ISOLATION AND CHARACTERIZATION OF BROMELAIN ENZYME FROM PINEAPPLE AND ITS UTILIZATION AS ANTI-BROWNING AGENT

A THESIS

BY

SHAMPA SARKAR
STUDENT ID. 1405205
SESSION: 2014-2015
SEMESTER: JULY- DECEMBER, 2015

MASTER OF SCIENCE (MS)
IN
FOOD PROCESSING AND PRESERVATION

DEPARTMENT OF FOOD PROCESSING AND PRESERVATION

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200, BANGLADESH

December, 2015

ISOLATION AND CHARACTERIZATION OF BROMELAIN ENZYME FROM PINEAPPLE AND ITS UTILIZATION AS ANTI-BROWNING AGENT

A THESIS

BY

SHAMPA SARKAR
STUDENT ID. 1405205
SESSION: 2014-2015
SEMESTER: JULY- DECEMBER, 2015

Submitted to the
Department of Food Processing and Preservation

Hajee Mohammad Danesh Science and Technology University, Dinajpur

In Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE (MS)
IN
FOOD PROCESSING AND PRESERVATION

DEPARTMENT OF FOOD PROCESSING AND PRESERVATION

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR-5200

December, 2015

ISOLATION AND CHARACTERIZATION OF BROMELAIN ENZYME FROM PINEAPPLE AND ITS UTILIZATION AS ANTI-BROWNING AGENT

A THESIS

BY

SHAMPA SARKAR
STUDENT ID. 1405205
SESSION: 2014-2015
SEMESTER: JULY- DECEMBER, 2015

Approved as to style and contents by:

Dr. Maruf Ahmed	**N.H.M. Rubel Mozumder**
Associate Professor	Assistant Professor
Supervisor	Co-Supervisor

Chairman of the Examination Committee
And
Chairman, Department of Food Processing and Preservation

Hajee Mohammad Danesh Science and Technology University
Dinajpur-5200, Bangladesh

December, 2015

DEDICATED TO MY BELOVED PARENTS

ACKNOWLEDGEMENT

All praises to the Almighty and Merciful God Who enabled me to complete the thesis for the degree of Masters of Science (MS) in Food Processing and Preservation.

I feel a proud pleasure and privilege to express my deepest sense of gratitude to my honorable supervisor, Dr. Maruf Ahmed, Associate Professor and Chairman, Department of Food Processing and Preservation, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for his valuable guidance, undaunted inspiration, continuous suggestions and instructions, throughout the entire period of this research as well as preparing this manuscript.

I also express my respect to the honorable co-supervisor N.H.M Rubel Mozumder, Assistant Professor, Department of Food Science and Nutrition, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for his co-operation, guidance, valuable suggestion and encouragement during the research work.

Special thanks are due to Dr. M. Harun-or-Rashid, Senior Scientific Officer, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh for his generous help in conducting the experiment and providing lab facilities in BINA.

Thankful appreciation is due to Md. Kabir Hossain, Lab attendant, Department of Food Processing and Preservation, Hajee Mohammad Danesh Science and Technology University, Dinajpur, who helped me in the laboratory during the study.

I also thankful to Amaena Begam, MLSS, Department of Food Processing and Preservation, Hajee Mohammad Danesh Science and Technology University, Dinajpur, for her co-operation in respect of different aspects.

Special thanks to my friends Alo Moni, Abu Saeid, Debashis Kumer Dutta Roy, Moriom Akter and Tanny Saha for their co-operation, inspirations for the successful completion of my thesis.

Finally, I express my ever gratefulness and indebtedness to my parents for their great sacrifice, endless prayer, blessing and support that delight the way of my higher education.

December, 2015 *The author*

ABSTRACT

Bromelain is a complex mixture of proteases and non-proteases components found in pineapple (*Ananas cosmosus*). The objective of this study was to find out a suitable extraction and purification process. Then characterize the pulp and stem bromelain enzyme and compare the effectiveness of those enzyme as anti-browning agent with commercial anti-browning agents. Both the stem and pulp extracted with sodium citrate buffer had higher protein content 1.178 and 0.332 mg/ml, respectively. Enzyme activity of stem bromelain was higher in extracted with sodium citrate buffer (0.0031 U/ml) and enzyme activity of pulp bromelain was higher in distilled water extraction (0.0085 U/ml). Bromelain with ethanol precipitation had higher protein content and enzyme activity than ammonium sulfate. The results showed that ammonium sulfate precipitation was achieved higher purification fold (1.76) and activity yield 27.53%. Bromelain showed a maximum activity at pH 8.5 and at 50°C temperature for 30 min. Stem bromelain with 1% concentration had better anti-browning activity than ascorbic acid and citric acid. There was no significant difference between ammonium sulfate and ethanol precipitation in terms of purification fold and yield. Although ethanol had achieved a lower purification fold but it had higher protein content and enzyme activity and it seems to be more suitable for bromelain recovery, based on process time, low ionic strength and low cost.

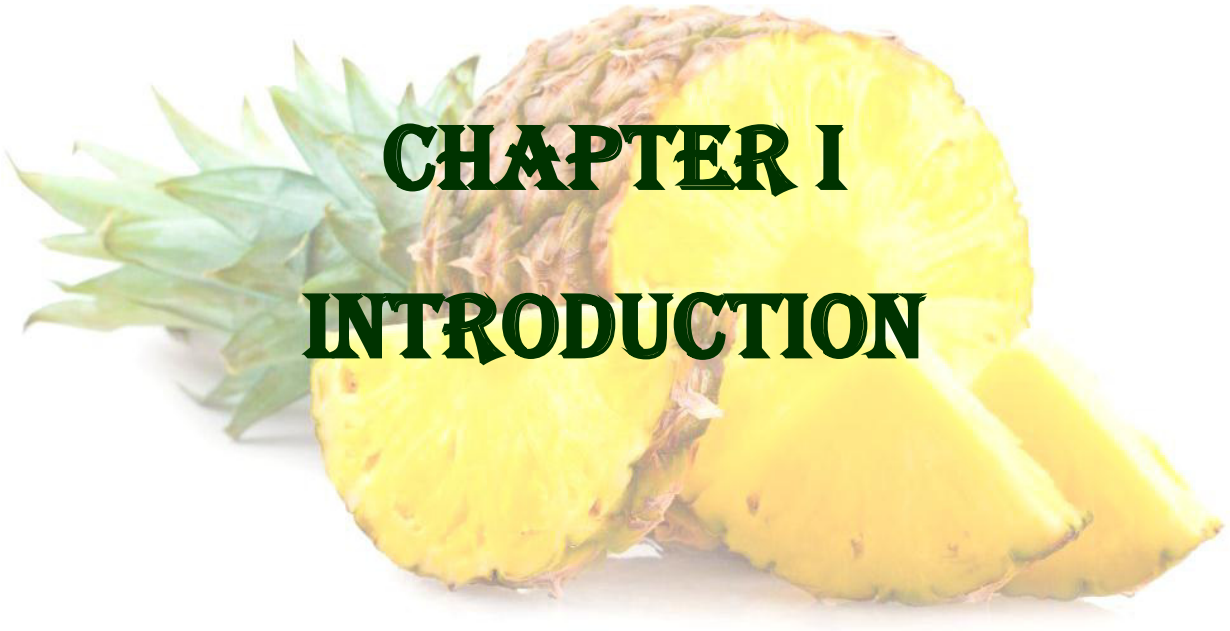
CONTENTS

CONTENT (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES



CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

In recent year, enzymes production has been increased due to their numerous industrial and therapeutic applications. Proteases are the most significant of all industrial enzymes which represent about 60% of all commercial enzymes worldwide (Leary *et al*., 2009). In the field of biotechnology and medicine, plant proteases have been gaining unique attention due to their exploitable properties. The most recognized plant proteases with greater commercial values are papain from *Carica papaya*, ficin from *Ficus spp*. and bromelain from pineapple plant (*Ananas comosus*) (Dubey *et al*., 2007).

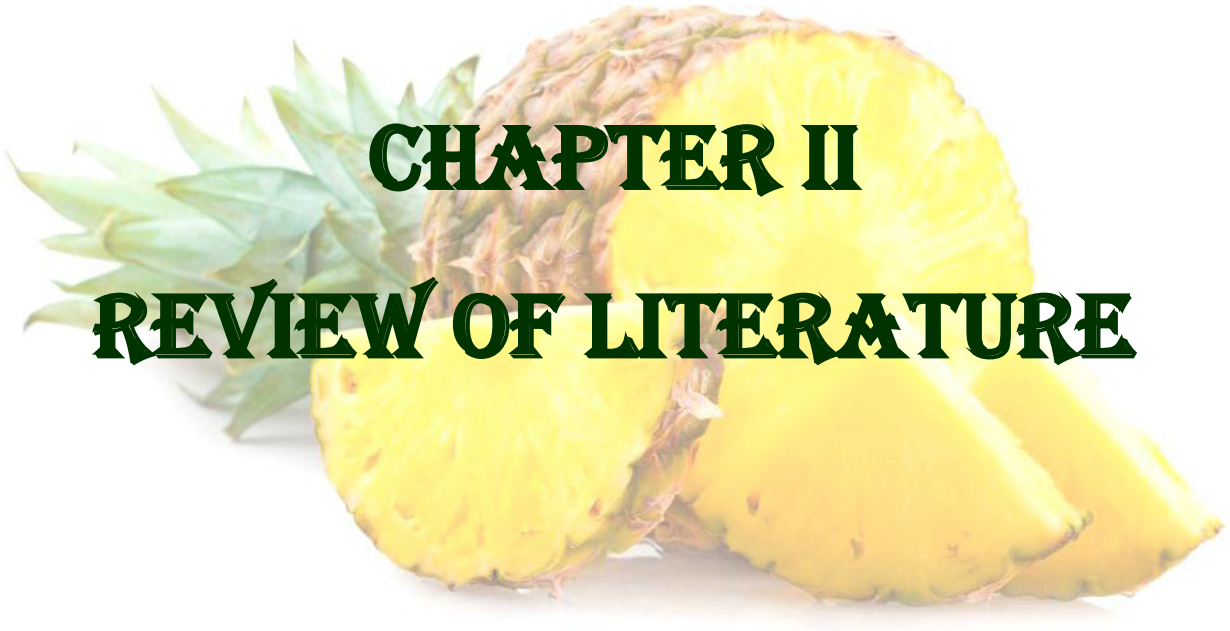
Pineapple (*Ananas cosmosus*) belongs to the Bromeliaceace family and is widely grown in Cuba, Hawaii, West Indies, Philippines, Malaysia, Brazil, Mexico and India. The production of pineapple also in Bangladesh is increasing day by day. During 2007-08, total production of pineapple in country was 210.3 thousand metric tons from which was increased to 218.6 thousands metric tons during 2010-11 (BBS, 2011). The quality of pineapple fruits is evaluated by their physical features externally, like as peel colour, size and shape of the fruit. It also internally conferred by broad range of constituents, especially by the high levels of sugars and proteolytic enzymes, particularly bromelain (Abilio *et al*., 2009). Bromelain is a crude aqueous extract of protein digesting enzymes from stems and immature fruits of pineapple (Hale *et al*., 2005a; Hebbar *et al*., 2008) and constitutes with an unusually complex mixture of different thiol endopeptidase, being able to break peptide bonds, separating proteins and amino acids (Srinath *et al*., 2012). Usually Stem bromelain (EC 3.4.22.32) and fruit bromelain (EC 3.4.22.33) are the two main enzymes extracted from stem and fruit respectively (Babu *et al*., 2008). Bromelain is also present in pineapple wastes such as core, peel, crown & leaves in relatively smaller quantities as compared to those in the stem (Hebbar *et al*., 2008). Stability of the bromelain enzyme is influenced by numerous factors such as pH, temperature and time. It is stable at pH 3.0-6.5 and the effective temperature range is 40°C-65°C with the optimum being 50°C-60°C. Calcium chloride, cysteine, bisulphate salt and benzoate can activate bromelain. It is inhibited by mercury ion, silver ion, copper ion, antitrypsin, statin A and B, iodoacetate (Gautam *et al*., 2010). The isoelectric point of stem bromelain is 9.5 and fruit bromelain is 4.6 (Rabelo *et al*., 2004). Bromelain is made up of 212 amino acids and molecular weight is 33 kDa (Gautam *et al*., 2010).

Previous works have been used ethanol (Paulo *et al*., 2012), ammonium sulfate (Srinath *et al*., 2012) and cold distilled water for isolation and purification of bromelain. Various modern technique like affinity chromatography, ion exchange, gel filtration, aqueous two phase extraction and extraction by using reversed micelle are also be used to extraction and purification of various enzyme from different sources (Desai, 2000). But these techniques are complicated and production cost is so high (Hans *et al*., 2011; Hutti-Kaul *et al*., 2003). So, ammonium sulfate precipitation and ethanol precipitation are frequently used due to simple equipment requirements, low energy needs and easy scale up. Ethanol precipitation is a promising technique and also could be recycled in the final process by simple distillation (Golunski *et al*., 2011).

Bromelain has numerous applications in the food industry as well as in pharmaceutical industry. It has been used for meat tenderization, solubilization of grain proteins, stabilization of beer, baking cookies, production of protein hydrolysates, softening skins in leather and textiles (Walsh, 2002). It is also used as a phytomedical compound and demonstrates, in *vitro* and in *vivo*, anti-inflammatory, antiedemic (Hale *et al*., 2005b; Braun *et al*., 2005), anti-thrombotic (Ley *et al*., 2011), anti-cancer and anti-metastatic properties (Chobotova *et al*., 2010; Bhui *et al*., 2009), in addition to strong immunogenicity (Hale *et al*., 2002) and wound healing in burns (Rosenberg *et al*., 2009). Bromelain also act as an anti- browning agents (Tochi *et al*., 2009). Using anti-browning agents such as sulphites is a way to the prevention of enzymatic browning in fruit juices but it has health effect (Anon, 1991). So, the most attractive way to inhibit browning would be by natural methods (Laurila *et al.,* 2002). Pineapple fruit proteases-bromelain is some of the proposed natural agents that have inhibitory effect on poly phenol oxidase (PPO) (Tochi *et al*., 2009). However, the inhibitory effect of stem bromelain as anti-browning agent was found to be minimal (Tochi *et al*., 2009). Therefore, in point of economical and technical aspects it is necessary to find out the suitable extraction and purification process for bromelain enzyme. A few information is available about effectiveness of extraction and purification of bromelain enzyme from stem and pulp of pineapple using ethanol and ammonium sulfate. To my knowledge, no one compare the effectiveness of stem and pulp bromelain enzyme as an anti-browning agent in apple juice.

Therefore, the purposes of the present investigation were to:-

- Extract the bromelain enzyme from pulp and stem of pineapple using distilled water, citric phosphate buffer and sodium citrate buffer.
- Investigate the effects of various purification processes such as ethanol and ammonium sulfate precipitation on bromelain enzyme from stem and pulp of pineapple.
- Characterize the pulp and stem bromelain enzyme and compare the effectiveness of the both enzyme as anti-browning agent on apple juice.



CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 Origin and production of pineapple in worldwide

Cultivated pineapple (*Ananas comosus* (L.) Merrill, which is now called *Ananas comosus* var *comosus*) belongs to the family *Bromeliaceae*. The pineapple is the leading edible member of *Bromeliaceae* which embraces about 2,000 species, mostly epiphytic and many strikingly ornamental. Pineapple is now the third most important commercial tropical fruit crop in the world. The processing of pineapple has made the fruit well known even in the temperate parts of the world. The following graph shows the top producing countries of the world.

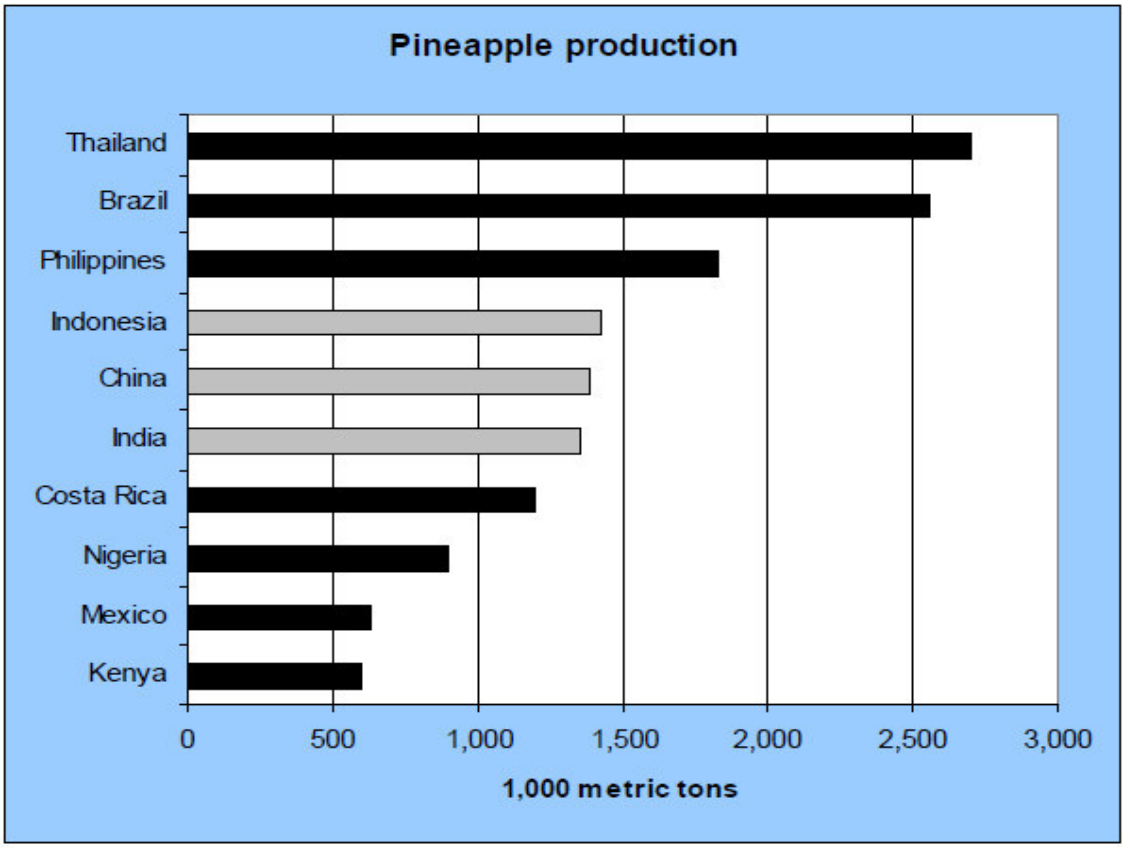


Figure 2.1 Top producers of pineapples in 2006 (USDA, Economic Research Service, 2009)

2.2 Pineapple production in Bangladesh

Pineapple is an important fruit crops among all other minor crops in Bangladesh. Pineapples abundantly grow in many districts, namely Tangail, Rangamati, Chittagong, Bandarban, Dhaka, Mymensingh, Khagrachari, Sylhet and Moulvibazar. At least ninety varieties of pineapple are cultivated in the world. In Bangladesh, however, three varieties of pineapple are mostly grown such as Giant Kew, Honey Queen and Ghurasal. Among all the fruits produced in the country, pineapple ranks 4th in terms of total cropping area and production. During 2007-08, total production of pineapple in the country was 210.3 thousand metric tons from which was increased to 218.6 thousands metric tons during 2010-11 (BBS, 2011). The total cultivation area and annual production of Pineapple in Bangladesh during last eight years are shown in Table 2.1:

Table 2.1 Acreage, Production & Yield rate of Pineapple during the year 2003-04 to 2011-12

Year	Acreage (in '000')	Production (in '000' M. Tons)	Yield rate (M. Tons) per acre
2003-04	41.5	212.8	5.1
2004-05	45.7	234.9	5.1
2005-06	42.3	253.8	6.0
2006-07	41.9	238.4	5.7
2007-08	39.4	210.3	5.3
2008-09	39.0	229.1	5.9
2009-10	39.6	234.5	5.9
2010-11	37.0	218.6	5.9
2011-12	34.5	180.9	5.2

Source: Yearbook of Agricultural Statistics of Bangladesh (2008, 2011 & 2012, BBS).

2.3 Nutritional value of pineapple

Pineapple (*Ananus cosmosus*, Bromeliaceae) is a wonderful tropical fruit having exceptional juiciness, vibrant tropical flavour and immense health benefits. Pineapple contains considerable calcium, potassium, fibre, and vitamin C. It is also good source of vitamin B1, vitamin B6, copper and dietary fibre. Pineapples are highly rich in the enzyme bromelain for which pineapples are great to eat as a digestion aid and also why pineapple juice has an anti-inflammatory effect (Joy, 2010). The nutritional value of pineapple given in the Table 2.3.

Table 2.2: Nutritional value of pineapple per 100g

Nutrients	Amount
Protein	0.54g
Carbohydrate	13.7g
Diatery fibre	1.4g
Energy	52 calories
Vitamin A	130 I.U
Iron	0.28mg
Calcium	16mg
Magnesium	12mg
Potasium	150mg
Phosphorus	11mg
Vitamin B_1	0.079mg
Vitamin B_2	0.031mg
Vitamin B_3	0.489mg
Vitamin B_6	0.110mg
Vitamin C	24mg
Zinc	0.10mg

Source : Joy (2010)

2.4 Enzyme

Enzyme are macromolecular biological catalysts. Enzymes accelerate or catalyze chemical reactions. The molecule at the beginning of the process are called substrates and the enzyme converts these into different molecule. Almost all metabolic processes in the cell need enzymes in order to occur at rates fast enuogh to sustain life. The set of enzyme made in a cell determines which metabolic pathways occur in that cell. Enzymes are known to catalyze more than 5,000 biochemical reaction type. Most enzymes are proteins,although a few are RNA molecule. Enzymes' specificity comes from their unique three-dimensional structures. Enzyme activity can be affected by other molecules: inhibitors are molecules that decrease enzyme activity and activators are molecules that increase activity. Many drugs and poisons are enzyme inhibitors. Some enzymes are used commercially, for example, in the synthesis of antibiotics. Some household products use enzymes to speed up chemical reactions: enzymes in biological washing powders break down protein, starch or fat stains on clothes and enzymes in meat tenderizer break down proteins into smaller molecules, making the meat easier to chew (Abraham & Sangeetha, 2006).

2.5 Enzyme present in pineapple

Plant proteases have been gaining unique attention in the field of biotechnology and medicine due to their exploitable properties. The most recognized plant proteases with greater commercial values are papain from *Carica papaya*, ficin from *Ficus spp*. and bromelain from pineapple plant (*Ananas comosus*) (Dubey *et al*., 2007). Bromelain is one of the protease enzymes found in the pineapple plant (*Ananas comosus*). Stem bromelain (SBM) (EC 3.4.22.32) is the major protease present in extracts of pineapple stem while fruit bromelain (FBM) (EC 3.4.22.3) is the major enzyme fraction present in the juice of the pineapple fruit (Kelly, 1996).

2.6 Bromelain enzyme from pineapple

Bromelain (EC 3.4.22.32) is a mixture of cysteine proteases and non-proteases components. Proteases constitute the major components of bromelain and include stem bromelain (80%), fruit bromelain (10%), and ananain (5%). Among non-protease components are phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates (Chobotova *et al*., 2009). Bromelain proteases are usually unstable and sensitive under stress conditions, such as elevated temperature, acidity, organic solvents and chemicals (Xue *et al*., 2010). The biochemical properties of bromelain have been studied comprehensively in order to enhance its various industrial and therapeutic applications. Bromelain activity can be determined under optimal pH and temperature conditions, with various substrates including casein, gelatin and synthetic substrates. SBM was found have molecular weight range (26-37), pH optimum range (6-7) and optimum temperature range of 50-60 º C (Kumar *et al*., 2011; Xue *et al*., 2010; Liang *et al*., 2011). On the other hand, FBM had been shown to have molecular weight range (24.5-32.5), pH optimum range (3-8) and optimum temperature range of 37-70 º C (Corzo *et al*., 2011; Ketnawa *et al.*, 2010, 2011a and 2011b, Kumar *et al*., 2011; Jutamongkon and Charoenrein, 2010; Liang *et al.,* 2011).There are numerous chemical methods that have been employed to modify the activity and stability of enzymes. These include cross-linking of the enzyme with chemicals, modification of the amino acid side chains of enzymes, the introduction of hydrophilic and hydrophobic groups, etc. (Xue *et al*., 2010).

2.7 Method of bromelain extraction and purification

Heinecke and Gotner (1957) reported that bromelain concentration was very high in pineapple stems and hence leading to its extraction and utilization as phytomedicinal compound. Unlike the pineapple fruit which is normally used as food, the stems are waste by-product and thus, very cheap source of bromelain (Tochi *et al*., 2008). Apart from the stem and fruit, it had also been discovered that other parts of the pineapple plant contain bromelain. For instance, bromelain was extracted from the peel, core, stem and crown of wastes from two pineapple cultivars (Ketnawa *et al*., 2012). The highest protein contents and proteolytic activity were obtained from the extracts of the crowns while lowest values were recorded from the stem of both cultivars. The commercially available product is most often made from stem bromelain, whereby the extract is removed from cooled pineapple juice through centrifugation, ultra filtration and lyophilization (Corzo *et al*., 2011). After the extraction processes, the crude extract containing the enzyme of interest is then subjected to various purification operations in order to remove contaminants that may interfere with the application of bromelain as well as to increase the specific activity of the enzyme. In the case of industrial enzymes, yield of the enzyme recovery rather than purity is the major concern. However, for specialty enzymes, purity is the main priority over the yield (Illanes, 2008).

The conventional procedures employed for bromelain extraction and purification are often tedious, and frequently result in low yields of the enzyme. Thus, novel purification techniques are highly needed to enhance the overall enzyme yields and at the same time to lessen the number of steps involved in the downstream processing of bromelain.

Considering the various applications of bromelain, some novel purification strategies have been recently employed for the enzyme extraction and purification. These comprise of aqueous two phase systems (Babu *et al*., 2008; Ketnawa *et al*., 2010; Ketnawa *et al*., 2011a, Ketnawa *et al*., 2011b; Ferreira *et al*., 2011; Rabelo *et al*., 2004), reversed micellar systems (Hebbar *et al*., 2008; Hebbar *et al*., 2012; Kumar *et al*.,2011) membrane processes (Doko *et al*., 2005) precipitation (Doko *et al*., 2005; Silvestre *et al*., 2011; Gautam, *et al*., 2010) and different chromatographic techniques (Gautam *et al*., 2010; Yin *et al*., 2011; Devakate *et al*., 2009).

Some of the methods are discussed below:

Isolation using reverse micellar system

Reverse micellar system (RMS) is a promising liquid-liquid extraction technique for downstream processing of biomolecules from dilute solutions. In addition, RMS has been greatly used for the enhancement of protein separations. It had been shown to provide outstanding conditions for protein separation. Some of the advantages obtained in using the RMS include higher sample loading capacity, simple operation and continuous preparation (Dong *et al*., 1999). There are many reports from the literature on the use on RMS for bromelain extraction from pineapple plant. For instance, reverse micellar systems were employed for the extraction and purification of bromelain from crude aqueous extract of pineapple wastes (core, peel, crown and extended stem) (Hebbar *et al*., 2008). In addition, RMS was reported to have been used for bromelain extraction from pineapple juice (Hemavathi *et al*., 2007). Besides, an attempt was made on the scale-up studies for phase transfer mode of reverse micellar extraction for the separation and purification of bromelain from pineapple waste (Hebbar *et al*., 2011). Similarly, an integrated approach was used for coupling RMS with ultrafiltration to enhance the overall efficiency of extraction and purification of bromelain from aqueous extract of pineapple core (Hebbar *et al*., 2012). Equally, optimization of the extraction of bromelain from pineapple fruit by reversed micelles had been reported (Fileti *et al*., 2007). In addition to this, the batch and continuous extraction of bromelain from pineapple juice by reversed micelles under optimized conditions was also studied (Fileti *et al*., 2009).

Bromelain extraction using aqueous two phase system (ATPS)

The aqueous two-phase systems have been widely used in bio separation. It is involved in the partitioning of proteins in aqueous two phase systems, depending on their physico-chemical properties. The great advantages of aqueous two phase extraction are based on volume reduction, rapid separations, high capacity, and mildness of the process. The technique can be used in the early purification stages and is relatively easier to scale-up. The ATPS technique is highly suitable for the extraction and purification of proteins that may prove difficult to purify with other existing techniques (Gupta *et al*., 1999). Many reports from the literature had also indicated the application of ATPS for bromelain separation and purification. For instance, ATPS was employed for the separation and purification of mixture of bromelain and polyphenol oxidase from the pineapple (Babu *et*

al., 2008). In addition, an attempt had been made on optimizing the extraction of bromelain from pineapple peel using ATPS (Ketnawa *et al*., 2010). In another study, the extraction of bromelain from pineapple peels using ATPS was investigated (Ketnawa *et al*., 2011a). In the same vein, Ferreira *et al*. (2011) had explored the use of thermodynamic equilibrium and applying of ATPS on the purification of bromelain extracted from pineapple.

Chromatography techniques

Chromatographic techniques have been widely and continuously developed for separation of bromelain from pineapple. Such techniques include ion exchange chromatography, affinity membrane chromatography, gel filtration chromatography and capillary electro chromatography (Babu *et al*., 2008; Devakate *et al*., 2009; Chen and Huang, 2004; Chen *et al*., 2008). All of these methods have been used successfully to separate bromelain from pineapple. For instance, a comparative study on the extraction, purification and evaluation of bromelain from stem and fruit of pineapple plant was carried out (Gautam *et al*., 2010). The purification of the enzyme preparations was achieved by the combinations of centrifugation, salt precipitation technique, dialysis and then ion exchange chromatography. The results revealed that chromatographic technique had greatly maintained the structural integrity of the purified bromelain. The use of precipitation and chromatographic techniques for the purification of the bromelain from pineapple fruit yielded purification fold almost 3.3 times of that from precipitation (Devakate *et al*., 2009).

Ammonium sulphate precipitation

Ammonium sulphate precipitation has been widely used to precipitate proteins in a partially purified form (Saxena *et al*., 2007). Ammonium sulphate is the most commonly used salt as it is cheap and sufficiently soluble. Ammonium sulphate concentration is usually quoted as percent saturation, assuming that the extract will dissolve the same amount of ammonium sulphate as pure water (Harris, 1989). Brovko *et al*. (1998) had used ammonium sulphate to separate proteins from phenols in cereal leaf extract. Barros *et al*. (2001) found that precipitation with ammonium sulfate is an effective way to produce substantial amounts of active proteases from the flowers of *C. cardunculus*. The bromelain in fruit of pineapple plant was 2.81-fold pure at 40-60% saturation level using ammonium sulphate precipitation (Devakate *et al*., 2009).

2.8 Application of bromelain

Bromelain has numerous applications in the food industry as well as in pharmaceutical industry. It has been used for meat tenderization, solubilization of grain proteins, stabilization of beer, baking cookies, production of protein hydrolysates, softening skins in leather & textiles (Walsh G., 2002). It is used as drugs for oral systemic treatment of inflammatory, blood-coagulation related & malignant diseases. It demonstrated antiedemateous, antithrombotic, anti-inflammatory and fibrinolytic activities (Maurer, 2001).

2.9 Health benefits of bromelain enzyme

Anti - inflammatory agent:

Botanicals such as *Ananas comosus* (Pineapple) and their extracts (bromelain) have been used clinically as anti-inflammatory agents in rheumatoid arthritis, soft tissue injuries, colonic inflammation, chronic pain and asthma (Maurer, 2001; Hale *et al.,* 2005b; Jaber, 2002) and are currently in use as anti-inflammatory agents (Ammon, 2002; Lemay *et al.,* 2004; Darshan and Doreswamy, 2004).

Bromelain as an anti-tumour agent:

Pharmacological agents with modulation of anti-inflammatory, proteolytic, platelet aggregation inhibition and prostaglandin synthesis have been considered to be beneficial in regulating tumour growth and its metastasis. Bromelain, with similar regulating actions, has shown protective properties on tumour cell growth retardation and lung metastasis (Tochi *et al*., 2008).

Bromelain promotes debridement of burns:

Burns are characterized by formation of an eschar, which is made up of burned and traumatized tissue. The eschar not only hinders accurate diagnosis of the burn's depth but also serves as a medium for bacterial growth and therefore a source of infection, contamination and sepsis of the injury and to the neighbouring originally undamaged tissues (Rosenberg *et al.,* 2004). Topical bromelain (35% in a lipid base) was reported to achieve complete debridement on experimental burns in rats in about 2 days, as compared with collagenase, which required about 10 days, with no side effects or damage to adjacent burned tissue (Klaue *et al.,* 1979).

Effects of bromelain on diarrhoea:

Diarrhoea is a major cause of illness and death in children and young animals (Roselli *et al.,* 2007). Bromelain has been demonstrated to have antidiarrhoea activity (Thomson *et al.,* 2001).

Bromelain gives strong immunogenicity:

Bromelain has been shown to remove T-cell CD44 molecules from lymphocytes among other bromelain sensitive molecules (Hale *et al.,* 2002). Roep *et al.* (2002) reported that protease treatment reduced expression of cell surface receptors on T-cells and antigen-presenting cells. Roep *et al.* (2002) also suggested that the generation of soluble forms of adhesion molecules by proteolytic cleavage could act as an additional benefit for immonomudulatory function of protease treatment. However, they noted that the quality of immune activation plays an important role during chronic autoimmunity. Earlier, animal models for rheumatoid arthritis and Type 1 diabetes protease treatment prevented or delayed the onset of these diseases (Wiest-Ladenburger *et al*., 1997). Hale *et al*. (2004) unexpectedly found bromelain to exhibit strong immunogenicity following oral dosing. In further studies following this phenomenon, Hale *et al.* (2006) reported that repeated exposure was necessary for development of anti-bromelain antibodies, with exposure period ranging from 3 to 6 weeks on a dose dependent manner.

Bromelain application in dermatological disorders:

Bromelain among other fruit extracts from apricots, apples, peaches, pears, papayas, pomegranates, cherries, kiwis, tangerines and oranges have been described to play an important role in treating dermatological disorders (Murad, 2003). Ozlen, (1995) has disclosed a cosmetic composition containing at least one alpha-hydroxy acid, salicylic acid and at least one digestive enzyme derived from fruit. Preferably the digestive enzyme was a mixture of bromelain and papain. Bromelain was disclosed as being typically obtained from pineapple and papain was disclosed as being typically obtained from dry papaya latex. The compositions were allegedly useful for treating various cosmetic conditions or dermatological disorders, such as lack of adequate skin firmness, wrinkles and dry skin.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Immature fruits of honey queen variety of pineapple and stem (shown in Figure 3.1) were collected from research field of Agro-forestry Department, Hajee Mohammad Danesh Science and Technology University, Dinajpur. Coomassie brilliant blue, casein, bovine serum albumin (BSA) and others reagents were purchased from local market. All the buffers and reagents were of analytical grade.

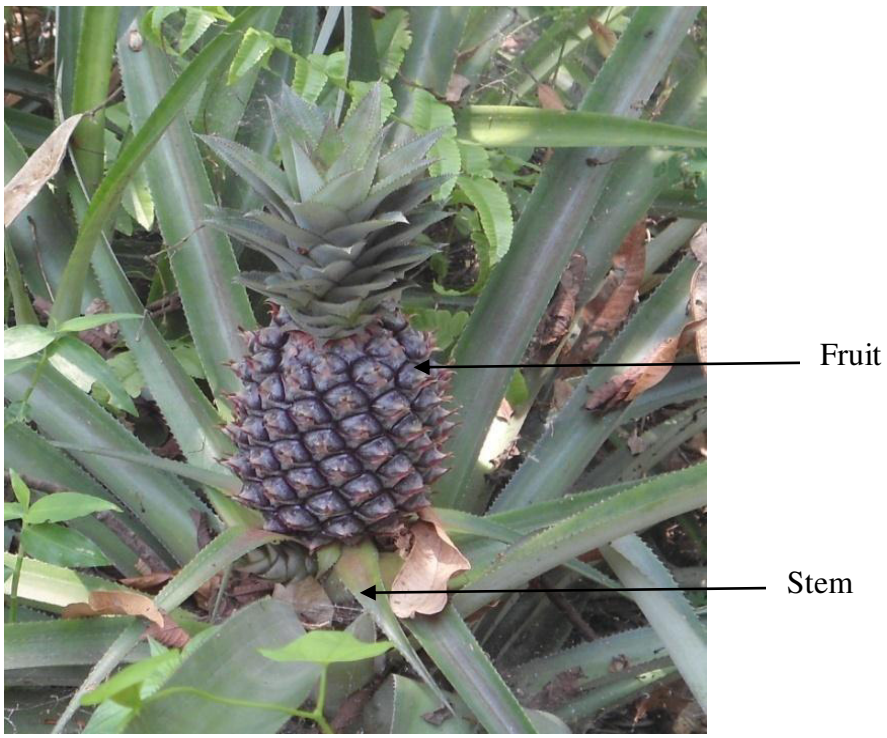


Figure 3.1 Honey queen pineapple

3.2 Preparation of crude extract from pineapple pulp and stem

3.2.1 Extraction by distilled water

Extraction by distilled water was done by following Paulo *et al*. (2012) method with some modification. The stem was separated by knife from pineapple fruit and washed with water to remove soil and dirt. After that pineapple fruits were peeled with a stainless steel knife to remove outer layer. Then cut into small pieces and crushed in a laboratory

blender (KA-735, konka, China) with distilled water (1:1 w/w) to get approximately 115 ml of juice. The juice was filtered through a muslin cloth to remove the fibrous material. The filtered juice was centrifuged (Biofuge 15R, USA) at 14000 rpm for 15 min at 4°C to remove pellet. The clear supernatant obtained was used as crude extract and stored at -20°C for further uses.

3.2.2 Extraction by sodium citrate buffer

Srinath *et al*. (2012) method was used for extraction of bromelain by sodium citrate buffer. The stem and small size of peeled pineapple were dried at 55°C at several hours using a cabinet drier. Ten gram of peeled pineapple and stem were blended in a blender (KA-735, konka, China) by adding 90 ml of pre-cooled sodium citrate buffer and filtered using a muslin cloth to remove fiber. After that it was centrifuged (Biofuge 15R, USA) at 5000 rpm for 15 min. The pellet was discarded and the supernatant used as crude protease was stored at -20°C for further uses.

3.2.3 Extraction by citric phosphate buffer

Bromelain extraction using citric phosphate buffer was followed according to Chaiwut *et al.* (2006) with some modification. Ten gram of the dried samples were soaked in 90 ml of 0.1 M citric phosphate buffer (pH 6.5) for 10 min and then filtered. The filtrate was centrifuged (Biofuge 15R, USA) with 14000 rpm at 4°C for 15 min and supernatant was collected and finally stored at -20°C for further uses.

3.3 Purification of crude protease

Crude protease which was collected from distilled water, sodium citrate buffer and citric phosphate buffer extraction, was taken for purification by using ammonium sulfate precipitation and ethanol precipitation.

3.3.1 Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed by the method as described by Srinath *et al*. (2012) with some modification. 15 ml of crude stem and pulp were mixed along with nine gram of ammonium sulfate to get 60% saturation under constant stirring using a magnetic stirrer for 10 min. The salt enriched solution was then subjected to centrifugation (Biofuge15R, USA) at 14000 rpm for 15 min and the precipitate was collected. After that, precipitate was dissolved with distilled water, sodium citrate buffer

and citric phosphate buffer respectively and then the solution was poured into dialysis tube and sealed. Then the dialysis tube was soaked in distilled water and kept overnight at 4°C. Distilled water was changed subsequently over the period of 12 hours.

3.3.2 Ethanol precipitation

Bromelain precipitation was performed according to methodology as described by Englard and Seifter (1990) with some modification. 15 ml of crude stem and pulp were taken and 98% cold ethanol was added drop wise until concentration of 70% (w/w) was reached. The solution was then centrifuged (Biofuge 15R, USA) at 14000 rpm for 15 min at 4°C and the resulting pellet was suspended in 1.0 ml of phosphate buffer (0.03M, pH-7.0) and stored at -8°C for further uses.

3.4 Determination of protein content

Protein content in the samples was measured by spectrophotometer according to Bradford method (Bradford MM, 1976) with little modification. 500 µl sample was taken into a falcon tube and diluted to 5 ml distilled water. Then 5 ml of Bradford reagent was added and mixed by vortex (KMC-1300V, Korea) for few minutes. The concentration of protein in the solution was determined from absorbance at 595 nm (T60 U, PG instrument, United Kingdom). Protein content was calculated on the basis of calibration curves of bovine serum albumin and expressed as mg/ml.

3.5 Enzyme activity assay

Enzyme activity was determined according to the Liggieri *et al.* (2009) with slight modification. Purified enzyme (0.1 ml) was mixed with 1.1 ml of 1% casein containing 0.1 M Tris–HCl buffer (pH 8.5). Then incubated at 42°C for 2 min and 1.8 ml of 5% trichloroacetic acid (TCA) was then added to stop the reaction. After that, centrifuged (MF-300, Korea) at 3000 rpm for 30 min and the absorbance of the supernatant were measured at 280 nm. The enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions (Manjunath *et al*., 2014).

3.6 Determination of specific activity, purification fold and yield

After determining protein content and enzyme activity, specific activity, purification fold and yield were determined by following formula

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity}}{\text{Protein content}}$$

$$\text{Purification fold} = \left(\frac{\text{Specific enzyme activity of purified bromelain}}{\text{Specific enzyme activity of crude extract}}\right)$$

$$\text{Yield (\%)} = \left(\frac{\text{Total enzyme activity of purified bromelain}}{\text{Total enzyme activity of crude extract}} \times 100\right)$$

3.7 Characterization of bromelain enzyme

3.7.1 pH stability of bromelain enzyme

The reaction mixture was prepared by mixing of 0.1 ml of the purified enzyme with 1.1 ml of 1% casein containing different pH buffers. The buffers of 0.1M Tris-HCl buffer (8.5 and 10), buffer 4 and buffer 7 were used. The reaction was carried out at room temperature. Then incubated at 42°C and stopped the reaction after 2 min by the addition of 1.8 ml of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 3000 rpm for 30 min and the absorbance of the supernatant was measured at 280 nm. The enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions.

3.7.2 Thermal stability of bromelain enzyme

The purified bomelain (0.1ml) was first incubated at 40°C, 50°C, 60°C, 70°C and 80°C for 30 min respectively. Then reaction mixture was prepared by mixing with 1.1 ml of 1% casein containing 0.1 M Tris–HCl buffer (pH 8.5). The reaction was carried out at room temperature. Then incubated at 42°C and stopped the reaction 2 min later by the addition of 1.8 ml of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 3000 rpm for 30 min and the absorbance of the supernatant measured at 280 nm. The enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions.

3.8 Application of bromelain enzyme on apple juice as natural anti-browning agents compare to commercial anti-browning agents

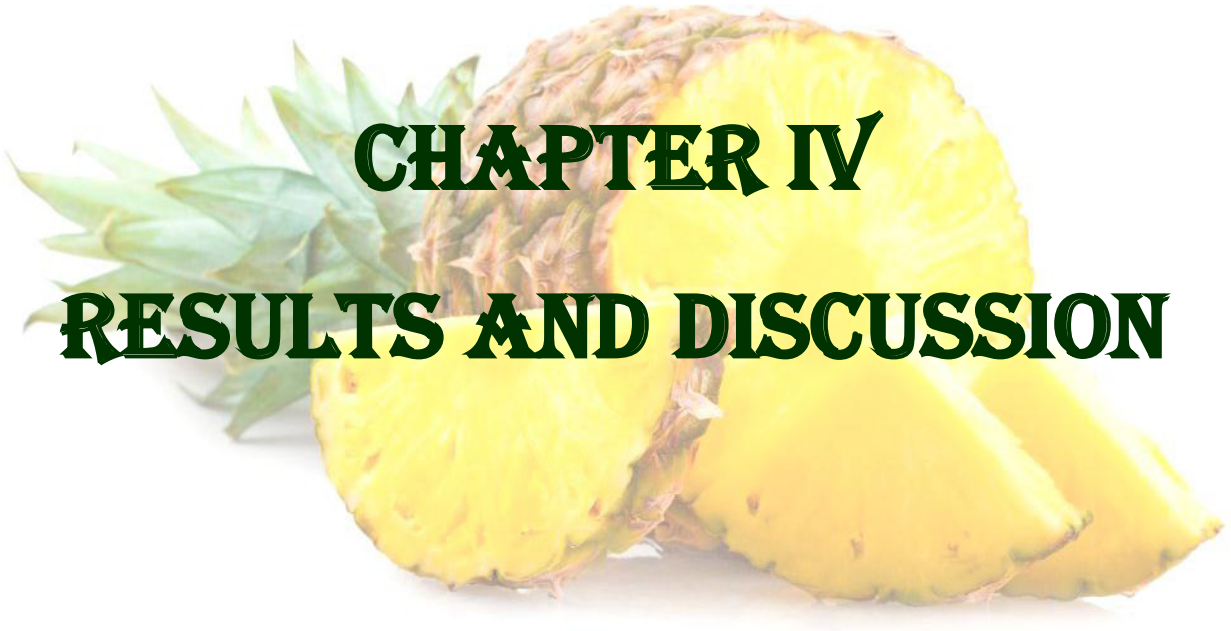
Fresh apples were purchased from local market and were washed, peeled, de-stoned and juiced using kitchen blender (KA-735, konka, China). 10 ml of juice samples were transferred into beakers containing anti-browning agents (L-cysteine, citric acid, ascorbic acid, pulp bromelain and stem bromelain) and stirred with a vortex for 10 sec (Ozoglu *et al*., 2002). The concentration of the commercial anti-browning agents was 0.1%, while concentrations of 0.1% and 1% were chosen for stem and pulp bromelain enzyme, respectively (Srinath *et al*., 2012). Without any anti-browning agent was used as control. The degree of browning was calculated by following formula (Lee *et al*., 2000)

$$\Delta L = (L^* \text{ value at initial} - L^* \text{ value at given time})$$

Where, L^*values were measure using a spectrophotometer (Minolta Camera, Tokyo, Japan).

3.9 Statistical Analysis

Each experiment was done in duplicate. The results were expressed as mean ± standard deviation and were analyzed by R (version 2.13.1). Significant differences between the means were determined by Duncan's Multiple Range test. $P \leq 0.05$ was considered as a level of significance.



CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Protein content, enzyme activity and specific activity of crude extract

Table 4.1 shows the effects of distilled water, citric phosphate buffer and sodium citrate buffer on protein content, enzyme activity and specific activity of bromelain enzyme from crude extract of pineapple stem and pulp. It was observed that the protein content of crude extract of pulp and stem ranged from 0.151- 0.332 mg/ml and 0.182-1.178 mg/ml, respectively. Protein content was significantly highest in sodium citrate buffer extract whereas in distilled water extraction recorded lowest protein content for both samples. This variation might be difference in protease composition between pulp and stem. These results were lower than pineapple pulp (0.871 mg/ml) extracted by sodium citrate buffer obtained by Srinath *et al*. (2012) and higher than that of stem and bark of ripe pineapple (0.23 mg/ml) extracted with deionised water (Paulo *et al*., 2012). This variation may be due to difference in source material used to extract enzyme. The enzyme activity of crude extracts was found 0.0040 - 0.0085 U/ml for pulp whereas for stem it was 0.0020- 0.0031 U/ml. Significantly maximum enzyme activity was observed at sodium citrate buffer (0.0031 U/ml) for stem, while maximum enzyme activity was found to be at distilled water (0.0085 U/ml) for pulp. These results opposed to Paulo *et al*. (2012) and Martins *et al*. (2014) who got enzyme activity of crude extract 2.86 U/ml and 16.25 U/ml, respectively. The highest specific activity of bromelain enzyme was found in stem (0.012 U/mg) and pulp (0.056 U/mg) extracted with citric phosphate buffer and distilled water, respectively. This might be due to the lower enzyme activity and higher protein concentration.

Table 4.1 Protein content (mg/ml), enzyme activity (U/ml) and specific activity (U/mg) of pulp and stem bromelain enzyme extracted by distilled water, citric phosphate buffer and sodium citrate buffer on bromelain extraction.

Samples	Extraction Methods	PC	EA	SA
Pulp	Distilled water	0.151 ± 0.004^{b}	0.0085 ± 0.0002^{a}	0.056 ± 0.0002^{a}
	Citric phosphate buffer	0.197 ± 0.001^{b}	0.0036 ± 0.0002^{b}	0.018 ± 0.0020^{b}
	Sodium citrate buffer	0.332 ± 0.008^{a}	0.0040 ± 0.0005^{b}	0.012 ± 0.0020^{b}
Stem	Distilled water	0.182 ± 0.004^{b}	0.0020 ± 0.0002^{b}	0.011 ± 0.0010^{a}
	Citric phosphate buffer	0.212 ± 0.005^{b}	0.0025 ± 0.0005^{ab}	0.012 ± 0.0030^{a}
	Sodium citrate buffer	1.178 ± 0.080^{a}	0.0031 ± 0.0006^{a}	0.003 ± 0.0003^{a}

PC = protein content, EA = enzyme activity, SA = Specific activity

Values are mean of duplicate analysis with standard deviation

a-b Means followed by different superscript in each coloum are significantly different among distilled water, citric phosphate buffer and sodium citrate buffer extraction ($p \leq 0.05$).

4.2 Ammonium sulfate and ethanol precipitation for bromelain purification

The protein content of pulp and stem bromelain ranged from 0.076-0.272 mg/ml (Table 4.2) and 0.121-0.499 mg/ml (Table 4.3) in ammonium sulfate precipitation, respectively and 0.136-0.302 mg/ml (Table 4.2) and 0.151-0.982 mg/ml (Table 4.3) in ethanol precipitation, respectively. Significantly highest protein content was observed at ethanol precipitation than ammonium sulfate precipitation. It might be due to low ionic strength of ethanol than ammonium sulfate. These values were higher than Martins *et al*. (2014), who reported 0.084 mg/ml protein in stem, bark and leaves after 30-70% ethanol precipitation and consistent with Paulo *et al*. (2012), who observed that 0.20 mg/ml protein remained in stem, bark of pineapple after 70% ethanol precipitation. The highest enzyme activity was found to be 0.0046 U/ml in pulp bromelain precipitated by ammonium sulfate and in ethanol it was 0.0048 U/ml extracted with distilled water. On the other hand, stem bromelain purified by ammonium sulfate and ethanol had highest enzyme activity in sodium citrate extraction. These results contrasted with Paulo *et al*. (2012) and Martins *et al*. (2014), who reported that enzyme activity after 70% ethanol precipitation was 2.77 U/ml and 15.96 U/ml, respectively. The specific activity was observed significantly higher in pulp (0.061 U/mg) and stem bromelain (0.015 U/mg) extracted by distilled water in ammonium sulfate precipitation (Table 4.2 and 4.3).A relatively much higher specific activity (189.5 U/mg and 13.85 U/mg) were reported by Paulo *et al*. (2012) and Martins *et al*. (2014) in the stem of the pineapple.

In case of ammonium sulfate precipitation, the purification fold and yield was ranged from 0.84- 1.09 and 7.48 - 11.14% for pulp bromelain, respectively (Table 4.2). The quantification results were consistent with the findings of Paulo *et al*. (2012) who found purification fold of 4.4 and 44% yield after bromelain precipitate with ammonium sulfate. Although highest purification fold was found in pulp extracted with distilled water (1.09) but activity yield was higher in pulp extracted with citric phosphate buffer (11.14 %) (Table 4.2).The highest purification fold was determined in stem extracted with sodium citrate buffer (1.76) and activity yield was highest in stem extracted with citric phosphate buffer (27.53%) (Table 4.3).These results were lower than fruit bromelain (2.97 fold) precipitated by ammonium sulfate obtained by Devakate *et al*. (2009).

In case of ethanol precipitation, the purification fold was ranged from 0.65- 0.91 and 1.13-1.22 in pulp and stem bromelain, respectively. The highest yield was 12.34% in pulp extracted with sodium citrate buffer and in case of stem, extracted with citric phosphate buffer was higher yield (14.14 %). The quantification results of this study were similar with the findings of Rabelo *et al*. (2004) who found the purification fold 1.25 purified with aqueous-two phase system. There was no significant difference between ammonium sulfate and ethanol precipitation in terms of purification fold and yield.

Table 4.2 Protein content (mg/ml), enzyme activity (U/ml), specific activity (U/mg), purification fold and yield (%) of pulp bromelain enzyme purified by ammonium sulfate and ethanol precipitation

Purification methods	Extraction methods	Parameters				
		PC	EA	SA	PF	Y
Ammonium Sulfate	Distilled water	$^{B}0.076 \pm 0.003^{b}$	$^{NS}0.0046 \pm 0.0006^{a}$	$^{NS}0.061 \pm 0.0090^{a}$	$^{NS}1.09 \pm 0.173^{a}$	$^{NS}7.48 \pm 0.39^{a}$
	Citric phosphate buffer	$^{NS}0.136 \pm 0.010^{b}$	$^{NS}0.0023 \pm 0.0007^{a}$	$^{NS}0.018 \pm 0.0060^{b}$	$^{NS}0.95 \pm 0.267^{a}$	$^{NS}11.14 \pm 0.87^{a}$
	Sodium citrate buffer	$^{NS}0.272 \pm 0.020^{a}$	$^{NS}0.0027 \pm 0.0006^{a}$	$^{NS}0.010 \pm 0.0014^{b}$	$^{NS}0.84 \pm 0.005^{a}$	$^{NS}10.01 \pm 1.07^{a}$
Ethanol	Distilled water	$^{A}0.136 \pm 0.003^{b}$	0.0048 ± 0.0001^{a}	0.036 ± 0.0021^{a}	0.65 ± 0.063^{a}	7.88 ± 0.18^{a}
	Citric phosphate buffer	0.166 ± 0.009^{b}	0.0028 ± 0.0003^{a}	0.017 ± 0.0007^{b}	0.91 ± 0.115^{a}	11.25 ± 3.51^{a}
	Sodium citrate buffer	0.302 ± 0.006^{a}	0.0028 ± 0.0010^{a}	0.010 ± 0.0035^{b}	0.83 ± 0.418^{a}	12.34 ± 8.75^{a}

PC = protein content, EA = enzyme activity, SA = Specific activity, PF = purification fold, Y = activity yield. Values are mean of duplicate analysis with standard deviation

a-b Means followed by different superscript in each coloum are significantly different among distilled water, citric phosphate buffer and sodium citrate buffer extraction ($p \leq 0.05$).

$^{A-B}$ Means followed by different superscript in each coloum are significantly different among extraction methods, between ammonium sulfate and ethanol ($p \leq 0.05$).

^{NS} means non significant.

Table 4.3 Protein content (mg/ml), enzyme activity (U/ml), specific activity (U/mg), purification fold and yield (%) of stem bromelain enzyme purified by ammonium sulfate and ethanol precipitation

Purification methods	Extraction methods	Parameters				
		PC	EA	SA	PF	Y
Ammonium Sulfate	Distilled water	$^{B}0.121 \pm 0.004^{b}$	$^{NS}0.0017 \pm 0.0009^{a}$	$^{NS}0.014 \pm 0.0084^{a}$	$^{NS}1.26 \pm 0.631^{a}$	$^{NS}14.93 \pm 5.40^{a}$
	Citric phosphate buffer	$^{NS}0.166 \pm 0.004^{b}$	$^{NS}0.0024 \pm 0.0006^{a}$	$^{NS}0.015 \pm 0.0035^{a}$	$^{NS}1.31 \pm 0.598^{a}$	$^{NS}27.53 \pm 5.26^{a}$
	Sodium citrate buffer	$^{B}0.499 \pm 0.018^{a}$	$^{NS}0.0025 \pm 0.0002^{a}$	$^{NS}0.005 \pm 0.0007^{a}$	$^{NS}1.76 \pm 0.463^{a}$	$^{NS}14.31 \pm 3.91^{a}$
Ethanol	Distilled water	$^{A}0.151 \pm 0.003^{b}$	0.0019 ± 0.0002^{a}	0.012 ± 0.0014^{a}	1.13 ± 0.272^{a}	13.95 ± 6.46^{a}
	Citric phosphate buffer	0.182 ± 0.010^{b}	0.0025 ± 0.0007^{a}	0.014 ± 0.0035^{a}	1.22 ± 0.578^{a}	14.14 ± 6.53^{a}
	Sodium citrate buffer	$^{A}0.982 \pm 0.006^{a}$	0.0030 ± 0.0002^{a}	0.003 ± 0.0000^{a}	1.16 ± 0.127^{a}	13.08 ± 2.19^{a}

PC = protein content, EA = enzyme activity, SA = Specific activity, PF = purification fold, Y = activity yield. Values are mean of duplicate analysis with standard deviation

a-b Means followed by different superscript in each coloum are significantly different among distilled water, citric phosphate buffer and sodium citrate buffer extraction ($p \leq 0.05$).

$^{A-B}$ Means followed by different superscript in each coloum are significantly different among extraction methods, between ammonium sulfate and ethanol ($p \leq 0.05$).

^{NS} means non significant.

4.3 Temperature and pH effect on bromelain

The enzyme activity increased with increasing temperature until it reached 50°C, where it began to decline rapidly (Figure 4.1) for both stem and pulp. Thus the enzyme is inactivated at high temperature due to the partial unfolding of its molecule (Klomklao *et al*., 2007). This result is compatible with those reported by Srinath *et al*. (2012) where maximum enzyme activity was found at 50°C. Liang *et al*. (1999) reported that the optimum temperature for the activity of bromelain was found to be at about 55°C. The result is contrasted with the ones described by Ketnawa *et al*. (2012), Valles *et al*. (2007) and Koh *et al*. (2006). At those studies, the maximum activity was reached at 60, 63 and 60ºC, respectively. As the temperature increases, more molecules have kinetic energy to undergo the reaction. After the temperature is raised above the optimum temperature, a biochemical threshold, the systems energy is so high that peptide bonds and disulfide bonds are disrupted, therefore inactivating the enzymes.

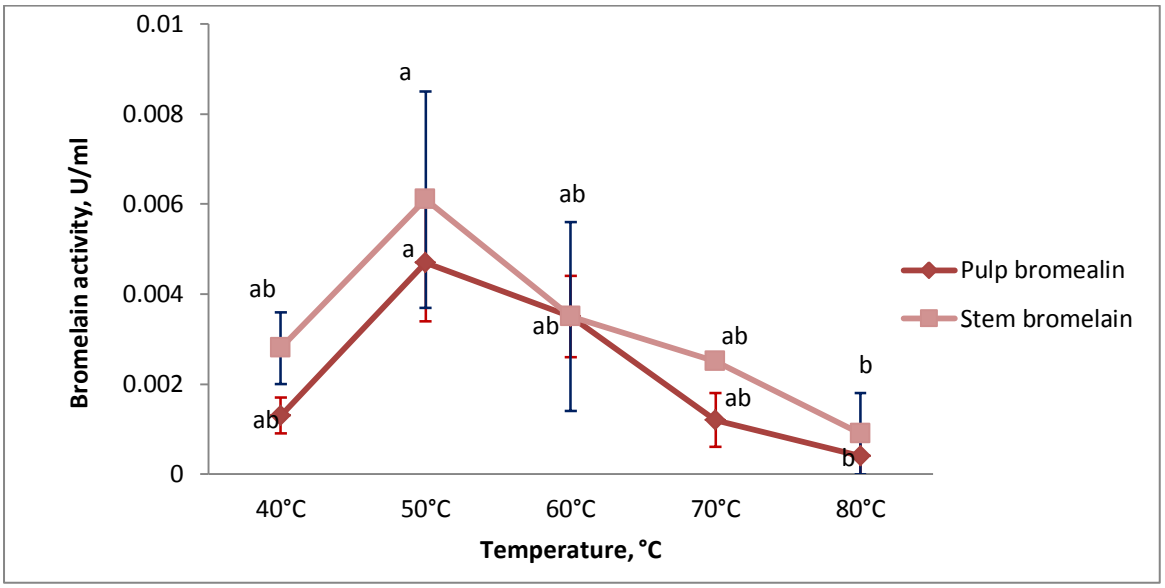


Figure 4.1 Effect of temperature on bromelain activity

[a-b] Means followed by different superscript in each sample are significantly different among various temperature ($p \leq 0.05$).

The stability of bromelain incubated with various pH buffers is shown in Figure 4.2. Both samples of bromelain were able to retain most of the activity at pH 8.5. The enzyme activity slightly reduced in the acidic area, while it was dramatically lost at pH above 8.5. This result is similar to Liang *et al*. (1999), who reported that the bromelain has a wider pH range for optimum activity at 6.8- 9.0. Ketnawa *et al*. (2012) described that high enzyme activity was observed in pH ranging from 6.5 to 8.0, and maximum activity was

near pH 7.0. Under the very acidic and alkaline pH, the charge repulsion associates with a decrease in electrostatic bonds (Benjakul *et al*., 2003).

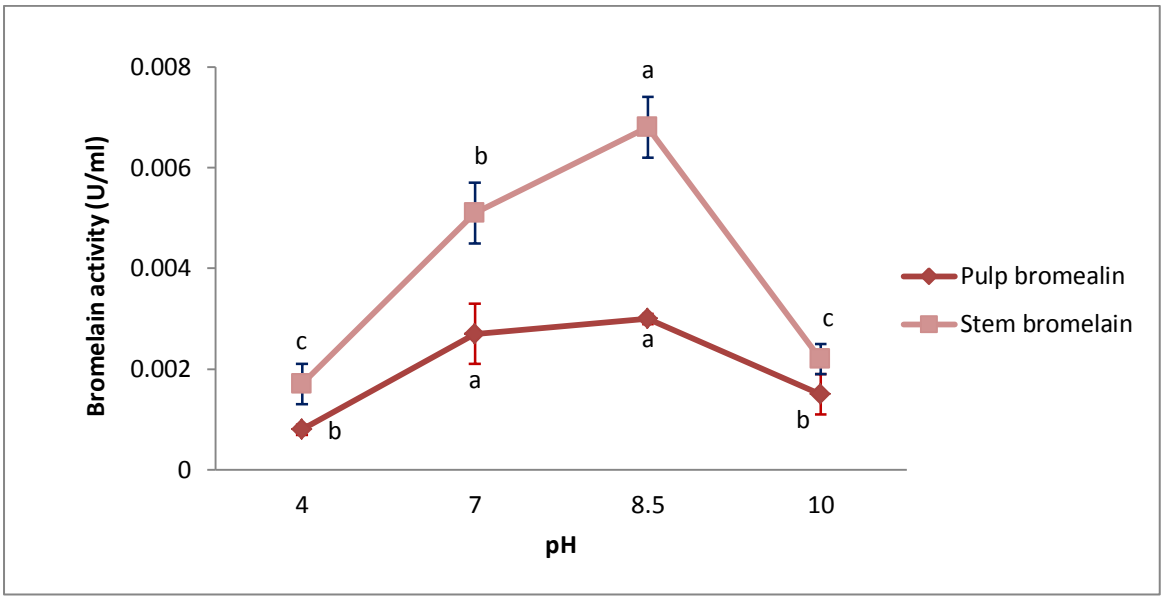


Figure 4.2 Effect of pH on bromelain activity

[a-c] Means followed by different superscript in each sample are significantly different among various pH ($p \leq 0.05$).

4.4 Comparing bromelain as anti-browning agent on apple juice with commercial anti-browning agents

From the Figure 4.4, we see that bromelain is a weak anti browning agent when compared with other. Amongst the anti-browning agents L-cysteine was significantly most effective followed by citric acid, ascorbic acid, pulp and stem bromelain. These results are compatible with those reported by Tochi *et al*. (2009) who said that L-cysteine is a more effective browning inhibitor as compared to either ascorbic acid or stem bromelain. The bromelain extracted from pineapple stem was significantly more capable for preventing browning than the bromelain extracted from the pulp. This can be explained by the fact that protein content was more in stem part than pulp part. Lower concentration 0.1% of stem bromelain was not so potent anti-browning agent whereas higher concentration 1% stem bromelain proved to be better anti-browning agent than ascorbic acid and citric acid. Similar result was found by Srinath *et al*. (2012), who said that bromelain extracted from the fruit pulp had better anti-browning agent than ascorbic acid and acetic acid with a concentration of 1%. Because, the effectiveness of ascorbic acid as anti-browning agent is temporary (Komthong *et al*., 2007) and it can be easily oxidized by endogenous enzyme, as well as decomposed by auto oxidation (Ozoglu and Bayindirli, 2002).

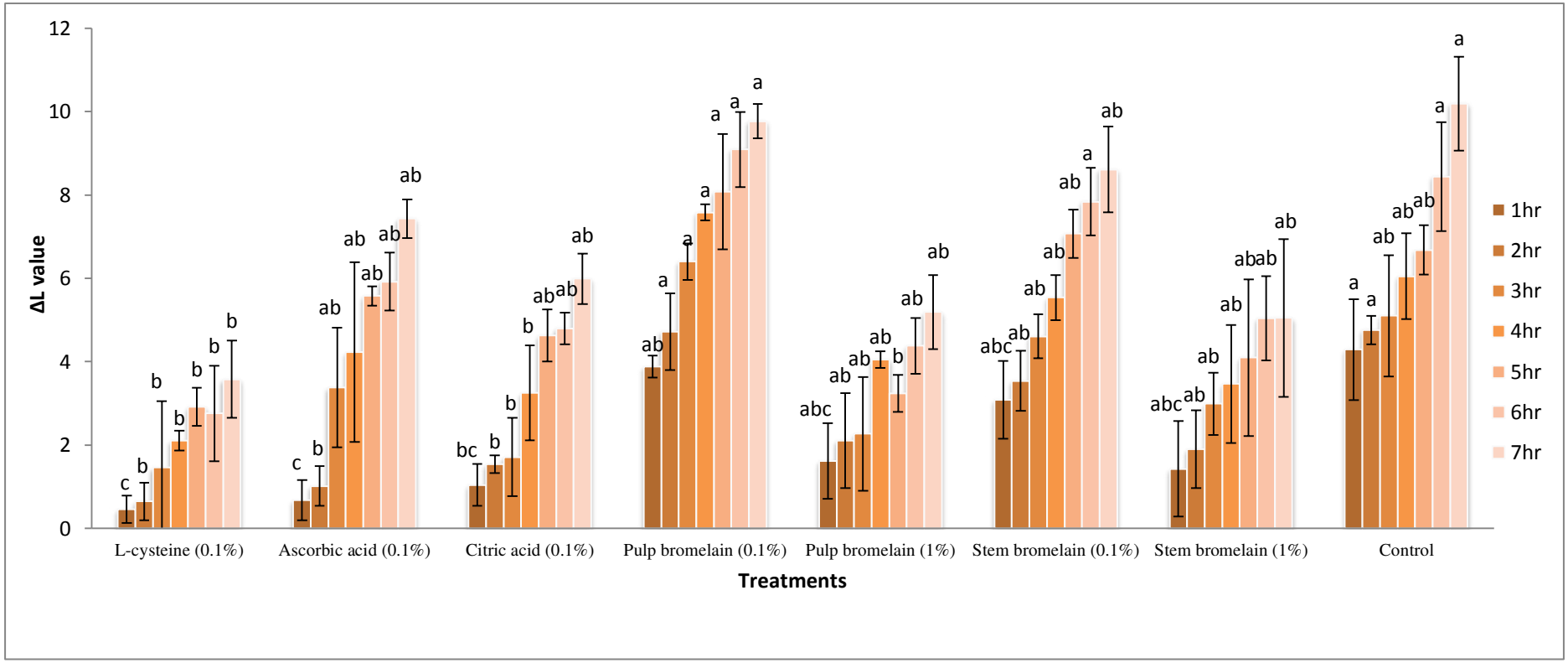
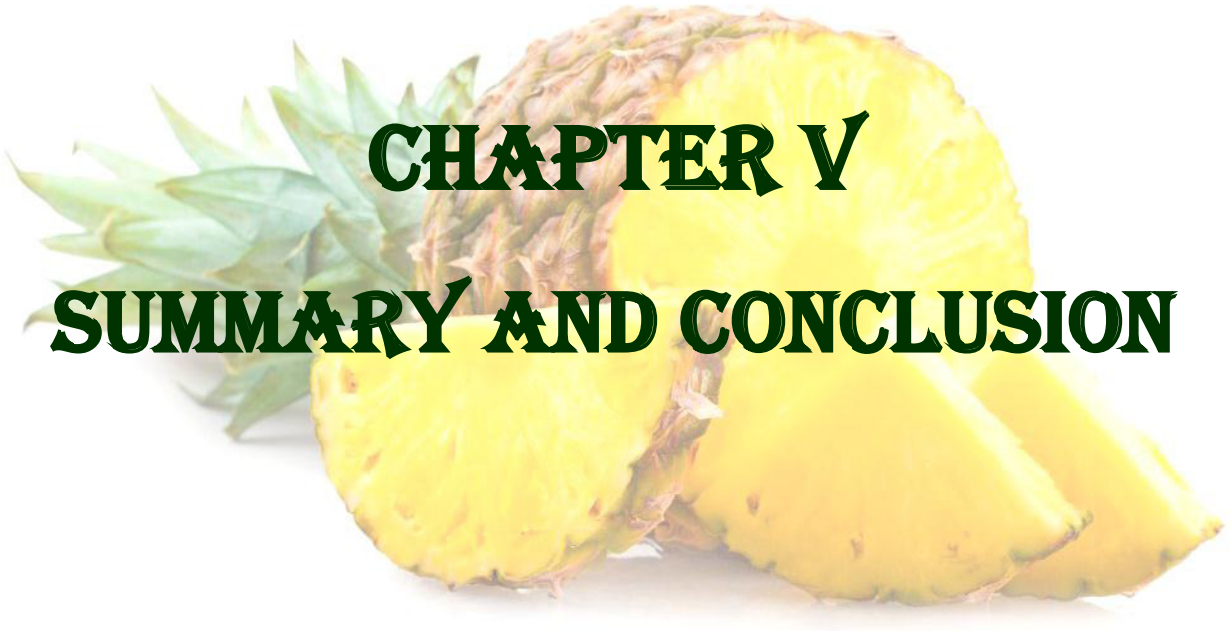


Figure 4.4 Browning of apple juice at different time interval treated with commercial anti-browning agents and bromelain enzyme

[a-c] Means followed by different superscript in each hour are significantly different among various anti-browning agents ($p \leq 0.05$).



CHAPTER V

SUMMARY AND CONCLUSION

CHAPTER V

SUMMERY AND CONCLUSION

In the present study, the bromelain was extracted from pineapple stem and pulp with distilled water, citric phosphate buffer and sodium citrate buffer and purified by ammonium sulfate and ethanol. The highest protein content was in bromelain extracted with sodium citrate buffer and lowest was in bromelain extracted with distilled water. Enzyme activity was higher in stem bromelain extracted with sodium citrate buffer whereas enzyme activity was higher in pulp bromelain extracted with distilled water. Bromelain with ethanol precipitation had higher protein content and enzyme activity but lower purification fold and yield than ammonium sulfate precipitation. Bromelain showed a maximum activity at pH 8.5 and at 50°C temperature for 30 min. As observed stem bromelain at a concentration of 1% had the potential to be a better agent than ascorbic acid and citric acid. Although ethanol had achieved a lower purification fold but it had higher protein content and enzyme activity and it seems to be more suitable for bromelain recovery, based on process time, low ionic strength and low cost.

REFERENCES

REFERENCES

Abílio, G.M.F., Holschuh, H.J., Bora, P.S., and Oliveira, E.F.D., (2009). Extração, atividade da bromelina eanálise de alguns parâmetros químicos emcultivares de abacaxi. Revista Brasileria Fruticultura, 31**:**1117-1121.

Abraham, T.E., and Sangeetha K.K., (2006). Chemical modification of papain for use in alkaline medium. p 80-92.

Ammon, H.P., (2002). Boswellic acids (components of frankincense) as the active principle in treatment of chronic inflammatory disease. Wiener Medizinische Wochenschrift, 152:373-378.

Anon., (1991). Sulphites banned. Food Ingredients Process, International. 11:11-11.

Babu, B.R., Rastogi, N.K., and Raghavarao, K.S.M.S., (2008). Liquid- liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. Chemical Engineering and Processing, 47: 83-89.

Barros, R.M., Ferreira, C.A., Silva, S.V., and Malcata, F.X., (2001). Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate. Enzyme and Microbial Technology, 29: 541-547.

Benjakul, S., Visessanguan, W., and Leelapongwattana, K., (2003). Purification and characterization of heat-stable alkaline proteinase from bigeye snapper (*Priacanthus macracanthus*) muscle. Comparative Biochemistry and Physiology, 13: 579-591.

Bhui, K., Prasad, S., George, J., and Shukla, K., (2009). Bromelain inhibits COX-2 expression by blocking the activation of MAPK regulated NF-kappa B against skin tumor initiation triggering mitochondria. Cancer Letters, 282: 167–176.

Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Braun, J.M., Schneider, B., and Beuth, H.J., (2005). Therapeutic use, efficiency and safety of the proteolytic pineapple enzyme Bromelain-POS in children with acute sinusitis in Germany, In Vivo 19: 417–422.

Brovko, F.A., and Zagranichnaya, T.K., (1998). Separation of proteins from phenols in cereal leaf extract by hydrophobic interaction- ammonium sulfate fractionation. Plant Physiology and Biochemistry, 36: 773-777.

Chaiwut, P., Nitsawang, S., Shank, L., and Kanasawud, P., (2007). A comparative study on properties and proteolytic components of papaya peel and latex proteases. Chiang Mai Journal of Science, 34: 109-118.

Chen, D., and Huang, S., (2004). Fast separation of bromelain by polyacrylic acid-bound iron oxide magnetic nanoparticles. Process Biochemistry, 39: 2207–2211.

Chen, T. Su. S., Nie, H., and Zhu, L., (2008). Display: Design and selection of functional binding peptides ligands application for isolation of bromelain using affinity chromatography. Journal of Biotechnology, 136S, 620S–632S.

Chobotova, K., Vernallis, A.B., and Abdul Majid, F.A., (2009). Bromelain's activity and potential as an anti-cancer agent: Current evidence and perspectives. Cancer Letters, 20: 1-9.

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

Corzo, C.A., Waliszewski, K.N., and Welti-Chanes, J., (2011). Pineapple fruit bromelain affinity to different protein substrates. Food Chemistry, 133: 631– 635.

Darshan, S., and Doreswamy, R., (2004). Patented anti- inflammatory plant drug development from traditional medicine. Phytotherapy Research, 18: 343-357.

Desai, M.A., (2000). Downstream Processing of Proteins: Methods and Protocols, first edition, Humana Press, New Jersey.

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, B., Bassani, V., Casadebaig, J., Cavailles, L., and Jacob, M., (2005). Preparation of proteolytic enzyme extracts from *Ananas comosus* L. Merr. fruit juice using semi permeable membrane, ammonium sulfate extraction, centrifugation and freeze-drying processes. International Immunopharmacology, 4: 783–79.

Dong, H., Gao, S., Han, S., and Cao, S., (1999). Purification and characterization of a *Pseudomonas sp.* Lipase and its properties in non-aqueous media. Applied Microbiology and Biotechnology, 30: 251–256.

Dubey, V.K., Pande, M., Singh, B.K., and Jagannadham, M.V., (2007). Papain-like proteases: applications of their inhibitors. African Journal of Biotechnology, 6: 1077–1086.

Englard, S., and Seifter, S., (1990). Precipitation techniques.in: Guide to protein purification, Editors. Deutscher, M.P., Academic Press, San Diego, USA.

Ferreira, F.J., Santana, J.C.C., and Tambourgi, E.B., (2011). The effect of pH on bromelain partition from *Ananas comosus* by PEG4000/Phosphate ATPS. Brazilian Archives Biology and Technology, 1: 125-132.

Fileti, A.M., Fischer, G.A., and Tambourgi, E.B., (2007). Optimizing bromelain extraction by reversed micelles from pineapple fruit. Chemical Engineering Transactions, 11: 989-994.

Fileti, A.M.F., Fischer, G.A., Santana, J.C.C., and Tambourgi, E.B., (2009). Batch and continuous extraction of bromelain enzyme by reversed micelles. Brazilian Archives Biology and Technology, 52: 1225 -1234.

Gautam, S.S., Mishra, S.K., Dash, V., Amit, K.G., and Rath, G., (2010). Comparative study of extraction, purification and estimation of bromelain from stem and fruit of pineapple plant. Thai Journal of Pharmaceutical Science, 34: 67-76.

Golunski, S., Astolfi, V., Carniel, N., Oliveira, D., Di Luccio, M., Mazutti, M.A., and Treichel, H., (2011). Ethanol precipitation and ultrafiltration of inulinases from Kluyveromyces marxianus. Separation and Purification Technology, 78: 261–265.

Gupta, R., Bradoo, S., and Saxena, R.K., (1999). Aqueous two-phase systems: an attractive technology for downstream processing of biomolecules. Current Science, 77: 520–523.

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: 183–190.

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

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

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

Hale, L.P., David, J.F., and Herman, F.S., (2006). Oral immunogenicity of the plant proteinase bromelain. International Immunopharmacology, 6: 2038-2046.

Hans-Jorg, B., and Stephan, P., (2011). Industrial Scale Natural Products Extraction, first edition. Wiley-VCH, Weinheim.

Harris, E.L.V., (1989). Concentration of the extract. In: Harris, E.L.V and Angal, S. (Editors), Protein Purification Methods: A Practical Approach. Oxford University Press, New York, p 125-174.

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 waste. Bioresourse Technology, 99: 4896-4902.

Hebbar, H.U., Hemavathi, A.B., Sumana, B., and Raghavarao, K.S.M.S., (2011). Reverse micellar extraction of bromelain from pineapple (*Ananas comosus* l. Merryl) waste: scale-up, reverse micelles characterization and mass transfer studies. Separation Science and Technology, 46: 1656-1664.

Hebbar, H.U., Hemavathi, A.B., Sumana, B., and Raghavarao, K.S.M.S., (2012). Separation and purification of bromelain by reverse micellar extraction coupled ultrafiltration and comparative studies with other methods. Food Bioprocess Technology, 5: 1010–1018.

Heinicke, R.M., and Gortner, W.A., (1957). Stem bromelain – a new protease preparation from pineapple plants. Economic Botany, 11: 225–234.

Hemavathi, A.B., Hebbar, H.U., and Raghavarao, K.S.M.S., (2007). Reverse micellar extraction of bromelain from *Ananas comosus* L. Merryl. Journal of Chemical Technology & Biotechnology, 82: 985-992.

Hutti-Kaul, R., and Mattiasson, B., (2003). Isolation and purification of proteins, first edition. Marcel Dekker, New York.

Illanes, A., (2008). Enzyme production. In: Enzyme biocatalysis. Brazil Springer Science and Business Media V.B., p 57-106.

Jaber, R., (2002). Respiratory and allergic diseases: From upper respiratory tract infections to asthma. Primary Care, 2: 231-261.

Joy, P.P., (2010). Benefits and uses of pineapple. Pineapple Research Station, Kerala Agricultural University, Kerala, India.
[http://www.kau.edu/prsvkm/Html/BenefitsofPA.htm]

Jutamongkon, R., and Charoenrein, S., (2010). Effect of temperature on the stability of fruit bromelain from smooth cayenne pineapple. Kasetsart Journal (Natural Science), 44: 943– 948.

Kelly, G.S., (1996). Bromelain: A Literature Review and Discussion of its Therapeutic Applications. Alternative Medicine Review, 1: 243-257.

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

Ketnawa, S., Chaiwut, P., and Rawdkuen, S., (2011a). Aqueous Two-phase Extraction of Bromelain from Pineapple Peels (_PhuLae‘ cultv.) and Its Biochemical Properties. Food Science and Biotechnology, 20: 1219- 1226.

Ketnawa, S. Chaiwut, P., and Rawdkuen, S., (2011b). Extraction of bromelain from pineapple peels. Food Science and Technology International, 4: 395-402.

Ketnawa, S., Chaiwut, P., and Rawdkuen, S., (2012). Pineapple wastes: A potential source for bromelain extraction. Food and Bioproducts Processing, 90: 385-391.

Klaue, P., Dilbert, G., and Hinke, G., (1979). In: Bromelain: Biochemistry, pharmacology and medical use. Cellular and Molecular Life Sciences, 58: 1234-1245.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., and Simpson, B.K., (2007). Purification and characterisation of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). Food Chemistry, 100: 1580–1589.

Koh, J., Kang, S. M., Kim, S. J., Cha, M. K., and Kwon, Y. J., (2006). Effect of pineapple protease on the characteristics of protein fibers. Fibers and Polymer, 7:180-185.

Komthong, P., Igura, N., and Shimoda, M., (2007). Effect of ascorbic acid on the odours of cloudy apple juice. Food Chemistry, 100: 1342-1349.

Kumar, S., Hemavathi, A.B., and Hebbar, H.U., (2011). Affinity based reverse micellar extraction and purification of bromelain from pineapple (*Ananas comosus L. Merryl*) waste. Process Biochemistry, 46: 1216–1220.

Laurila, E., and Ahvenainen, R., (2002). Minimal processing of fresh fruits and vegetables. In: fruits and vegetable processing-improving quality, Wim, J. (Editors). CRC Press, Washington. DC, p 1-22.

Leary, D., Vierros, M., Hamon, G., Arico, S. and Monagle, C., (2009). Marine genetic resources: a review of scientific and commercial interest. Marine Policy, 33:183–94.

Lee, C.Y., Son, S.M., and Moon, K.D., (2000). Inhibitory effects of various antibrowning agents on apple slices. Food Chemistry, 73: 23-30.

Lemay, M., Murray, M.A., Davies, A., Roh-Schmidt, H., and Randolph, R.K., (2004). *In vitro* and ex vivo cyclooxygenase inhibition by a hops extract. Asian Pacific Journal of Clinical Nutrition, 13: S110.

Ley, C.M., Tsiami, A., Ni, Q., and Robinson, N., (2011). A review of the use of bromelain in cardiovascular diseases. Chinese Journal of Integrative Medicine, 9: 702–710.

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

Liang, H.Y., Li, M., Shi, M., Liao, A.P., and Wu, S.R.C., (2011). Study on the Stability of Fruit Bromelain. Advanced Materials Research, 421: 19-22.

Liggieri C., Obregón W., Sebastián T., and Priolo N., (2009). Biochemical analysis of a papain-like protease isolated from the latex of Asclepias curassavica L. 41: 154-162.

Manjunath, H.M., Chandrashekhar, G.J., Vijay, R., and Mahesh, M., (2014). Comparative Analysis of Papain from Different varieties of Papaya Plant Latex. International Journal of Agricultural and Food Science, 4: 123-127.

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

Martins, B.C., Rescolino, R., Coelho, D.F., Zanchetta, B., Tambourgi, E.B., and Silveir, E., (2014). Characterization of Bromelain from *Ananas Comosus* Agroindustrial Residues Purified by Ethanol Factional Precipitation. The Italian Association of Chemical Engineering, p 781-786.

Murad, H., (2003). Method of treating dermatological disorders with fruit extracts. In: Patent, U.S. (Edition). http://www.freepatentsonline.com/. U.S., 1-38.

Ozlen, S.N., (1995). Cosmetic composition containing alpha hydroxyacids, salicyclic acid and enzyme mixture of bromelain and papain. In: Patent, U.S. (Editors). http://www.freepatentsonline.com/. United States: Longevity Network Ltd. Handerson, Nev 1-6.

Ozoglu, H., and Bayindirli, A., (2002). Inhibition of enzymic browning in cloudy apple juice with selected antibrowning agents. Food Control, 13: 213-221.

Paulo, A.G.S., Antônio, F.M.V., Maria T.S.C., Adalberto P.J, and Maria G.C., (2012). Purification of bromelain from pineapple wastes by ethanol precipitation. Separation and Purification Technology, 98: 389–395.

Rabelo, A.P.B., Tambourgi, E.B., and Pessoa, J. A., (2004). Bromelain partitioning in two-phase aqueous systems containing PEO-PPO-PEO block copolymers. Journal of Chromatography, B, 807: 61-68.

Roselli, M., Britti, M.S., Le, H.I., Marfaing, H., Zhu, W.Y., and Mengheri, E., (2007). Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic *Escherichia coli* K88 in pig intestinal cells. Toxicology *in vitro,* 21: 224-229.

Roep, B.O., Van den Engel, N.K., VanHalteren, A.G.S., Duinkerken, G., and Martin., (2002). Modulation of autoimmunity to beta-cell antigens by proteases. Diabetologia, 45: 686-692.

Rosenberg, L., Lapid, O., Berezovsky, A., Glesinger, R., Krieger, Y., Silberstein, E., Sagi, A., Judkins, K., and Singer, A.J., (2004). Safety and efficacy of a proteolytic enzyme for enzymatic burn de´bridement: A preliminary report, Burns, 30: 843-850.

Rosenberg, L., Barezovski, A., Gurfinkel, R., Silverstein, E., and Krieger, Y., (2009). Safety & efficacy of bromelain extract in debriding deep burns: recent summary of all available data, Burns, 35: S17.

Saxena, L., Iyer, B.K., and Ananthanarayan, L., (2007). Three phase partitioning as a novel method for purification of ragi (*Eleusine coracana*) bifunctionalamylase/protease inhibitor. Process Biochemistry, 42: 491-495.

Silvestre, M.P.C., Carreira, R.L., Silva, M.R., Corgosinho, F.C., Monteiro, M.R.P., and Morais, H.A., (2011). Effect of pH and temperature on the activity of enzymatic extracts from pineapple peel. Food and Bioprocess Technology, p 1824- 1831

Srinath, R., Ramalingam,C., and Islam, N.N., (2012). Isolation and characterization of Bromelain from pineapple (*Ananas Comosus*) and comparing its anti-browning activity on apple juice with commercial antibrowning agents. Food Science, 45:7822-7826.

Thomson, A.B., Keelan, M., Thiesen, A., Clandinin, M.T., Ropeleski, M., and Wild, G.E., (2001). Small bowel review: normal physiology. Part1. Digestive Diseases and Science, 46: 2567-2587.

Tochi, B.N., Zhang, W., Ying, X., and Wenbin, Z., (2008). Therapeutic Application of Pineapple Protease (Bromelain): A review. Pakistan Journal of Nutrition, 7: 513-520.

Tochi, B.N., Wang, Z., Xu, S.Y., and Zhang, W., (2009). Effect of stem bromelain on the browning of apple juice. American Journal of Food Technology, 4: 146-153.

USDA Economic Research Service. Accessed 30 Nov 2009. http://www.ers.usda.gov/data/fruitvegphyto/Data/fr-pineapples.xls

Valles, D., Furtado, S., and Cantera, A.M., (2007). Characterization of news proteolyticenzymes from ripe fruits of Bromelia antiacantha Bertol. (Bromeliaceae). Enzyme and Microbial Technology, 40: 409-413.

Walsh, G., (2002). Protein biochemistry and biotechnology (1st edition). New York: John Wiley and Sons.

Wiest-Ladenburger, U., Richter, W., Moeller, P., and Boehm, B.O., (1997). Protease treatment delays diabetes onset in diabetes - prone nonobese diabetic (NOD) mice. International Journal of Immunotherapy, 13: 75-78.

Xue, Y., Wu, C., Branford-White, C.J., Ning, X., Nie, H., and Zhu, L., (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.

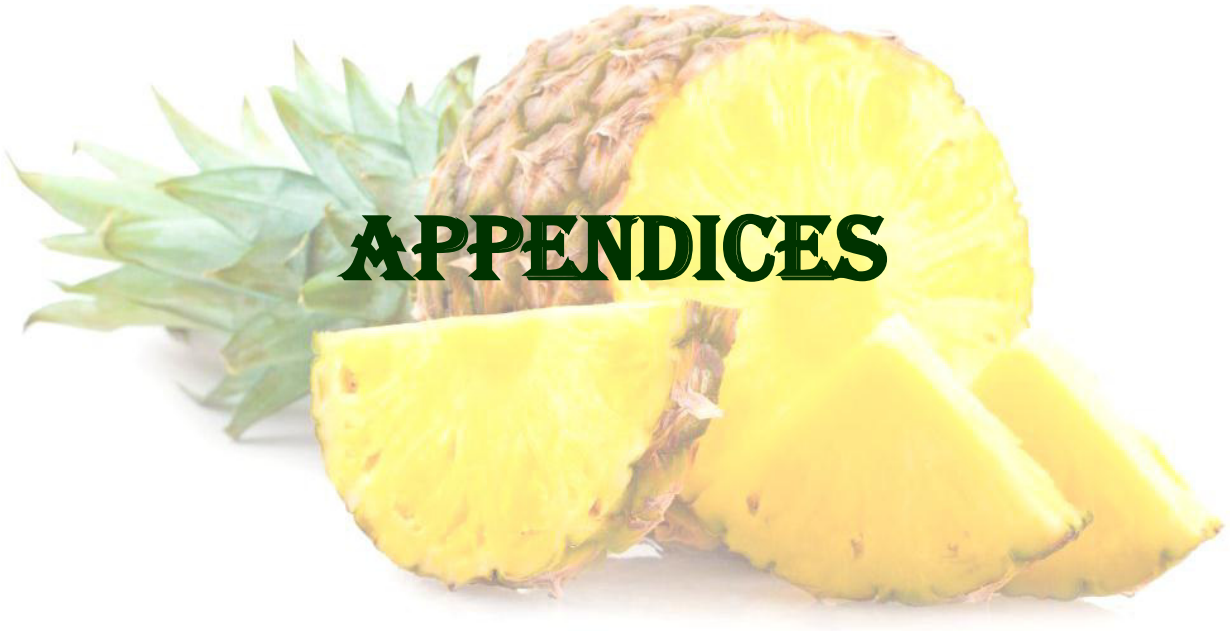
Yearbook of Agriculture Statistics of Bangladesh, (2008). Bangladesh Bureau of Statistics.

Yearbook of Agriculture Statistics of Bangladesh, (2011). Bangladesh Bureau of Statistics.

Yearbook of Agriculture Statistics of Bangladesh, (2012). Bangladesh Bureau of Statistics.

Yin, L., Sun, C.K., Han, X., Xu, L., Xu, Y., Qi, Y., and Peng, J., (2011). Preparative purification of bromelain (EC 3.4.22.33) from pineapple fruit by high-speed counter-current chromatography using a reverse-micelle solvent system. Food Chemistry, 129: 925–932.

APPENDICES



APPENDICES

Appendix I

Protein concentration (mg/ml), enzyme activity (U/ml) and specific activity (U/mg) of crude extract from pulp and stem from pineapple

Samples	Extraction Methods	PC	EA	SA
Pulp	Distilled water	0.154 0.148	0.0086 0.0083	0.0558 0.0561
	Citric phosphate buffer	0.198 0.196	0.0034 0.0038	0.0172 0.0194
	Sodium citrate buffer	0.338 0.326	0.0036 0.0043	0.0107 0.0132
Stem	Distilled water	0.184 0.179	0.0018 0.0021	0.0098 0.0117
	Citric phosphate buffer	0.215 0.208	0.0021 0.0028	0.0098 0.0135
	Sodium citrate buffer	1.121 1.235	0.0027 0.0035	0.0024 0.0028

PC = Protein content, EA = Enzyme activity, SA = Specific activity

Appendix II

Protein concentration (mg/ml), enzyme activity (U/ml) and specific activity (U/mg) of purified pulp bromelain from pineapple

Samples	Extraction Methods	PC	EA	SA
Ammonium Sulfate	Distilled water	0.078 0.074	0.0042 0.0050	0.054 0.068
	Citric phosphate buffer	0.143 0.129	0.0018 0.0028	0.013 0.022
	Sodium citrate buffer	0.257 0.287	0.0023 0.0031	0.009 0.011
Ethanol	Distilled water	0.138 0.134	0.0047 0.0049	0.034 0.037
	Citric phosphate buffer	0.172 0.159	0.0030 0.0026	0.017 0.016
	Sodium citrate buffer	0.306 0.298	0.0036 0.0020	0.012 0.007

Appendix III

Protein concentration (mg/ml), enzyme activity (U/ml) and specific activity (U/mg) of purified stem bromelain from pineapple

Samples	Extraction Methods	PC	EA	SA
Ammonium Sulfate	Distilled water	0.123 0.118	0.001 0.0024	0.008 0.020
	Citric phosphate buffer	0.169 0.163	0.0028 0.0020	0.017 0.012
	Sodium citrate buffer	0.486 0.512	0.0026 0.0023	0.005 0.004
Ethanol	Distilled water	0.153 0.149	0.0020 0.0017	0.013 0.011
	Citric phosphate buffer	0.189 0.175	0.0030 0.0020	0.016 0.011
	Sodium citrate buffer	0.986 0.978	0.0028 0.0031	0.003 0.003

Appendix IV

Purification Fold (PF) and yield (%) of purified pulp bromelain from pineapple

Samples	Extraction Methods	PF	Yield
Ammonium Sulfate	Distilled water	0.968 1.212	7.75 7.20
	Citric phosphate buffer	0.756 1.134	11.76 10.52
	Sodium citrate buffer	0.841 0.833	9.25 10.76
Ethanol	Distilled water	0.610 0.700	7.75 8.00
	Citric phosphate buffer	0.988 0.825	13.73 8.77
	Sodium citrate buffer	1.121 0.530	18.52 6.15

Appendix V

Purification Fold (PF) and yield (%) of purified stem bromelain from pineapple

Samples	Extraction Methods	PF	Yield
Ammonium Sulfate	Distilled water	0.816 1.709	11.11 18.75
	Citric phosphate buffer	1.734 0.888	31.25 23.81
	Sodium citrate buffer	2.083 1.428	17.07 11.54
Ethanol	Distilled water	1.326 0.940	18.52 9.38
	Citric phosphate buffer	1.633 0.815	18.75 9.52
	Sodium citrate buffer	1.250 1.071	14.63 11.53

Appendix VI

Bovine Serum Albumin Standard Curve for protein determination

Serial No	BSA (mg/ml)	Absorbance at 595nm
1	0.5	0.107
2	1	0.115
3	1.5	0.136
4	2	0.158
5	4	0.219

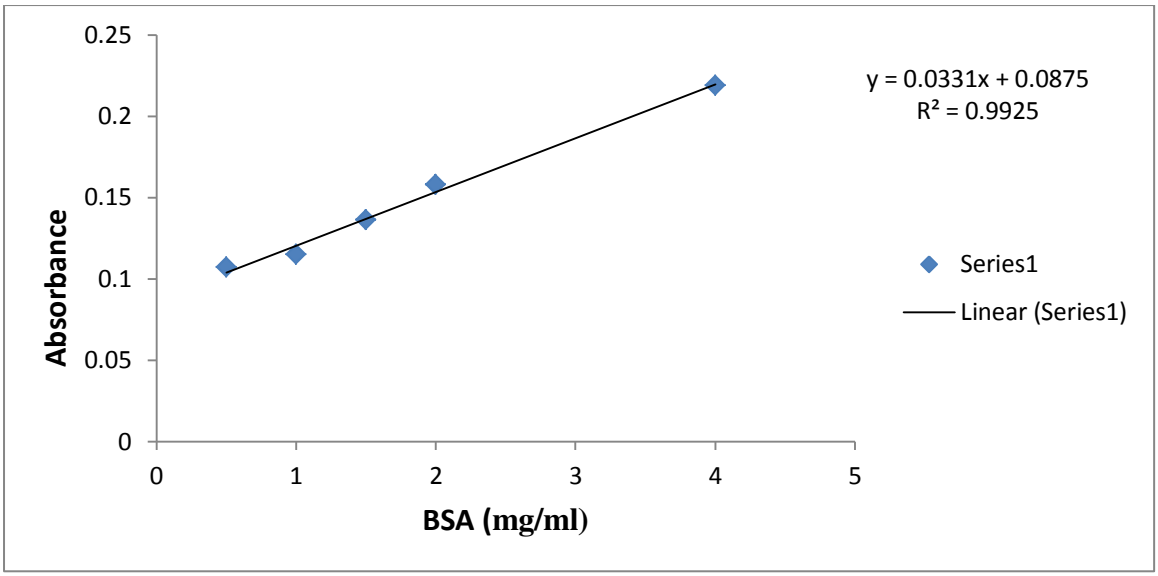


Figure: Bovine Serum Albumin Standard Curve

Y = 0.0331x + 0.0875

Here,

Y = Absorbance

x = Protein content (mg/ml)

Appendix VII

Browning of apple juice at different time interval treated with commercial anti-browning agents and bromelain enzyme

Treatments	Time (hr)						
	1	2	3	4	5	6	7
L-cysteine (0.1%)	0.69 0.23	0.97 0.33	2.59 0.33	1.94 2.28	3.24 2.60	3.57 1.95	2.92 4.23
Ascorbic acid (0.1%)	1.02 0.34	1.35 0.68	2.36 4.39	2.70 5.75	5.74 5.41	6.41 5.43	7.76 7.10
Citric acid (0.1%)	0.68 1.39	1.69 1.39	2.37 1.04	4.05 2.44	5.07 4.18	5.07 4.53	6.42 5.57
Pulp bromelain (0.1%)	3.69 4.06	5.37 4.06	6.71 6.09	7.71 7.44	9.06 7.10	9.73 8.46	10.06 9.47
Pulp bromelain (1%)	2.26 0.98	2.91 1.30	3.23 1.30	4.19 3.91	3.55 2.93	4.85 3.91	5.82 4.56
Stem bromelain (0.1%)	3.74 2.42	4.05 3.03	4.98 4.24	5.92 5.15	7.48 6.66	8.41 7.27	9.34 7.88
Stem bromelain (1%)	2.24 0.62	2.56 1.24	3.52 2.47	4.47 2.47	5.43 2.77	5.75 4.32	6.39 3.71
Control	5.15 3.44	4.52 5.00	6.13 4.07	6.78 5.32	7.10 6.26	9.36 7.51	10.98 9.39