitate

Bromelain is primarily obtained from pineapple and both of the fruit and enzyme are usually applied in food processing, pharmaceutical and biotechnological industry. Heating is one of the unavoidable stress conditions and this external factor applied in sterilization and manufacturing might affect enzymatic activity and other nutraceutical properties of bromelain. Information on effect of temperature and thermal stability of bromelain is of great importance for preserving its nutraceutical properties and also the improvement in fruit and enzyme processing. The improvement can be done by application of optimum conditions or manipulating operating conditions to minimize heat denaturation effect on enzymatic activity.

In the following sections, effect of temperature, denaturation rate constant, half life and kinetics of thermal inactivation of free bromelain and bromelain-polyphenol precipitate are discussed.

Interaction of polyphenols and bromelain is believed to provide an alternative method for giving the enzyme some protective characteristics related to thermal

stability and enzymatic activity. These enzymes are inactivated and the extent of protection effect exerted by cashew leaf extract polyphenols could be determined by comparison of the residual enzymatic activity and antioxidant activity between free and precipitated bromelain. Bromelain in the precipitate with polyphenols is hypothesized to have good heat resistance, which is not achievable by using free bromelain.

4.2.1 Thermal Stability of Free and Precipitated Bromelain at Incubation Temperature of 60 °C

In this section, thermal stability test was conducted at incubation temperature of 60 °C. Some previous studies reported that the optimum enzymatic activity of bromelain was attained at this temperature. Xue *et al.* (2010) showed that maximum enzyme activity of native bromelain and modified bromelain with anhydride groups was obtained at 60 °C and high activity was observed at incubation temperatures between 50 °C to 70 °C. Study on effect of temperature on bromelain activity of clarified bromelain and tea-polyphenol-complexed bromelain in pineapple juice indicated the maximum activity was at 55 °C (Liang *et al.*, 1999).

Free bromelain and precipitated bromelain are referred to bromelain in Josapine juice extract (without mixing with cashew leaf extract) and bromelain-cashew polyphenol precipitate respectively. Josapine juice extract with free bromelain and precipitated bromelain (cashew-josapine precipitate and cashew-bromelain precipitate) were incubated at 60 °C for 180 minutes. Analysis of treated sample was assayed every 30 minutes. The experimental results on residual bromelain activity, protein content, antioxidant activity and phenolic content were discussed in the following sections.

4.2.1.1 Bromelain Activity, Denaturation Rate Constant and Half Life

Figure 4.16 and Figure 4.17 depicted relative bromelain activity of free bromelain and precipitated bromelain after incubation at 60 °C at various 30 minutes time intervals. It could be seen in Figure 4.16, bromelain activity of 1.5% and 1.0% cashew-josapine precipitates did not vary significantly with increasing incubation duration up to 150 minutes and 120 minutes respectively. Activity of free bromelain started to decrease gradually from the beginning of incubation. 1.5% and 1.0% precipitated bromelain retained about 72% and 55% of the original activity after 180 min-incubation, whereas free bromelain exhibited only about 16%. 0.5% cashew-josapine precipitate lost activity from 15 minutes to 60 minutes at which was followed by a stable line and its decrease at 15-60 minutes was more significant than free bromelain. At 180 minutes, 0.5% precipitate had a same remaining activity as 1.0% precipitate. There was no activity observed with negative control, cashew leaf extract.

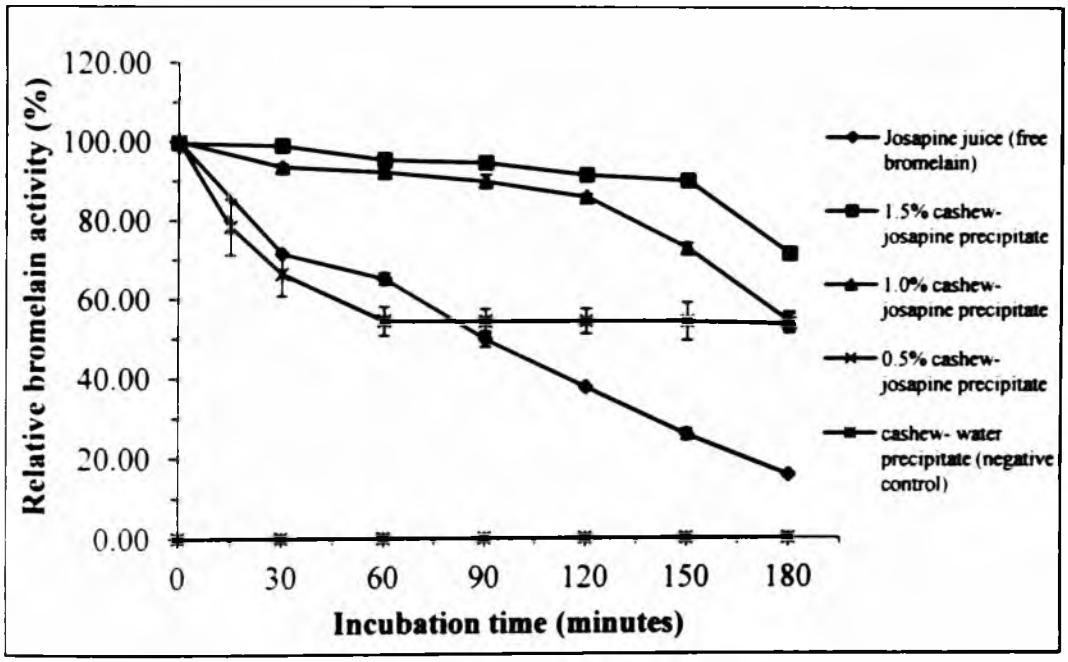


Figure 4.16 Relative bromelain activity of free bromelain in Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

In Figure 4.17, there was a slight reduction in bromelain activity of 1.5% and 1.0% positive control precipitates at the beginning of incubation and high activities of about 74% and 70% were remained respectively after 180 min-incubation. Likewise in Jospaine juice extract, activity of 0.4% bromelain solution decreased continuously with increasing incubation time and about 28% activity was retained after 180 min-incubation.

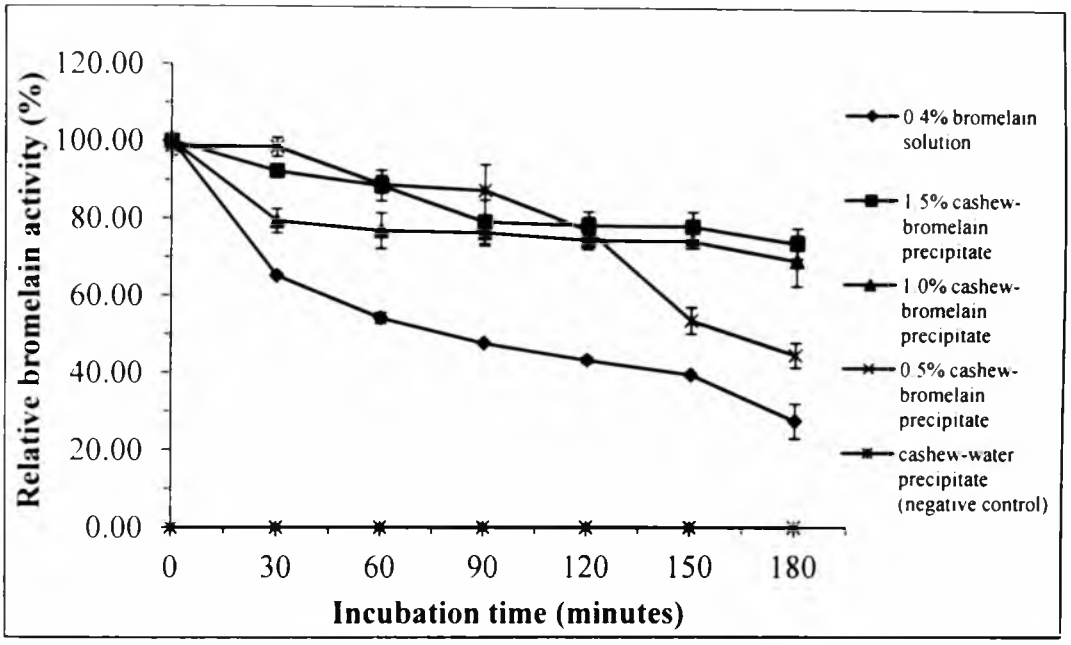


Figure 4.17 Relative bromelain activity of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

In contrast, solution either Josapine juice extract or 0.4% bromelain solution containing free bromelain was highly susceptible to heat denaturation whereas precipitated bromelain showed a marked thermal stability. Precipitation of bromelain with cashew leaf extract polyphenol improved its thermal stability and high concentration of cashew extract (1.5% and 1.0%) in precipitate gave a high thermal stabilization. Interaction of cashew extract polyphenols with bromelain might protect enzyme's active site and reduce heat denaturation effect on bromelain.

Thermal stability of a sample is determined by its rate of denaturation. Plots of logarithm of residual bromelain activity versus incubation time at 60 °C for free

bromelain and precipitated bromelain were made (Figure 4.18 and Figure 4.19). The straight line plot exhibited first order characteristic with slope of line equal to –k. The equation can be arranged as $\ln [v] = -kt + \ln[v_o]$, where [v] is residual activity and k is rate constant. Since bromelain activity decreases with incubation time, the reaction is denaturation. Denaturation reaction can be described with rate constant which is derived from the slope of first order reaction. The rate constant was assigned as denaturation rate constant (k) in this section. Denaturation rate constant and half life could be calculated from linear slope of ln [v] versus incubation time and presented in Table 4.4. The extent of heat denaturation on bromelain was measured by denaturation rate constant.

As shown in Figure 4.18, heat denaturation of free bromelain in Josapine juice, 1.5% and 1.0% cashew-josapine precipitates was described by single fist order reaction within 180 minutes. 0.5% precipitate exhibited two kinetics - First order denaturation reaction occurred in duration range of 15 and 60 minutes and no further denaturation reaction was observed from 60 to 180 minutes. Therefore, the measured denaturation rate constant of 0.5% precipitate was derived from the first order reaction at 15-60 minutes. In the case of free bromelain in bromelain solution and positive control precipitate, denaturation reaction of all samples exhibited single first order kinetic (Figure 4.19).

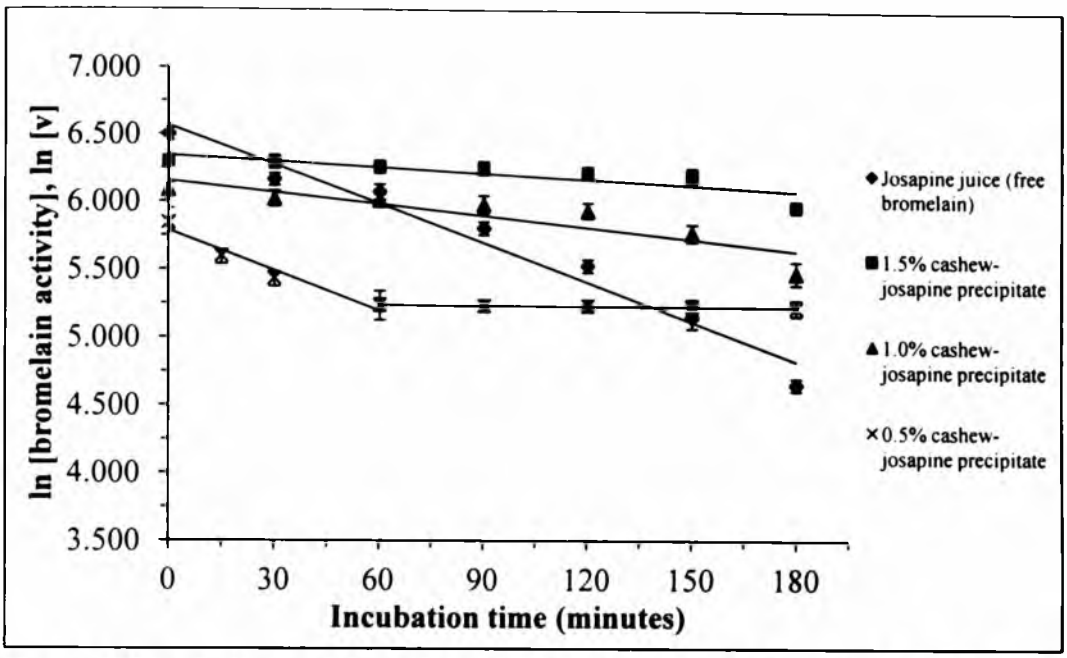


Figure 4.18 ln [bromelain activity] of free bromelain in Josapine juice extract and cashew-josapine precipitated bromelain at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

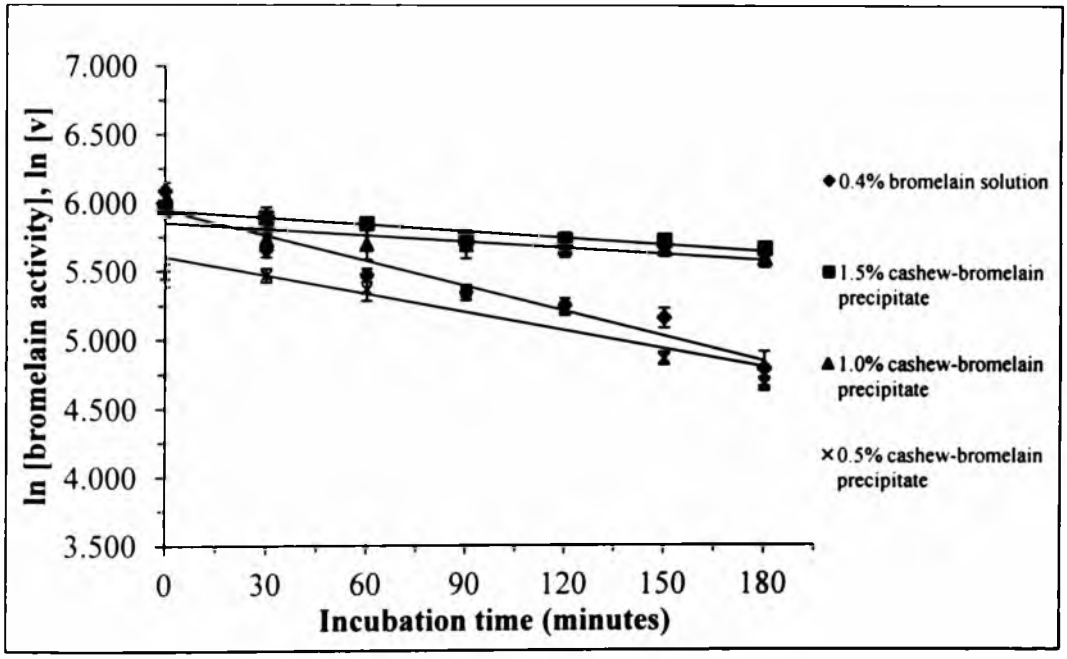


Figure 4.19 ln [bromelain activity] of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

From Table 4.4, 1.5% and 1.0% cashew-josapine precipitates exhibited high stability by having low calculated denaturation rate constants which were 1.4 x 10^{-3} min^{-1} and 2.8 x 10^{-3} min^{-1} respectively ($p<0.05$). Free bromelain in Josapine juice extract and 0.5% cashew-josapine precipitate had higher rate constants (9.7 x 10^{-3} min^{-1} and 9.8 x 10^{-3} min^{-1} respectively) in which indicate a more rapid denaturation of enzyme and no significant difference in k was found. As depicted from denaturation rate constant (k), 1.5% and 1.0% cashew-josapine precipitated bromelain had about 7.0 and 3.5 times increase in its stability, respectively, at 60 °C as compared to free bromelain and 0.5% precipitated bromelain. It could also be said that heat damage effect on bromelain was several times diminished after complexing with 1.5% and 1.0% cashew leaf extract. Since free bromelain in Josapine juice extract and 0.5% cashew-josapine precipitate had similar rate constants, 0.5% cashew extract was insufficient to protect bromelain from heat denaturation. Likewise, 1.5% and 1.0% cashew-bromelain precipitates (positive control) showed an increased thermostability of about 3 and 4 times compared to 0.5% precipitate and free bromelain in bromelain solution respectively. However, this fold of enhanced thermostability was lower than those obtained for cashew-josapine precipitate.

Table 4.4 Denaturation rate constant and half life for bromelain activity of free bromelain in Jospaine juice extract, cashew-josapine precipitated bromelain, 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3). Means with different letters within same column indicate a significant difference at $p<0.05$.

Sample	Denaturation rate constant, k (min^{-1})	Half life, t ½ (min)
Josapine juice extract	0.0097 ± 0.0001 a	71.71 ± 0.43 a,d
1.5% cashew-josapine precipitate	0.0014 ± 0.0001 b	484.10 ± 19.06 b
1.0% cashew-josapine precipitate	0.0028 ± 0.0002 c	245.11 ± 13.01 c,d
0.5% cashew-josapine precipitate	0.0098 ± 0.0008 a	71.05 ± 5.82 a,d
0.4% bromelain solution	0.0061 ± 0.0005 d	114.77 ± 9.40 d
1.5% cashew-bromelain precipitate	0.0016 ± 0.0003 b	443.74 ± 84.68 b
1.0% cashew-bromelain precipitate	0.0015 ± 0.0003 b	473.93 ± 96.91 b
0.5% cashew-bromelain precipitate	0.0044 ± 0.0006 e	157.96 ± 19.55 d

Overall, precipitation of bromelain with cashew leaf extract especially of high concentration (1.5% and 1.0%) brought about enhancement of thermal stability and reservation of enzymatic activity.

4.2.1.2 Protein Content and Decrease Rate Constant

As depicted in Figure 4.20, both Josapine juice extract and cashew-josapine precipitated bromelain showed a decrease in protein content as incubation duration increased at 60 °C. Figure 4.21 was a plot of logarithm of relative protein content against incubation time at 60 °C where the data points were derived from Figure 4.20. Since protein content of sample decreased with increasing incubation time, therefore the rate of protein content reduction was named as decrease rate constant in this section. Decrease rate constant for protein content of each sample was calculated from the slope of straight line. As could be seen from data of decrease rate constant in Table 4.5, heat damage effect on protein content was about 2.21 times reduced after precipitation josapine bromelain with cashew extract of 1.5% concentration. 1.0% cashew-josapine precipitate also had a moderate stability, by displaying 1.35 times lower decrease rate constant as compared to Josapine extract. For 0.5% cashew-josapine precipitate, decrease in protein content was described with two first order reactions with different rate constants (Figure 4.21). By comparing the decrease rate constant of Jospaine juice extract and 0.5% precipitated bromelain in duration range of 0-120 minutes (Table 4.5), moderate stabilization in protein content was observed with 0.5% precipitate since it displayed about 1.23 times lower constant than that of free bromelain. However, protein content of 0.5% precipitated bromelain was readily to be denatured at incubation times above 120 minutes (Figure 4.20).

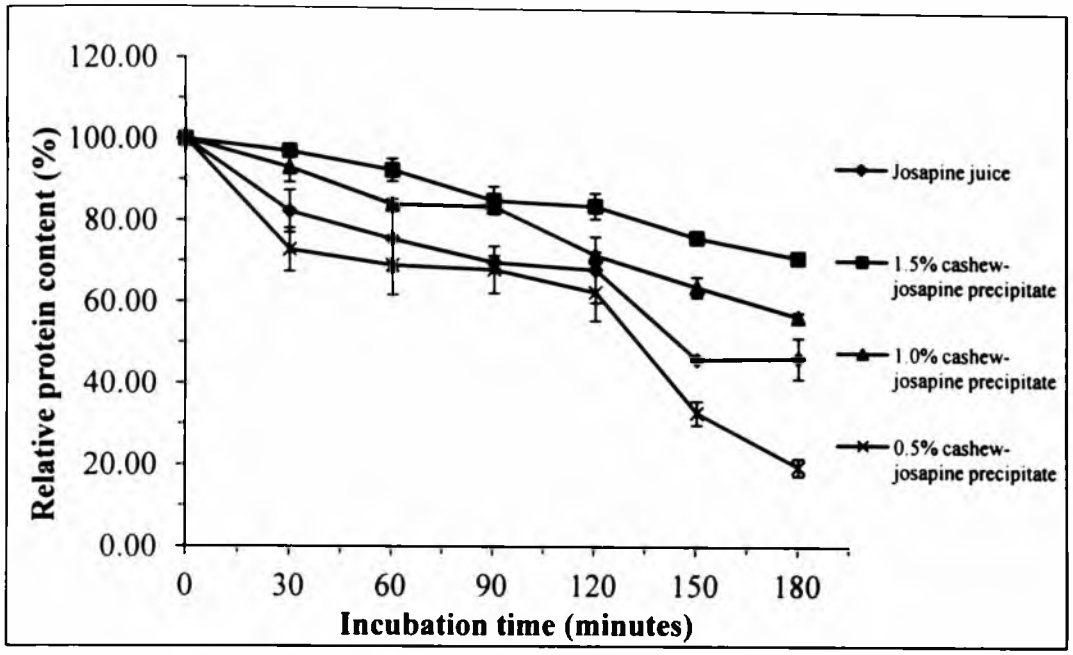


Figure 4.20 Relative protein content of Josapine juice extract and cashew-josapine precipitated bromelain at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

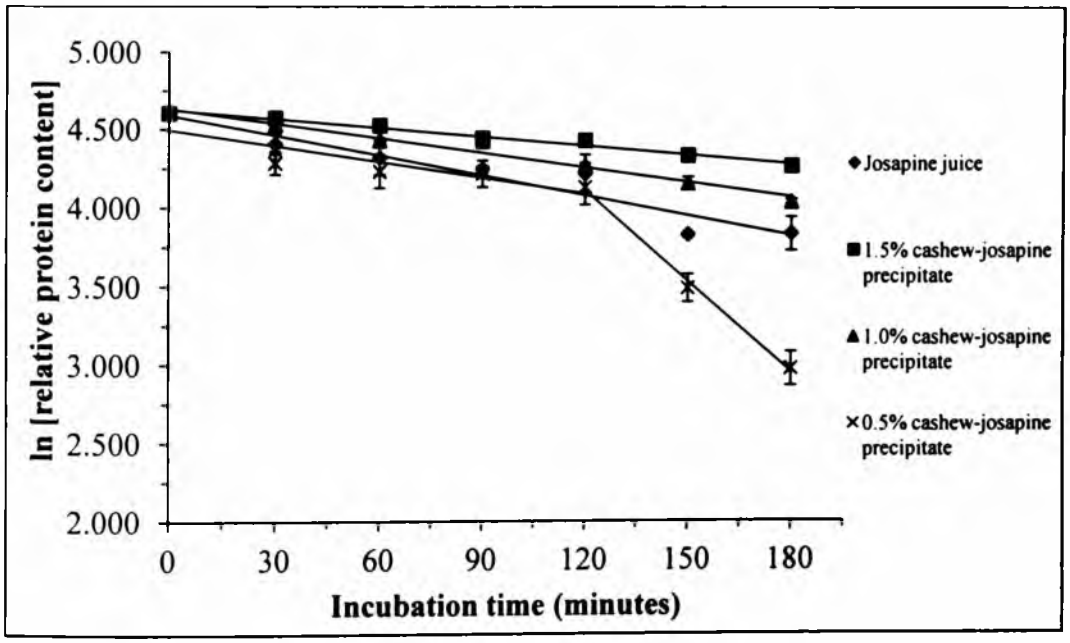


Figure 4.21 ln [relative protein content] of Josapine juice extract and cashew-josapine precipitated bromelain at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

Table 4.5 Decrease rate constant for protein content of Josapine juice extract, cashew-josapine precipitated bromelain, 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at 60 °C for stated incubation duration. Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

Sample	Incubation time range (min)	Decrease rate constant (min^{-1})
Josapine juice extract	0-180	0.0042 ± 0.0006 a,f
1.5% cashew-josapine precipitate	0-180	0.0019 ± 0.0001 b,d,f
1.0% cashew-josapine precipitate	0-180	0.0031 ± 0.0001 a,f
0.5% cashew-josapine precipitate	0-120	0.0034 ± 0.0006 a,b,f
0.4% bromelain solution	0-60	0.0127 ± 0.0014 c
1.5% cashew-bromelain precipitate	0-180	0.0014 ± 0.0001 d,e
1.0% cashew-bromelain precipitate	0-180	0.0023 ± 0.0001 e,f
0.5% cashew-bromelain precipitate	0-180	0.0031 ± 0.0002 f

In Figure 4.22, protein content of 0.4% bromelain solution decreased dramatically within 60 min-incubation and became stable after 60 minutes. Decrease rate constants calculated in the duration range of 0-60 minutes for 0.4% bromelain solution was analyzed. 1.5% and 1.0% positive control precipitates proved more stable than 0.5% precipitated bromelain. Means of constant of 1.5% and 1.0% precipitates were significant different ($p<0.05$) as compared to 0.5% precipitate. Positive control precipitated bromelain with 1.5%, 1.0% and 0.5% cashew extracts increased its stability in protein content at 60 °C compared to free bromelain. Decrease rate constants for 0.4% bromelain solution and cashew-bromelain precipitate were presented in Table 4.5. Figure 4.23 was a plot of logarithm of relative protein content versus incubation time for 0.4% bromelain solution and cashew-bromelain precipitated bromelain.

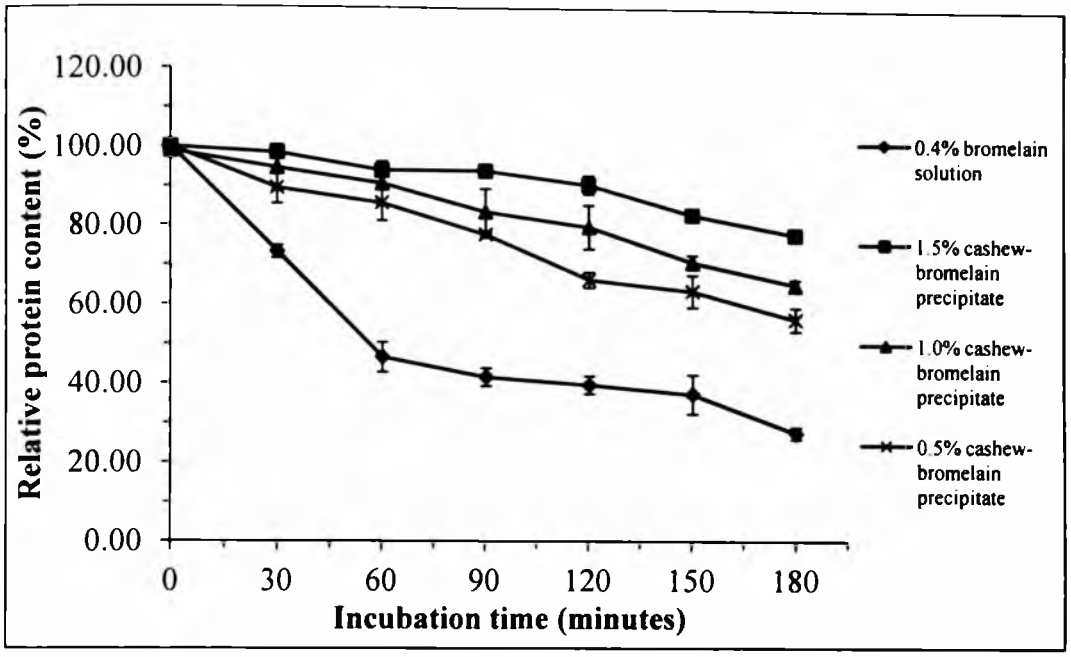


Figure 4.22 Relative protein content of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

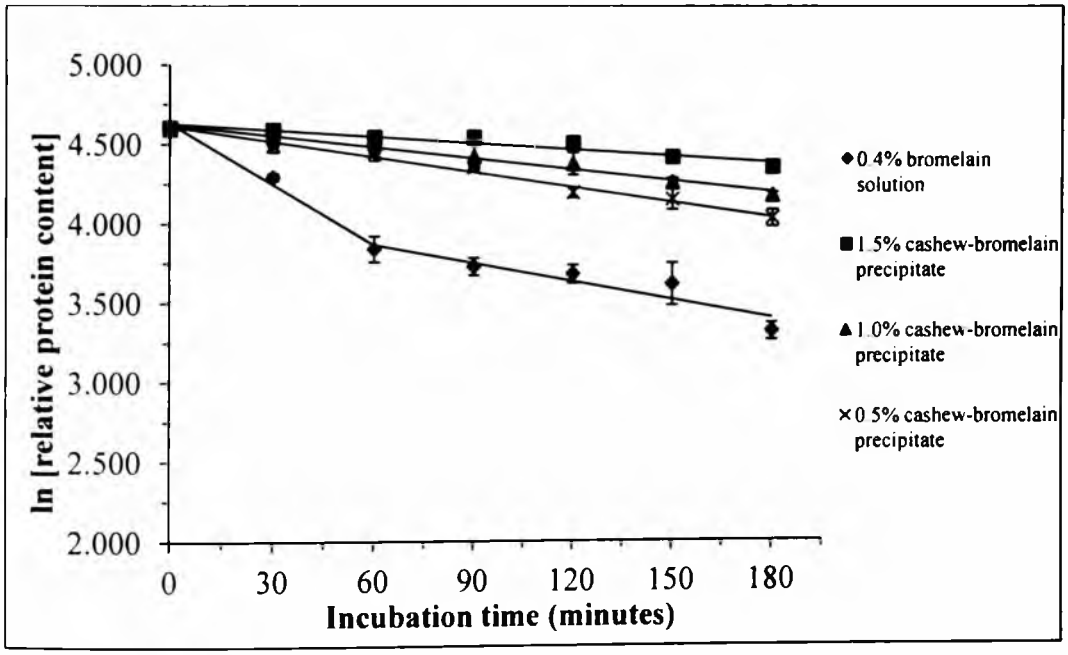


Figure 4.23 ln [relative protein content] of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

Environment conditions such as continuous heating on enzyme at elevated temperature will distort conformation of protein, disrupt disulphide bonds and

noncovalent interaction and results in protein deformation and denaturation. Based on BCA protein assay, proteins reduce Cu^{2+} to Cu^{+}. BCA could detect the amount of Cu^{+} by development of purple colour which is proportional to protein concentration. Denatured protein might not be able to reduce copper ion. It is supposed that only conformational intact proteins could be detected by BCA assay. In addition, presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) is responsible for colour formation with BCA (Walker, 2002).

Results indicated that residual protein content was higher for precipitated bromelain particularly with 1.5% cashew leaf extract polyphenols than for free bromelain solution at 60 °C. It could be explained that complexing of bromelain with cashew polyphenols could diminish adverse effect of incubation temperature on conformation and functionality of enzyme, suggesting an enhanced thermal stability in accordance with the above-mentioned thermal stability studies on bromelain activity.

4.2.1.3 Antioxidant Activity

Results on DPPH scavenging activity in thermal stability analysis at 60 °C were presented in Figure 4.24 and Figure 4.25. It was obvious from these plots that cashew leaf extract (negative control), precipitated bromelain with 1.5% and 1.0% cashew extract were stable in antioxidant activity throughout 180 min-incubation. In Figure 4.24, 1.5% and 1.0% cashew-josapine precipitates maintained their great activity of above 85%. The DPPH scavenging activities of 1.5% and 1.0% precipitates did not show any significant difference at each 30 min-incubation interval. Antioxidant activity of Josapine juice extract preserved at about 60% for 120 minutes and further increase in incubation time caused about 15% reduction. For 0.5% cashew-josapine precipitate, a drop of about 12% in activity was measured in 90-120 minutes. Both 0.5% precipitate and Josapine juice attained a similar residual activity of about 45% after 180 min-incubation. In contrast in Figure 4.25, a stable activity (~65%) was observed with 0.5% cashew-bromelain precipitate (positive control).

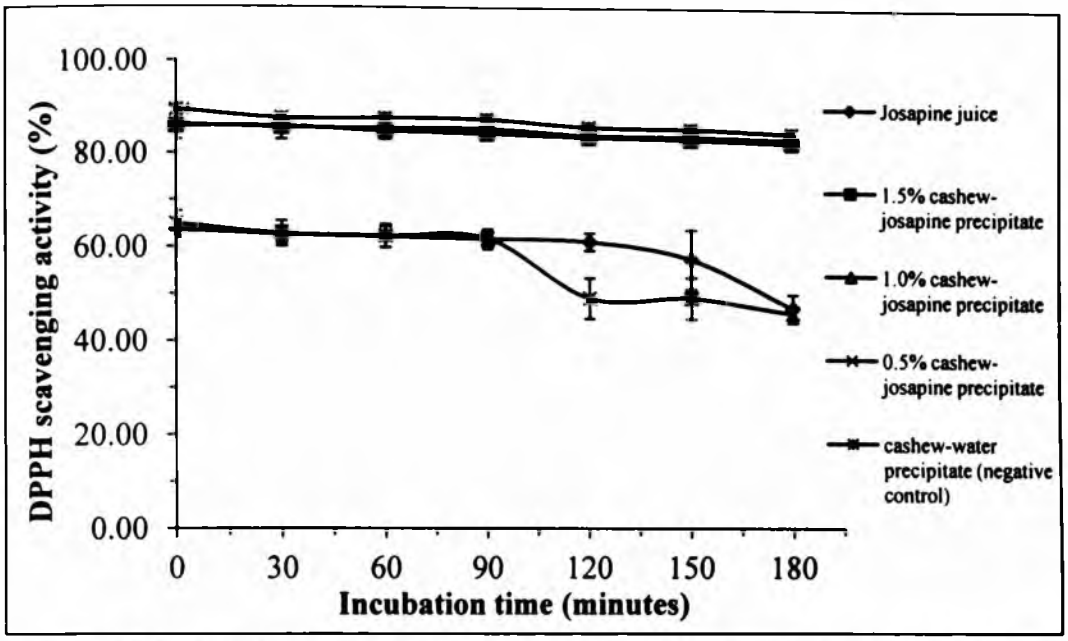


Figure 4.24 DPPH scavenging activity of Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

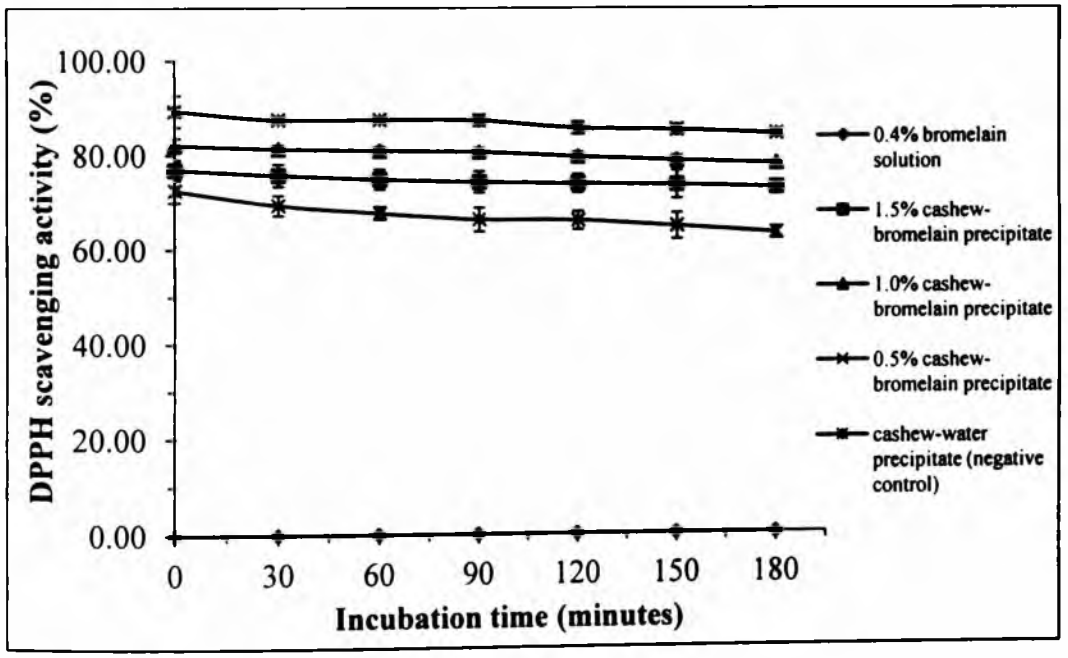


Figure 4.25 DPPH scavenging activity of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

Relative higher and more stable DPPH scavenging activity was found in cashew-josapine precipitated bromelain except 0.5% cashew-josapine precipitate, as compared to Josapine juice extract. The difference in stability might be due to the presence of cashew leaf extract polyphenols in precipitate. Polyphenols might exhibit a greater tolerance to heat than did proteins in pineapple juice and therefore the antioxidant activity of bromelain in complexing with polyphenols was stabilized and not severely inhibited by continuous heating.

4.2.1.4 Phenolic Content

Polyphenolic content of a sample is a major contributor to its antioxidant activity. In Figure 4.26, all cashew-josapine precipitated bromelain showed a great drop in phenolic content at the beginning of incubation at 60 °C. While at incubation time greater than 30 minutes, the phenolics decreased slightly with the increase of time. Cashew leaf extract (negative control) exhibited a stable line in phenolic content up to 150 minutes and further increase in time caused a slight decrease. Results on phenolic content for positive control precipitate were shown in Figure 4.27. Phenolic content of cashew leaf extract appeared to be more stable relative to continuous heat denaturation at 60 °C as compared to precipitated bromelain.

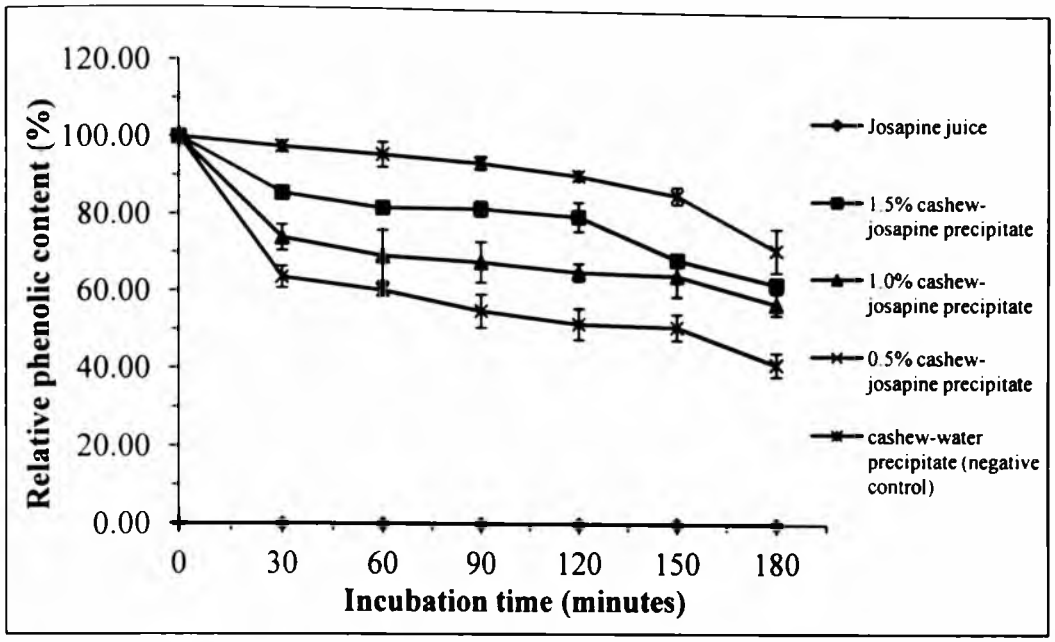


Figure 4.26 Relative phenolic content of Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

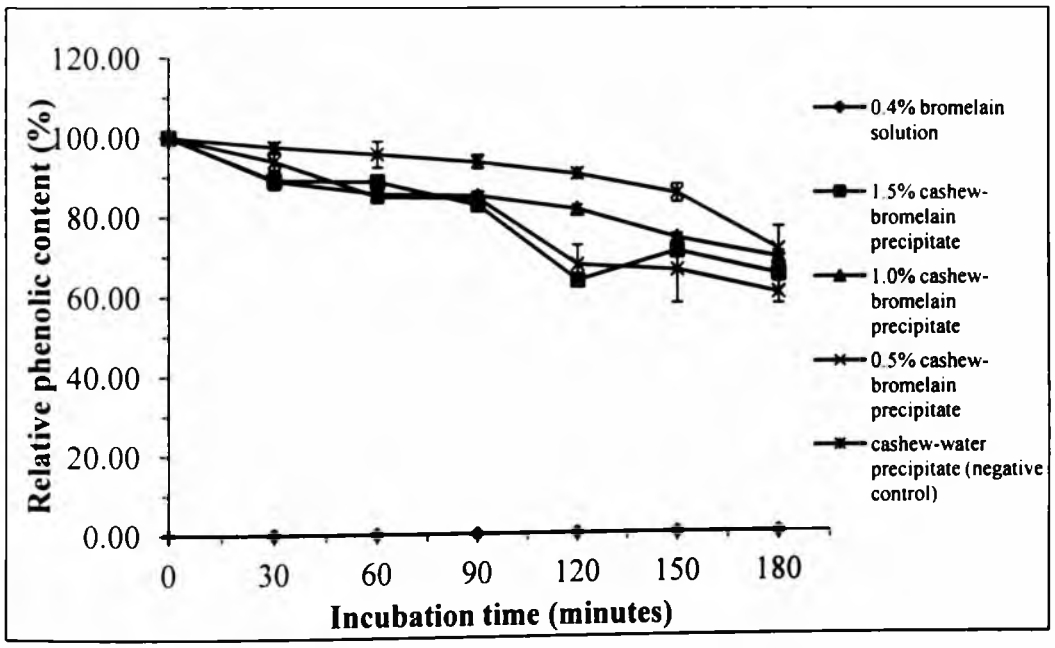
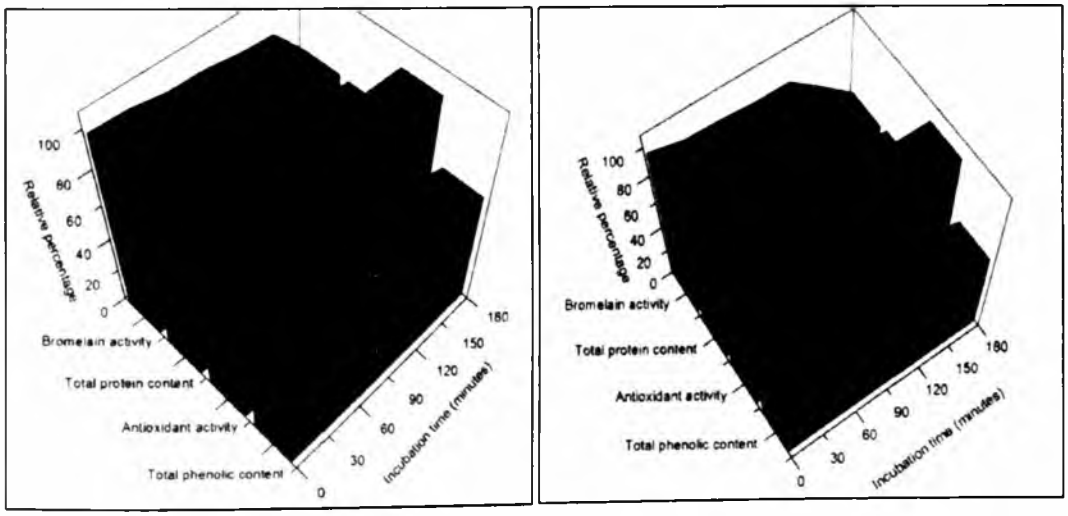


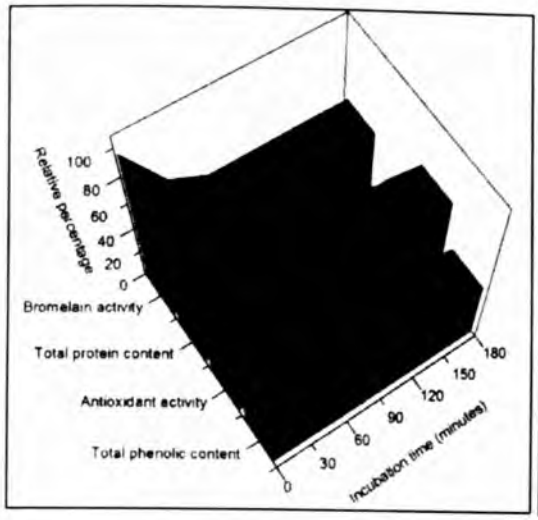
Figure 4.27 Relative phenolic content of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at 60 °C for 180 minutes. Indicated values are expressed as mean ± SD (n=3).

It was observed that the pattern of phenolic content at 60 °C (Figure 4.26 and Figure 4.27) was not in accordance with those obtained for DPPH scavenging activity (Figure 4.24 and Figure 4.25). It could be explained, take an example of 1.5% cashew-josapine precipitate in Figure 4.26, the lowest phenolic content of 5.623 mg GAE/ml was found after 180 minutes incubation at 60 °C and this phenolic concentration might accounted for 82.45% DPPH scavenging activity under the same condition. This value of antioxidant activity at 180 minutes was only about 3% less than the activity of no heat treated 1.5% precipitate (85.77%). Therefore, a decrease in phenolic content of a sample would not affect its performance of antioxidant activity. However, it depends on the maximum antioxidant capacity a certain amount of phenolic content could exhibit.

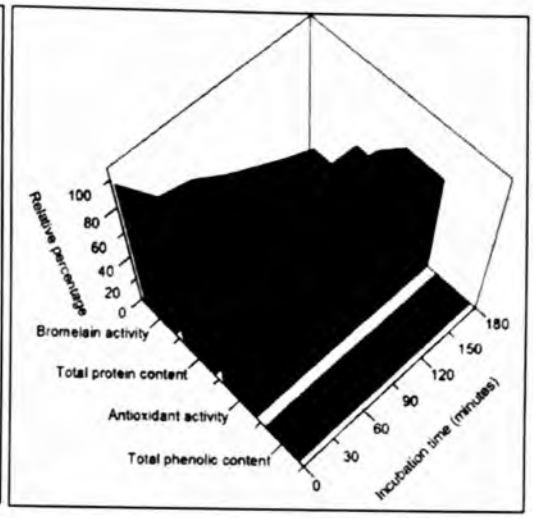
Figure 4.28 presented the bromelain activity, protein content, antioxidant activity and phenolic content of each cashew-josapine precipitated bromelain, Josapine juice extract and cashew-water precipitate (negative control) obtained at 60 °C for 180 minutes.



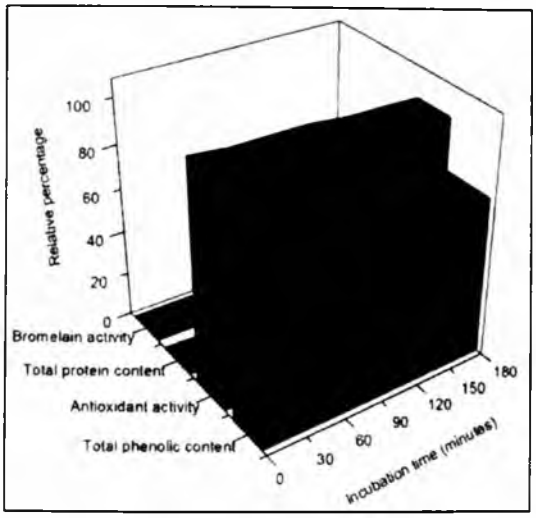
(a) 1.5% cashew-josapine precipitate (b) 1.0% cashew-josapine precipitate



(c) 0.5% cashew-josapine precipitate



(d) Josapine juice extract



(e) Cashew-water precipitate

Figure 4.28 Properties of 1.5%, 1.0%, 0.5% cashew-josapine precipitates, Josapine juice extract and cashew-water precipitate at 60 °C for 180 minutes. Indicated values are expressed as mean (n=3).

Strength of association between 2 variables (bromelain activity versus protein content and antioxidant activity versus phenolic content) was determined by running Pearson product moment correlation (Details on Pearson correlation are elaborated in section 3.11). The relationship was quantified by correlation coefficient, r and P value for correlation coefficient. The associations between above stated variables for each cashew-josapine precipitate, Josapine juice extract and cashew leaf extract (negative control) in response to incubation temperature at 60 °C for 180 minutes were presented in Appendix B, Table B.1.

In Table B.1, between bromelain activity and protein content, 1.0% cashew-josapine precipitate was found to have greater positive correlation as compared to 1.5% and 0.5% precipitates by displaying higher positive r value. Josapine extract exhibited the strongest positive linear relationship. In the view on another set of variables, 1.5% cashew-josapine precipitate and cashew leaf extract displayed a strong positive association between antioxidant activity and phenolic content. No relationship of these variables was observed with Josapine extract. The greater the variables are correlated, the smaller the P value.

Matrix of scatter graphs for Pearson correlation for 1.5% cashew-josapine precipitate, Josapine extract and cashew leaf extract between all combinations of variables in response to 60 °C, 180 minutes incubation was presented in Appendix C, Figure C.1 (Details on scatter matrix are elaborated in section 3.11). Take an example in Figure C.1 (a), the third scatter graph in second row in the martrix presented association between bromelain activity and protein content of 1.5% cashew-josapine precipitate. Scatter points are plot in orientation of right upward position, indicating a positive relationship. Narrow elipse indicates scatter points are running through the straight correlation line. No scatter point indicates there is no association between corresponding variables.

Effect of an elevated temperature (60 °C) on four properties (bromelain activity, protein content, antioxidant activity and phenol content) on free bromelain and precipitated bromelain for 180 min-incubation has been described. It was suggested that precipitation of bromelain with cashew leaf extract polyphenols could make the enzyme more resistant to continuous heat damage effect which exerts on its properties. Discussion on thermal stabilization by interaction of proteins with polyphenols will be elaborated in the following paragraphs.

Stem bromelain exists as a single polypeptide chain which is folded into two domains and the structure is stabilized by disulphide bond and hydrogen bond. The cysteine catalytic groups and other residues located at active site between two domains are important in substrate specificity, catalysis and structural maintenance (Details on molecular structure and catalytic active site of bromelain are elaborated

in section 2.1.1 and 2.1.2). Conformational stability of enzyme is crucial in preserving its catalytic activity. Heating process will alter conformation of structural domains which is favorable relative to catalytic activity, break stabilizing interaction, unravel and distort binding sites of reactive groups. These structural changes in active sites of enzyme lead to protein denaturation, loss in functionality and catalytic activity (Rawlings *et al.*, 2007).

Thermal stability describes the ability of enzyme to exhibit resistance against thermal unfolding at elevated temperature (Georis *et al.*, 2000). Complexing of bromelain with polyphenols is the method used in this study for conferring the enzyme with enhanced stability. Based on the earlier studies on driving forces in formation of protein-polyphenol complex, the interaction between proteins and polyphenols is driven by hydrophobic interaction and hydrogen bonding (Details on interaction bonding between proteins and polyphenols are elaborated in section 2.7.3.2). Daniel (1996) reported that thermal stabilization could be made by addition of noncovalent bonds including hydrogen bonds, hydrophobic interaction and salt bridge to stabilize native enzyme. Since the interaction between pineapple bromelain and cashew polyphenols might involve hydrophobic and hydrogen bonding, free bromelain could be stabilized by these additional putting of noncovalent bonds.

It is essential to note that involvement of the above-mentioned interaction bonding could stabilize the folded state of protein and increase its conformational stability (Matthews, 1993). Conformational rigid protein is more resistant to thermal unfolding and protein denaturation (Daniel *et al.*, 1996). Therefore, bromelain-polyphenol complex with an increased conformational stability is hypothesized to be more thermostable as compared to free bromelain. In this study, the precipitated bromelain appeared to be more heat resistant than free bromelain. The involvement of additional noncovalent bonds in the interaction of polyphenols and proteins could explain why precipitated protein had great thermal stability.

Liang *et al.* (1999) has reported that one of the factors for improved thermal stability of tea-polyphenol-complexed bromelain was antioxidant properties of tea polyphenols. Several studies have shown that high temperature facilitates

bromelain precipitated bromelain. As shown in Figure 4.29, enzymatic activity of free bromelain in Josapine juice extract decreased gradually with increasing temperature from 25 °C to 95 °C and apparent no differ in activity at 95 °C-105 °C was observed. Free bromelain in Josapine juice retained about 20% of its total activity after incubation at 105 °C. A large reduction in activity was measured between 85 °C and 95 °C, which occupied half of the total activity loss (40% activity loss out of total 80%).

In contrast to free bromelain in Josapine juice extract, all cashew-josapine precipitated bromelain showed an activity increase as incubation temperature increased from 25 °C to 85 °C (Figure 4.29). While at temperature above 85 °C, activity of precipitated bromelain decreased dramatically and at 95 °C it was ~35%-45%. The activity loss was about 55-65% at 85 °C-95 °C but at 95 °C-105 °C, it did not differ appreciably. After incubation at 105 °C, 1.5%, 1.0% and 0.5% cashew-josapine precipitates retained approximately 35%, 40% and 55% of total activity respectively, which was 15%-35% more than free bromelain activity. The temperature for maximum activity of 1.5%, 1.0% and 0.5% precipitated bromelain was from 75 °C to 85 °C, whereas that of free bromelain was 25 °C. These results indicated that precipitated bromelain showed a good heat resistance and it was able to adapt to a wide temperature range by displaying an increasing activity in the range of 25 °C-85 °C. The activities for 1.5%, 1.0% and 0.5% precipitated bromelain were elevated approximately 15%, 30% and 43% respectively within this range, whereas free bromelain in Josapine juice was gradually inactivated under same condition. Rate of enzyme-catalyzed reaction will increase with temperature to a maximum level and further increase of temperature beyond optimum will cause declination of enzyme activity. Precipitated bromelain had a much higher optimum temperature of maximum activity than did free bromelain. Free bromelain was not able to withstand heat denaturation effect at temperature above 25 °C. Precipitated bromelain favored higher temperature as could be noticed from activity data at 65 °C, 75 °C and 85 °C. Bromelain precipitated with cashew leaf extract polyphenol produced an enhancement of its thermal stability at elevated temperature, particularly from 25 °C to 85 °C.

At temperature above 85 °C, cashew leaf extract in the precipitate did not exert any protection against heat denaturation effect since all precipitated bromelain exhibited a dramatic reduction. Same behavior in enzymatic reduction under the same condition was observed with free bromelain. It could suggest that heating at temperature beyond 85 °C caused a large alteration in conformational stability and functionality of bromelain enzyme, leads to loss of enzymatic activity.

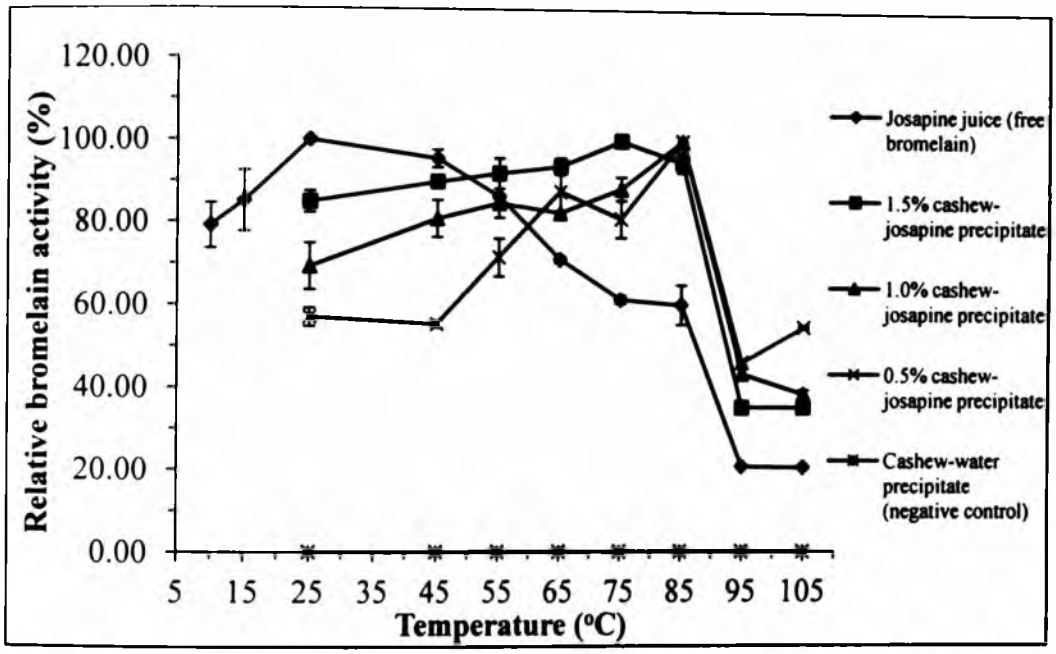


Figure 4.29 Relative bromelain activity of free bromelain in Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

In the case of positive control sample (Figure 4.30), bromelain activity of 0.4% bromelain solution (free bromelain) was in bell-shaped curve at 25 °C-105 °C and the maximum activity was obtained at 65 °C. At temperature above 65 °C, the activity reduced gradually and at 105 °C it retained ~26%. For 1.0% and 0.5% cashew-bromelain precipitates, likewise in cashew-josapine precipitate, the activity was elevated about 20% as temperature increased up to 85 °C. An increase in temperature beyond optimum caused a dramatic inactivation of enzyme with ~65-70% activity loss observed at 95 °C. 1.0% and 0.5% precipitated bromelain had optimum temperature of 85 °C and 0.4% bromelain solution exhibited highest activity at 65 °C. It was observed that 1.5% cashew-bromelain precipitate preserved its original activity as temperature increased up to 105 °C. There was only a slight

drop in activity measured at 85 °C-95 °C. Further studies deal with 1.5% cashew-bromelain precipitate need to be carried out for validation of its thermostability at elevated temperature.

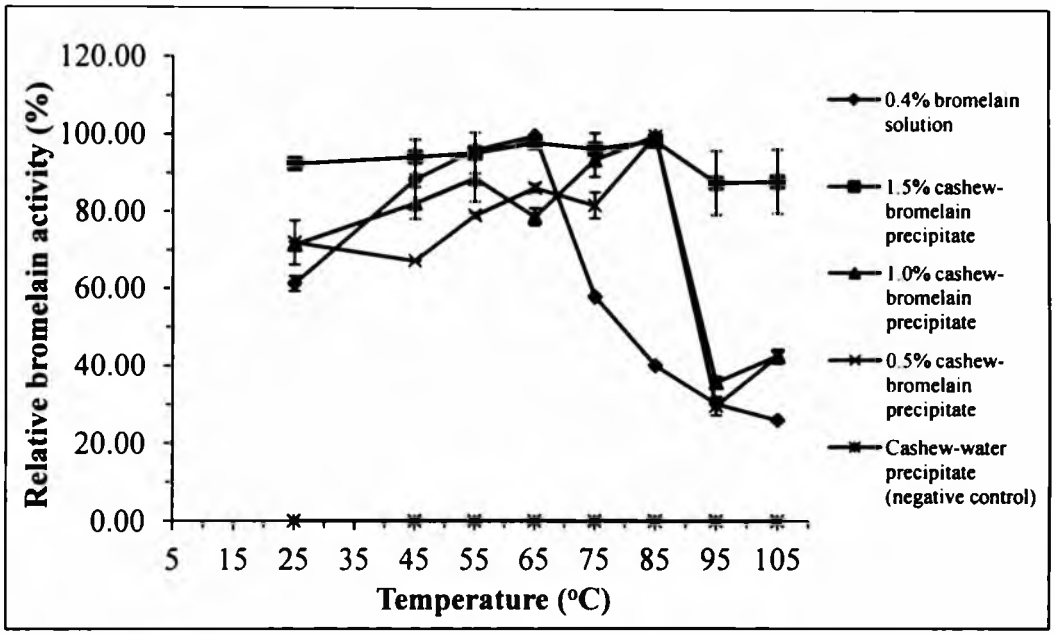


Figure 4.30 Relative bromelain activity of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

It would be interesting to notice that as incubation temperature was risen to 85 °C above, the enzymatic activity decreased sharply for either free bromelain or precipitated bromelain. In the plot of logarithm of relative bromelain activity versus incubation temperature for free bromelain in Josapine juice and cashew-josapine precipitated bromelain (Figure 4.31), variation of bromelain activity could be categorized into three parts which were located at 25 °C-85 °C, 85 °C-95 °C and 95 °C-105 °C. In temperature range of 25 °C-85 °C, the data points in plot for each sample could be fitted with straight line. Gradient of the straight line equation (term of 'constant' is used for the following analysis) could indicate the relation between bromelain activity and increasing temperature or effect of temperature on enzymatic activity.

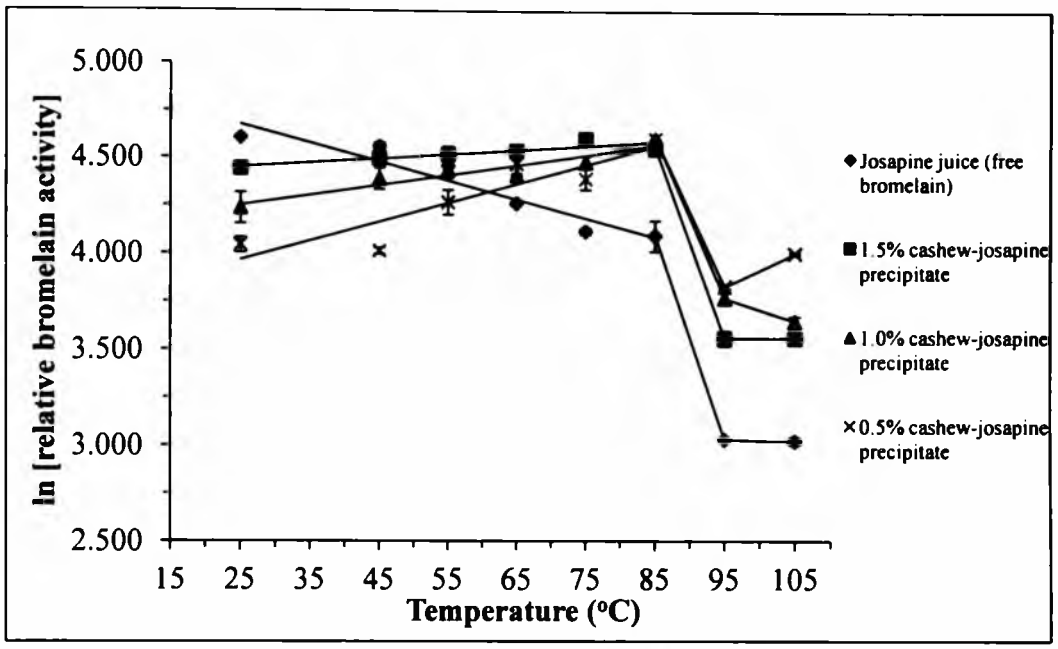


Figure 4.31 ln [relative bromelain activity] of free bromelain in Josapine juice extract and cashew-josapine precipitated bromelain at various temperatures. Indicated values are expressed as mean ± SD (n=3).

Constants for variation of bromelain activity in temperature range of 25 °C-85 °C for free bromelain and precipitated bromelain were presented in Table 4.6. Negative constant indicates activity decreases with increasing temperature or temperature inversely affects activity. The data in Table 4.6 signified that free bromelain in Josapine juice extract was readily denatured as the temperature was increased. In contrast, increased temperature enhanced bromelain-catalyzed reaction of 1.5%, 1.0% and 0.5% cashew-josapine precipitated bromelain, corroborating great resistance against thermal denaturation was exhibited by precipitated bromelain. The constant of cashew-josapine precipitate was positive value and it was decreased with increasing concentration of cashew extract in precipitate. 1.5% precipitated bromelain exhibited a smaller value of constant, indicating the effect of temperature in the range of 25 °C-85 °C was smaller as compared to 1.0% and 0.5% precipitates. Means of constant of cashew-josapine precipitates and Josapine juice exhibited significant difference at $p<0.05$. It could be noticed that 0.5% cashew-josapine precipitate had about 1.89 and 4.35 times higher constant than that of 1.0% and 1.5% precipitates respectively. It showed that activation of enzymatic activity by elevating temperature was more significant with 0.5% precipitated bromelain than 1.0 % and 1.5% precipitates.

Table 4.6 Constants of variation of bromelain activity with stated incubation temperature for free bromelain in Jospaine juice extract, cashew-josapine precipitated bromelain, 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control). Indicated values are expressed as mean ± SD (n=3). Means with different letters indicate a significant difference at $p<0.05$.

Sample	Temperature range	Constant
Josapine juice extract	25 °C-85 °C	-0.0098 ± 0.0010 a
1.5% cashew-josapine precipitate	25 °C-85 °C	0.0023 ± 0.0002 b
1.0% cashew-josapine precipitate	25 °C-85 °C	0.0053 ± 0.0007 c
0.5% cashew-josapine precipitate	25 °C-85 °C	0.0100 ± 0.0010 d
0.4% bromelain solution	25 °C-65 °C	0.0126 ± 0.0009 e
1.5% cashew-bromelain precipitate	25 °C-85 °C	0.0011 ± 0.0001 b
1.0% cashew-bromelain precipitate	25 °C-85 °C	0.0049 ± 0.0002 c
0.5% cashew-bromelain precipitate	25 °C-85 °C	0.0055 ± 0.0009 c

Plot of ln [relative bromelain activity] versus temperature for 0.4% bromelain solution and positive control precipitated bromelain was shown in Figure 4.32. As shown in Table 4.6, the extent of effect of increasing temperature ranged from 25 °C-85 °C on activity for 1.0% and 0.5% cashew-bromelain precipitates was similar, while great activity activation was observed with 0.4% bromelain solution at 25 °C-65 °C. 1.0% and 0.5% precipitates did not show a significant difference.

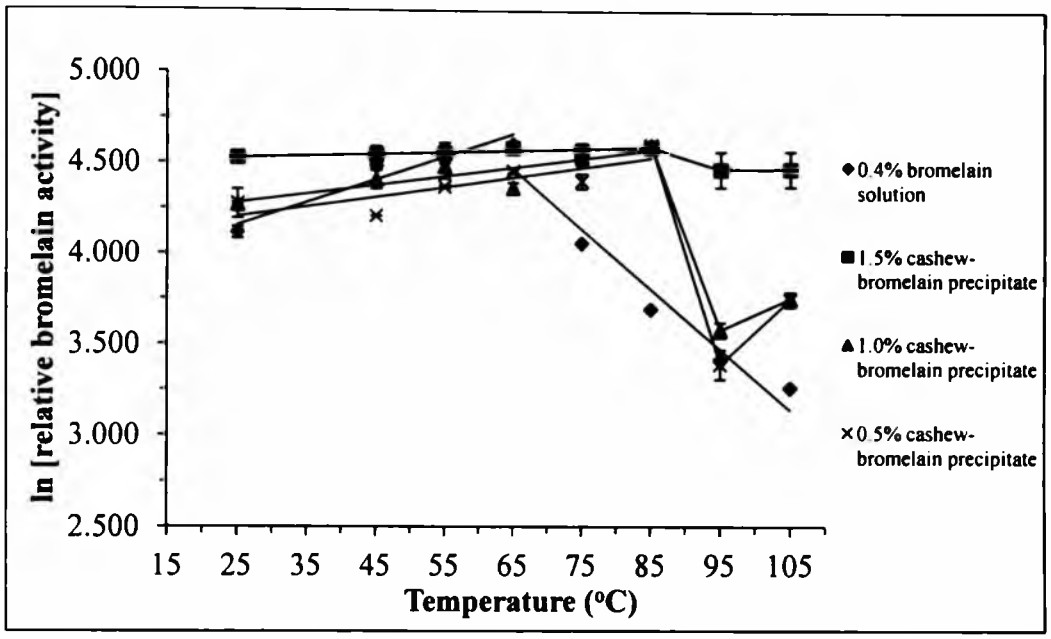


Figure 4.32 ln [relative bromelain activity] of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

Overall, optimum temperature of maximum activity was 75 °C-85 °C for precipitated bromelain and 25 °C for free bromelain in Josapine juice extract. Precipitated bromelain showed a better tolerance to heat denaturation by displaying a stable and increasing activity over wide elevated temperature range, as compared to free bromelain. Its increased thermal stability might be due to the stabilization effect associated with cashew polyphenols complexing which will be discussed in detail later.

4.2.2.2 Protein Content

Figure 4.33 and Figure 4.34 illustrated the effect of temperature on protein content. In Figure 4.33, the protein content of all three cashew-josapine precipitated bromelain was found to follow a general trend where it increased with incubation temperature and started to decrease as temperature was risen beyond optimum. This trend was also similar to the variation of protein observed with positive control precipitate (Figure 4.34). The temperature was optimum from 75 °C to 85 °C for precipitated bromelain. Except for Josapine juice extract and 1.5% cashew-

bromelain precipitate, a trend similar to the variation of protein content for precipitated bromelain and 0.4% bromelain solution was observed with bromelain activity, which had been reported in the last section. For Josapine juice extract, instead of reduction with temperature observed with its bromelain activity, variation of protein appeared stable but did not increase linearly in temperature range from 25 °C to 85 °C. Further increase in temperature beyond 85°C caused a reduction in protein content (Figure 4.33).

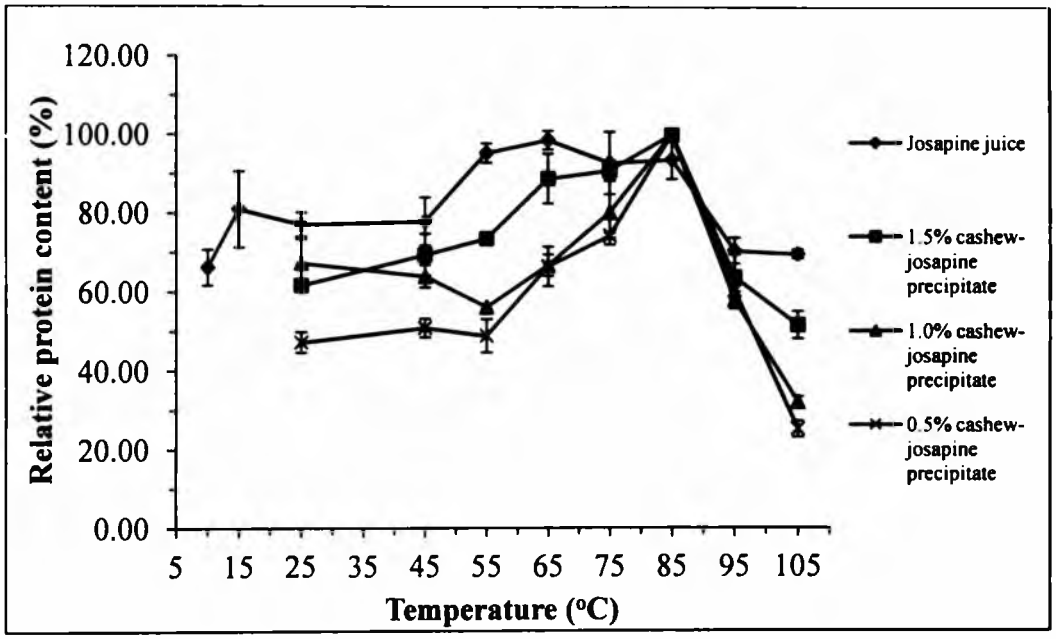


Figure 4.33 Relative protein content of Josapine juice extract and cashew-josapine precipitated bromelain at various temperatures. Indicated values are expressed as mean ± SD (n=3).

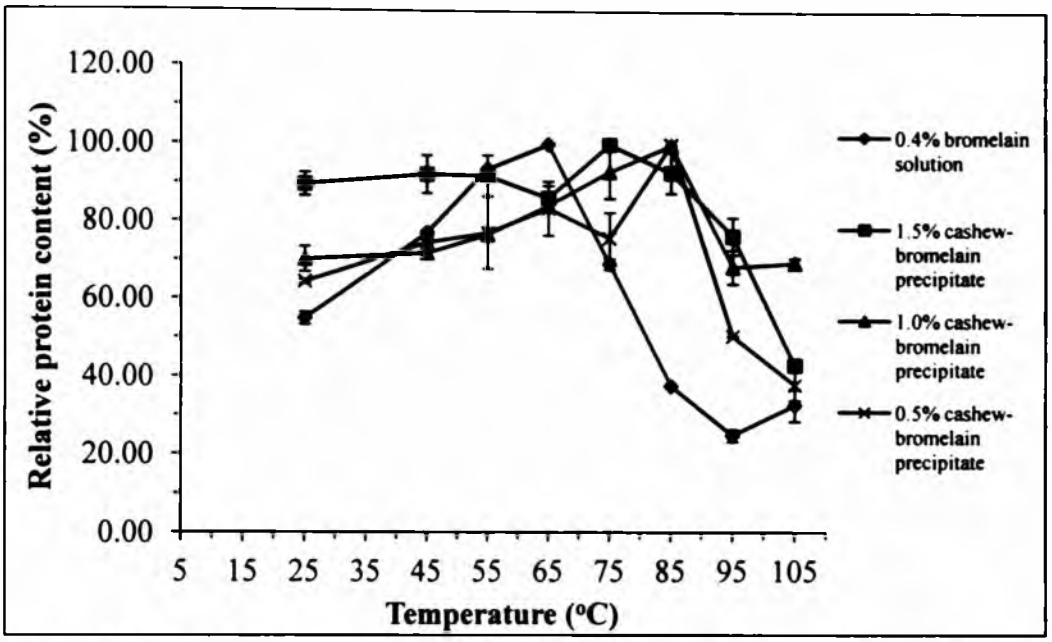


Figure 4.34 Relative protein content of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

Similar pattern of variation was observed with protein content and bromelain activity with incubation temperature for precipitated bromelain. It further suggested that bromelain was the protein precipitated with cashew polyphenols, therefore increasing temperature had the same trend of effect on protein content and bromelain activity. In the case of Josapine juice, difference in variation of these two properties with temperature might be explained in terms of difference in analyzed substrate between enzymatic activity assay and protein assay. For example, only functional active bromelain in Josapine juice exhibits bromelain activity, whereas protein content is contributed by various proteins in addition to bromelain. Bromelain is highly susceptible to heat denaturation and bromelain activity is less thermal stable than did protein content.

4.2.2.3 Antioxidant Activity

Results on effect of temperature on DPPH scavenging activity were presented in Figure 4.35 and Figure 4.36. In Figure 4.35, for Josapine juice extract, the activity

did not vary significantly at temperature from 25 °C to 65 °C. However, a sudden drop of about 25% was measured at 65 °C-75 °C. 1.5% and 1.0% cashew-josapine precipitates had a high and stable activity at temperature from 25 °C to 95 °C and small reduction of 5% and 10% at 105°C respectively. It could be observed that thermal treatment on cashew leaf extract (negative control) caused a slight increase in DPPH scavenging activity at temperature range of 75 °C-95 °C. The overall antioxidant activity of cashew extract was stable after incubation at various elevated temperatures. Antioxidant activity of 0.5% cashew-josapine precipitate decreased slightly with increasing temperature from 25 °C to 105 °C and at 105°C, activity loss was ~24%. In Figure 4.36, each of three cashew-bromelain precipitates exhibited high stability at increasing temperature.

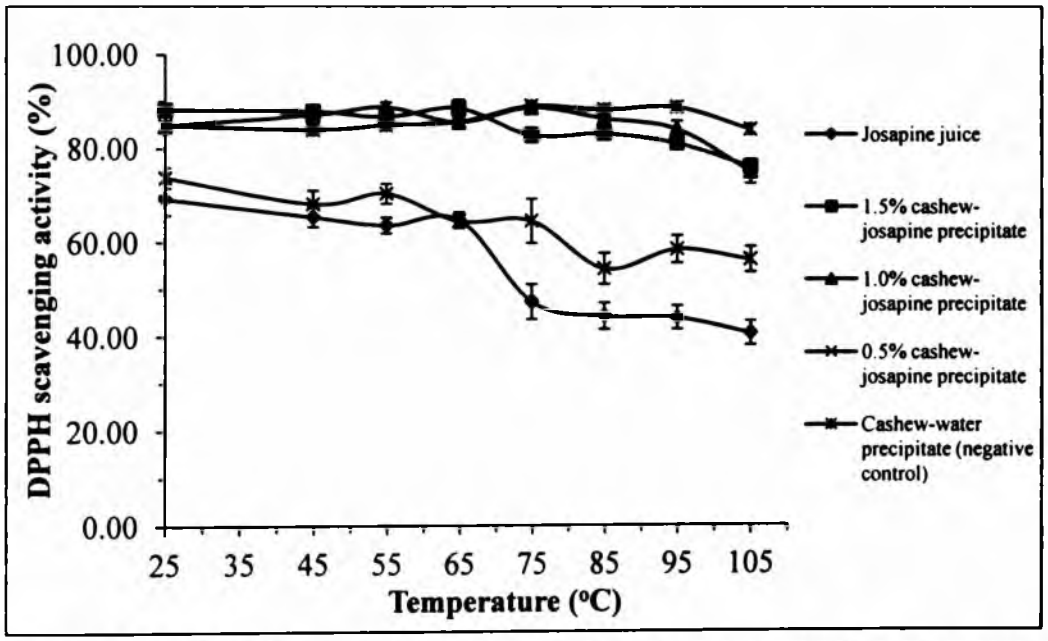


Figure 4.35 DPPH scavenging activity of Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

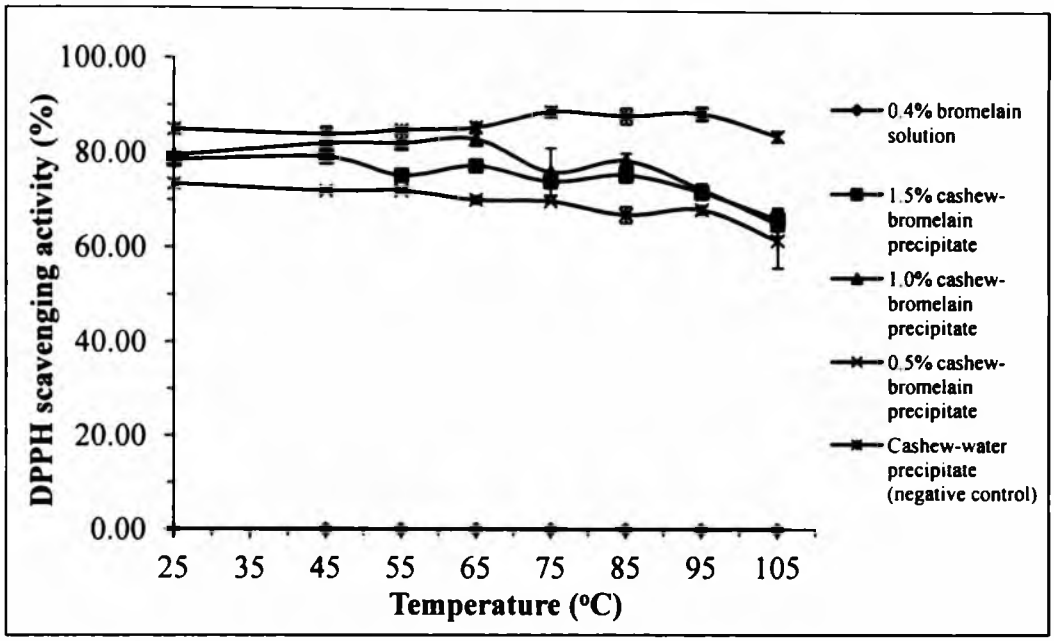


Figure 4.36 DPPH scavenging activity of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

From the results obtained above, complexing of bromelain with cashew polyphenols might not only elevate the antioxidant activity of Josapine juice but also stabilize it through reduction of heat damage effect caused by elevated temperature. 1.5% and 1.0% cashew-josapine precipitated bromelain had high and stable antioxidant capacity comparable with cashew leaf extract. Activation of antioxidant activity of cashew leaf extract by elevated incubation temperature was observed and it could be due to generation of polyphenolic compounds with stronger radical scavenging ability after progressive oxidation of plant extract polyphenols at high temperature (Manzocco *et al.*, 1998). Besides, cashew leaf extract polyphenols was shown to have great heat resistance by preserving its antioxidant activity over wide elevated temperature range.

4.2.2.4 Phenolic Content

As could be seen in Figure 4.37, thermal treatment caused a decrease in phenolic content of cashew-josapine precipitated bromelain. The decrease was not linear and large drop in phenolics was observed at 65 °C-75 °C for 1.5% precipitate, 25 °C-55 °C for 1.0% precipitate and 25 °C-45 °C for 0.5% precipitate. Cashew leaf extract showed a slight decrease but relatively stable at 25 °C-95 °C, at which followed by about 11% reduction at 95 °C-105 °C. In comparison with cashew-josapine precipitated bromelain, positive control precipitate showed a relative greater stability in phenolic content (Figure 4.38).

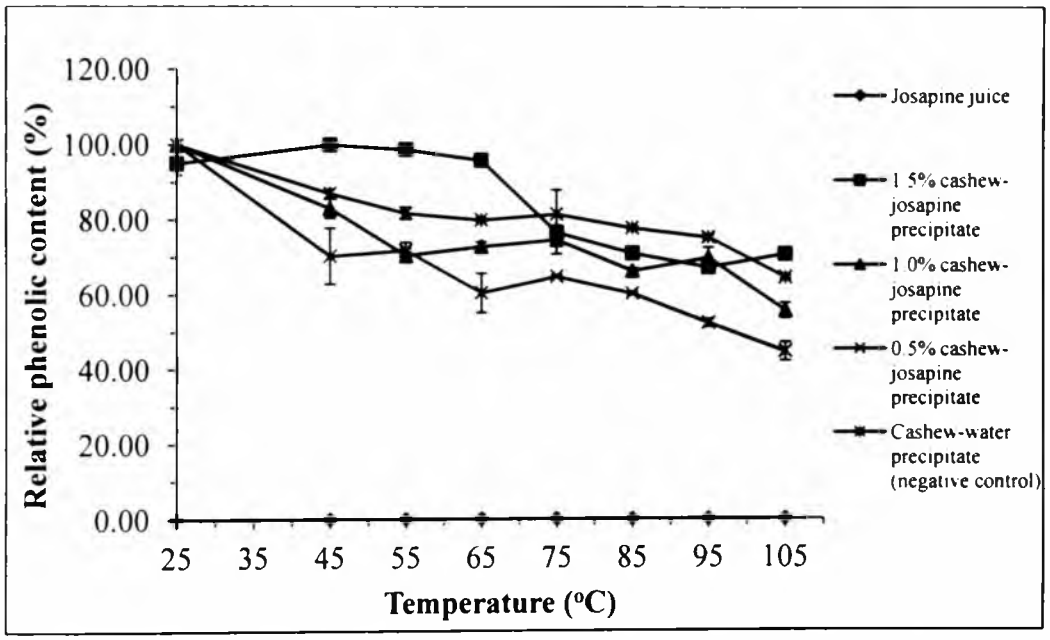


Figure 4.37 Relative phenolic content of Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

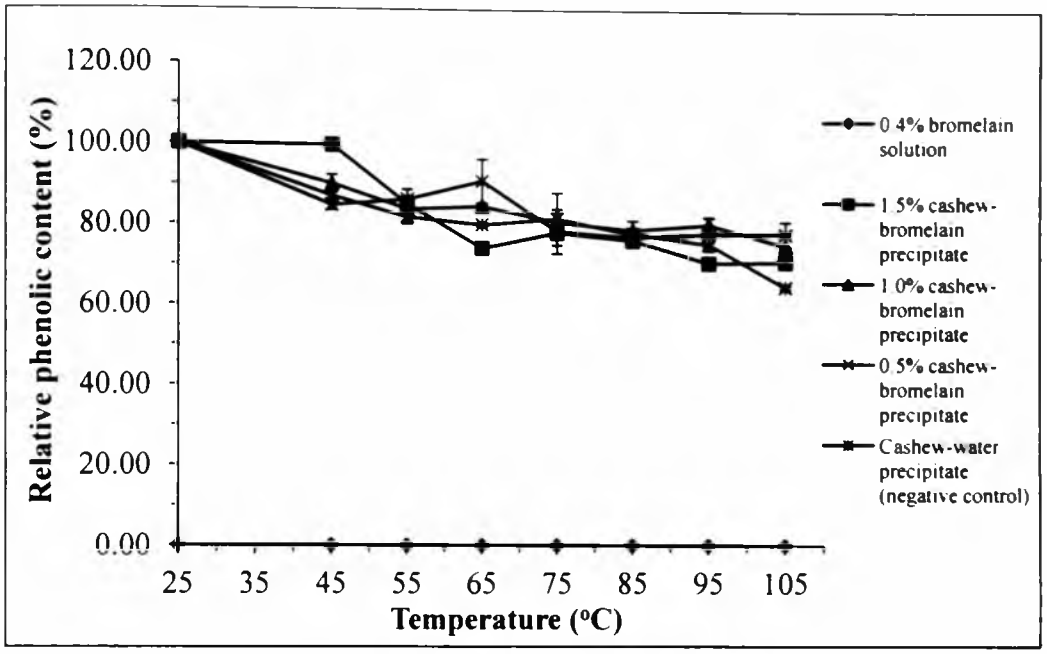
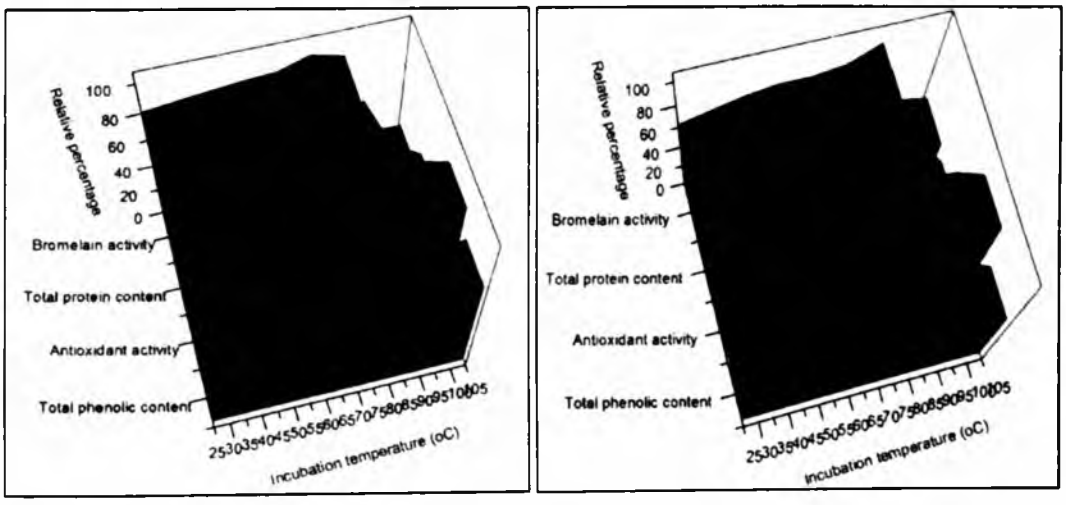
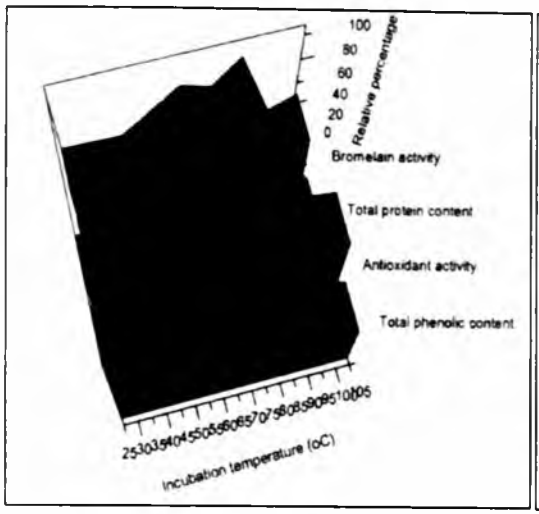


Figure 4.38 Relative phenolic content of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various temperatures. Indicated values are expressed as mean ± SD (n=3).

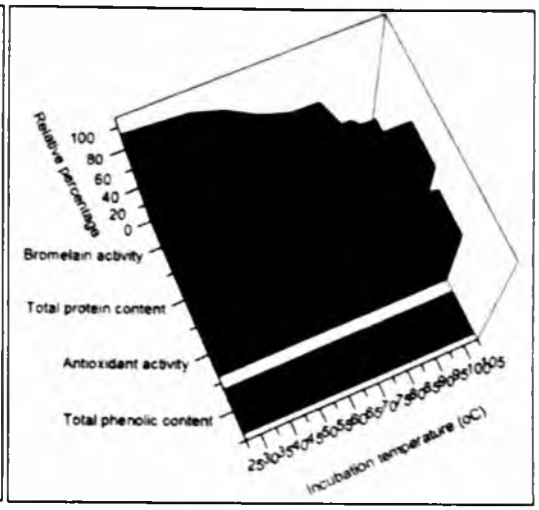
Figure 4.39 presented bromelain activity, protein content, antioxidant activity and phenolic content for each tested sample in variation of incubation temperature.



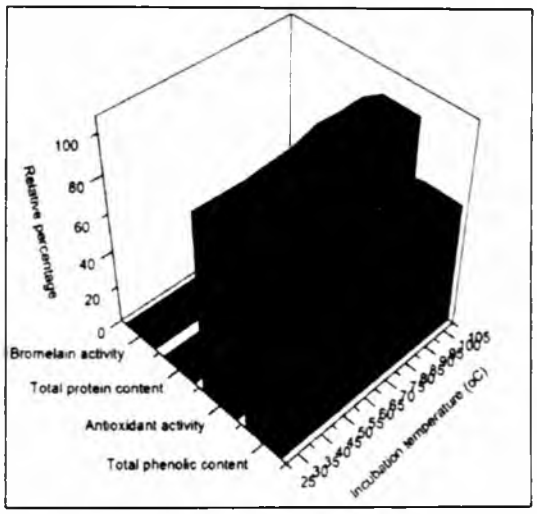
(a) 1.5% cashew-josapine precipitate (b) 1.0% cashew-josapine precipitate



(c) 0.5% cashew-josapine precipitate



(d) Josapine juice extract



(e) Cashew-water precipitate

Figure 4.39 Properties of 1.5%, 1.0%, 0.5% cashew-josapine precipitates, Josapine juice extract and cashew-water precipitate at various temperatures. Indicated values are expressed as mean (n=3).

Correlation between stated pairs of variables of tested sample in response to various incubation temperatures ranged from 25 °C to 105 °C was shown in Appendix B, Table B.2. Moderate correlation between bromelain activity and protein content was observed with cashew-josapine precipitate. Weak correlation was found on Josapine juice extract since the trend of variation of these variables was not consistent at temperature range of 25 °C-85 °C. 1.5% and 0.5% cashew-josapine precipitates had a great association between antioxidant activity and phenolic content. However, negative relationship was observed with cashew leaf extract since phenolic content decreased gradually while antioxidant activity

remained stable at temperature range of 25 °C-105 °C. Scatter matrix for Pearson correlation obtained from this section was presented in Appendix C, Figure C.2.

Overall, based on the results obtained from analysis of effect of various temperatures on bromelain's nutraceutical properties, bromelain-polyphenol precipitate had an increased and stable bromelain activity over a wide temperature range and maximum activity was attained at 75 °C-85 °C. In contrast, started from 25 °C, free bromelain in Josapine juice showed an activity declination. Antioxidant activity of free bromelain was elevated and stabilized against heat denaturation at elevated temperature after precipitation with cashew leaf extract polyphenols.

Proteins/ enzymes are sensitive to elevated temperature. Extreme temperature will cause thermal unfolding of enzymes by disrupting stabilizing interaction or noncovalent linkage, results in protein deformation. The opening up of enzyme structure increases the extent of damage on reactive groups through irreversible degradative reaction. Therefore loss in conformational integrity of enzyme facilitates degradative reactions and results in protein inactivation (Daniel *et al.*, 1996) (Details on effect of temperature on conformational stability of protein are elaborated in section 2.8).

In this study, variation in stability in response to heat noticed for free and precipitated bromelain might probably be due to the difference in enzyme's conformational structure. Daniel *et al.* (1996) observed that thermal degradative process is found to be slower at high temperature for conformational intact proteins. It has been mentioned in the discussion part (section 4.2.1) that interaction of bromelain with cashew polyphenols will generate a complex with high conformational integrity due to the involvement of hydrophobic and hydrogen bonding. Therefore conformational stable bromelain-polyphenol complex is hypothesized to have a great heat resistance against increased temperature. Another explanation is cross-linking of polyphenolic compounds and polypeptide chain will alter the impact of external environment factors on structure and functionality of enzyme to an extent.

High temperature is one of the operation conditions employed for food and beverage processing, pharmaceutic product manufacturing and other industrial application. Complexed bromelain with great thermostability could preserve its bromelain activity, thereby maintaining this health benefit characteristic, while inhibiting the growth and proliferation of microbe during processing and sterilization.

It could be found that cashew leaf extract polyphenols well preserved its DPPH scavenging activity at elevated temperature, indicating its strong resistance against heat damage effect. This thermal stable antioxidant capacity, which is attributed by cashew polyphenols, gives bromelain-polyphenol complex another nutraceutical properties in addition to its potential enzymatic activity.

4.2.3 Estimation of Activation Energy and Thermodynamic Parameters

Protein inactivation is the process whereby its conformational structure alters without the disruption of covalent bonds. It was found that bromelain activity of precipitated bromelain remained stable in the temperature range of 25 °C-85 °C and as the temperature was increased to 85 °C above, the enzymatic activity for either free bromelain and precipitated bromelain decreased sharply. Therefore, in the following section, free bromelain in Josapine juice extract and 1.0% cashew-josapine precipitated bromelain were inactivated at 80 °C, 82 °C, 85 °C, 87 °C and 90 °C to study the inactivation profile (activation energy and thermodynamic parameters) of bromelain in response to both elevated temperature and incubation time. The residual activities of free and precipitated bromelain were presented as ln [relative bromelain activity] versus time in response to incubated temperature (Figure 4.40 and Figure 4.41 respectively) and the denaturation rate constants and half life derived from these plots were presented in Table 4.7.

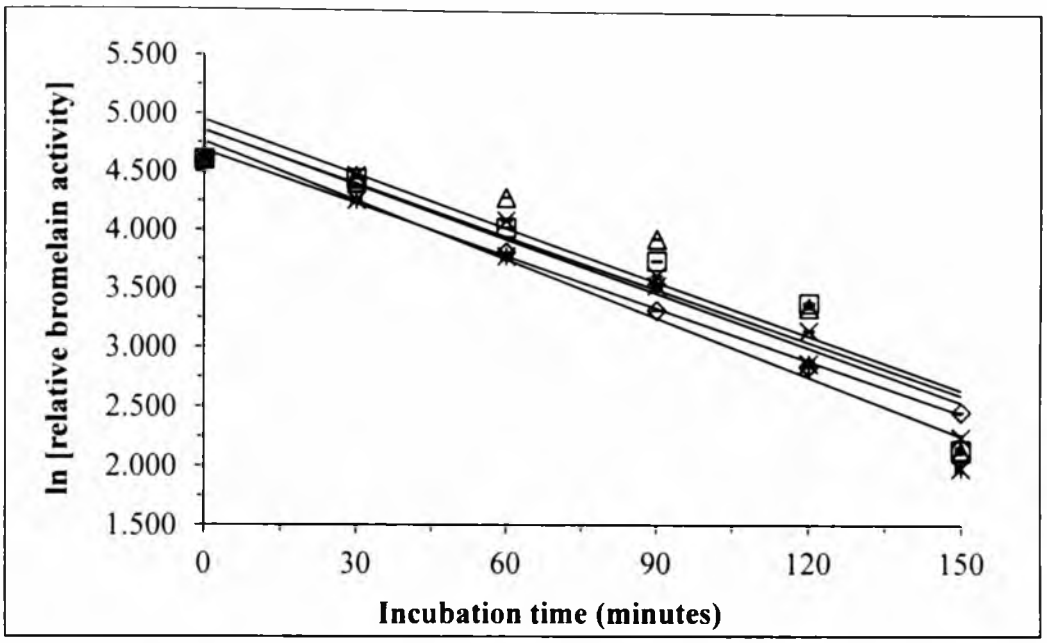


Figure 4.40 ln [relative bromelain activity] of free bromelain in Josapine juice extract at various temperatures (◊ 80 °C, □ 82 °C, Δ 85 °C, × 87 °C and * 90 °C) in variation of incubation time. Indicated values are expressed as mean ± SD (n=3).

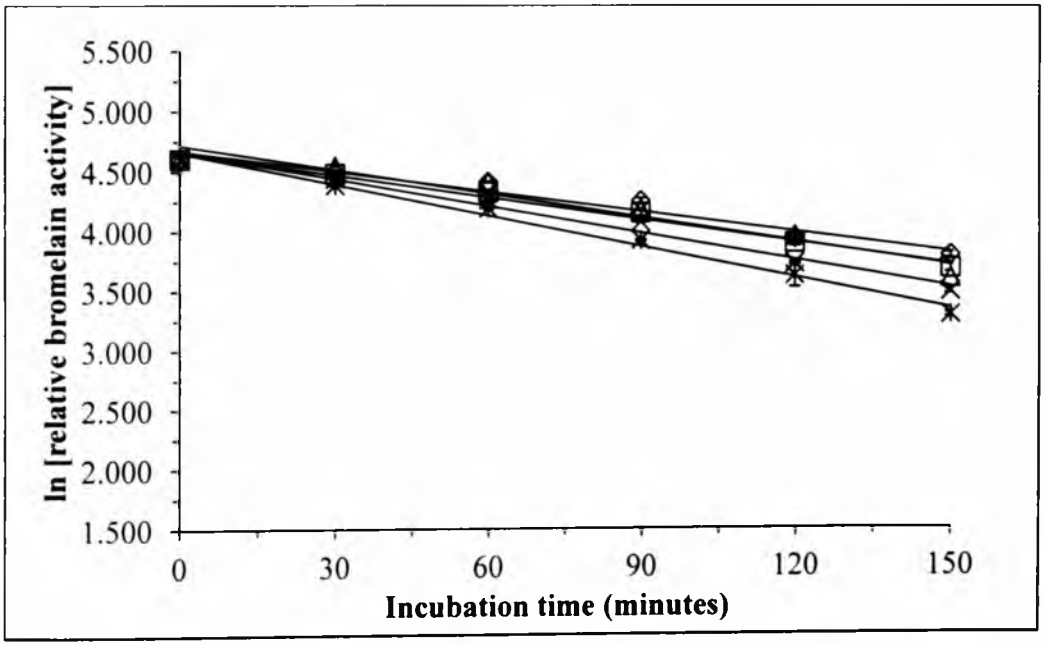


Figure 4.41 ln [relative bromelain activity] of 1.0% cashew-josapine precipitated bromelain at various temperatures (◊ 80 °C, □ 82 °C, Δ 85 °C, × 87 °C and * 90 °C) in variation of incubation time. Indicated values are expressed as mean ± SD (n=3).

Table 4.7 Denaturation rate constant and half life of free bromelain in Jospaine juice extract and 1.0% cashew-josapine precipitated bromelain at various temperatures in variation of incubation time. Indicated values are expressed as mean ± SD (n=3). Means with different letters within same column indicate a significant difference at $p<0.05$.

	Josapine juice extract (free bromelain)		**1.0% cashew-josapine precipitated bromelain**	
Temperature (°C)	**Denaturation rate constant, k (min^{-1})**	**Half life, t ½ (min)**	**Denaturation rate constant, k (min^{-1})**	**Half life, t ½ (min)**
80	0.0149 a	46.52 a	0.0056 ± 0.0002 a	124.58 ± 3.45 a
82	0.0150 ± 0.0001 a	46.21 ± 0.31 a	0.0062 ± 0.0002 b	112.45 ± 2.81 b
85	0.0153 ± 0.0001 b	45.31 ± 0.30 b	0.0066 b	105.02 c
87	0.0154 ± 0.0001 b	45.01 ± 0.29 b	0.0075 ± 0.0001 c	92.43 ± 1.23 d
90	0.0167 ± 0.0001 c	41.59 ± 0.14 c	0.0086 ± 0.0004 d	80.38 ± 3.25 e

For both free and precipitated bromelain (Table 4.7), it was found that the inactivation rate constant increased with incubation temperature, indicating bromelain activity was inhibited by heat denaturation effect at increasing temperature. 1.0% precipitated bromelain had 1.94 to 2.66 times lower inactivation rate constant at temperature range of 80 °C-90 °C than that reported from free bromelain, suggesting the precipitation of bromelain with cashew leaf extract polyphenols produced an enhancement of its thermal stability. Half life of bromelain at each tested temperature was calculated based on the rate constant and half life equation.

Arrhenius plot measures the dependency of inactivation rate constant on temperature, which is a plot of ln (k) versus 1/T for determination of thermal inactivation for free bromelain and precipitated bromelain (Figure 4.42 and Figure

4.43). Activation energy, E could be derived from the Arrhenius plot according to Arrhenius equation (section 3.6.3). Free bromelain in Josapine juice extract and 1.0% precipitated bromelain were found to have activation energy of 10.847 $kJmol^{-1}$ and 45.487 $kJmol^{-1}$ respectively (Table 4.8). Precipitated bromelain with 1.0% cashew polyphenols had about 4.19 times higher activation energy of inactivation, indicating it exhibited more resistance against thermal unfolding and denaturation effect at elevated temperature.

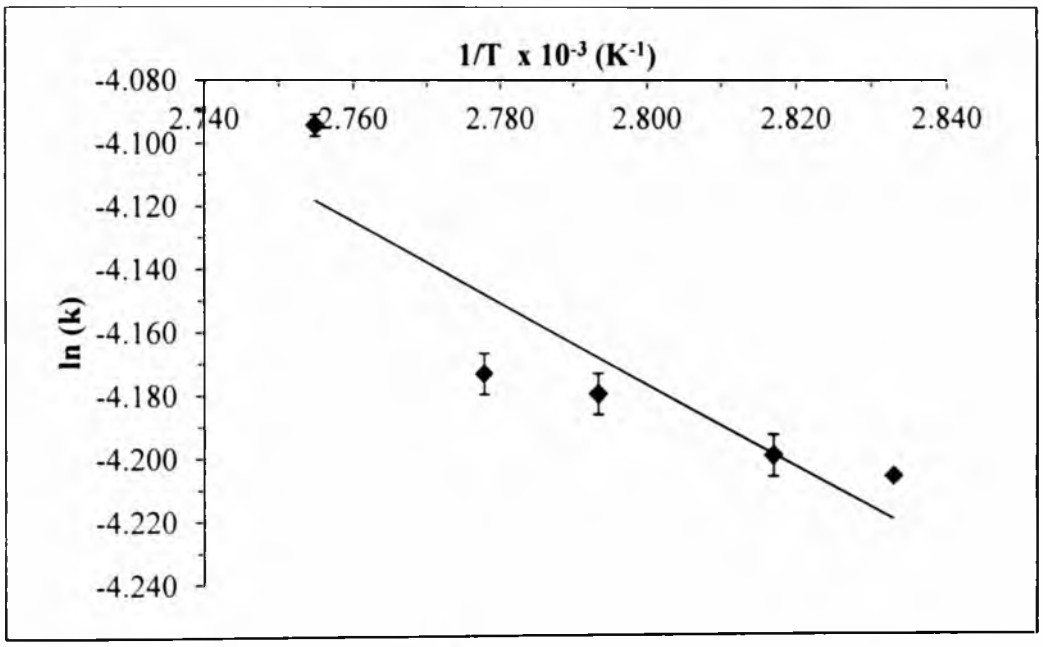


Figure 4.42 Arrhenius plot for free bromelain in Josapine juice extract. Indicated values are expressed as mean ± SD (n=3).

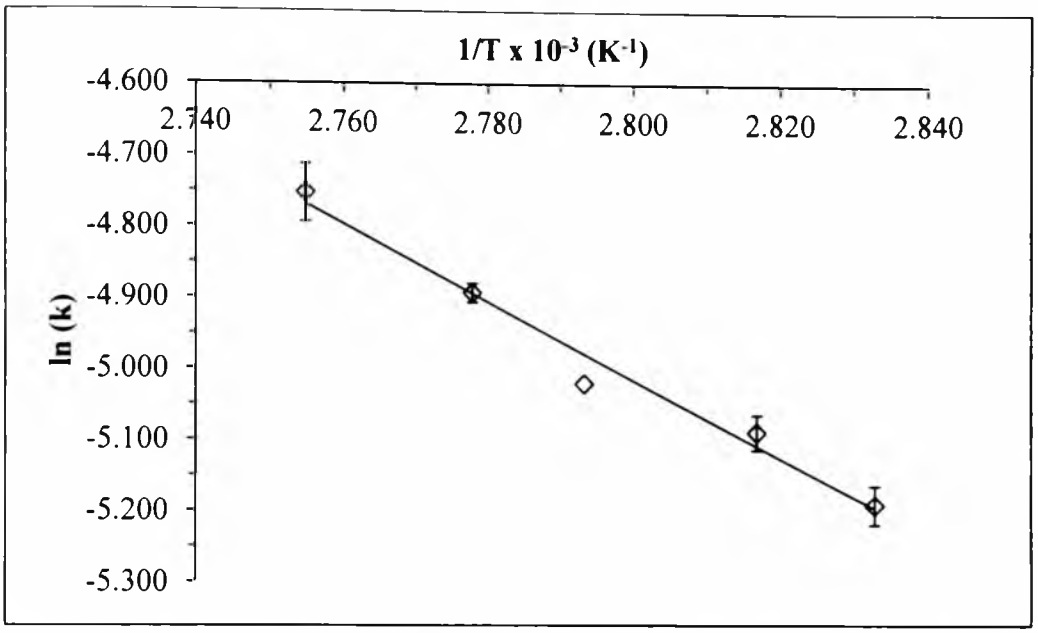


Figure 4.43 Arrhenius plot for 1.0% cashew-josapine precipitated bromelain. Indicated values are expressed as mean ± SD (n=3).

Table 4.8 Activation energy of free bromelain in Josapine juice extract and 1.0% cashew-josapine precipitated bromelain. Indicated values are expressed as mean ± SD (n=3).

Sample	**Josapine juice extract**	**1.0% cashew-josapine precipitated bromelain**
Activation energy, E ($kJmol^{-1}$)	10.847 ± 0.390	45.487 ± 1.144

Thermodynamic parameters ($\Delta H^{\bullet}$, $\Delta G^{\bullet}$, $\Delta S^{\bullet}$) were calculated according to absolute reaction rate equations (section 3.6.3) regarding the thermal inactivation of bromelain (Table 4.9).

Table 4.9 Thermodynamic parameters of free bromelain in Josapine juice extract and 1.0% cashew-josapine precipitated bromelain. Indicated values are expressed as mean ± SD (n=3). Means with different letters within same column indicate a significant difference at $p<0.05$.

	kJmol^{-1}		
	Josapine juice extract (free bromelain)		
Temperature (K)	**ΔH^***	**ΔG^***	**ΔS^***
353	7.912±0.390 a	79.026 a	-201.458±1.105 a
355	7.895±0.390 a	79.471±0.020 b	-201.622±1.048 a
358	7.870±0.390 a	80.109±0.019 c	-201.784±1.140 a
360	7.854±0.390 a	80.554±0.019 d	-201.944±1.034 a
363	7.829±0.390 a	81.011±0.010 e	-201.605±1.104 a
	1.0% cashew-josapine precipitated bromelain		
Temperature (K)	**ΔH^***	**ΔG^***	**ΔS^***
353	42.552±1.144 a	81.970±0.081 a	-111.515±3.075 a
355	42.536±1.144 a	82.095±0.073 a	-111.436±3.073a
358	42.511±1.144 a	82.611 b	-112.013±3.196 a
360	42.494±1.144 a	82.707±0.040 b	-111.703±3.113 a
363	42.469±1.144 a	82.998±0.122 c	-111.650±2.984 a

Activation enthalpy (ΔH^*) is energy associates with disruption of noncovalent bonds during thermal denaturation of enzymes (Daniel, 1996). As shown in Table 4.9, at 80°C, 1.0% precipitated bromelain had 5.38 times higher values of ΔH^* as compared to Josapine free bromelain, corroborating free bromelain might be more susceptible to alteration and disruption of stabilizing bond including hydrophobic bonding which is essential in maintaining the folded stated of enzyme. ΔH^* for both free and precipitated bromelain decreased slightly with increasing temperature, signifying alteration of conformation of enzyme is in response to temperature (Bhatti *et al.*, 2006). A slight increase in ΔG^* (free energy of inactivation) was observed after precipitation of bromelain with 1.0% cashew extract. 1.0% cashew-josapine precipitated bromelain showed a higher value of some thermodynamic parameters including E, ΔH^* and ΔG^* than those obtained for free bromelain, indicating complexing of bromelain with cashew polyphenols increases its thermal stability.

This result was in accordance with the study on chemical modification of turnip peroxidase (Quintanilla-Guerrero *et al.*, 2008). Complexed bromelain became more thermostable and the reason might be its enhanced conformational stability in the result of addition of noncovalent bonds for example hydrophobic, hydrogen bonding during interaction of proteins and polyphenols.

Molecular disorderness or activation entropy ($\Delta S^{\bullet}$) would increase during protein unfolding at high temperature. Negative value of $\Delta S^{\bullet}$ observed with both Josapine free bromelain and 0.1% precipitated bromelain (Table 4.9) indicated that the disorderness might be negligible at such high temperature. Invertase from *Fusarium solani* also showed negative $\Delta S^{\bullet}$ between 50 °C and 70 °C (Bhatti *et al.*, 2006). Xue *et al.* (2010) reported that thermostable anhydride modified bromelain exhibited lower entropy of activation as compared to free bromelain.

1.0% cashew-josapine precipitated bromelain was shown to be more thermostable by displaying higher values of activation energy (E), activation enthalpy ($\Delta H^{\bullet}$) and free energy of inactivation ($\Delta G^{\bullet}$) as compared to free bromelain in Josapine juice extract.

4.3 Effect of pH on Enzymatic and Antioxidant Activities of Free Bromelain and Bromelain-polyphenol Precipitate

pH condition of solution is an essential factor in determination of protein stability. Proteins/ enzymes become unstable at extremes of pH due to the alternation of overall charge by acidity and basicity, results in increased charge repulsion, protein denaturation and decreased stability (Matthews, 1993). Stem bromelain is accepted as a therapeutic protein and when administered orally, it is exposed to acidic or alkaline condition at where digestion and absorption occur. Besides, bromelain is widely applied in food and beverage, pharmaceutical processing industries. Therefore, study on pH stability is very important to access its enzymatic, structural or nutraceutical properties under acidic/ alkaline conditions.

In following sections, bromelain activity, protein content, antioxidant activity and phenolic content of free bromelain and bromelain-polyphenol precipitate were compared over a wide range of pH (pH 3-10) to study pH stability profile of bromelain on these properties. Bromelain in complexing with cashew polyphenols is hypothesized to have improved stability under extreme conditions in terms of pH.

4.3.1 Bromelain Activity

Figure 4.44 showed the variation of relative bromelain activity of free bromelain in Josapine juice extract and cashew-josapine precipitated bromelain at different pH values. Free bromelain showed a slight increase in activity as pH increased up to pH 7. However, further increase in pH beyond pH 7 caused an enzyme inactivation with ~32% activity loss at pH 10. In contrast, precipitated bromelain exhibited increased and stable activity at alkaline pH range with pH optimum from 8 to 10. As could be seen in Figure 4.45, relative activity of free bromelain determined at pH 3-7 was higher as compared to all three precipitated bromelain whereas precipitated bromelain had a tendency for increased pH stability at alkaline pH values. 1.5% and 1.0% cashew-josapine precipitates showed a similar slope of increasing line between pH 3 and pH 8. While 0.5% precipitate had a greater gradient, indicating increasing pH over this range had a larger influence on its bromelain activity. The trend of activity variation with pH for free bromelain in Jospaine juice and cashew-josapine precipitated bromelain was coincided with the results for positive control sample (Figure 4.46 and Figure 4.47).

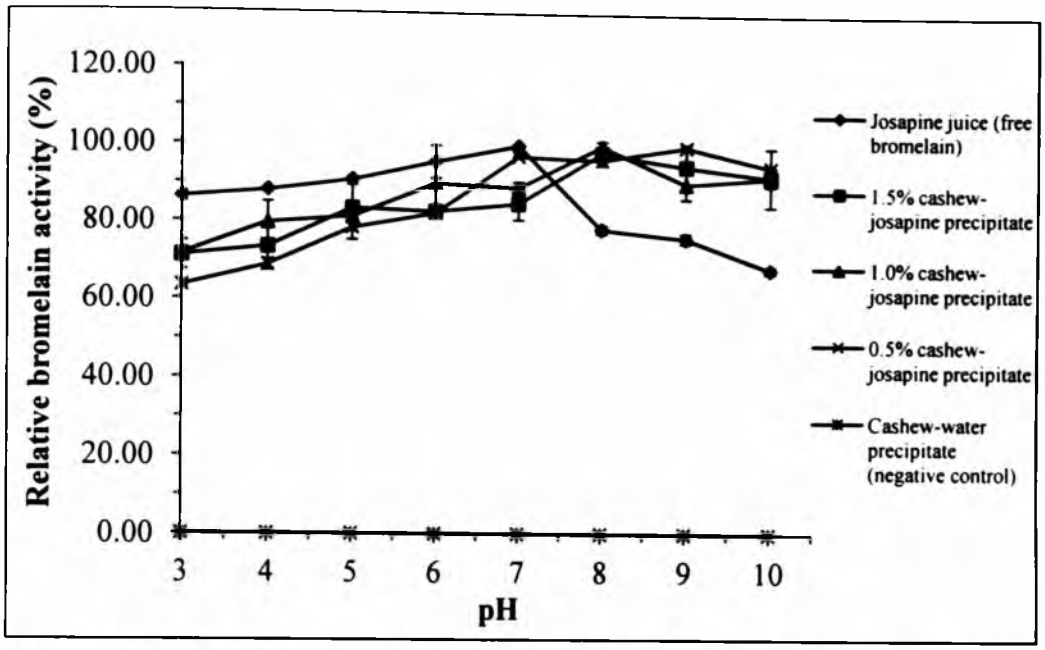


Figure 4.44 Relative bromelain activity of free bromelain in Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

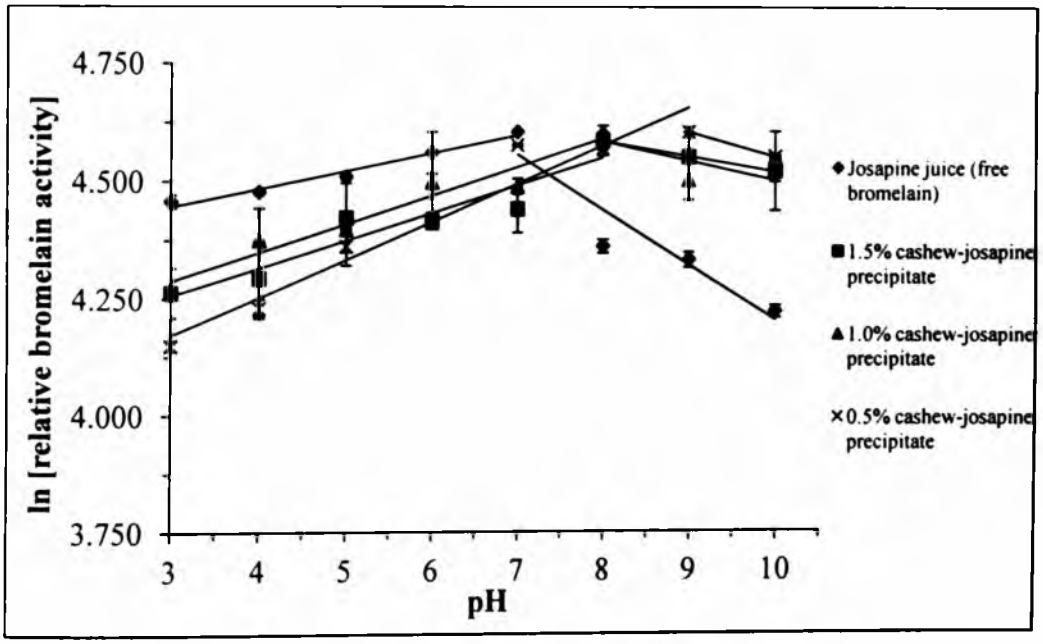


Figure 4.45 ln [relative bromelain activity] of free bromelain in Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

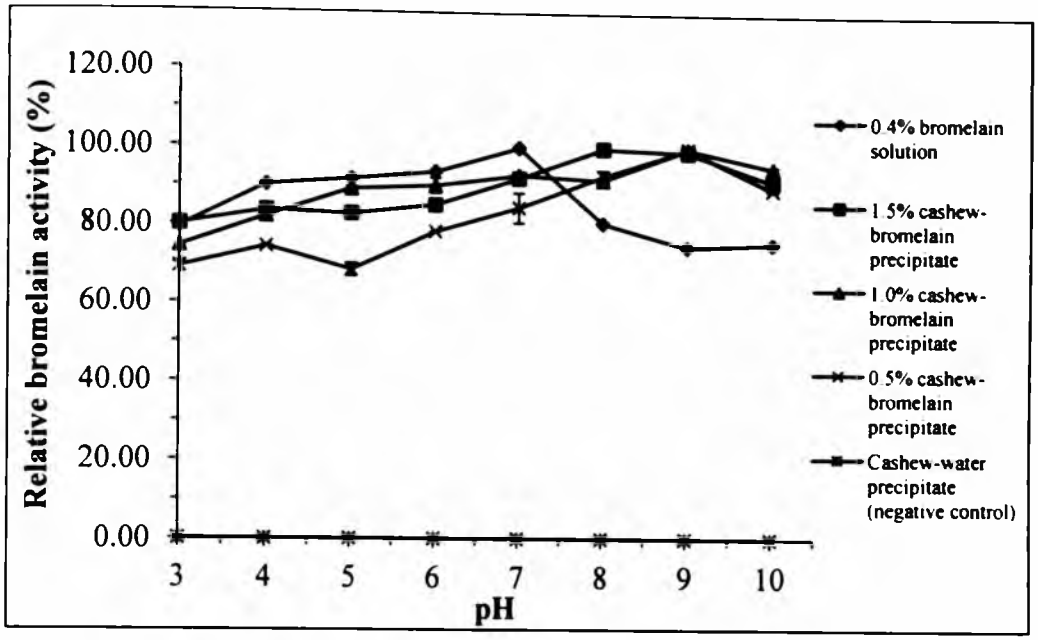


Figure 4.46 Relative bromelain activity of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

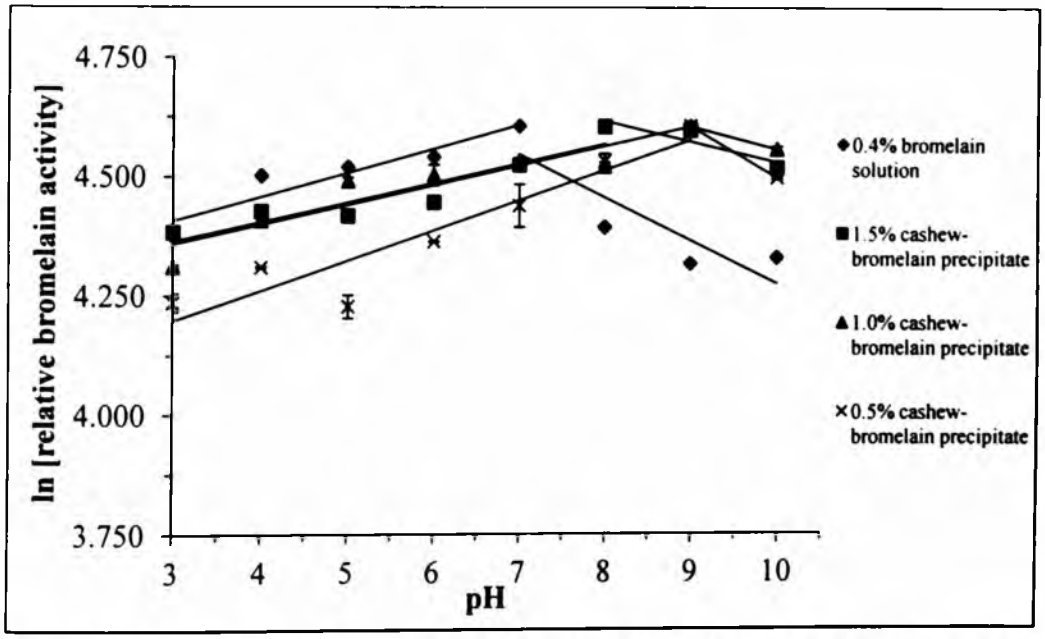


Figure 4.47 ln [relative bromelain activity] of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

Based on the result obtained, alkaline pH caused a reduction in bromelain activity of free bromelain. It has been reported that alkaline pH accelerates

deactivation process of enzyme and it may be due to disulphide exchange at cysteine residues (Naidu and Panda, 2003). Cysteine is reactive group of bromelain which is important in catalysis and enzyme's structural maintenance. Destruction on this catalytic group would result in activity loss. Cross linking of polyphenol with bromelain would alter the influence of alkaline environment condition on catalytic groups of bromelain to an extent.

It was shown that optimum pH of free bromelain in bromelain activity was shifted about 1 to 2 points towards alkaline pH after precipitation with cashew leaf extract polyphenols. Free bromelain was able to adapt to acidic condition whereas precipitated bromelain displayed high stability at alkaline region. Free bromelain and precipitated bromelain will have a performance in preserving their enzymatic activity better than each other in their respective favorable pH regions. It could not be said that bromelain in complexing with polyphenols has an improved pH stability, however, it provides an alternative environmental application in which a alkaline stable enzyme is required.

4.3.2 Protein Content

Similar plots as bromelain activity were shown for protein content of free bromelain solution and precipitated bromelain in variation of pH (Figure 4.48 and Figure 4.49). In Figure 4.48, optimum pH of maximum protein content was found to be pH 7 for Jospaine juice extract, pH 9 for 1.5% cashew-josapine precipitate and pH 8 for 1.0% and 0.5% cashew-josapine precipitates. Variation of capacity corresponded to pH with both measures of bromelain activity and protein content showed some differences. It did not appear linear increase in protein content for all three precipitated bromelain when pH increased from pH 3 to pH 8, with a large increase within pH 7-8 for 1.5% precipitate and pH 6-8 for 1.0% precipitate. Besides, the protein content of precipitates decreased as incubation pH increased beyond optimum, while all precipitated bromelain displayed high bromelain activity over wide alkaline pH region (pH 8-10).

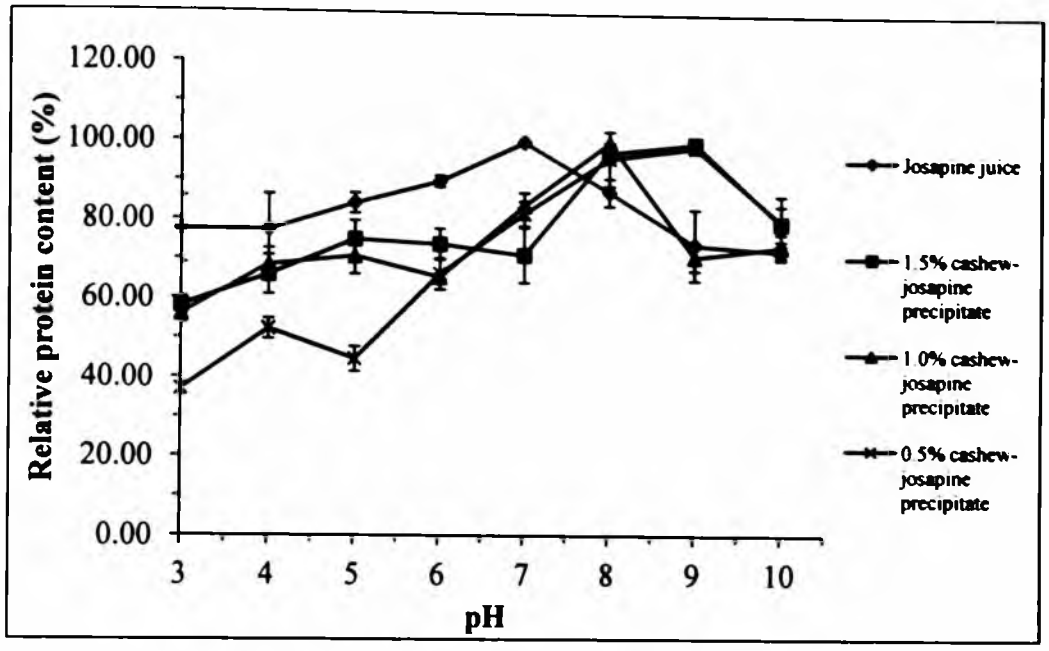


Figure 4.48 Relative protein content of Josapine juice extract and cashew-josapine precipitated bromelain at various pH. Indicated values are expressed as mean ± SD (n=3).

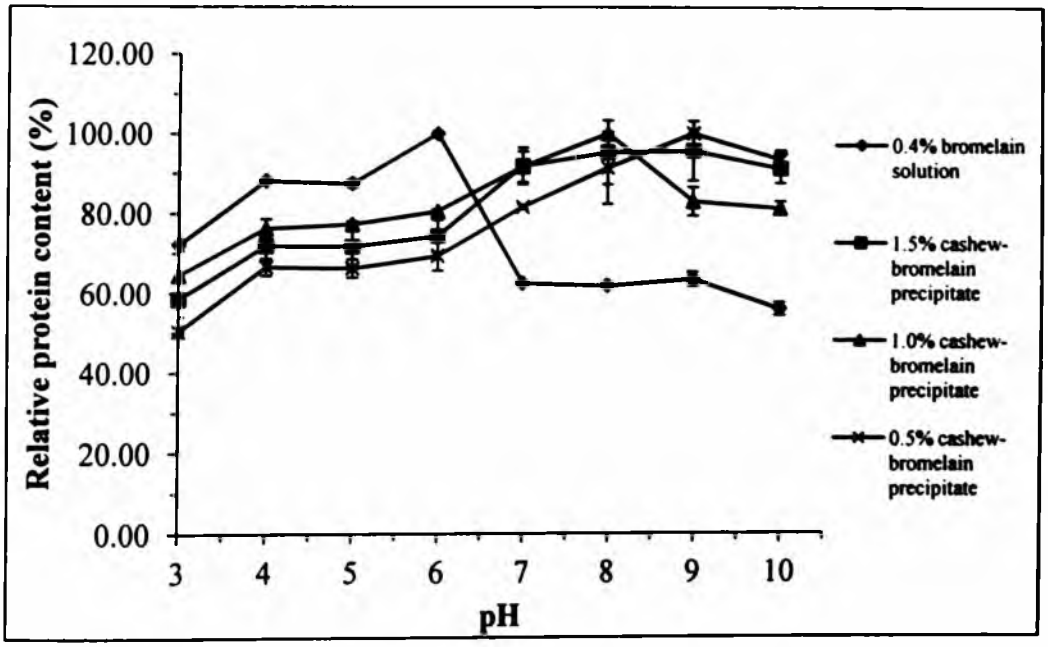


Figure 4.49 Relative protein content of 0.4% bromelain solution and cashew-bromelain precipitated bromelain (positive control) at various pH. Indicated values are expressed as mean ± SD (n=3).

In the case of positive control sample (Figure 4.49), the optimum pH of protein content for 0.4% bromelain solution was attained at pH 6. 1.5% and 0.5%

cashew-bromelain precipitate exhibited high stability in protein content over alkaline range of pH 8-10. 1.0% positive control precipitate had a similar trend as its corresponding sample precipitate.

4.3.3 Antioxidant Activity

Josapine juice extract was shown to have 70.04% DPPH scavenging activity in section 4.1.2, however pH buffer solution strongly inhibited its antioxidant activity by at least 30% (Figure 4.50). Degree of inhibition for juice extract exhibited a bell-shaped trend with increasing pH values from 3 to 7. The scavenging capacity of juice determined at optimum pH (pH 4) was only about 40% and no activity was detected at pH 7-10. In contrast, it was found that cashew-josapine precipitated bromelain retained high and stable antioxidant activity within pH 3-6, showing that precipitated bromelain was a more effective inhibitor of DPPH scavenging than Josapine juice in response to pH variation. However, the activities of all three precipitated bromelain were reduced dramatically at pH above 6 and as pH increased to 8, a negligible activity was observed (Figure 4.50). Increase in pH tolerance was observed with cashew leaf extract, which showed an increased and higher scavenging activity than precipitated bromelain up to pH 7. Also, its activity was sharply reduced as pH increased beyond optimum. Similar effect of pH on antioxidant activity was shown for positive control precipitate (Figure 4.51).

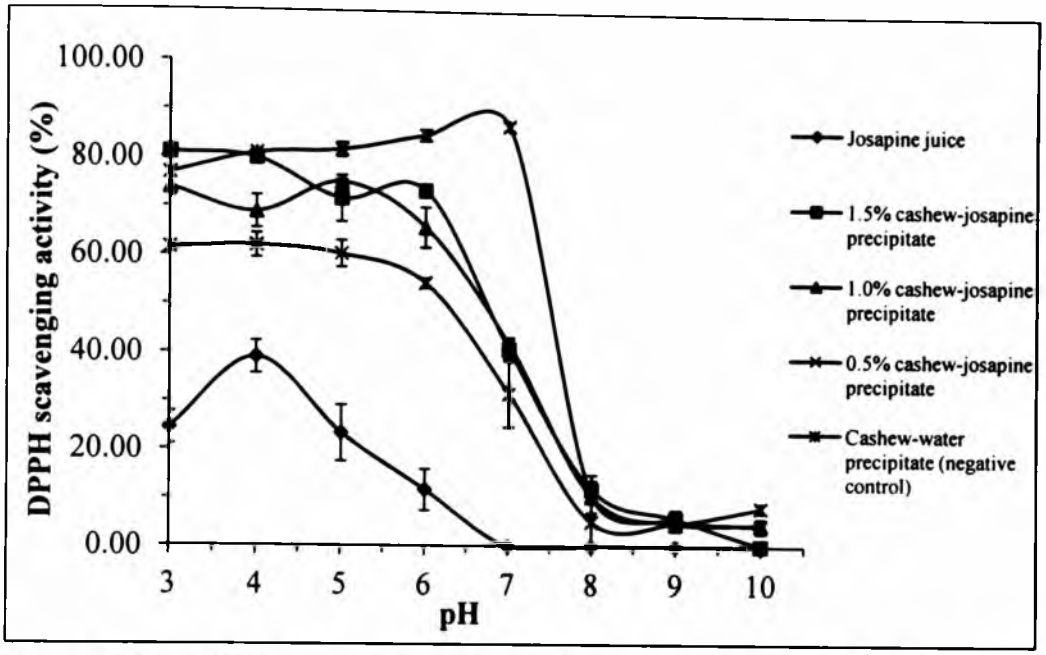


Figure 4.50 DPPH scavenging activity of Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

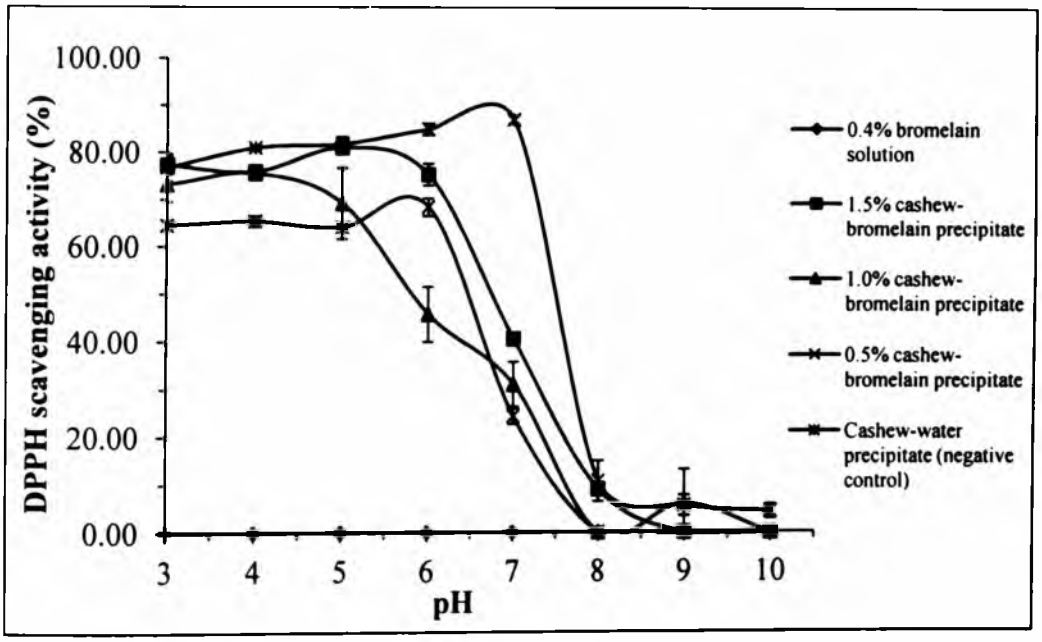


Figure 4.51 DPPH scavenging activity of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

Results indicated that complexing of bromelain with cashew leaf extract enhances and stabilizes DPPH scavenging activity at acidic and neutral conditions.

The enhanced activity might be attributed by cashew extract polyphenols. Neither Josapine juice extract nor precipitated bromelain exhibited antioxidant activity at alkaline region. Cashew leaf extract showed an activity increase with pH in acidic region. Variation/ magnitude of scavenging activity noticed for precipitated bromelain and cashew leaf extract may be due to the degree to which cashew polyphenolic groups are ionized (Riedl and Hagerman, 2001). Precipitated bromelain was found to have lower activity than cashew leaf extract in pH range of 3-7. It was because interaction with proteins will reduce the scavenging activity of polyphenols against free radical (Riedl and Hagerman, 2001).

4.3.4 Phenolic Content

Studies on effect of pH on phenolic content revealed a similar pattern as DPPH scavenging activity plot reported above, corroborating a correlation existed between antioxidant activity and phenolic content among precipitated bromelain. In Figure 4.52, in pH range of 3-6, cashew leaf extract and 0.5% cashew-josapine precipitate had similar, high and stable phenolic content, whereas 1.5% and 1.0% precipitates showed moderate pH stability with some reduction in phenolic content observed at pH 3-4. Either precipitated bromelain or cashew leaf extract had a dramatic phenolic content decrease when the samples were exposed to pH above 6. At pH 10, 0.5% cashew-josapine precipitate retained 26.18% of total phenolics, which was ~10.8% and 6.6% more than that of 1.0% precipitate and cashew leaf extract respectively. No phenolic content was detected at pH 10 for 1.5% precipitate. In Figure 4.53, phenolic content of all three positive control precipitates were stable up to only pH 5 and further increase in pH caused a sharp reduction with 100% content loss at pH 8.

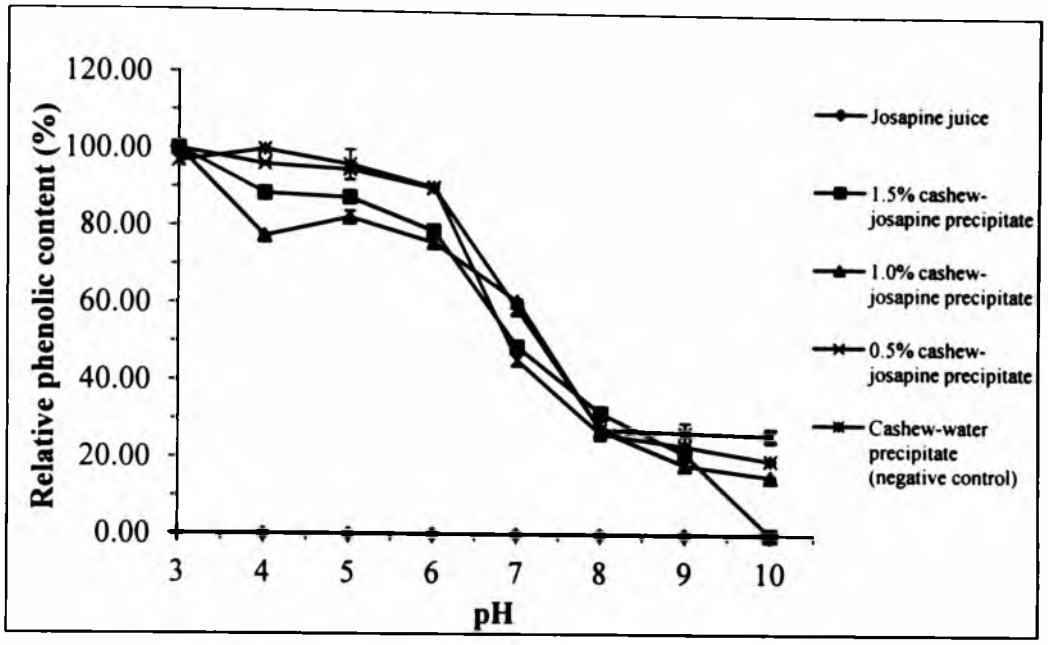


Figure 4.52 Relative phenolic content of Josapine juice extract, cashew-josapine precipitated bromelain and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

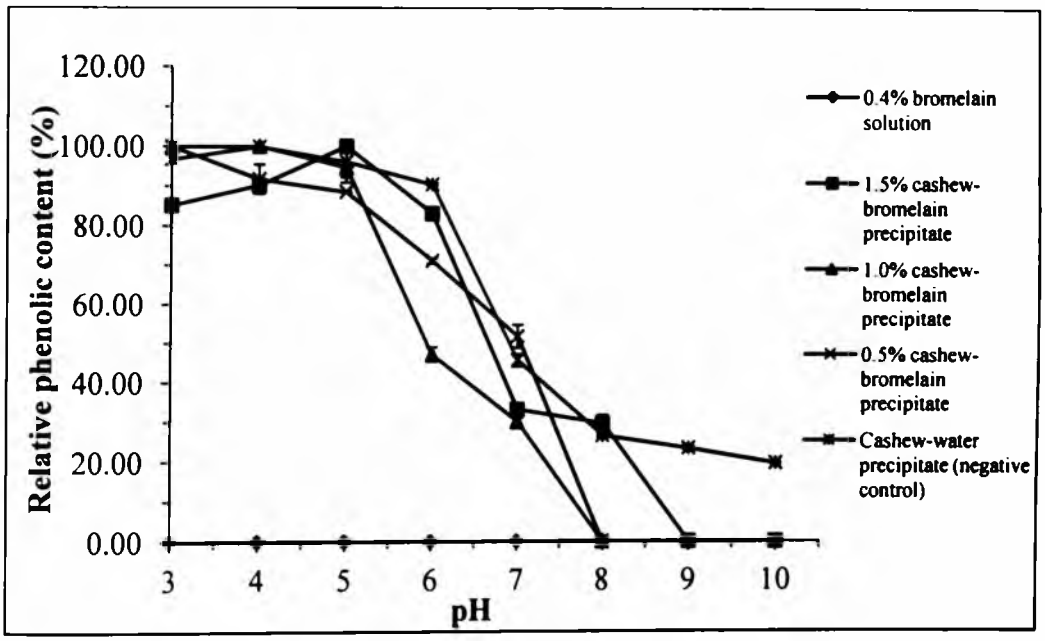
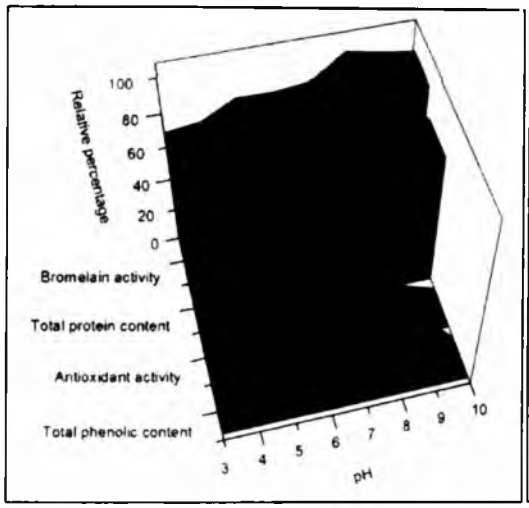


Figure 4.53 Relative phenolic content of 0.4% bromelain solution, cashew-bromelain precipitated bromelain (positive control) and cashew-water precipitate (negative control) at various pH. Indicated values are expressed as mean ± SD (n=3).

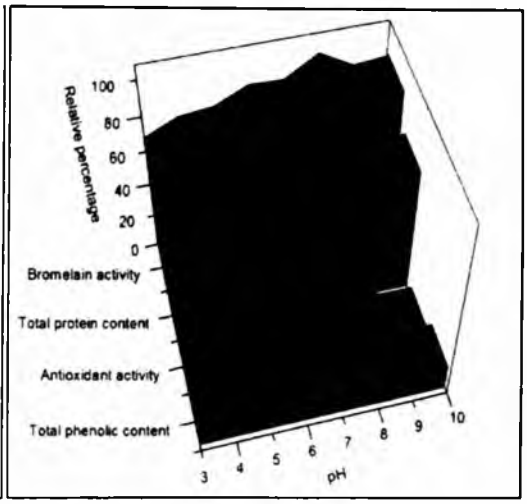
Similar effect of pH on DPPH scavenging activity and phenolic content further supported that cashew leaf extract polyphenols might be the major

contributors of antioxidant capacity of precipitated bromelain and the pH stability of phenolic content of samples determined their variation of antioxidant activity.

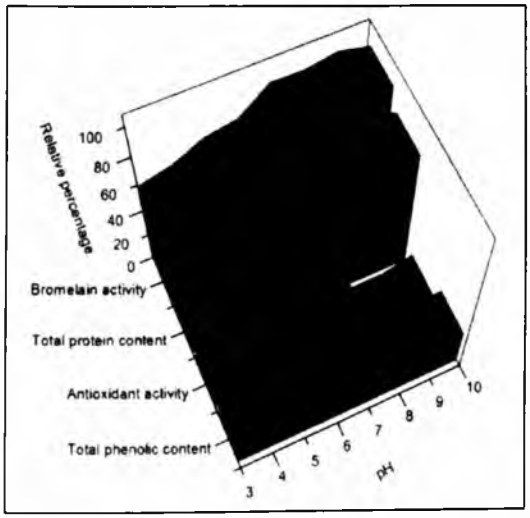
Figure 4.54 combined the results of four properties in variation with pH for free bromelain solution, precipitated bromelain and negative control. The relation among the properties for each sample could be clearly observed from this figure.



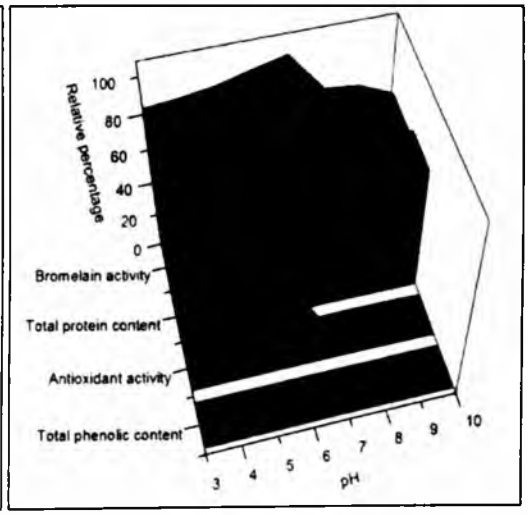
(a) 1.5% cashew-josapine precipitate



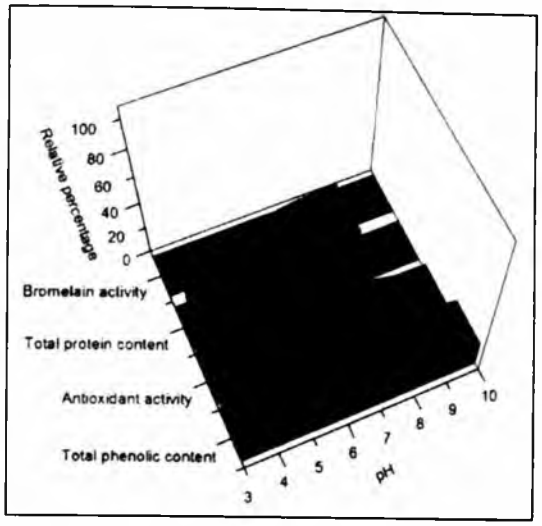
(b) 1.0% cashew-josapine precipitate



(c) 0.5% cashew-josapine precipitate



(d) Josapine juice extract



(e) Cashew-water precipitate (negative control)

Figure 4.54 Properties of 1.5%, 1.0%, 0.5% cashew-josapine precipitates, Josapine juice extract and cashew-water precipitate at various pH. Indicated values are expressed as mean (n=3).

Pearson correlation between variables in response to various pH (pH 3-10) was stated in Appendix B, Table B.3. Great positive correlation between pairs of stated variables was observed with cashew-josapine precipitate, especially the association between antioxidant activity and phenolic content. Moderate relationship between bromelain activity and protein content was observed with Josapine juice extract. Scatter matrix for Pearson correlation on the effect of pH was shown in Appendix C, Figure C.3.

In addition to temperature, pH also influences the conformation of protein by disrupting electrostatic interactions between amino acids that favoring the folded state, increasing intramolecular charge repulsion and leads to protein deformation. It has been reported that extreme pH increases Coulombic forces of repulsion that overcome the stability forces such as hydrophobic forces, salt bridges, drives the formation of unfolded protein (Fink *et al.*, 1994; Ahmad *et al.*, 2004). Besides, change in pH alters the ionization of essential reactive amino acids and ionizable nonessential residues located at active site, directly and indirectly affect catalytic activity of enzyme (Details on effect of pH on conformational stability of protein are elaborated in section 2.8).

As stated above, high and low pH facilitate thermal unfolding and protein degradation. Conformational intact protein will have a great stability against pH denaturation. Therefore, the objective of complexing of bromelain with cashew leaf extract polyphenols is to improve pH stability by displaying high enzymatic activity. Results obtained from this section indicated that precipitated bromelain had increased bromelain activity only at alkaline region whereas free bromelain was stable in activity at acidic and neutral conditions. Precipitated bromelain did not exhibit high enzymatic activity over wide pH range but its activity was not severely inhibited by acidic/ neutral pH. Its pH stability in alkaline condition makes it good alternative for environmental and biotechnological applications. Another unique property found in this section is precipitated bromelain had a much stronger, stable antioxidant activity in acidic region as compared to Josapine juice extract. These two features suggested that bromelain-polyphenol precipitate may have a better performance than did free bromelain, thereby tolerating high/ low pH conditions while displaying stable enzymatic activity and antioxidant capacity.

Both bromelain and cashew leaf extract polyphenols in bromelain-polyphenol complex were proven to have therapeutic properties. Based on some *in vitro* studies, stem bromelain could be absorbed across intestinal epithelium and exerts pharmacological effect in blood and immune system (Details on pharmacological effect of bromelain are elaborated in section 2.2). Tannins within polyphenol-protein complex retains high radical scavenging activity in gut lumen to reduce oxidative stress (Riedl and Hagerman, 2001). In this study, precipitated bromelain exhibited somewhat pH stability in terms of enzymatic and antioxidant activity. Because of its therapeutic utility and pH stability, it is supposed that, when taken orally, bromelain-polyphenol complex could encounter the condition in digestive system to which it is exposed and at the same time, exhibit its potential therapeutic properties *in vivo*.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In the current study, polyphenols of ethanolic cashew leaf extract was mixed with pineapple juice which is containing free bromelain and the precipitate of bromelain-polyphenol was obtained. We focus on determination of nutraceutical properties for example bromelain activity and antioxidant activity of bromelain-cashew leaf polyphenol precipitate and the variation of these properties with external conditions to obtain its thermal and pH stability profile.

Ethanolic cashew leaf, mangosteen fruit rind and cengal wood chipping extracts were found to possess phenolic content and antioxidant capacity. Among these plant extracts, cashew leaf extract displayed the highest DPPH scavenging activity (EC=0.030 mg/ml) and phenolic content (628.70 mg GAE/g) and therefore it was selected as a source of polyphenols to separate and precipitate bromelain in clarified Josapine juice. Josapine juice extract was found to have bromelain activity of 621.92 U/ml, protein content of 14.41 mg/ml and DPPH scavenging activity of 70.04%.

After mixing cashew leaf extract with Josapine juice extract, the supernatant was separated from precipitate formed in each sample. The % recovery of bromelain activity from Josapine juice on precipitate was hyperbolic increased with concentration of cashew extract and optimum recovery (79.35%) was attained at 1.0% cashew leaf extract in juice extract. Bromelain might be successfully

recovered from Josapine juice extract on precipitate by forming bromelain-polyphenol precipitate after adding cashew leaf extract.

Bromelain-polyphenol precipitate was then analyzed with bromelain activity, protein content, antioxidant activity and phenolic content. 1.5%, 1.0% and 0.5% cashew-josapine precipitated bromelain were found to retain 63.14%, 63.39% and 39.32% of total bromelain of Josapine juice extract respectively, indicating bromelain activity was somewhat retained and stabilized in bromelain-polyphenol precipitate. Precipitated bromelain containing higher content of cashew leaf extract was shown to retain greater bromelain activity. In addition, decrease in bromelain activity measured in supernatant was proved to be correlated with increase in precipitate bromelain activity in each sample. In BCA protein assay, protein content measured in precipitated bromelain had a same behavior as precipitate bromelain activity in response to concentration of cashew extract and it further supported that bromelain might be the protein precipitated with cashew polyphenols. For antioxidant activity, precipitated bromelain had an increased activity as compared to Josapine juice extract (~70%), especially with 1.5% and 1.0% precipitates (~85%) and this increase might be attributed by antioxidant capacity of cashew leaf extract. Interaction of polyphenols and protein affects the antioxidant capacity of polyphenols since precipitated bromelain had DPPH scavenging activity lower than did cashew leaf extract itself. Phenolic content of precipitated bromelain increased with concentration of cashew leaf extract within the precipitate and it suggested phenolic compounds might be the major contributors to its antioxidant activity.

At 60 °C, precipitated bromelain with 1.5% and 1.0% cashew extracts were found to have higher thermal stability in bromelain activity by displaying lower denaturation rate constants (0.0014 min^{-1} and 0.0028 min^{-1} respectively), which were about 7.0 and 3.5 times lower than that of free bromelain in Josapine juice extract and 0.5% precipitated bromelain (0.0097 min^{-1} and 0.0098 min^{-1} respectively). Precipitated bromelain especially those contained high concentrations of cashew extract (1.5% and 1.0%) maintained relative higher and more stable DPPH scavenging activity (~85%) as compared to Josapine juice extract which showed activity declination from 60% to 45% during 180 minutes incubation. These results indicated that precipitation of bromelain with cashew leaf extract polyphenols

improved its thermal stabilization with well preservation of enzymatic and antioxidant activities against continuous heat damage effect.

All precipitated bromelain displayed a stable and increasing bromelain activity over a wide temperature range from 25 °C to 85 °C while free bromelain in Josapine juice extract showed an activity declination started from 25 °C. The temperature for maximum activity of precipitated bromelain was 75 °C-85 °C whereas that of free bromelain in Josapine juice extract was 25 °C. The result indicated bromelain in complexing with cashew polyphenols had a good heat resistance against high temperature-caused protein denaturation. However, the enzymatic activity for either free bromelain or precipitated bromelain decreased sharply as incubation temperature increased to 85 °C above. Thermal treatment by various temperatures did not cause significant reduction on antioxidant activity of precipitated bromelain especially those in precipitation with 1.5% and 1.0% cashew extracts, whereas antioxidant activity of Josapine juice extract and 0.5% precipitate decreased gradually with increasing incubation temperature.

Based on the results obtained from analysis of thermodynamic parameters, precipitated bromelain with 1.0% cashew extract was found to have 4.19 times higher activation energy of inactivation (E) as compared to free bromelain in Josapine juice extract. In addition, precipitated bromelain had 5.39 – 5.42 times higher enthalpy of activation (ΔH^{*}) and 1.03 times free energy of inactivation (G^{*}) than that reported from free bromelain at temperature range of 80 °C – 90 °C. The observed higher value of thermodynamic parameters including E, ΔH^{*} and G^{*} provided empirical support on resistance of precipitated bromelain against thermal inactivation.

In the study on effect of pH, precipitated bromelain exhibited increased and stable bromelain activity at alkaline region with pH optimum from 8 to 10 while free bromelain in Josapine juice extract favored acidic and neutral conditions. For antioxidant activity, precipitated bromelain displayed a much more stable and stronger activity than juice extract in pH range of 3-6, indicating precipitation of bromelain with cashew polyphenols increased its pH stability in term of antioxidant

activity at acidic region. However, the activity was dramatically reduced when either precipitated bromelain or Josapine juice extract was exposed to neutral and alkaline pH.

Bromelain-cashew leaf polyphenol precipitate had great thermal stability and pH stability by displaying high and stable enzymatic activity (bromelain activity) and antioxidant activity (DPPH radical scavenging activity) as compared to free bromelain in Josapine juice extract. This improvement was obviously observed with precipitated bromelain with high concentrations of cashew leaf extract (1.5% and 1.0% precipitates). In considerations of concentration of cashew leaf extract in precipitate, activity and stability, 1.0% cashew extract in Josapine juice extract generates an optimum precipitate with relative high enzymatic activity, antioxidant activity and stability in terms of temperature and pH, since all these properties similar to the precipitate with 1.5% cashew extract.

Overall, precipitation of bromelain with cashew polyphenols was shown to have stabilized enzymatic activity, increased antioxidant activity and great stability in conditions in terms of temperature and pH.

5.2 Recommendation

As we know, bromelain has been clinically evidenced to have several therapeutic applications especially its anti-inflammatory, antithrombotic, anticancerous properties and cashew polyphenols is well known for its antioxidant activity (Details on pharmacological actions of bromelain and polyphenols are elaborated in section 2.2, 2.4.2 and 2.5.2). Therefore, it would be essential to evaluate the therapeutic properties of bromelain-polyphenol complex *in vitro/ in vivo* for its bioavailability. Interaction of bromelain with plant polyphenols has the potential to influence the biological activity and physiological actions of each of these two characters depending upon the resultant structure and functionality. Immunogenicity of bromelain-polyphenol complex within murine gastrointestinal tract could be studied in the future to evaluate the stimulation of immune response

following long term exposure of complexed bromelain, or whether the ingested bromelain-polyphenol complex retains its proteolytic activity and antioxidant activity *in vivo*. Besides, acute, subacute toxicity and genotoxicity assay could also be carried out to evaluate the safety and toxicological effect of bromelain-polyphenol complex.

Cashew leaf was ethanolic extracted for polyphenols. The composition of cashew polyphenols might be identified and characterized by liquid chromatography coupled to mass spectrometry analysis in future. This phytochemical substance screening is important because the antioxidant activity of plant extract is correlated with concentration and structural properties of major constituents of polyphenols. Therefore, measurement of total phenolic content is insufficient to determine the characteristic of phenolic content in contributing to antioxidant activity of plant extract. Further, properties of binding and precipitation of polyphenols in protein-polyphenol complex depends on its structural properties. Identification of individual polyphenol components could suggest whether the polyphenolic compounds in plant extract contain components of high protein binding and precipitating affinity.

Interaction between proteins and polyphenols could be investigated on H-NMR to determine the specificity and strength of binding interaction. Binding of galloyl rings of polyphenols with proline rich polypeptide is evidenced by chemical shift changes observed on proline residue and its proceeding amino acid (Details on NMR studies on protein-polyphenol interaction are elaborated in section 2.7.1.2). Protein-polyphenol interaction could also be characterized on frontal analysis capillary electrophoresis by determination of association/ dissociation constant relating to complex formation (Papadopoulou and Frazier, 2004).

Current approaches for extraction and purification of bromelain from aqueous extract of pineapple including two phase partitioning, reverse micellar extraction and conventional ammonium sulfate precipitation (Doko *et al.*, 1991; Hebbar *et al.*, 2008; Ketnawa *et al.*, 2010). In this study, complexing of bromelain with plant polyphenols might be a novel method for separation of bromelain from pineapple extract. Bromelain in complexing with polyphenols is proved to exhibit stable

enzymatic activity. Intensive studies need to be done on recovery of bromelain from bromelain-polyphenol complex without remarkably affecting its biochemical properties. Interaction of proline rich polypeptide and polyphenols involves hydrophobic and hydrogen bonding, therefore, it is supposed that alternation of these interaction bindings would drive the dissociation of proteins and polyphenols.

Overall, complexing of bromelain in pineapple juice with polyphenols of cashew leaf extract is a useful strategy for providing the enzymes some new characteristics for example stabilized enzymatic activity under conditions in terms of temperature and pH and also increased antioxidant activity. The enhanced stability of complexed bromelain makes it a good alternative for food and beverage, pharmaceutical and biotechnological applications in which a stable enzyme is required. In addition to the stable health beneficial enzymatic activity, complexed bromelain is coupled with significant antioxidant activity which is attributed by cashew leaf extract polyphenols, thereby enhancing its therapeutic properties.

REFERENCES

Abas, F., Lajis, N.H., Israf, D.A., Khozirah, S. and Umi Kalsom, Y. (2006). Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables. *Food Chemistry.* 95 (4), 566-573.

Ahmad, B., Kamal, M.J. and Khan, R.H. (2004). Alkali-induced conformational transition in different domains of bovine serum albumin. *Protein Peptide Lett.* 4, 307-315.

Akinpelu, D.A. (2001). Antimicrobial activity of *Anacardium occidentale* bark. *Fitoterapia.* 72, 286-287.

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7915-7922.

Arts, M.J.T.J., Haenen, G.R.M.M., Wilms, L.C., Beetstra, S.A.J.N., Heijnen, C.G.M., Voss, H.P. *et al.* (2001). Interactions between flavonoids and proteins: effect on the total antioxidant capacity. *J. Agric. Food Chem.* 50, 1184-1187.

Arya, R., Babu, V., Ilyas, M. and Nassim, K.T. (1989). Phytochemical examination of the leaves of *Anacardium occidentale*. *Journal of the Indian Chemical Society.* 66, 67-68.

Asano, K., Ohtsu, K., Shinagawa, K. and Hashimoto, N. (1984). Affinity of proanthocyanidins and their oxidation products for haze forming proteins of beer and the formation of chill haze. *Agric. Biol. Chem.* 48, 1139-1146.

Asano, K., Shinagawa, K. and Hashimoto, N. (1982). Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J. Am. Soc. Brew. Chem.* 40, 147-154.

Assuncao, R.B. and Mercadante, A.Z. (2003). Carotenoids and ascorbic acid from cashew apple (*Anacardium occidentale* L.): variety and geographic effects. *Food Chemistry.* 81, 495-502.

Atoui, A.K., Mansouri, A., Boskou, G. and Kefalas, P. (2005). Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chemistry*. 89, 27-36.

Bacon, J.R. and Rhodes, M.J.C. (2000). Binding affinity of hydrolyzable tannins to parotid saliva and to proline-rich proteins derived from it. *J. Agric. Food Chem.* 48, 838-843.

Balasubramanian, R., Lakshiminarayanan, A.V., Sabesan, M.N., Tegoni, G., Venkatesan, K. and Ramachandran, G.N. (1971). Studies on the conformation of amino acids. VI. Conformation of the proline ring as observed in crystal structures of amino acids and peptides. *Int. J. Protein Res.* 3, 25-33.

Baldwin, E.P. and Matthews, B.W. (1994). Core-packing constraints, hydrophobicity and protein design. *Curr. Opin. Biotechnol.* 5, 396-402.

Baron, M., Norman, D.G. and Campbell, I.D. (1991). Protein modules. *Trends Biochem. Sci.* 16, 13-17.

Baxter, N.J., Lilley, T.H., Haslam, E. and Williamson, M.P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry*. 36, 5566-5577.

Belitz, H.D., Grosch, W. and Schieberle, P. (2009). *Amino acids, peptides, proteins.* In Belitz, H.D., Grosch, W. and Schieberle, P (Eds.). *Food Chemistry*. (1-85). London: Springer.

Belloc, C., Lu, H., Soria, C., Fridman, R., Legrand, Y. and Menashi, S. (1995). The effect of platelets on invasiveness and protease production of human mammary tumor cells. *Int. J. Cancer*. 60, 413-417.

Bennick, A. (1982). Salivary proline-rich proteins. *Mol. Cell. Biochem.* 45, 83-99.

Bennick, A. (2002). Interaction of plant polyphenols with salivary proteins. *Crit. Rev. Oral Biol. Med.* 13 (2), 184-196.

Bhattacharya, R. and Bhattacharyya, D. (2007). Preservation of natural, stability of fruit "bromelain" from *Ananas comosus* (pinapple). *J. Food Biochem.* 33, 1-19.

Bhatti, H.N., Asgher, M., Abbas, A., Nawaz, R. and Sheikh, M.A. (2006). Studies on kinetics and thermostability of a novel acid invertase from *Fusarium solani*. *J. Agric. Food Chem.* 54, 4617-4623.

Biggerstaff, J.P., Weidow, B., Dexheimer, J., Warnes, G., Vidosh, J., Patel, S. *et al.* (2008). Soluble fibrin inhibits lymphocyte adherence and cytotoxicity against

tumor cells: implications for cancer, metastasis and immunotherapy. *Clin. Appl. Thromb. Haemost.* 14, 193-202.

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*. 56 (11), 317-333.

Brito, E.S., Araujo, M.C.P., Lin, L.Z. and Harnly, J. (2007). Determination of the flavonoid components of cashew apple (*Anacardium occidentale*) by LC-DAD-ESI/MS. *Food Chemistry*. 205, 1112-1118.

Cao, G., Sofic, E. and Prior, R.L. (1996). Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* 44, 3426-3431.

Castell, J.V., Friedrich, G., Kuhn, C.S. and Poppe, G.E. (1997). Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. *Am. J. Physiol.* 273, 139-146.

Cavalcante, A.A.M., Rubensam, G., Picada, J.N., Silva, E.G., Moreira, J.C.F. and Henriques, J.A.P. (2003). Mutagenicity, antioxidant potential, and antimutagenic activity against hydrogen peroxide of cashew (*Anacardium occidentale*) apple juice and cajuina. *Environmental and Molecular Mutagenesis*. 41, 360-369.

Cecchi, H.M. and Rodriguez-Amaya, D.B. (1981). Carotenoid composition and vitamin A value of fresh and pasteurized cashew apple juice. *Journal of Food Science*. 46, 147-149.

Charlton, A.J., Baxter, N.J., Khan, M.L., Moir, A.J.G., Haslam, E., Davies, A.P. *et al.* (2002). Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* 50, 1593-1601.

Charlton, A.J., Baxter, N.J., Lilley, T.H., Haslam, E., Mcdonald, C.J. and Williamson, M.P. (1996). Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats. *FEBS Letters*. 382, 289-292.

Chobotova, K., Vernallis, A.B. and Majid F.A.A. (2010). Bromelain's activity and potential as an anti-cancer agent: Current evidence and perspectives. *Cancer Letters*. 290(2), 148-156.

Cirelli, M.G. and Smyth, R.D. (1963). Effects of bromelain antiedema therapy on coagulation, bleeding and prothrombin times. *J. New Drugs*. 3, 37-39.

Clarke, S. (1987). Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Peptide Protein Res.* 30, 808-821.

Cook, N.C. and Samman, S. (1996). Flavonoids: chemistry, metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem.* 7, 66-76.

Coppens d' Eeckenbrugge, G. and Leal, F. (2003). *Morphology, anatomy and taxonomy.* In Bartholomew, D.P., Paull, R.E. and Rohrbach, K.G. (Eds.). *The pineapple: botany, production and uses.* (13-32). Wallingford, Oxon: CABI.

Corden, J.L. (1990). Tails of RNA polymerase II. *Trends Biochem. Sci.* 15, 383-387.

Daniel, R.M. (1996). The upper limits of enzyme thermal stability. *Enzyme and Microbial Technology.* 19, 74-79.

Daniel, R.M., Dines, M. and Petach, H.H. (1996). The denaturation and degradation of stable enzymes at high temperatures. *Biochem. J.* 317, 1-11.

Davey, M.R., Sripaoraya, S., Anthony, P., Lowe, K.C. and Power, J.B. (2007). *Pineappple.* In Pua, E.C. and Davey, M.R. (Eds.). *Biotechnology in Agriculture and Forestry, Vol. 60 Transgenic Crops V.* (pp. 97-127). London: Springer-Verlag Berlin Heidelberg.

Desser, L. and Rehberger, A. (1990). Induction of tumor necrosis factor in human peripheral-blood mononuclear cells by proteolytic enzymes. *Oncology.* 47, 475-477.

Desser, L., Rehberger, A., Kokron, E. and Paukovits, W. (1993). Cytokine synthesis in human peripheral blood mononuclear cells after oral administration of polyenzyme preparations. *Oncology.* 50, 403-407.

Devakate, R.V., Patil, V.V., Waje, S.S. and Thorat, B.N. (2009). Purification and drying of bromelain. *Separation and Purification Technology.* 64, 259-264.

Doko, M.B., Bassani, V., Casadebaig, J., Cavailles, L. and Jacob, M. (1991). Preparation of proteolytic enzyme extracts from *Ananas comosus* L., Merr. fruit juice using semipermeable membrane, ammonium sulfate extraction, centrifugation and freeze-drying processes. *International Journal of Pharmaceutics.* 76, 199-206.

Dufresne, C.J. and Farnworth, E.R. (2001). A review of latest research findings on the health promotion properties of tea. *Journal of Nutritional Biochemistry.* 12, 404-421.

Fernandez, J. and Lilley, T.H. (1992) Aqueous solutions containing amino acids and peptides. Part 28. Enthalpy of interaction of some amides with glycine and α-alanine: Interactions of the zwitterionic group of α-amino acids with hydrophobic groups and peptide group. *J. Chem. Soc. Faraday Trans.* 88, 2503-2509.

Fink, A.L., Calciano, I.J., Goto, Y., Kurotsu, Y. and Palleros, D.R. (1994). Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry.* 33, 12504-12511.

Floyd, R.A. (1999). Neuroinflammatory processes are important in neurodegenerative diseases: a hypothesis to explain the increased formation of reactive oxygen and nitrogen species as major factors involved in neurodegenerative disease development. *Free Radical Biology and Medicine.* 26, 1346-1355.

Franca, F., Lago, E.L. and Marsden, P.D. (1996). Plants used in the treatment of leishmanial ulcers due to Leishmania (Viannia) braziliensis in an endemic area of Bahia Brazil. *Rev. Soc. Bras. Med. Trop.* 29, 229-232.

Frankel, E.N., Kanner, J., German, J.B., Parks, E. and Kinsella, J.E. (1993). Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *The Lancet.* 341, 454-457.

Freitas, V.D. and Mateus, N. (2001). Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* 49, 940-945.

Gansau, J.A., Goh, K.S. and How, S.E. (2009). Primary screening for natural inhibitors against eukaryotic signal transduction from selected medicinal plants in Sabah, Malaysia. *Borneo Science.* 24, 37-46.

Georis, J., Esteves, F.L., Brasseur, J.L., Bougnet, V., Devreese, J.L.B., Giannotta, F. *et al.* (2000). An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: structural basis and molecular study. *Protein Sci.* 9, 466-475.

Goncalves, J.L.S., Lopes, R.C., Oliveira, D.B., Costa, S.S., Miranda, M.M.F.S., Romanos, M.T.V. *et al.* (2005). In vitro anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. *Journal of Ethnopharmacology.* 99, 403-407.

Goodwin, J.S. and Brodwick, M. (1995). Diet, aging, and cancer. *Clinics in Geriatric Medicine.* 11, 577-589.

Grabowska, E., Eckert, K., Fichtner, I., Schulze-Forster, K. and Maurer, H.R. (1997). Bromelain proteases suppress growth, invasion and lung metastasis of B16F10 mouse melanoma cells. *Int. J. Oncol.* 11, 243-248.

Ha, T.J. and Kubo, I. (2005). Lipoxygenase inhibitory activity of anacardic acids. *Journal of Agricultural and Food Chemistry.* 53, 4350-4354.

Hagerman, A.E. and Butler, L.G. (1980). Determination of protein in tannin-protein complexes. *J. Agric. Food Chem.* 28, 944-947.

Hagerman, A.E. and Butler, L.G. (1981). The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* 256, 4494-4497.

Hagerman, A.E. and Klucher, K.M. (1986). Tannin-protein interactions. *Prog Clin Biol Res.* 213, 67-76.

Hagerman, A.E., Rice, M.E. and Ritchard, N.T. (1998). Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4→8) catechin (procyanidin). *J. Agric. Food Chem.* 46, 2590-2595.

Hale, L.P. (2004). Proteolytic activity and immunogenicity of oral bromelain within the gastrointestinal tract of mice. *International Immunopharmacology.* 4, 255-264.

Hale, L.P., Fitzhugh, D.J. and Staats, H.F. (2006). Oral immunogenicity of the plant proteinase bromelain. *International Immunopharmacology.* 6, 2038-2046.

Hale, L.P., Greer, P.K. and Sempowski, G.D. (2002). Bromelain treatment alters leukocyte expression of cell surface molecules involved in cellular adhesion and activation. *Clinical Immunology.* 104 (2), 183-190.

Hale, L.P., Greer, P.K., Trinh, C.T. and Gottfried, M.R. (2005a). Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease. *Clin. Immunol.* 116, 135-142.

Hale, L.P., Greer, P.K., Trinh, C.T. and James, C.L. (2005b). Proteinase activity and stability of natural bromelain preparations. *International Immunopharmacology.* 5, 783-793.

Harrach, R., Gebauer, F., Eckert, K., Kunze, R. and Maurer, H.R. (1994). Bromelain proteinases modulate the CD44 expression on human Molt4/8 leukemia and SK-Mel 28 melanoma cells in vitro. *Int. J. Oncol.* 5, 485-488.

Harrach, T., Eckert, K., Schulze-Forster, K., Nuck, R., Grunow, D. and Maurer, H.R. (1995). Isolation and partial characterization of basic proteinases from stem bromelain. *Journal of Protein Chemistry.* 14 (1), 41-52.

Haslam, E. (1996). Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J.Nat. Prod.* 59, 205-215.

Haslam, E. (1998). *Practical Polyphenolics.* Cambridge, UK: Cambridge University Press.

Hebbar, H.U., Sumana, B. and Raghavarao, K.S.M.S. (2008). Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes. *Bioresource Technology*. 99, 4896-4902.

Heinicke, R.M., van der Wal, L. and Yokoyama, M. (1972). Effect of bromelain on human platelet aggregation. *Experientia*. 28, 844-845.

Himejima, M. and Kubo, I. (1991). Antibacterial agents from the cashew, *Anacardium occidentale*, nut shell oil. *Journal of Agricultural Food Chemistry*. 39, 418-421.

Hodgson, J.M. (2008). Tea flavonoids and cardiovascular disease. *Asia Pac. J. Clin. Nutr*. 17 (S1), 288-290.

Ikram, E.H.K., Khoo, H.E., Jalil, A.M.M., Ismail, A., Idris, S, Azlan, A., *et al.* (2009). Antioxidant capacity and total phenolic content of Malaysian underutilized fruits. *Journal of Food Composition Analysis*. 22, 388-393.

Jutamongkon, R. and Charoenrein, S. (2010). Effect of temperature on the stability of fruit bromelain from Smooth Cayenne pineapple. *Kasetsart J. (Nat. Sci.)*. 44, 943-948.

Kamath, V. and Rajini, P.S. (2007). The efficacy of cashew nut (*Anacardium occidentale* L.) skin extract as a free radical scavenger. *Food Chemistry*. 103, 428-433.

Kamphuis, I.G., Drenth, J. and Bakes, E.N. (1985). Thiol proteases. Comparative studies based on the high-resolution structures of papain and actinidin, and on amino acid sequence information for cathepsins B and H, and stem bromelain. *J. Mol. Biol*. 182, 317-329.

Kamtchouing, P., Sokeng, S.D., Moundipa, P.F., Watcho, P., Jatsa, H.B. and Lontsi, D. (1998). Protective role of *Anacardium occidentale* extract against streptozotocin-induced diabetes in rats. *Journal of Ethnopharmacology*. 62 (2), 95-99.

Kawamoto, H., Nakatsubo, F. and Murakami, K. (1995). Quantitative determination of tannin and protein precipitates by high performance liquid chromatography. *Phytochemistry*. 40, 1503-1505.

Kelly, G.S. (1996). Bromelain: A literature review and discussion of its therapeutic applications. *Alternative Medicine Review*. I (4), 243-257.

Ketnawa, S., Rawdkuen, S. and Chaiwut, P. (2010). Two phase partitioning and collagen hydrolysis of bromelain from pineapple peel *Nang Lae* cultivar. *Biochemical Engineering Journal*. 52, 205-211.

Khan, R.H., Rasheedi, S. and Haq, S.K. (2003). Effect of pH, temperature and alcohols on the stability of glycosylated and deglycosylated stem bromelain. *Indian Academy of Sciences*. 28 (6), 709-714.

Kogel, I. and Zech, W. (1985). The phenolic acid content of cashew leaves (*Anacardium occidentale L.*) and of the associated humus layer, Senegal. *Geoderma*. 35(2), 119-125.

Konan, N.A. and Bacchi, E.M. (2007). Antiulcerogenic effect and acute toxicity of a hydroethanolic extract from the cashew (*Anacardium occidentale* L.) leaves. *Journal of Ethnopharmacology*. 112, 237-242.

Konan, N.A., Bacchi, E.M., Lincopan, N., Varela, S.D. and Varanda, E.A. (2007). Acute, subacute toxicity and genotoxic effect of a hydroethanolic extract of the cashew (*Anacardium occidentale* L.). *Journal of Ethnopharmacology*. 110, 30-38.

Kornsteiner, M., Wagner, K.H. and Elmadfa, I. (2006). Tocopherols and total phenolics in 10 different nut types. *Food Chemistry*. 98 (2). 381-387.

Kozubek, A. and Tyman, J.H. (1999). Resorcinolic lipids, the natural nonisoprenoid phenolic amphiphiles and their biological activity. *Chem. Rev*. 99, 1-25.

Kozubek, A., Zamowski, R., Stasiuk, M. and Gubernator, J. (2001). Natural amphiphilic phenols as bioactive compounds. *Cellular and Molecular Biology Letters*. 6, 351-355.

Kubo, I., Ha, T.J., Tsujimoto, K., Tocoli, F.E. and Green, I.R. (2008). Evaluation of lipoxygenase inhibitory activity of anacardic acids. *Z. Naturforsch [C]*. 63, 539-546.

Kubo, I., Masuoka, N., Ha, T.J. and Tsujimoto, K. (2006). Antioxidant activity of anacardic acids. *Food Chemistry*. 99 (3), 555-562.

Kubo, I., Muroi, H. and Himejima, M. (1993). Structural-antibacterial activity relations of anacardic acids. *Journal of Agricultural and Food Chemistry*. 41, 1016-1019.

Kubo, I., Nihei, K. and Tsujimoto, K. (2003). Antibacterial action of anacardic acids against Methicillin Resistant *Staphylococcus aureus* (MRSA). *Journal of Agricultural and Food Chemistry*. 51, 7624-7628.

Kubo, J., Lee, J.R. and Kubo, I. (1999). Anti-*helicobacter pylori* agents from the cashew apple. *Journal of Agricultural and Food Chemistry.* 47, 533-537.

Kudi, A.C., Umoh, J.U., Eduvie, L.O. and Gefu, J. (1999). Screening of some Nigerian medicinal plants for antibacterial activity. *Journal of Ethnopharmacology.* 67, 225-228.

Liang, H.H., Huang, H.H. and Kwok, K.C. (1999). Properties of tea-polyphenol-complexed bromelain. *Food Research International.* 32, 545-551.

Lipinski, B. and Egyud, L.G. (2000). Resistance of cancer cells to immune recognition and killing. *Med. Hypotheses.* 54, 456-460.

Liyana-Pathiranan, C.M. and Shahidi, F. (2005). Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum L*) as affected by gastric pH condition. *J. Agric. Food Chem.* 53, 2433-2440.

Luiz-Ferreira, A., Cola-Miranda, M., Barbastefano, V., Hiruma-Lima, C.A., Vilegas, W. and Souza Brito, A.R. (2008). Should Anacardium humile St. Hil be used as an antiulcer agent? A scientific approach to the traditional knowledge. *Fitoterapia.* 79, 207-209.

MacArthur, M.W. and Thornton, J.M. (1991). Influence of proline residues on protein conformation. *J. Mol. Biol.* 218, 397-412.

Maciel, M.I., Hansen, J.T., Aldinger, S.B. and Labows, J.N. (1986). Flavor chemistry of cashew apple juice. *Journal of Agricultural and Chemistry.* 34, 923-927.

Makkar, H.P.S., Dawra, R.K. and Singh, B. (1988). Determination of both tannin and protein in a tannin-protein complex. *J. Agric. Food. Chem.* 36, 523-525.

Manhart, Akomeah, N.R., Bergmeister, H., Spittler, A., Ploner, M. and Roth, E. (2002). Administration of proteolytic enzymes bromelain and trypsin diminish the number of CD4+ cells and interferon-gamma response in Peyer's patches and spleen in endotoxemic balb/c mice. *Cell. Immunol.* 2, 113-119.

Manning, M.C., Patel, K. and Borchardt, T.T. (1989). Stability of protein pharmaceuticals. *Pharm. Res.* 6, 903-918.

Manzocco, L., Anese, M. and Nicoli, M.C. (1998). Antioxidant properties of tea extracts as affected by processing. *Lebensmittel-Wissenschaftund-Technologie.* 31, 694-698.

MARDI, Horticulture Research Centre (1996). *Josapine, the world's first commercial pineapple hybrid.* [Brochure]. Malaysia: MARDI.

Martin, M.J., Motilva, V. and Alarcon de la Lastra, C. (1993). Quercetin and naringenin: Effects on ulcer formation and gastric secretion in rats. *Phytotherapy Research.* 7, 150-153.

Masuoka and Kubo (2004). Characterization of xanthine oxidase inhibition by anacardic acids. *Biochimica et Biophysica Acta.* 1688, 245-249.

Matthews, B.W. (1993). Structural and genetic analysis of protein stability. *Ann. Rev. Biochem.* 62, 139-160.

Maurer, H.R. (2001). Bromelain: biochemistry, pharmacology and medical use. *Cellular and Molecular Life Sciences.* 58, 1234-1245.

McManus, J.P., Davis, K.D., Beart, J.E., Gaffney, S.H., Lilley, T.H. and Haslam, E. (1985). Polyphenol interactions. Part 1. Introduction: some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J Chem Soc Perkin Trans II.* 9, 1429-1438.

McMurrough, I., Kelly, R. and Byrne, J. (1992). Effect of the removal of sensitive proteins and proanthocyanidins on the colloidal stability of lager beer. *J. Am. Soc. Brew. Chem.* 50, 67-76.

Meda, A., Lamien, C.E., Romito, M., Millogo, J. and Nacoulma, O.G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry.* 91, 571-577.

Mehansho, H., Butler, L.G. and Carlson, D.M. (1987). Dietary tannins and salivary proline-rich proteins: interactions, induction and defense mechanisms. *Ann. Rev. Nutr.* 7, 423-440.

Mehta, P. (1984). Potential role of platelets in the pathogenesis of tumor metastasis. *Blood.* 63, 55-63.

Mirdawati, M., Siti Zalita, A.T. and Abdul Majid, F.A. (2010). Non-destructive sterilization process of pineapple juice. *7th International Pineapple Symposium 2010.* 13-15 July. Johor, Malaysia, 41-42.

Morita, A.H., Uchida, D.A., Taussig, S.J., Chon, S.C. and Hokama, Y. (1979). Chromatographic fractionation and characterization of the active platelet aggregation inhibitory factor from bromelain. *Arch. Int. Pharmacodyn.* 239, 340-350.

Mota, M.L.R., Thomas, G. and Barbosa Filho, J.M. (1985). Anti-inflammatory actions of tannins isolated from the bark of *Anacardium occidentale* L. *Journal of Ethnopharmacology.* 13 (3), 289-300.

MPIB, Malaysian Pineapple Industry Board (2008). *Malaysian's Pineapple Cultivar.* [Brochure]. Malaysia: MPIB.

Mulkay, P. and Jerumanis, J. (1983). Effects of molecular weight and degree of hydroxylation of proanthocyanidins on the colloidal stability of beer. *Cerevisia.* 8, 29-35.

Munch, O. and Tritsch, D. (1990). Irreversible thermoinactivation of glucoamylase from *Aspergillus niger* and thermostabilizatin by chemical modification of carboxyl groups. *Biochim. Biophys. Acta.* 1041, 111-116.

Munzig, E., Eckert, K., Harrach, T., Graf, H. and Maurer, H.R. (1994). Bromelain protease F9 reduces the CD44 mediated adhesion of human peripheral blood lymphocytes to human umbilical vein endothelial cells. *FEBS Lett.* 351, 215-218.

Murray, N.J, Williamson, M.P., Lilley, T.H. and Haslam, E. (1994). Study of the interaction between salivary proline-rich proteins and a polyphenol by ^{1}H-NMR spectroscopy. *Eur. J. Biochem.* 219, 923-935.

Naidu, G.S.N. and Panda, T. (2003). Studies on pH and thermal deactivation of pectolytic enzymes from *Aspergillus niger*. *Biochemical Engineering Journal.* 16, 57-67.

Narayana, K.R., Reddy, M.S., Chaluvadi, M.R. and Krishna, D.R. (2001). Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian Journal of Pharmacology.* 33, 2-16.

Noroozi, M., Angerson, W.J. and Lean, M.E.J. (1998). Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am. J. Clin. Nutr.* 67, 1210-1218.

Oh, H.I., Hoff, J.E., Armstrong, G.S. and Haff, L.A. (1980). Hydrophobic interaction in tannin-protein complexes. *J. Agric. Food Chem.* 28, 394-398.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R. and Simons, A. (2009). Anacardium occidentale L. [CD-ROM]. *Agroforestree Database: a tree reference and selection guide version 4.0*, 1-5. World Agroforestry Centre, ICRAF.

Ozawa, T., Lilley, T.H. and Haslam, E. (1987). Polyphenol interactions: astringency and the loss of astringency in ripening fruit. *Phytochemistry.* 26, 2937-2942.

Page, M.I. and Jencks, W.P. (1971). Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. *Proc. Natl. Acad. Sci. U.S.A.* 68, 1678-1683.

Papadopoulou, A. and Frazier, R.A. (2004). Characterization of protein-polyphenol interactions. *Trends in Food Science and Technology.* 15, 186-190.

Paramashivappa, R., Phani Kumar, P., Vithayathil, P.J. and Srinivasa Rao, A. (2001). Novel method for isolation of major phenolic constituents from cashew (*Anacardium occidentale* L.) nut shell liquid. *Journal of Agricultural Food Chemistry.* 49, 2548-2551.

Pereira, J.M., Severino, R.P., Vieira, P.C., Fernandes, J.B., da Silva, M.F., Zottis, A. *et al.* (2008). Anacardic acid derivatives as inhibitors of glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi. Bioorg. Med. Chem.* 19, 8889-8895.

Pothitirat, W., Chomnawang, M.T., Supabphol, R. and Gritsanapan, W. (2009). Comparison of bioactive compounds content, free radical scavenging and anti-acne inducing bacteria activities of extracts from the mangosteen fruit rind at two stages of maturity. *Fitoterapia.* 80, 442-447.

Quintanilla-Guerrero, F., Duarte-Vazquez, M.A., Tinoco, R., Gomez-Suarez, M., Garcia-Almendarez, B.E., Vazquez-Duhalt, R. *et al.* (2008). Chemical modification of turnip peroxidase with methoxypolyethylene glycol enhances activity and stability for phenol removal using the immobilized enzyme. *J. Agric. Food Chem.* 56, 8058-8065.

Rangan, T.S. (1984). Pineapple. Handbook of plant cell culture. *Crop Species.* 3, 373-382.

Rawlings, N.D., Morton, F.R. and Barrett, A.J. (2007). *An introduction to peptidases and the Metrops database.* In Polaina, J. and MacCabe, A.P. (Eds.). *Industrial Enzymes.* (161-179). London: Springer.

Razali, N., Razab, R., Junit, S.M. and Aziz, A.A. (2008). Radical scavenging and reducing properties of extracts of cashew shoots (*Anacardium occidentale*). *Food Chemistry.* 111, 38-44.

Riedl, K.M. and Hagerman, A.E. (2001). Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* 49, 4917-4923.

Ritonja, A., Rowan, A.D., Buttle, D.J., Rawlings, N.D., Turk, V. and Barrett, A.J. (1989). Stem bromelain: amino acid sequence and implications for weak binding of cystatin. *FEB.* 247 (2), 419-424.

Rowan, A.D., Buttle, D.J. and Barrett, A.J. (1988). A novel cysteine proteinase found in pineapple stem. *Arch. Biochem. Biophys.* 267, 262-270.

Rowan, A.D., Buttle, D.J. and Barrett, A.J. (1990). The cysteine proteinases of the pineapple plant. *Biochem. J.* 266, 869-875.

Runnie, I., Salleh, M.N., Mohamed, S., Head, R.J. and Abeywardena, M.Y. (2004). Vasorelaxation induced by common edible tropical plant extracts in isolated rat aorta and mesenteric vascular bed. *Journal of Ethnopharmacology.* 92 (2-3), 311-316.

Salah, N., Miller, N.J., Parganga, G., Tifburg, L., Bolwell, G.P. and ice-Evan, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics.* 322, 339-346.

Schmourio, G., Mendonca-Filho, R.R., Alviano, C.S. and Costa, S.S. (2005). Screening of anti-fungal agents using ethanol precipitation and bioautography of medicinal and food plants. *Journal of Ethnopharmacology.* 96 (3), 563-568.

Siebert, K.J. and Lynn, P.Y. (1998). Comparison of polyphenol interactions with PVPP and haze-active protein. *J. Am. Soc. Brew. Chem.* 56, 24-31.

Siebert, K.J., Troukhanova, N.V. and Lynn P.Y. (1996). Nature of polyphenol-protein interactions. *J. Agric. Food Chem.* 44, 80-85.

Singh, B., Kale, R.K. and Rao, A.R. (2004). Modulation of antioxidant potential in liver of mice by kernel oil of cashew nut (*Anacardium occidentale* L.) and its lack of tumor promoting ability in DMBA induced skin papillomagenesis. *Indian Journal of Experimental Biology.* 42, 372-377.

Spanos, G.A. and Wrolstad, R.E. (1992). Phenolics of apple, pear and white grape juices and their changes with processing and storage – A review. *J. Agric. Food Chem.* 40, 1478-1487.

Steinberg, D. (1991). Antioxidants and atherosclerosis: a current assessment. *Circulation.* 84, 1420-1425.

Subramaniam, V., Gardner, H. and Jothy, S. (2007). Soluble CD44 secretion contributes to the acquisition of aggressive tumor phenotype in human colon cancer cells. *Exp. Mol. Pathol.* 83, 341-346.

Takahashi, N., Yasuda, Y., Goto, K., Miyake, T. and Murachi, T. (1973). Multiple molecular forms of stem bromelain. Isolation and characterization of two closely related components, SB1 and SB2. *Journal of Biochemisty.* 74, 355-373.

Taussig, S.J. and Batkin, S. (1988). Bromelain, the enzyme complex of pineapple (Ananas comosus) and its clinical application: An update. *Enthnopharmacol.* 22, 191-203.

Tochi, B.N., Wang, Z., Xu, S.Y. and Zhang, W. (2008). Therapeutic application of pineapple protease (Bromelain): A review. *Pakistan Journal of Nutrition.* 7(4), 513-520.

Tomizawa, H., Yamada, H., Wada, K. and Imoto, J. (1995). Stabilization of lysozyme against irreversible inactivation by suppression of chemical reactions. *J. Biochem. (Tokyo).* 117, 635-640.

Toyomizu, M., Sugiyama, S., Jin, R.L. and Nakatsu, T. (1993). α-Glucosidase and aldose reductase inhibitors: constituents of cashew, *Anacardium occidentale*, nut shell liquid. *Phytotherapy Research.* 7, 252-254.

Trevisan, M.T.S., Pfundstein, B., Haubner, R., Wurtele, G., Spiegelhalder, B. and Bartsch, H. *et al.* (2006). Characterization of alkyl phenols in cashew (*Anacardium occidentale*) products and assay of their antioxidant capacity. *Food and Chemical Toxicology.* 44, 188-197.

Turkmen, N., Sari, F. and Velioglu, Y.S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chemistry.* 99, 835-841.

Venkatachalam, M. and Sathe, S.K. (2006). Chemical composition of selected edible nut seeds. *Journal of Agricultural and Chemistry.* 54, 4705-4714.

Walker, J.M. (2002). *The Bicinchoninic acid (BCA) assay for protein quantitation.* In Walker, J.M. (Ed.). *The protein protocols handbook, 2nd edition.* (11-14). Totowa, NJ: Humana Press Inc.

Williamson, M.P. (1994). The structure and function of proline-rich regions in proteins. *Biochem. J.* 297, 249-260.

Wolfe, K., Wu, X. and Liu, R.H. (2003). Antioxidant activity of apple peels. *J. Agric. Food Chem.* 51, 609-614.

Wu, H.C., Shiau, C.Y., Chen, H.M. and Chiou, T.K. (2003). Antioxidant activities of carnosine, anserine, some free amino acids and their combination. *Journal of Food and Drug Analysis.* 11, 148-153.

Xu, C., Zhang, Y., Cao, L. and Lu, J. (2010). Phenolic compounds and antioxidant properties of different grape cultivars grown in China. *Food Chemistry.* 119, 1557-1565.

Xue, Y., Wu, C.Y., Branford-White, C.J., Ning, X., Nie, H.L. and Zhu, L.M. (2010). Chemical modification of stem bromelain with anhydride groups to enhance its stability and catalytic activity. *Journal of Molecular Catalysis B: Enzymatic.* 63, 188-193.

Yon-Kahn, J. and Herve, G. (2010). *Examples of structure-function relationships in enzymatic systems.* In Yon-Kahn, J. and Herve, G (Eds.). *Molecular and Cellular Enzymology.* (451-541). London: Springer.

Yu, J., Ahmedna, M. and Goktepe, I. (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chemistry.* 90, 199-206.

Zadernowski, R., Czaplicki, S. and Naczk, M. (2009). Phenolic acid profiles of mangosteen fruits (*Garcinia mangostana*). *Food Chemistry.* 112, 685-689.

Zheng, W. and Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* 49, 5165-5170.