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THESIS

FACTORS AFFECTING THE STABILITY OF FRUIT BROMELAIN IN SMOOTH CAYENNE PINEAPPLE (*Ananas Comosus*)

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A Thesis Submitted in Partial Fulfillment of
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Bromelain is the collective name for closely related proteolytic enzymes found in stem and fruit tissue of the plant family Bromeliaceae, of which pineapple, *Ananas comosus*, is the best known. The medicinal properties of pineapple bromelain have been primarily associated with the proteolytic activity of bromelain. This research had objectives to study the relationship of bromelain activity and total soluble solids, total acidity in Smooth Cayenne type pineapple. Moreover, optimal pH, the effect of temperature on stability of bromelain , kinetics of bromelain and the effect of sugars and polyol on the thermal stability of fruit bromelain were studied. An inverse relationship appeared to exist between the bromelain activity and total soluble solids in pineapples, especially ($R^2 = 0.597$) for 14.1–16.0 °Brix. In other words, within this °Brix range, more mature pineapple exhibited low bromelain activity. There was no relationship between total acidity and bromelain activity. Thermal inactivation of bromelain in temperature range of 40-80 °C, the calculated activation energy (*E*a) for bromelain was 313.18 ± 57.44 kJ/ mol. Pineapple juice exhibited high bromelain activity (higher than 2,000 CDU/g) in a broad pH range (6 –10), with two maximum at pH 8.0 and 10.0. For kinetic studies, we found that V_{max} 65.78 ± 7.79 CDU/min/mg protein and K_m 4.00 ± 0.07 x 10^{-5} mol/l. Heating at 60 °C was selected in the study of effect of sugars and polyol on the thermal stability of bromelain**.** Denaturation temperature of bromelain with and without solutes using differential scanning calorimetry (DSC) were determined and used to elucidate the results. The % residual activity indicated the increased thermal stability of the enzyme in all the solutes. The thermal stability of bromelain increased with incremented concentrations of sucrose. Trehalose was the best stabilizer of bromelain, followed by sorbitol and sucrose, respectively. Therefore, if we want to preserve bromelain activity in pineapple juice from heat treatment. Trehalose should be added to pineapple juice before heat treatment.

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FACTORS AFFECTING THE STABILITY OF FRUIT BROMELAIN IN SMOOTH CAYENNE PINEAPPLE (*Ananas Comosus*)

INTRODUCTION

Nowadays, consumers are increasingly interested in health and nutrition and consequently fruits and vegetables are consumed much more than in previous years. Pineapple is a fruit that is planted all over many areas in Thailand, and is generally consumed fresh. Bromelain is an enzyme beneficial for health that is naturally found in pineapples. It has long been used as a medicinal substance by several native cultures and has been chemically known since 1876 (Taussig and Batkin, 1988). Pineapple bromelain has been used commercially as a meat-tenderizing enzyme and as a nutraceutical. Bromelain has also demonstrated the following characteristics: (i) interference with the growth of malignant cells; (ii) inhibition of platelet aggregation; (iii) fibrinolytic action; (iv) anti-inflammatory processes; and (v) skin debridement (Rohrbach *et al*., 2003). The medicinal properties of bromelain have been primarily associated with the proteolytic activity of bromelain (Hale *et al*., 2005).

Total soluble solids, mainly sugars, are often used as an indicator of fruit maturity and quality. In pineapple total soluble solids can vary by 40 g/liter or 4 °Brix from the more mature, sweeter basal tissue to the crown end of the fruit, and decline slightly after harvest. Bromelain activity remained high during fruit

development and declines during ripening (Paull and Chen, 2003). Yoon *et al*. (2006) found a positive relationship between acid phosphatase and °Brix in cherries. Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on the charge distribution on both substrate and, in particular, enzyme molecules. Charge distribution on proteins is determined by the state of the ionisable side-chains of their constituent amino acids, which in turn is dependent on pH. Some enzymes exhibit activity over a broad range of pH values. However, in most cases, enzymes are active over a very narrow range of pH values; this pH optimum varying between different enzymes (Tucker, 1995).

The processed pineapple products are likely to be ineffective proteolytically. This inactivation of enzymes may arise from harsh conditions of sterilization, precipitation and/or autodigestion. Several studies have shown that the stability of enzymes may be increased by the addition of solute. The increase in thermal stabilities of glucose oxidase added to trehalose (Paz-Alfaro *et al.* 2009). The thermal stability of purified tomato pectin methylesterase (PME) increased six-fold in the presence of sucrose when compared to 0.1 M citrate buffer (Guiavarc'h *et al*. 2003). Papain activity at 75 °C as a function of time was greater in the presence of sucrose than for sorbitol (Sathish *et al.* 2007). Information on the thermal stability and solute effect on thermal stability of fruit bromelain is of great importance for pineapple processing, particularly for those who are interested in preserving bromelain activity in their products.

Hypothesis of this study were:

1. The relationship of total acidity, total soluble solids and bromelain activity.
2. Thermal stability, optimum pH and kinetics of bromelain.
3. Pineapple juice added solutes could preserve bromelain activity from thermal processing.

OBJECTIVES

The Objectives of this study were:

1. To study the relationship of total acidity, total soluble solids and bromelain activity.
2. To study thermal stability of bromelain
3. To study the optimal pH of bromelain
4. To determine Michaelis constants (K_m) and maximum reaction velocity (V_{max}) of bromelain
5. To study the effects of solutes on thermal stability of bromelain

LITERATURE REVIEWS

1. Bromelain

Bromelain was originally only extracted from Hawaiian pineapple stems but now is manufactured in Taiwan, Thailand, Brazil and Puerto Rico (Rohrbach *et al*., 2003).

The name 'bromelain' was originally given to the mixture of proteases found in the juice of the stem and fruit of pineapple (*Ananas comosus*). Even now, bromelain is still used as the collective name for enzymes found in various members of the Bromeliaceae family. The major endopeptidase present in extracts of plant stem is termed 'stem bromelain', whereas the major enzyme fraction found in the juice of the pineapple fruit is named 'fruit bromelain'. Some other minor cysteine endopeptidases (ananain, comosain) are also found in the pineapple stem (Grzonka *et al*., 2007).

Bromelain was the sulfhydryl proteases (also called the thiol proteases and cysteine proteases) that have in common the ability to hydrolyze the peptide bonds of proteins and their inhibition by sulfhydryl reagents. A great many other proteolytic enzymes are also inhibited by sulfhydryl reagents and may eventually be shown to have similar kinetic properties to the best understood of these enzymes, papain. The group of enzymes includes the higher plant enzymes, papain (EC 3.4.22.2), ficin

(EC 3.4.22.3), and bromelain (EC 3.4.22.4), and the microbial enzyme Streptococcus protease, EC 3.4.22.10) (Whitaker, 1994).

2. Characteristics of Bromelain

The major cysteine proteinase is fruit bromelain (EC 3.4.22.33), a non-glycosylated proteinase that is immunologically distinct from the glycosylated stem bromelain (EC 3.4.22.32). Fruit bromelain has an estimated molecular weight of between 23 and 31 kDa by different laboratories. The fruit bromelain is an acidic protein and its isoelectric point (pI) is 4.6, different from the basic stem bromelain, the pI of which is 9.6. The amino-terminal end of the fruit bromelain, has an additional alanine (Yamada *et al*., 1976). The sequence around the reactive cysteine is the same. Fruit bromelain has a 20% cross-reactivity with anti-stem bromelain (Paull and Chen, 2003).

Fruit bromelain, the major endopeptidase present in the juice of the pineapple fruit. It has much higher proteolytic activity compared to stem bromelain and a broader specificity for peptide bonds (Grzonka *et al*., 2007).The relative activity of stem bromelain and fruit bromelain with casein were 9.2 and 23.7, respectively (Ota *et al*., 1964). Fruit bromelain FA2, the main proteinase component of the juice of pineapple fruit, has been purified and characterized. Molecular weight is 31,000. Isoelectric point (pI) is 4.6. FA2 gave only alanine phenylthiohydantoin upon amino-terminal group. Stepwise degradation yielded the amino-terminal sequence Ala-Val-Pro-Gln-Ser-Ile-Asp-Trp-Arg-Asp-Tyr-Gly-Ala. The amino acid composition of FA2

was not markedly different from that of stem bromelain, except for a much smaller lysine content and a smaller alanine content relative to glycine in FA2.

FA2 contained neither amino sugars nor neutral carbohydrates as determined by several methods, so FA2 is not a glycoprotein. By labeling the reactive cysteine residue with [^{14}C] iodoacetate, the following partial amino acid sequence has been determined. Asn-Glx-Asn-Pro-Cys-Gly-Ala-CYS (Yamada *et al*., 1976).

Stem bromelain contains seven cysteines, one of which is involved in catalysis. The other six are associated in pairs forming three disulphide bridges. The crystal structure of stem bromelain has not yet been reported. Stem bromelain can be purified from dried pineapple stem powder by cation-exchange or affinity chromatography method (Grzonka *et al*., 2007). Pure stem bromelain is stable when stored at -20 °C. The pH optimum for bromelain activity is 6 – 8.5 for most of its substrate, and the temperature optimum rage of this enzyme is 50 to 60 °C (Helmut, 1998; Grzonka *et al*., 2007). Cysteine is commonly used as an activating compound for bromelain. Other thiols being less effective. Stem bromelain has high proteolytic activity for protein substrates, with a preference for polar amino acids. It has strong preference for Z-Arg-Arg-NHMee among small molecule substrates. It is scarcely inhibited by chicken cystatin and very slowly inactivated by E-64 (Grzonka *et al*., 2007).

By high-resolution fast protein liquid chromatography (FPLC) and other biochemical methods, basic stem bromelain, ananain, comosain and acidic thiol-

proteinases have been isolated from curde bromelain, partially or fully sequenced and characterized in more detail as Table 1.

Table 1 Cysteine proteinases (bromelains) from pineapples (*Ananas comosus*)

Name (EC number)	Abbreviation	MW. (Dalton)	Isoelectric point	Sequences	Glycosylation
From pineapple stems: Stem bromelain (EC 3.4.22.32)	F4andF5	23,800	>10	completely sequenced (212 amino acids)	glycosylated
Ananain (EC 3.4.22.31)	F9	23,464	>10	completely sequenced (216 amino acids)	not glycosylated
Comosain	F9b	24,509	>10	N-term.sequence	glycosylated
	SBA/a	23,550	4.8	N-term.sequence	highly glycosylated
	SBA/b	23,560	4.9	N-term.sequence	highly glycosylated
From pineapple fruits:					
Fruit bromelain (EC 3.4.22.33)		23,000	4.6	N-term.sequence	not glycosylated

Source: Maurer (2001)

They mainly comprise glycosylated multiple enzyme species of the papain superfamily with different proteolytic activities, molecular masses between 20 and 31 kDa, and isoelectric points between > 10 and 4.8. Two major basic proteinases, F4 and F5, were further characterized and showed molecular masses of 24,397 and 24,472 Da, respectively. In addition, numerous, different protein fractions were obtained by means of various biochemical methods [SDS-polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), multicathodal-PAGE]. Among the basic proteinases, one fraction (F9, ananain) reveals the highest specific proteinase activity, is not glycosylated and has a molecular mass of 23,427 Da. The enzymatic activities comprise a wide spectrum with pH optima between 5.5 and 8.0. The substrate spectrum is similarly broad, extending from synthetic low molecular mass amides and dipeptides up to high molecular substrates such as fibrin, albumin, casein, angiotensin II, bradykinin. Bromelain preferentially cleaves glycyl, alanyl and leucyl bonds (Maurer, 2001).

3. Structure of Bromelain

The similar among the sulfhydryl proteases extends to the amino acid sequence around the essential cysteinyl and histidyl residues of the active site (Table 2). The active site Cys and His residues are at positions 25 and 159, respectively, in papain. Residues 25 and 159 are the cysteine and histidine residues that form the catalytic thiolateimidazolium pair. They have in common a broad pH optimum between pH 6 and 7.5 and their activity appears to be dependent on two prototropic groups with p*K*a values near 4 and 8.5. The enzymes are quite heat stable

up to 60 to 80 °C at neutral pH (Whitaker, 1994). The complete amino acid sequence of stem bromelain exists as a single polypeptide chain with 211 or 212 residues, depending on the presence or absence of the N-terminal Ala (Ritonja *et al.,* 1989). The thiolateimidazolium pair at the active site of stem bromelain is likely to have a different conformation from that in papain. The geometry and reactivity of the catalytic site of bromelain are different from papain that bind cystains tightly and react rapidly with E-64 (Ritonja *et al*., 1989).

Table 2 Amino acid sequences around he essential sulfhydryl group and histidine in several sulfhydryl proteolytic enzymes.

Sources	**Amino acid sequences**
Bromelain, stem	Asn-Gln-Asp-Pro-Cys-Gly-Ala- **Cys*** -Trp-
Papain	- Pro-Val-Lys-Asn-Gln-Gly- Ser-Cys-Gly-Ser-**Cys*** - Trp-
Ficin	- Pro- lLe-Arg-Gln-Gln-Gly- Gln-Cys-Gly-Ser-**Cys*** -Trp-
Bromelain, stem	-**His*** -Ala-Val-Thr-Ala-lLe-Gly-Tyr-
Papain	-Val-Gly-Pro-Cys-Gly-Asn-Lys-Val-Asp-**His*** -Ala-Val-Ala-Ala-Val-Gly-Tyr-
Ficin	-Thr-Gly-Pro-Cys-Gly-Thr-Ser-Leu-Asp-**His*** -Ala-Val-Ala-Leu-

Source: Whitaker (1994)

Evidence for involvement of the sulfhydryl group in the active site includes (a) loss of activity when the sulfhydryl group is modified or cysteine is replaced by another amino acid, (b) the spectrophotometric observation and isolation of an acylenzyme intermediate that has all the properties of a thioester, and (c) kinetic

studies which indicate involvement of a group with a p*K*a value of 8.3 and a ΔH ion value of 5.1 kcal/mol.

A second group involved in the active site of papain is either a carboxyl group, a histidyl group, or both. In the x-ray diffraction pattern the nearest carboxyl group is 7.5 °A away from the essential sulfhydryl group. This distance is too far, by about 3 °A, to permit the carboxyl group (Asp-158) to be a catalytic group unless there is a conformational change in the active site on binding substrate. The carbonyl group of Asn-175 is a better candidate.

An alternative explanation is that the carbonyl group of Asn-175 is in the active site of papain but is hydrogen bonded to the imidazole group of His-159, thereby facilitating involvement of His-159 in the hydrolysis. This is substantiated by (a) the x-ray diffraction pattern of the resting enzyme, in which a histidyl residue (No. 159) is found to be 4.8 °A away from the essential sulfhydryl group, and (b) by chemical modification with the bifunctional reagent, dibromoacetone. This reagent reacts first with the very reactive sulfhydryl group but then the other bromo group reacts with histidyl-159 (Figure 1). Because of the small size of the dibromoacetone, the histidyl residue most certainly must be close to the sulfhydryl group.

Based on kinetic evidence and on the isolation of a thiolester intermediate, the reaction pathway for papain can be described by the general reaction (Figure 2) (Whitaker, 1994).

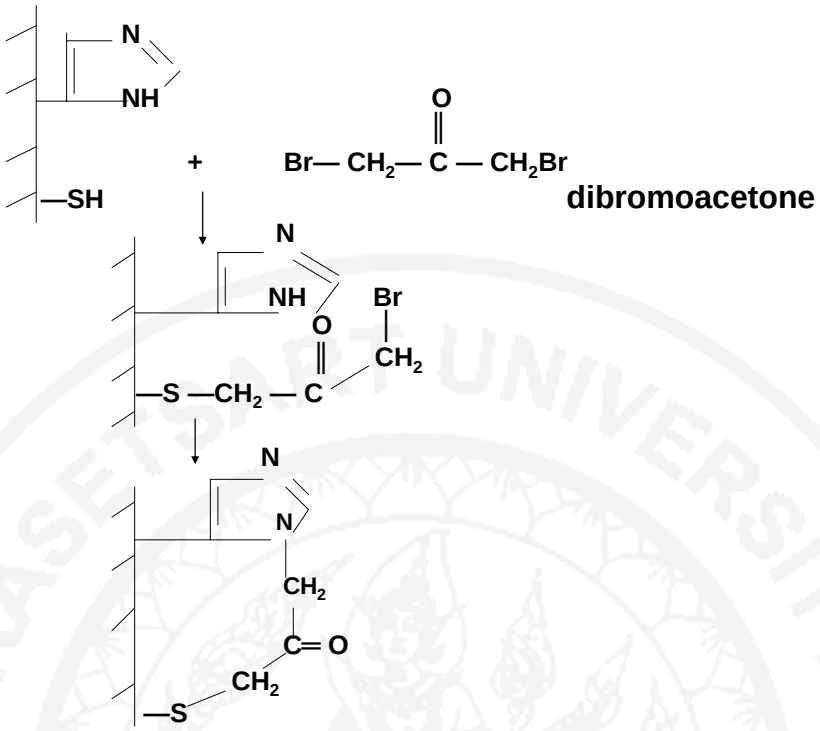


Figure 1 Chemical modification with the bifunctional reagent, dibromoacetone.
Source: Whitaker (1994)

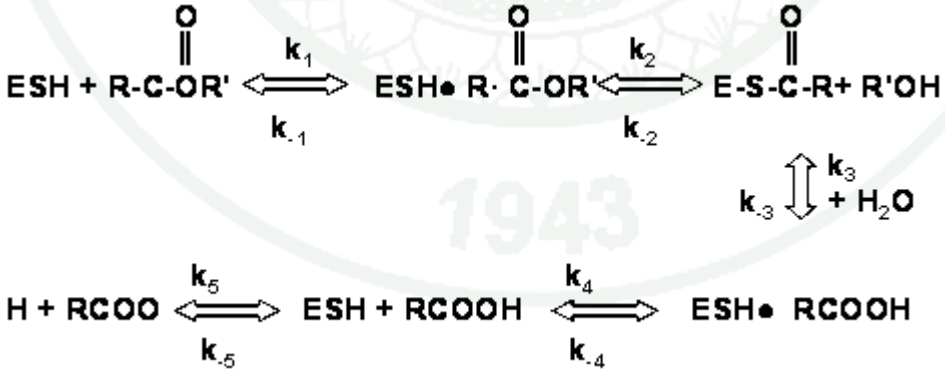


Figure 2 Kinetic evidence of papain and on the isolation of a thiolester intermediate
Source: Whitaker (1994)

4. Bromelain; pharmacology and medical uses

Commercial bromelain preparations are evaluated according to their proteolytic activity. The platelet aggregation inhibitory and antiinflammatory action seem to be related to the protease activity. However, other effects such as inhibition of tumor cell growth and metastasis as well as debridement of burns are associated with other nonproteinolytic components contained in bromelain. Thus, the determination of the proteolytic activity alone may not be sufficient to completely characterize the pharmacological properties of bromelain (Maurer, 2001).

Bromelain also contains peroxidase, acid phosphatase, several protease inhibitors and organically bound calcium and remains stable over a wide range of pH 2 to 9 which explains why its activity has been found to be effective over the entire gastrointestinal tract. Available evidence indicates bromelain is well absorbed orally with its therapeutic effects being enhanced in a dose dependent manner. It has been demonstrated to be safe and an effective food supplement. However, all the mechanisms of its action remain unresolved (Tochi *et al*., 2008).

4.1 Pharmacodynamics of Bromelain

4.1.1 Bromelain Promotes the Absorption of Antibiotic Drugs

It has been known for a number of years that bromelain is capable of enhancing the tissue permeability of penicillins and tetracyclins after oral

administration. This increases absorption and leads to an improved diffusion after subcutaneous and intramuscular application of the antibiotics. Higher serum and tissue levels are obtained, and side effects are reduced (Maurer, 2001).

4.1.2 Bromelain Affects Blood Coagulation and Fibrinolysis

In the inflammatory animal models, bromelain increased the fibrinolytic activity in a dose-dependent manner. An increase of prothrombin and partial thromboplastin time as well as a decrease of ADP – induced platelet aggregation. All these effects were clearly dose dependent and related to the proteolytic acitivity of bromelain, since inactivation of the enzyme abolished the effects. Oral administration of bromelain to healthy persons, particularly those with high platelet counts, significantly lowers the ADP-induced aggregation of platelets (Maurer, 2001).

4.1.3 Bromelain Prevents Edema Formation and Reduces Existing Edema

Several groups have provided significant evidence for both the edema-protective and edema-reducing efficacy of bromelain in a variety of classical animal experiments. Among them, papain another cysteine proteinase, was ineffective in all experimental models, whereas bromelain induced 41% (carrageenin) and 45% (dextran) inhibition of edema formation. In addition, bromelain showed the strongest edema-protective efficacy of all drugs tested, such as indometacin,

acetylsalicylic acid, aescin, oxyphenbutazon, and so on. It was concluded that bromelain increases tissue permeability by fibrinolysis and promotes reabsorption of edema fluid into blood circulation (Maurer, 2001).

4.1.4 Bromelain Achieves Debridement of Burns

Burns are characterized by formation of an eschar, which is made up burned and traumatized tissue. The eschar not only hinders accurate diagnosis of the burn ‘s depth but also serve as a medium for bacterial growth and therefore a source of infection, contamination and sepsis of the injury and to the neighbouring originally undamaged tissue (Rosenberg *et al*., 2004).

Enzymatic debridement has been suggested with experimental runs giving positive results. Topical bromelain (35% in a lipid base) has achieved 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 (Maurer, 2001).

4.1.5 Effects of Bromelain on Malignant Growth

First observations on the effects of bromelain on cancer patients were reported on beneficial effects following oral administration of bromelain to cancer patients. After treatments with relatively high doses for several weeks and months,

respectively, they noted remarkable remissions of malignant tumors with negligible side effects.

Bromelain inhibits the proliferation of different tumor cells in vitro. The inhibitory activity can be traced neither to the proteolytic nor to the peroxidase activity or to the platelet aggregation-inhibitory activity. The concentration-dependent inhibitory activity of bromelain crude extract and bromelain fractions on various tumor cells in vitro (Maurer, 2001).

4.2 Drug Safety

Bromelain is considered to be nontoxic and without side effects; therefore it can be used without concern in daily doses from 200 up to 2000 mg for prolonged periods time. Bromelain has shown therapeutic benefit in doses as small as 160 mg/day, but the best results occur when staring at a dose of 750 mg/day (Maurer, 2001). 2 g of fresh pineapple fruit needed to supply substrate activity equivalent to 1 mg stem bromelain. Previous studies of bromelain in humans have used doses from 1 to 12 g/day (Castell *et al*., 1997). Obtaining the stem bromelain activity of this dose solely from the fruit would therefore require consumption of 2,000 – 24,000 g fresh pineapple fruit per day (Hale *et al.,* 2005).

5. Bromelain Activity in Pineapple

5.1 Proteolytic Activity of Bromelain

A widely used method in assay of proteolytic activity of bromelain is the change in the trichloroacetic acid (TCA) solubility of a protein when it is subjected to the action of a proteolytic enzyme. As a proteolytic enzyme acts on a protein, the amount of TCA-soluble peptides produced is proportional to the amount of enzyme and time of action. The amount of TCA-soluble products formed can be determined by measuring the absorbance of the supernatant liquid at 280 nm. The method is fast and precise but does not give the number of peptide bonds hydrolyzed (Whitaker, 1994).

Activity of stem bromelain extracted from *Ananas comosus* of the Smooth Cayenne variety by different extraction. Acetone extraction method had 7,256 CDU/g (Orwin, 1984). Vaccum evaporater and ultrafiltration method had 18,030 CDU/g (Theerakulkait, 1985). Ammonium sulphate sedimentation and freeze dryer method had 5,750.77 CDU/g (Supaniuda *et al.,* 2008). Activity of stem bromelain extracted from *Ananas comosus* L. Merryl cv. Kew by reverse micellar extraction had 681.16 CDU/g (Hebbar *et al*., 2008). Activity of fruit bromelain extracted from unripe fruits of *Bromelia balansae* Mez had 17 Ucas/g (Pardo *et al*., 2000). Ripe fruits of *Bromelia antiacantha* Bertol had 200 Ucas/g (Valles *et al*., 2007) and immature fruits of *Pseudananas macrodontes* (Morr.) Harms had 10.73 Ucas/g (Natalucci *et al.,* 1996).

5.2 Relationship between Enzyme Bromelain Activity and Total Soluble Solids

Bromelain activity remained high during fruit development and declines during ripening. It seems reasonable to speculate that bromelain is transformed to another protein which may have a different metabolic role, such as the flavor-producing enzymes (Paull and Chen, 2003). The volatile flavor constituents are first formed at the time bromelain activity is dropping (Gortner and Singleton, 1965). The major sugars in mature fruit are sucrose, glucose and fructose and the peak in sucrose concentration is attained at full-yellow stage and then declines. Fruit sugars continued to increase through to senescence, unless the fruit is harvested. Total soluble sugar content is low during fruit growth and composed mainly of glucose and fructose. Glucose is at a slightly higher concentration than fructose during the early stages of fruit development. Sucrose accumulated rapidly 6 weeks before commercial harvest and ultimately exceeds the glucose and fructose concentration. Fructose and glucose continue to increase postharvest. In addition, sucrose accumulated more in the fruitlet than in the interfruitlet tissue until the last 2 weeks of fruit development, when sucrose accumulation rate in the interfruitlet tissue was greater than in the fruit let. Total soluble solids, mainly sugars, are often used as an indicator of fruit maturity and quality. Total soluble solids can vary by 40 g/l or 4 °Brix from the more mature, sweeter basal tissue to the crown end of the fruit, and decline only slightly after harvest (Paull and Chen, 2003).Yoon *et al*.(2006) found that a positive relationship between acid phosphatase and °Brix in cherries. Acid phosphatase appears to play an important role in the metabolism of carbohydrates in cherries. Areas and Lajolo

(1981) reported that as starch was transformed into soluble sugar (sucrose, glucose, fructose) acid phosphatase was increased during banana ripening. In contrast, acid phosphatase activity decreased while starch synthesis increased (Goswami and Borthakur, 1996).

5.3 Relationship between Bromelain Activity and Total Acidity

Juice pH declines from 3.9 to 3.7 as fruit approach the full-yellow stage and increases as the fruit senesces, with titratable acidity showing the opposite trend. The flesh acidity increases distally from the central core (4 mEq /100 ml) out wards to 10 mEq/100 ml) and a major portion (65 – 70%) of the total non-volatile acids occurs as free organic acids. The two major non-volatile organic acids are citric and malic. Malic acid can vary from 18% to 30% of total acids in pineapple and does not vary markedly between the cool-and warm-season crops. Citric acid (28 – 66% of total acids) is lower in Smooth Cayenne fruit harvested in the warm season and tends to vary primarily with stage of fruit development (Paull and Chen, 2003). There were not found any researches about relationship between total acidity of pineapple and bromelain activity in pineapple. Other reports were relationship between total acidity and enzyme in citrus fruits and cherries. Marsh *et al*., (2001) reported that acid limes had the lowest level of pyrophosphatase acivity, while sweet limes and moderately acid Valencia oranges contain the highest and intermediate levels of pyrophosphatase activity, respectively. Yoon *et al*. (2006) found that an inverse relationship appeared to exist between the acid phosphatase activity and the acidity in cherries.

6. Optimal pH of Enzyme

An enzyme has maximum activity only within a narrow pH range. The pH optimum of an enzyme is dependent on a number of experimental parameter, including time of reaction, temperature, nature and concentration of substrate, nature and concentration of buffer, ionic strength of medium, and purity of enzyme preparation, among others (Whitaker, 1994). Valles *et al*. (2007) found that pineapple fruit juice exhibited high caseinolytic activity (higher than 80%) in a broad pH range (5 – 9), with two maximum: at pH 6.0 and 9.0. Whereas, Liang *et al.* (1999) found that pineapple fruit juice have optimum pH 6.8 – 9. The optimal pH of bromelain from commercial (stem bromelain) for casein is 6 – 8 (Helmut, 1998).

Factors responsible for the effect of pH on enzyme-catalyzed reactions can be devided into two categories: (a) those that influence the stability of the enzyme, and (b) those that influence binding and catalysis.

The effect of pH on catalytic activity should be clearly distinguished from the effect of pH on stability of enzymes. Whitaker (1994) defined the effect of pH on the catalytic activity of an enzyme to mean the effect of pH on the ionization of prototropic groups involved in the active site of an enzyme. The prototropic groups (groups capable of ionizing) in the active site of and enzyme may be involved (a) in maintaining proper conformation of the active site, (b) in binding of substrate to enzyme, or (c) in transformation of substrate to products. These prototropic groups are located on the side chains of the acidic and basic amino acid residues (Whitaker,

1994). Prototropic group that may be involved in enzyme catalysis was sulfhydryl group in cysteinyl residue.

$$-SH \leftrightarrow -S^- + H^+ \qquad pKa\ 8.0\text{-}8.5. \qquad \Delta H \text{ ion } 6.5 - 7.0 \text{ kcal/mol}$$

The similar among the sulfhydryl proteases extends to the amino acid sequence around the essential cysteinyl and histidyl residues of the active site. The active site Cys and His residues are at positions 25 and 159, respectively (Whitaker, 1994).

Proteins are more stable against denaturation at their isoelectric point than any other pH. At neutral pH, most proteins are negatively charged, and a few are positively charged. Since the net electrostatic repulsive energy is small compared to other favorable interactions, most proteins are stable at around neutral pH. However, at extreme pH values, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecule. The degree of unfolding is greater at extreme alkaline pH values than it is at extreme acid pH values. The former behavior is attributed to ionization of partially buried carboxyl, phenolic and sulfhydryl groups, which cause unraveling of the polypeptide chain as they attempt to expose themselves to the aqueous environment. pH – induced denaturation is mostly reversible. However, in some cases, partial hydrolysis of peptide bonds, deamidation of Asn and Gln, destruction of sulfhydryl groups at alkaline pH, or aggregation can result in irreversible denaturation of proteins (Damodaran, 1996).

7. Enzyme Kinetics

Two main approaches have been used to explain chemical reactivity: thermodynamics and kinetics. The kinetics, or time course, of processes can provide any information on the rate or mechanism of a chemical reaction. For this purpose it is necessary to construct a mathematical model that embodies the hypothesized mechanisms. Whether or not the solutions of the resulting equations are consistent with the experimental data will either prove or disprove the hypothesis (Marangoni, 2003).

Enzyme kinetics is the study of enzymes in action. Enzyme activity is affected by various parameters such as: enzyme concentration, substrate concentration. Kinetics provide a systematic approach for the analysis and quantification of the effect of these parameters on enzyme activity. In many cases the mathematical models, for simplicity, must assume ideal conditions of the enzyme reaction. Often these condition are unrelated to those encountered in practice, but in most cases the kinetic models are a good indication of enzyme action in *situ* (Tucker, 1995).

7.1 Enzyme concentration

Enzyme velocities are linear with time. Enzyme reactions considered over a considerable period of time are definitely not linear but follow a general trend as illustrated in Figure 3. The initial rate may be linear, but eventually the rate begins to decline. These reasons for reduced activity with time are often complex but a major

cause, presuming a closed system, is obviously depletion of substrate. During the early stages of the reaction when substrate concentration [S] can be assumed to be constant, the enzyme reaction should be zero order with respect to product (P) formation; i.e.

$$\frac{d\,P}{d\,t} = k_O$$

where k_O = the zero order rate constant for the reaction.

However, in reality, the concentration of substrate decreases with time, the reaction follows first-order kinetics, and the change in rate of product formation is given by Equation;

$$\frac{d\,P}{d\,t} = k_1\,(S - P)$$

where k_1 = first order rate constant for the reaction (Tucker, 1995).

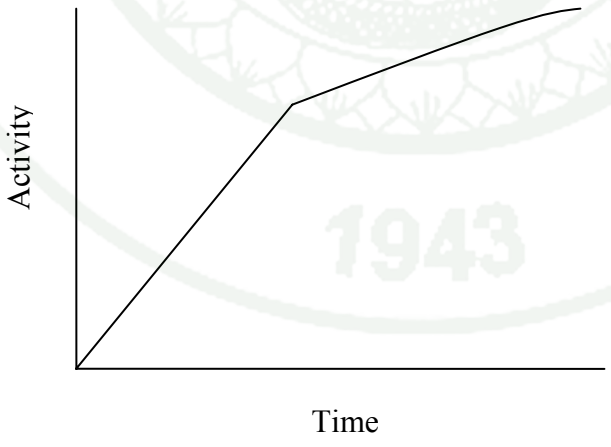


Figure 3 Progress of enzyme activity with time.
Source: Modified from Tucker (1995)

7.2 Substrate concentration

The rate of an enzyme-catalysed reaction is obviously dependent on substrate concentration. However, it is apparent from consideration of the reaction that the relationship between the velocity of the reaction (*V*) and concentration of substrate [S] will not be linear. This arises due to the mechanism of enzyme action in which the enzyme forms a one-to-one stoichiometric complex with its substrate and it is only this complex that can breakdown to give the product (P). Thus as substrate concentration [S] increases the enzyme becomes saturated and hence activity tends towards a maximum as shown in Figure 4. Thus it is expected that for unimolecular reactions the initial velocity will be directly proportional to substrate concentration [S] only at the lower range of [S] and as [S] becomes progressively larger the initial velocity approaches a maximum (V_{max}) It is usual to determine initial velocities in kinetic studies since, as shown in the proceeding section, velocity is not always linear with time (Tucker, 1995).

While it is obvious from actual experimental measurements that velocity-substrate curves tend to follow the trend shown in Figure 5 for invertase, the actual determination of V_{max} by this method is difficult. However, it is possible to derive mathematical equations to model the relationship between *V* and [S]. One such simple model for a unimolecular reaction was developed by Michaelis and Menton. For the purpose of this model the enzyme-substrate interaction is considered to occur as shown in Equation (1).

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_3} E + P \quad \text{.....(1)}$$

Where E = free enzyme; ES = enzyme – substrate complex; and k_1, k_2 and k_3 are the rate constants for the formation of ES, release of S or release of P, respectively. The following assumptions have to be made:

1. The concentration of substrate must be much larger than that of the enzyme.

2. The concentration of P must be effectively zero such that there is no reverse reaction.

3. The rate constant for the release of product (k_3) must be much slower than that for the release of substrate (k_2) from the enzyme. Thus k_3 must represent in effect the limiting rate for the reaction as a whole and E and ES are essentially at equilibrium.

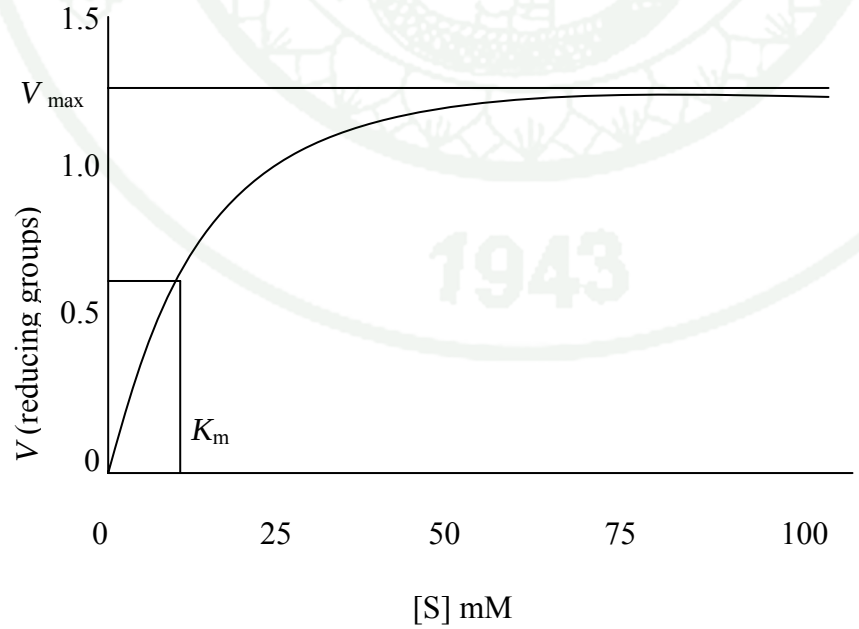


Figure 4 Effect of sucrose concentration on the activity of invertase
Source: Modified from Tucker (1995).

In general, these assumptions are true. Substrate is almost always in large excess, initial velocity readings ensure [P] approximates to zero and the catalytic step or k_3 is usually rate-limiting.

Since E and ES are in equilibrium, an equilibrium expression as in Equation (2);

$$k_1[E][S] = k_2 [ES] \quad \text{.....(2)}$$

$$[E] = \frac{[ES]\, k_2}{k_1 [S]} \quad \text{.....(3)}$$

However, total enzyme in the reaction e must be equal to the sum of [E] (free enzyme) and [ES] (enzyme-substrate complex) thus e = [E] + [ES]. If we substitute this for [E] in Equation (3), the equation (4) can be generated , which can be rearranged to form Equation (5)

$$e = \frac{[ES]\, k_2}{k_1 [S]} + [ES] \quad \text{.....(4)}$$

$$e = [ES] \left(1 + \frac{k_2}{k_1[S]} \right) \quad \text{.....(5)}$$

Since k_3 is the rate-limiting constant, the overall rate of the reaction (V) is equal to k_3 [ES]. Thus substituting V/k_3 for [ES] in Equation (5) and rearranging to form Equation (6)

$$V = \frac{k_3e}{1 + \frac{k_2}{k_1[S]}} = \frac{k_3e[S]}{[S] + \frac{k_2}{k_1}} \quad \text{..............................(6)}$$

Since k_1 and k_2 are constants, k_2/k_1 is also constant and is referred to as the Michaelis constant or K_m. Also k_3e represents the maximum possible rate of the reaction (all of the enzyme in the form ES, i.e. saturated) and is usually written as V_{max}. Substituting these new terms in Equation (6) arrive at the Michaelis-Menton equation-Equation (7).

$$V = \frac{V_{max}[S]}{[S] + K_m} \quad \text{... (7)}$$

This model related to the type of curve obtained experimentally as in Figure 5. When [S] is much lower than Km, Equation 7 approximates to $V = V_{max}[S]/K$m and thus for small values of [S], V is proportional to [S]. However, as [S] increases and K_m becomes insignificant in comparison, Equation (7) will approximate to $V = V_{max}$. This is essentially the situation predicted by considering how enzymes work and approximates to the experimentally derived cures as shown in Figure 5 (Tucker, 1995).

K_m, which is independent of enzyme concentration, and V_{max} which is dependent on enzyme concentration and thus usually expressed as 'Units' or katals of activity per milligram protein, or other convenient measure of enzyme amount. When enzyme concentration (molar) was equal, V_{max} value was shown the efficiency of enzyme reaction.

K_m is 'that substrate concentration that gives half maximum rate' This can easily be seen from Michaelis-Menten equation that set [S] = K_m the equation becomes:

$$V = \frac{V_{max}}{2}$$

However, in reality K_m is the ratio of the two rate constant k_1 and k_2, i.e., the constants for the formation or breakdown of ES without any catalysis. It therefore gives an indication of the enzyme affinity for its substrate and thus how 'quickly' the reaction will approach saturation as [S] increases, therefore, a low K_m value indicates an enzyme that will work efficiently (i.e. with a high V) at a low substrate levels and *vice versa* at high substrate levels. Thus, two enzymes with the same V_{max} but widely different K_m values will have significantly different efficiencies during processing. Enzyme with the low K_m will maintain a higher reaction rate as substrate levels decline, presuming that enzyme stability is maintained with time (Tucker, 1995).

7.3 Enzyme kinetic of bromelain

K_m of stem bromelain for BAEE (benzoyl-L-arginine ethyl ester) is much higher than that for BAA (benzoyl-L-argininamide). The value for the latter is 1.2 x 10^{-3} M, while that for BAEE is 0.17 M, or 142 times the value for BAA (Inagami and Murachi, 1963).

The kinetic features of bromelain, however, the 140-fold difference of k_3 between BAEE and BAA indicates that the process common to the two substrates cannot be rate limiting for both. It also seems to be improbable that the difference of the rate of initial binding of BAEE and BAA is large enough to explain the difference in k_3 in view of the similarity of the size and gross structure of the two molecules. Therefore, a mechanism involving three or more steps (Inagami and Murachi, 1963).

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES_1 \overset{k_2}{\leftrightarrow} ES_2 + P_1 \overset{k_3}{\leftrightarrow} E + P_2$$

$$\frac{d[P_2]}{dt} = \frac{k_3([E]_o[S]}{[S] + K_m}$$

where $[E]_o$ is the total concentration of enzyme and k_3 and K_m are will be required.

$$k_3 = \frac{k_2 k_3}{k_2 + k_3}$$

$$K_m = \frac{(k_{-1} + k_2)}{k_1} \frac{(k_2)}{k_2 + k_3}$$

8. Thermal Stability of Enzyme

An enzyme is a protein that is a large and complicated molecule. Stability of an enzyme is a function not only of temperature but also of pH, ionic strength and

nature of buffer, presence or absence of substrate, concentration of enzyme as well as other proteins in the system, time of incubation, and the presence or absence of activators and inhibitors. Data on the temperature stability of an enzyme have meaning only when all of these factors are controlled and stated explicitly (Whitaker, 1994).

The thermal stability of enzymes is quite variable. Only a few enzymes lose their catalytic activity at 0 °C, while some are even resistant to high temperatures, at least for a short time. In a few cases enzyme stability is lower at 0-10 °C than in a medium temperature range (20-40 °C). Lipase and alkaline phosphatase in milk are thermolabile, whereas acid phosphatase is relatively stable. Therefore, alkaline phosphatase (its activity is easier to determine than that of lipase) is used to distinguish raw from pasteurized milk. Peroxidase is the last enzyme in the potato tuber to be thermally inactivated. Such inactivation patterns are often found among enzymes in vegetables. In such cases, peroxidase is a suitable indicator for controlling the total inactivation of all the enzymes as is required in assessing the adequacy of a blanching vegetables process (Belitz and Grosch, 1987).

8.1 Effect of temperature on stability of enzyme

In general, enzymes are more stable the lower the temperature. There are a few enzymes that are more unstable at 0 to 10 °C than at 20 to 30 °C because of the types of bonds holding the subunits together. There are also great differences among enzymes in their susceptibility to heat. For example, beef liver catalase is unstable at

35 °C, whereas ribonuclease can withstand exposure to 100°C for a few minutes. Adenylate kinase can be boiled for a long time without loss of activity. Milk Alkaline phosphatase and plant peroxidase are both relatively heat stable near pH 7 and are used in the dairy and processing industries for measuring adequacy of pasteurization and blanching, respectively. Because of their high-temperature stability, other enzymes and microorganisms are destroyed by heat by the time these enzymes are inactivated. Some correlation may be drawn between the size and complexity of an enzyme and its susceptibility to heat. In general, those enzymes that have molecular weights ranging from 12,000 to 50,000, are composed of single polypeptide chains, and have disulfide bonds are more resistant to heat treatment. The larger an enzyme and the more complex its structure, the more susceptible is to high temperatures (Whitaker, 1994).

There are usually three reasons that temperature effects on enzymes are studied (a) to determine stability of the enzyme; (b) to determine the activation energy, *E*a, of the enzyme-catalyzed reaction; and (c) to determine the chemical nature of the essential prototropic groups in the active site of the enzyme. The design of the experiments is not different, except the buffer must always be made up to have the same pH at all temperatures used. This requires the buffer to be made up at the temperature to be used. Otherwise, the pH of the buffer is an uncontrolled variable (Whitaker, 1996).

8.1.1 Stability of the enzyme determination

Stability of enzyme can be determined in the following manner. Tubes with a fixed concentration of enzyme (similar to those to be used in the kinetic experiments), in the buffer adjusted to pH desired when made up at each temperature to be used, are incubated at the selected temperatures (usually starting at 25 °C). Aliquots are removed at various times, substrate in a buffer near the pH optimum is added, and the activity left is determined at a constant temperature and pH. The control enzyme activity (100%) is determined on enzyme maintained at 0 °C. Since the rate of loss of activity is usually first order for pure enzyme (no isozymes present). The slope of the line is *k*, the rate constant for denaturation of the enzyme as Figure 5 (Whitaker, 1996).

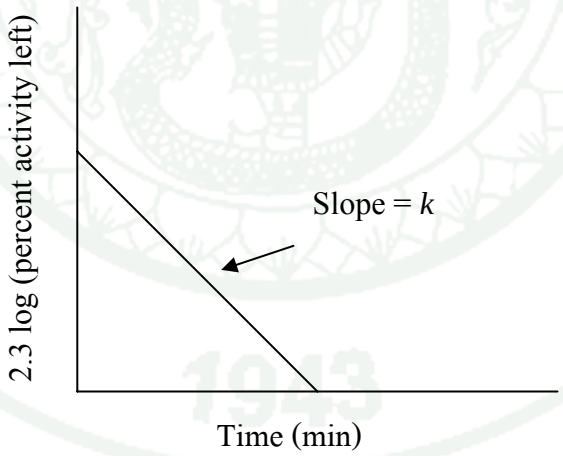


Figure 5 Rate of denaturation of an enzyme at constant temperature.
Source: Adapted from Whitaker (1996)

8.1.2 The activation energy (*Ea*) determination

To determine activation energies, two plots are required, the first is a plot of experimentally determined product concentration versus time at various temperatures, and the second a plot of log *k*, the zero-order reaction rate constant versus 1/*T* (Whitaker, 1996).

The rate of an enzyme-catalysed reaction, as for all chemical reactions, increases with temperature. The relationship between rate and temperature is best described by the Arrhenius equation. The dependence of the specific reaction rate constant, *k*, on temperature is given by (Whitaker, 1996).

$k = \mathrm{A}\, e^{-Ea/RT}$

which may be rewritten as taking logarithms of both sides of equation.

$$\mathrm{Log}\ k = \log \mathrm{A} - \frac{Ea}{2.3\ \mathrm{R}T}$$

where *k* is specific reaction rate constant at some temperature, *T*(K), and A and *Ea* are empirical constants. *Ea* is usually referred to as the Arrhenius activation energy and A is called the frequency factor or Arrhenius factor.

Ea is best evaluated by plotting log k versus 1/*T* (Figure 6) This plot is valid for the effect of temperature on any rate process.

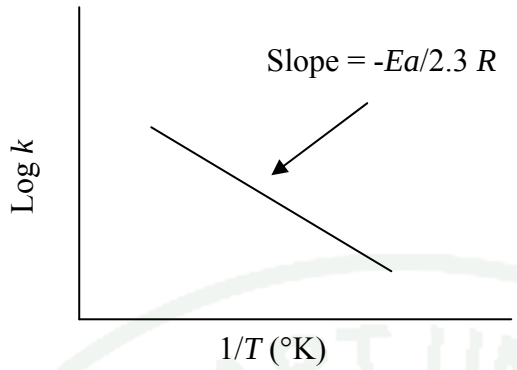


Figure 6 Effect of temperature on rate constant of a reaction.
Source: Whitaker (1996)

Ea can be defined as the minimum quantity of energy that a reactant molecule must have in order to be converted to a product molecule (Whitaker, 1994). In general, activation energies for transformation of reactants to products (catalysis) in enzyme-catalyzed reactions are within the range 6000 to 15,000 cal/mol or 25.1 – 62.8 kJ/mol while activation energies for denaturation of enzymes are within the range 50,000 to 15,0000 cal/mol or 209.2 – 627.5 kJ/mol. Therefore, enzymes will be relatively stable at lower temperatures. But at higher temperatures denaturation will become very rapid because relatively larger numbers of molecules have sufficient energy to achieve the denatured state. This is illustrated in Table 3, where *Ea*, the activation energy, is considered to be 6000 and 60,000 cal/mol for conversion of reactants to products and denaturation, respectively. At 60 °C the rate of conversion of conversion of reactant to product is only 11.4 times faster than at -10 °C, while the rate of denaturation is 3.16×10^{10} faster at 60 °C than at -10 °C (Whitaker, 1994).

Table 3 Relative effect of temperature on rate of transformation of reactants [(1)] and on rate of denaturation [(2)].

Temperature (°C)	Relative rates at *Ea* of:	
	6,000 cal/mol[(1)]	60,000 cal/mol[(2)]
-10	1.0	1.0
0	1.55	7.94×10^{1}
20	3.24	1.26×10^{5}
40	6.31	1.0×10^{8}
60	11.4	3.16×10^{10}

Source: Whitaker (1994)

Among the studies concerning the thermal stability of bromelain, most have been performed using commercial bromelain from the stems of pineapples (Yoshioka *et al*., 1991; Arroyo-Reyna and Hernandez-Arana, 1995; Gupta *et al*., 2007). Commercial bromelain from pineapple stems has been found to be completely inactivated by heating for 30 min at 60 °C (Yoshioka *et al*., 1991), while Gupta *et al*. (2007) found that bromelain retained 50% activity level after 20 min heating at 60 °C. Liang *et al*. (1999) found that bromelain from concentrated pineapple juice retained 50% of its initial activity after 60 min heating at 60 °C. Bromelain from frozen pineapple fruit had no activity loss when incubated at 37 °C for a period of 120 min; whereas at 45 °C almost 80% of activity remained. The enzyme was almost completely inactivated by heating for 60 min at 75 °C (Pardo *et al*., 2000). Valles *et al*. (2007) found that there was no bromelain enzyme activity loss when incubated at 37 °C for 180 min, or at 55 °C for 60 min; while after 30 min at 60 °C it

retained 80% of its initial activity. In these studies, the researchers used different purified bromelain and varieties of pineapples.

The *E*a of bromelain were 174.47 kJ/mol (Yoshioka *et al*., 1991) and 181 ± 35 kJ/mol (Arroyo-Reyna and Hernandez-Arana, 1995) which all these data was from commercial pure bromelain extracted from stem pineapple.

8.2 Effect of pH on stability of enzyme

Control of pH is often crucial and in many instances when the pH range suitable for processing does not coincide with the optima for the enzymes used, pH can become a limiting parameter. Besides limiting catalytic activity, pH also has a marked effect on enzyme stability. Thus enzymes not only exhibit a range of pH values within which they are active, but also within which they are stable. These two pH bands are not necessarily equivalent, indeed the stability range tends to be greater than the activity range. However, the effect of pH on stability, especially the combined interactive effect of pH and temperature, must be a consideration during processing (Tucker, 1995).

Most enzyme undergo irreversible denaturation in very acid and very alkaline solutions that affect the secondary, tertiary, and/or quaternary structures of enzyme. The pH at which this occurs varies with enzyme. Pepsin, the proteolytic enzyme of the stomach, is very rapidly inactivated at pH 7, whereas it is quite stable

at pH 2. Milk alkaline phosphatase is quite stable within the region pH 7 to pH 9, but above pH 9.5 it is rapidly inactivated (Whitaker, 1994).

9. Effects of Solutes on Thermal Stability of Enzyme

It has been common practice over many decades for biologists and biochemists to control the stability of enzymes and other proteins, as well as of assembled organelles, by the addition of solute at high concentration (typically at 0.3-10 M) (Timasheff, 1994). Nature selects molecules to act as solutes on the basis of the criteria: (1) that they should not affect specifically any enzyme or other cellular processes; (2) that they should not be electrically charged so as not to upset the electrostatic balance of cellular components; (3) that they should act as native protein structure stabilizers and be preferentially excluded from contact with cellular components so as not to perturb their structures. Molecules which satisfy universally the last two criteria are sugars, glycerol, many polyols, as well as many amino acids and their derivatives (Timasheff, 1992).

This research studied the effect of three solutes on bromelain activity. They were trehalose, sucrose and sorbitol.

9.1 Trehalose

Trehalose is a white, odorless powder with relative sweetness 45% that of sucrose. It is a bisacetal, nonreducing homodisaccharide in which two glucose units

are linked together in a a-1, 1-glycosidic linkage (a-D-glucopyranosyl-a-D-glucopyranoside; mycose, mushroom sugar) (Figure 7).

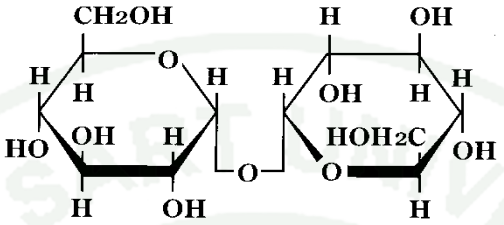


Figure 7 Structure of trehalose
Source: Richards and Dexter (2001)

Some of the properties of trehalose are listed in Table 4. Because of the inherent properties of trehalose, namely prevention of starch retrogradation and stabilization of proteins and lipids, it has proved quite useful in a number of industries including food processing, cosmetics, pharmaceutics, and so forth (Richards *et al*. 2003).

9.2 Sucrose

Sucrose is composed of an α-D- glucopyranosyl unit and a β-D-fructofuranosyl unit linked head to head (reducing end to reducing end) rather than by the usual head-to-tail linkage (Figure 8). Since it has no reducing end, it is a nonreducing sugar. Some of the properties of sucrose are listed in Table 4.

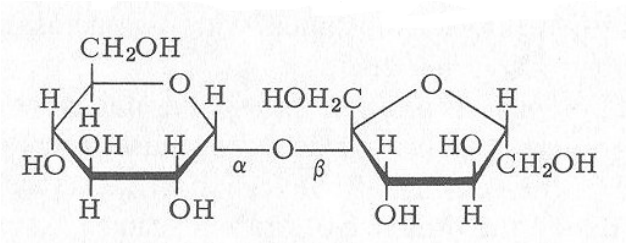


Figure 8 Structure of sucrose
Source: Modified from BeMiller and Whistler (1996)

There are two principal sources of commercial sucrose-sugar cane and sugar beets, Also present in sugar beet extract are a trisaccharide, raffinose, which has a D-galactopyranosyl unit attached to sucrose, and a tetrasaccharide, stachyose, which contains another D-galactosyl unit.

A portion of the water in any carbohydrate solution is nonfreezable. When the freezable water crystallizes, that is, forms ice, the concentration of solute in the remaining liquid phase increases, and the freezing point decreases. There is a consequential increase in viscosity of the remaining solution. Eventally, the liquid phase solidifies as a glass in which the mobility of all molecules becomes greatly restricted and diffusion-dependent reactions become very slow; because of the restricted motion, these glass-state water molecules cannot crystallize. In this way, carbohydrates function as cryoprotectants and protect against the dehydration that destroys structure and texture caused by freezing (BeMiller and Whistler, 1996).

9.3 Sorbitol

Sorbitol and mannitol are six-carbon, straight-chain polyhydric alcohols, meaning they have more than one hydroxyl group. Both sorbitol and mannitol have six hydroxyl groups and the same molecular formula, $C_6H_{14}O_6$. They are isomers of one another and have different molecular configurations (Figure 9).

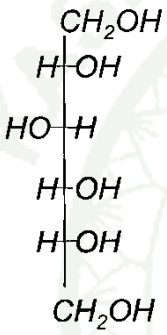


Figure 9 Structure of sorbitol
Source: Richards and Dexter (2001)

Sorbitol is produced from the catalytic hydrogenation of glucose. Crystalline sorbitol is made by further evaporating the sorbitol solution into molten syrup containing at least 99% solids. The molten syrup is crystallized into a stable crystalline polymorph that has one single melting point (99–101°C) and heat of fusion (42 cal/g, assuming 44 cal/g represents a fully crystallized crystalline sorbitol). The stable polymorph of sorbitol is known as gamma (γ) sorbitol. Sorbitol solution is hygroscopic, attracting and releasing moisture under varying humidity conditions, but it does so very slowly. Sorbitol provides improved moisture control and is more likely to maintain equilibrium in the surrounding environment. This slower rate of change in moisture content protects the food products in which sorbitol is used, thus maintaining

the quality of the product and extending the shelf-life. There is many different polymorphs of both sorbitol. The most stable polymorph of sorbitol is (γ)-sorbitol. The properties of γ-sorbitol are contained in Table 4. Sorbitol is widely accepted by the food and pharmaceutical industries as nutritive ingredients because of their ability to improve the taste and shelf-life of regular foods and special dietary products.

Use of the most stable polymorph ensures that the properties of the finished product will not change . Most of the commercially available sorbitol products are of the most stable polymorphs. Sorbitol is sweet, pleasant-tasting polyols. This characteristic makes them popular in confectionery and pharmaceutical taste-masking applications. Sorbitol is approximately 60% as sweet as sucrose. Sorbitol has a negative heat of solution, which gives them a cooling sensation in the mouth. Sorbitol's heat of solution is 26.5 cal/g (at 25°C) The caloric value of sorbitol is 2.6 cal/g in the United States. These are lower than the caloric value of sucrose, which is 4 cal/g (Richards and Dexter, 2001).

Table 4 Physical and chemical properties of solutes

Parameter	Trehalose[1]	Sucrose[2]	Sorbitol[2]
Sweetness	45% of sucrose	100%	60% of sucrose
Thermostability	120 °C	160 °C	>160 °C
Melting point	210.5 °C	186 °C	100 °C
Hygroscopicity	low	median	median
Solubility in water	68.9 g/100 g (20 °C)	67 g/100 g (25 °C)	75 g/100 g(25 °C)
Viscosity	8.2 cp (40 °C)	200 cp (25 °C)	80 cp (25 °C)

Source: [1]Richards *et al*. (2003)
[2]Lyn (2001)

9.4 Research on Effect of Solutes on Thermal Stability of Enzyme

Bromelain was the proteolytic enzyme that decreased in activity after heating. Therefore, it is necessary to find the way to preserve bromelain activity in pineapple juice during thermal treatment. Several studies have shown that stabilities of protein in aqueous solution may be increased by the addition of a solute. The decrease in activity of laccase after incubation for 24 h at 40 °C pH 4.5 was less in the presence of 0.05% trehalose than for the laccase without trehalose (Papinutti *et al*. 2008). Paz-Alfaro *et al.* (2009) studied the increase in thermal stabilities of glucose oxidase added to trehalose. Alpha-chymotrypsin was stabilized by sorbitol 1.24 M that retained around 50% of its activity in the 2-h incubation period (Simon *et al*. 2002). The thermal stability of purified tomato PME increased six-fold in the presence of sucrose when compared to 0.1 M citrate buffer (Gulavarch *et al*. 2003).

While, papain activity at 75 °C as a function of time was more in the presence of sucrose than for sorbitol (Sathish *et al.* 2007).

In contrast, Habib *et al.,* (2007) studied bromelain from stem and found that the decrease in activity of bromelain at 60 °C as a function of time was more in the presence of 1 M sucrose/trehalose than for the control sample. Trehalose inactivated bromelain more than sucrose.

The solutes interact with protein in a diverse way, depending upon the physicochemical properties of the proteins. The solutes are known to prevent the loss of enzyme activities (Sathish *et al*., 2007). The detailed and through studies of Timasheff and co-workers have proven that the ability of solutes to stabilize the structure and function of proteins is related to the preferential hydration macromolecules (Gekko *et al*., 1981; Timasheff and Arakawa, 1989). Preferential hydration is a thermodynamic phenomenon that reflects the inability of solute molecules to interact with proteins; thus, it leads to an exclusion of these solvent molecules from the protein surface. the protein has a higher affinity for water than for the solute (Timasheff and Arakawa, 1989).

10. Preferential Exclusion and Structure Stabilization

Preferential exclusion linked with stabilization of native protein structure. When a protein molecule is immersed into a solvent consisting of water and another chemical species (a co-solvent), the interactions between the protein and the solvent

components may lead to two possible situations: (1) the co-solvent is present at the protein surface in excess over its concentration in the bulk (this is what constitutes binding) ; (2) water is present in excess at the protein surface; this means that the protein has a higher affinity for water than for the co-solvent (this situation is referred to as preferential hydration, or preferential hydration, or preferential exclusion of the co-solvent). The cases are illustrated in Figure 10, which depicts schematically a dialysis equilibrium experiment. It is evident that the final equilibrium state reflects the relative affinities of the protein for water and the co-solvent (Timasheff, 1992).

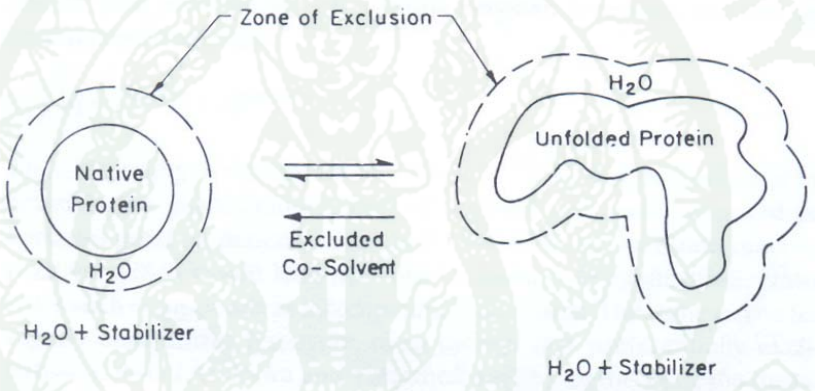


Figure 10 Pattern of protein-solvent interactions in the native and denatured equilibrium.
Source: Arakawa *et al*. (1990)

When the interaction is that of non-specific exclusion of the co-solvent by, for example, the increase of water surface tension by the co-solvent. Since in the asymmetric denatured state the surface of protein solvent contact is greater per protein molecule than in the compact native globular state, co-solvent exclusion is greater and the equilibrium is displaced toward native state.

11. Enzyme Denaturation Studies using Differential Scanning Calorimetry

A differential scanning calorimetry (DSC) measures the energy changes that occur as a sample is heated, cooled or held isothermally, together with the temperature at which these changes occur. One of the big advantages of DSC is that samples are very easily encapsulated, usually with little or no preparation, ready to be placed in the DSC, so that measurements can be quickly and easily made (Paul, 2008). The use of DSC to monitor conformational changes in the heat capacity of a protein system during heating provides a relatively simple and convenient technique for following protein denaturation. Commercially available differential scanning calorimeters, normally used with food systems, allow measurement of both reaction enthalpies (ΔH) and transition temperatures (Td); these two parameters are valuable in assessing how processing conditions affect protein conformation (Arntifield *et al.*,1990). Denaturation temperatures, (Td), and heats of denaturation, ΔHd, are often obtained with DSC. Most proteins denature at temperatures from 50 to 80 °C (Roos, 1995).

Protein denaturation is a process or a sequence of processes in which the spatial arrangement of the polypeptide chain within the protein molecule is changed from that typical of the native protein to a more disordered arrangement. Protein denaturation occurs over a temperature range that is specific for each protein and also dependent on other compounds and pH. The DSC thermogram for a single protein shows a fairly sharp endotherm at Td. The thermograms also show that the heat capacity of the native protein is lower than that of the denatured protein. The heat of

denaturation includes the heat of the conformational transition, the heat of ionization of the protein, and the heat of ionization of the buffer compound if denaturation occurs in a buffer solution. It should be noticed that the aggregation of protein molecules and breakup of hydrophobic interactions are exothermic processes that are associated with the total denaturation process (Roos, 1995).

DSC data for purified globular proteins such as ovalbumin, lysozyme, conalbumin and α-chymotrypsinogen, as well as the mixed system of egg white proteins, also demonstrated an increase in *Td* values in the presence of either glucose or sucrose, the greater increases again occurring with glucose. Furthermore, the extent of stablilization varied with the different proteins, suggesting the protein structure was a determining factor in the response to sugar environments. No Δ*H* values were reported due to baseline curvature in the presence of high sugar concentrations. With plant proteins, the same stabilization is evident; with vicilin, there is a linear increase in *Td* with sucrose concentration. There is no evidence of major changes in protein conformation as a result of the stabilization (no significant differences in Δ*H* values). As with the stabilizing salts, the influence of sugars on protein conformation is primarily through the indirect effect on hydrophobic interactions. Low levels of sugar increase the number of hydrophobic associations as evidenced by the decrease in surface hydrophobicity, and as sugar levels are further increased these interactions are strengthened (Arntifield *et al.*,1990).

Arroyo-Reyna and Hernandez-Arana (1995) used DSC to study the thermal denaturation of bromelain and found the denaturation peak at 59.3 °C .

Sathish *et al*. (2007) found that the thermal stability of papain increased in presence of xylose, sucrose, sorbitol and glycerol. Addition of 30% sorbitol increased the denaturation temperature of papain from 89 °C to 94 °C and induced the most thermal stabilization. While, Hedoux *et al*. (2009) reported that bovine serum albumin denatured at higher temperature in the presence of trehalose. The thermal denaturation temperature of bovine serum albumin increased with increasing sucrose content (Baier and McClements, 2006).

MATERIALS AND METHODS

Materials

1. Raw Material

Pineapples (*Ananas comosus*) of the Smooth Cayenne variety (Dole Thailand, Ltd.) from a supermarket during the fruiting season from December 2008 to May 2009. The shell colour was around 13-37% yellow eyes. The following are the shell color scale according to Paull and Chen (2003), which total soluble solids ranges had 8.0-16.0 °Brix of basal tissue.

Shell colour scale	0	Immature green
	1	0-12% yellow eyes
	2	13-37% yellow eyes
	3	38-62% yellow eyes
	4	63-87% yellow eyes
	5	88-100% yellow eyes
	6	Overripe

2. Apparatus

2.1 Confocal Laser Scanning Microscopy (CLSM)(Axio Imager MI, Carl Zeiss PTe Ltd, Germany)

2.2 Apparatus for pineapple juice preparation

2.2.1 Juice extractor (Hitachi Ltd., Tokyo, Japan)

2.3 Apparatus for the total soluble solids and acidity determination

2.3.1 Hand-held refractometer (model PAL-1 Atago#38 Tokyo, Japan).

2.3.2 pH meter (Jenco 6173 ,China)

2.3.3 Waring blender (model HGB2WT, USA)

2.4 Apparatus for the thermal stability

2.4.1 Water bath (Tempest HB25, Thailand)

2.4.2 Thermometer

2.4.3 Timer

2.5 Apparatus for the crude bromelain extract preparation

2.5.1 Refrigerated centrifuge (Sorvall RC 5C Plus, U.S.A.)

2.6 Apparatus for the enzyme analysis

2.6.1 Vortex (genie, scientific industries, INC. BOHEMIA, U.S.A.)

2.6.2 Hot plate magnetic stirrer (Cenco instrumenten , Breda, The Netherlands)

2.6.3 UV-visible spectrophotometer(Genesys10 UV Biomate 3,USA)

2.6.4 Glassware

2.7 Differential scanning calorimeter (PerkinElmer Pyris 1)

3. Chemical Reagents

3.1 Reagent for the confocal laser scanning microscopy

3.1.1 Rhodamine B hexyl ester perchlorate (Invitrogen)

3.1.2 95% ethanol (Merck)

3.2 Reagents for the enzyme analysis

3.2.1 Sodium dihydrogen orthophosphate (Analytical grade, Ajax)

3.2.2 Disodium hydrogen orthophosphate dodecahydrate (Analytical grade, Ajax)

3.2.3 Casein from bovine milk (Analytical grade , Fluka)

3.2.4 EDTA disodium salt (ethylenediaminetetra acetic acid disodium salt) (Analytical grade , Ajax)

3.2.5 L-Cysteine (Analytical grade , Fluka)

3.2.6 Trichloroacetic acid (TCA) (Analytical grade, Merck)

3.2.7 L-tyrosine (Analytical grade , Fluka)

3.2.8 Glycine (Analytical grade, Fisher)

3.2.9 Tris hydrochloride (Analytical grade , Fluka)

3.2.10 Commercial bromelain from the pineapple stem (Analytical grade, Fluka 16990) 2-5 U/mg (1 unit : the amount of enzyme which releases 1 μmol 4-nethophenol per minute at pH 4.6 and 25 °C (N_α –carbobenzoxy-L-lysine-4 nitrophenyl ester as substrate)

3.2.11 Bovine serum albumin (Analytical grade, Fluka)

3.2.12 Coomassie Brilliant Blue G-250 (Analytical grade, Fluka)

3.2.13 Ethanol 95% (Analytical grade, Merck)

3.2.14 85% phosphoric acid (Analytical grade, Ajax)

3.3 Reagents for the effect of solute on the thermal stability of bromelain

3.3.1 Sucrose (Food grade, MitrPhol, Thailand)

3.3.2 D-Sorbitol (Analytical grade, Fluka)

3.3.3 Trehalose (Food grade, Hayashibara, Japan)

Methods

1. Location of Bromelain in Pineapple Flesh

A solution of Rhodamine B (0.01% in 95% ethanol) was added to pineapple flesh prepared as cross section. After sample was loaded into a slide well and

observed for a location of fluorescent-labelled protein using the Confocal Laser Scanning Microscopy. An HeNe laser with an excitation wavelength 543 nm was used. CLSM digital image were acquired using the LSM 5 PAS-CAL program.

2. Pineapple Juice Preparation

Pineapples were washed, then peeled with an 80 mm diameter borer, and the cores were removed with a 30 mm diameter borer. Pineapple slices were cut into small pieces before separation of the pineapple juice with a juice extractor.

3. Crude bromelain extract extraction method and proteolytic Activity Assay

The method of a crude bromelain extract extracton and the proteolytic activity assays were Pardo *et al*.(2000) that method were shown in Appendix A1-A2.

Bromelain activity was calculated and expressed as casein digestion unit (CDU). A CDU is the amount of enzyme that will liberate one μg of tyrosine after one minute of digestion at 37 °C from a standard casein substrate solution at pH 7.0.

$$\text{CDU/g} = \frac{E - E_o \times b}{E_s \times a \times t} \times \frac{v}{w}$$

E = Absorbance at 280 nm of enzyme sample tube

E_o = Absorbance at 280 nm of enzyme blank tube

E_s = Absorbance at 280 nm of standard tyrosine = 0.0063

a = Crude bromelain extract volume = 1 ml

b = Total volume of crude bromelain extract and casein substrate solution and TCA = 30 ml

v = Total crude bromelain extract volume of pineapple juice in buffer = 50 ml

w = Weight of pineapple juice (g)

t = Reaction time = 10 min

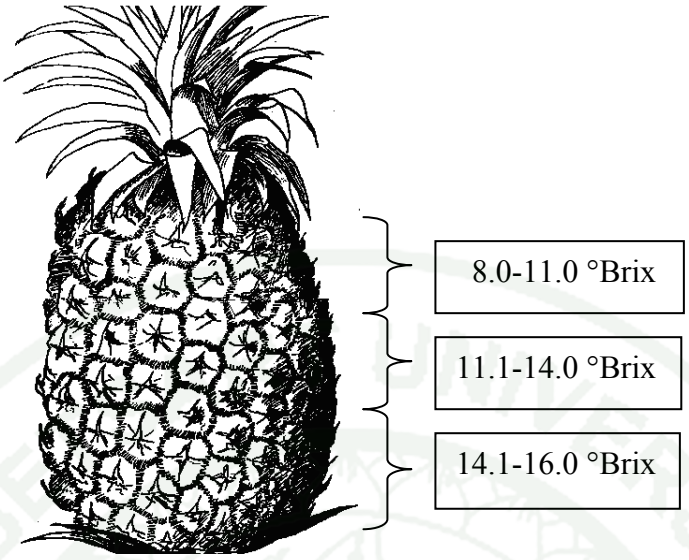
The calculation of recover method was shown in Appendix A5.

4. Protein Content Determination

Protein concentration was measured by the method of Bradford (Bradford, 1976), using bovine serum albumin as standard. The method was shown in Appendix A6. Specific activity of bromelain determination was shown in Appendix A7.

5. The Relationship between Enzyme Bromelain Activity and Total Soluble Solids

Pineapples were washed and peeled by a 80 mm diameter borer. The core was removed by a 30 mm diameter borer. Slices of 20 mm thick were cut perpendicularly to the fruit axis. Each slice was chopping. °Brix was measured with a hand-held refractometer (model PAL-1 Atago#38 Tokyo, Japan). Total soluble solids can vary from the more mature, sweeter basal tissue to the crown end of the fruit and total soluble solids was divided to three parts of fruit that were 8.0-11.0 °Brix, 11.1-14.0 °Brix and 14.1-16.0 °Brix.



The crude bromelain extracts were obtained by chopping and homogenizing fruits (8 g) for 1 min at 18,000 rpm in a Waring blender(model HGB2WT, USA) with 100 ml cold 0.1 M sodium phosphate buffer (pH 8.0, 5 °C) containing 5 mM EDTA and 25 mM cysteine. Homogenates (20 °C) were filtered through a two layers of gauze to remove plant debris and then centrifuged for 30 min 4 °C at 16,000 *g*. Supernatant was collected. 28 pieces of pineapples were used in the experiments. All measurements were done in duplicates.

6. The Relationship between Enzyme Bromelain Activity and Total Acidity

Pineapple juice that was measured total soluble solids and total acidity. Fruit acidity expressed as citric acid (g/100 g) was determined by titrating pineapple juice with 0.1 M NaOH to pH 8.2.

Crude bromelain extracts were obtained by chopping and homogenizing fruits (8 g) for 1 min at 18,000 rpm in a Waring blender (model HGB2WT, USA) with 100 ml cold 0.1 M sodium phosphate buffer (pH 8.0, 5 °C) containing 5 mM EDTA and 25 mM cysteine. Homogenates (20 °C) were filtered through two layers of gauze to remove plant debris and then centrifuged for 30 min 4 °C at 16,000 g. Supernatant was collected. The 28 pieces of pineapples were used in the experiments. All measurements were done in duplicates.

7. Optimal pH of Bromelain

The bromelain activity was assayed over the pH range of 3.0-13.0 (phosphate buffer 0.1 M pH 8.0 for pH 3.0-13.0 were adjusted with HCl 1 M and NaOH 1 M) at 37 °C for 10 min. All experiments were carried out in two trials. All measurements were done in duplicates. Each pH buffer solution was prepared as follow:

pH	3	4	5	6	7	8	9	10	11	12	13
Phosphate buffer pH 8.0(ml)	5	5	5	5	5	5	5	5	5	5	5
1 M HCl (ml)	4	3.8	3.4	2.8	1	-	-	-	-	-	-
1 M NaOH (ml)	-	-	-	-	-	-	-	0.4	0.6	0.8	1.0
0.1 M NaOH (ml)	-	-	-	-	-	-	3	-	-	-	-
Casein 1% w/v (ml)	11	11	11	11	11	11	11	11	11	11	11
Crude bromelain extract (ml)	1	1	1	1	1	1	1	1	1	1	1
TCA (ml)	18	18	18	18	18	18	18	18	18	18	18
Distilled water(ml)	-	0.2	0.6	1.2	3	-	1	3.6	3.4	3.2	3.0

8. Determination of Michaelis Constants (K_m) and Maximum Reaction Velocity (V_{max}) of Bromelain

The activities of bromelain were assayed at different casein concentrations (0.125%, 0.25%, 0.375%, 0.5%, 0.75%, 1%, 1.25%, 1.5% and 1.75% respectively). All experiments were carried out in two trials. The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were obtained from the plot of enzyme activity (CDU/g) and casein concentration [S]. All measurements were done in duplicates.

9. Thermal Stability of Bromelain

Pineapple juice (6 ml in each test tube) was incubated at different temperatures ranging from 40 to 80 °C for 0, 8, 12 and 60 min. The test tubes were quenched cool at 4 °C in an ice-water bath. Crude bromelain extracts were prepared from these cooled pineapple juice samples, Residual bromelain activity was measured by the proteolytic activity assay method and the suitable temperature for the study of the effect of solutes on thermal stability was chosen. All experiments were carried out in two trials. All measurements were done in duplicates.

The kinetics of thermal inactivation of the pineapple juice was studied. From a semi-logarithmic plot of the residual activity as a function of time, the inactivation rate constants (k) were calculated. Activation energy (Ea) for the thermal inactivation was calculated from the slope of the Arrhenius plot.

10. Effect of Solutes on Thermal Stability of Bromelain

The samples were prepared with sucrose, sorbitol and trehalose solutions at 1.3 M concentration in pineapple juice and only the dilution of sucrose in pineapple juice was prepared at 0.6 M concentration. The time course of the irreversible thermoinactivation was measured initially by incubation of 6 ml of pineapple juice in thermal baths at 60°C. At the regular intervals (4, 8, 12, 16 and 20 min) samples were removed and immediately cooled in ice-water baths; before the measurement of the enzyme activity of the samples. The enzyme activity of the same pineapple juice with solutes, kept on ice, was considered as the control sample (100%). All experiments were carried out in two trials. All measurements were done in duplicates.

11. Enzyme Denaturation Studies Using Differential Scanning Calorimetry

Bromelain solution (1%) in distilled water and bromelain solution with 1.3 M sucrose, sorbitol and trehalose were prepared. Denaturation temperature (onset, peak and conclusion temperature) of bromelain samples were investigated using DSC (PerkinElmer Pyris 1) equipped with an Intracooler subambient accessory. Nitrogen gas was used as the purge gas at a flow rate of 20 mL min^{-1} during calibration and measurements. Samples, 25-30 mg each, were weighed in stainless steel pans and hermetically sealed. An empty pan was used as reference sample.

The samples were heated from 30 to 140 °C at 10 °C/min to determine the denaturation temperature of bromelain. Onset, peak and conclusion temperatures were

obtained from PerkinElmer software. DSC measurements were carried out in duplicates.

12. Statistical analysis

The experiments were a completely randomized design and the difference in means was determined by the Duncan News Multiple Range Test. The data were analyzed using SPSS 15.0 for Windows.

13. Places

Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University

14. Duration

March 2007 to December 2009

RESULTS AND DISCUSSION

The results and discussion are reported in 10 sections that are:

Section 1 : Location of bromelain in pineapple flesh

Section 2 : Protein content determination

Section 3 : The relationship between bromelain activity and total soluble solids

Section 4 : The relationship between bromelain activity and total acidity

Section 5 : Optimal pH of bromelain

Section 6 : Determination of Michaelis constants (K_m) and maximum reaction velocity (V_{max})

Section 7 : Thermal stability of bromelain

Section 8 : Effects of solutes on thermal stability of bromelain

Section 9 : Enzyme denaturation studies using differential scanning calorimetry

1. Location of Bromelain in Pineapple Flesh

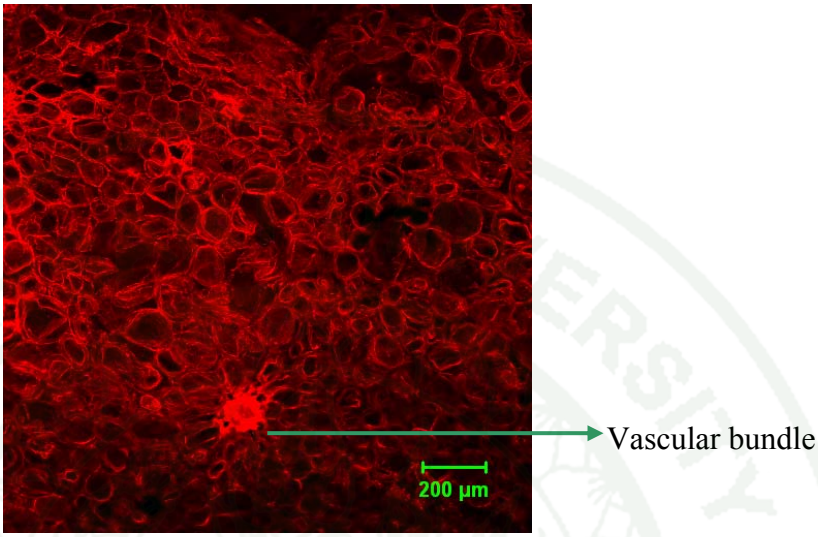


Figure 11 Confocal laser scanning micrographs of pineapple flesh. Proteins were stained with Rhodamine B, appeared in red fluorescence. Bars = 200 µm.

Rhodamine (fluorescent reagent) is reactive towards amine groups on proteins inside cells appeared in red fluorescence (Anonymous, 2010). The protein fractions in pineapple flesh fluoresced in red colour under CLSM. The protein fractions or bromelain located on cell wall, epidermis and vascular bundle, but parenchyma had little protein fraction. It can be seen from the figure that protein fractions concentrated at vascular bundle. Stem of pineapple had a lot of bromelain (Collins, 1968). It is expected that the most of bromelain was at vascular bundle.

2. Protein Content Determination

Table 5 Total activity, total protein and specific activity of crude bromelain extract

Sample	Total activity (CDU/g)	Total protein (mg protein/g)	Specific activity (CDU/mg protein)
Crude bromelain extract	1,634.37 ± 103.07	3.40 ± 0.19	480.70.27 ± 3.80

From Table 5, Total protein from pineapple juice from fruit was 3.40 ± 0.19 mg/g. This result correlated to the nutrition data which found that protein in pineapple juice was 4 mg/g (Anonymous, 2010). But it was different from pineapple juice from unripe fruits of *Bromelia balansae* Mez that the pineapple had total protein 9.6 mg/g (Pardo *et al*., 2000). Hebbar *et al*. (2008) found that total protein from core, peel crown and stem of pineapple from *Ananas comosus* L. Merryl cv. Kew with total soluble solids had 7 – 9 °Brix were 12.06, 22.06, 12.71 and 23.98 mg/g, respectively.

From this study, specific activity in crude extract (phosphate buffer pH 8.0) obtained from pineapple juice of fruit (*Ananas comosus* L. Merryl cv. Smooth Cayenne, 13 –15 °Brix) was 480.70.27 ± 3.80 CDU/mg. The specific activity was different from Hebbar *et al*. (2008) that specific activity in crude extract (phosphate buffer pH 6.5) obtained from core, peel crown and stem of pineapple (*Ananas comosus* L. Merryl cv. Kew, 7 – 9 °Brix) were 56.15, 18.49, 71.52 and 28.40 CDU/mg protein, respectively.

3. The Relationship between Enzyme Bromelain Activity and Total Soluble Solids

Total soluble solids, mainly sugars, are often used as an indicator of fruit maturity and quality. Total soluble solids of pineapple can vary by 40 g/liter or 4 °Brix from the more mature, sweeter basal tissue to the crown end of the fruit, and decline only slightly after harvest (Paull and Chen, 2003). Yoon *et al.* (2006) found that a positive relationship between acid phosphatase and °Brix in cherries. Acid phosphatase appears to play an important role in the metabolism of carbohydrates in cherries. Areas and Lajolo (1981) reported that as starch was transformed into soluble sugar (sucrose, glucose, fructose), acid phosphatase was increased during banana ripening. In contrast, acid phosphatase activity decreased while starch synthesis increased (Goswami and Borthakur, 1996).

Pineapples were washed and peeled by a 80 mm diameter borer. The core was removed by a 30 mm diameter borer. Slices of 20 mm thick were cut perpendicularly to the fruit axis. Each slice was chopping. °Brix was measured with a hand-held refractometer. Fifty slices of pineapples were used in the experiments. All measurements were done in duplicates.

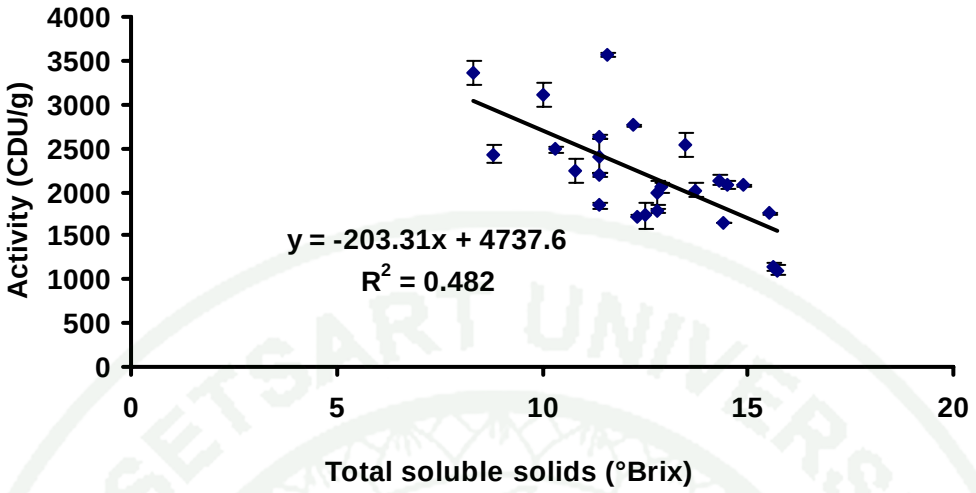


Figure 12 The relationship between enzyme bromelain activity and total soluble solids of pineapples from fruit (8 –16 °Brix).

Figure 12 and Appendix B1 was undertaken to determine the relationship between bromelain activity and total soluble solids in Smooth Cayenne type pineapple. Many pineapple fruits had ripe and unripe fruits from supermarket. The total soluble solids of pineapples were between 8.0 °Brix and 16 °Brix. The plot of bromelain activity and total soluble solids showed a negative slope, and the value of coefficient of determination (R^2) for bromelain activity at different total soluble solids levels was 0.482. The research approached to Paull and Chen (2003) found that bromelain activity remain high during fruit development and declines during ripening speculated that bromelain is transformed to another protein which may have a different metabolic role, such as the flavor-producing enzymes. Gortner and Singleton (1965) found that the volatile flavor constituents are first formed at the time bromelain activity is dropping.

However, the value of coefficient of determination (R^2) for bromelain activity at different total soluble solids (0.482) was low. Three ranges of total soluble solids were divided as Figure 13, 14 and 15. The value of coefficient of determination (R^2) at three total soluble solids ranges (8 – 11.0, 11.1 – 14.0 and 14.1 – 16.0 °Brix) were 0.338, 0.095 and 0.597, respectively. An inverse relationship appeared to exist between the bromelain activity and total soluble solids in pineapples, especially the R^2 value for 14.1–16.0 °Brix was 0.597. In other words, within this °Brix range, more mature pineapple exhibited low bromelain activity. Therefore, the sweet pineapples of 14.1–16.0 °Brix had low bromelain activity (2134.724 ± 57.54 ─ 1107.238 ± 49.47 CDU/g) compared to lower total soluble solids between 8 and 14 °Brix of pineapples (3351.64 ± 136.42 ─ 2022.05 ± 82.25 CDU/g).

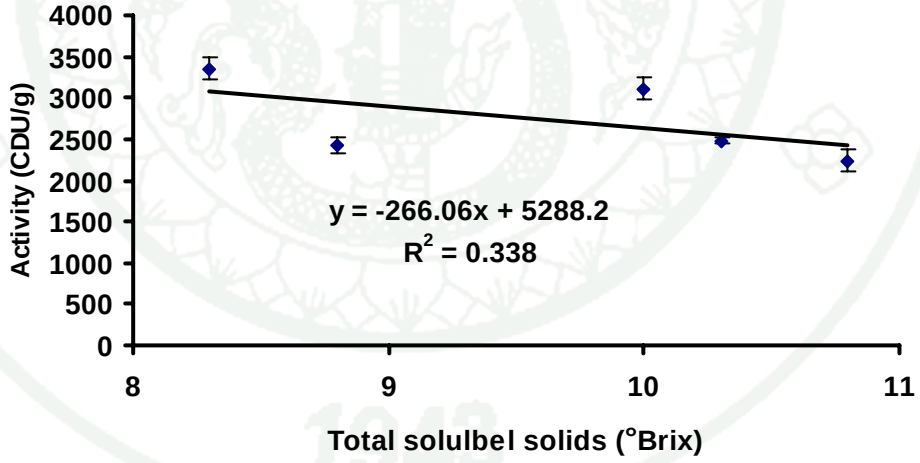


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Figure 13 The relationship between total soluble solids 8.0 –11.0 °Brix and bromelain activity.

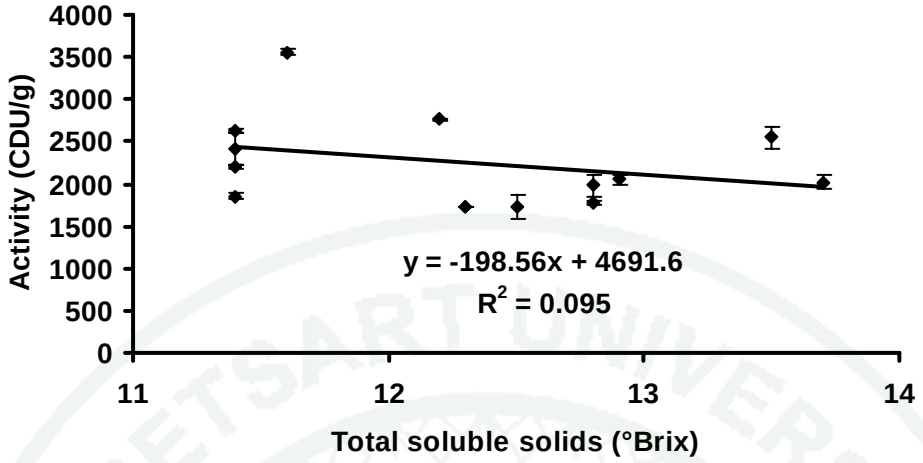

Figure 14 The relationship between total soluble solids 11.1–14.0 °Brix and bromelain activity.

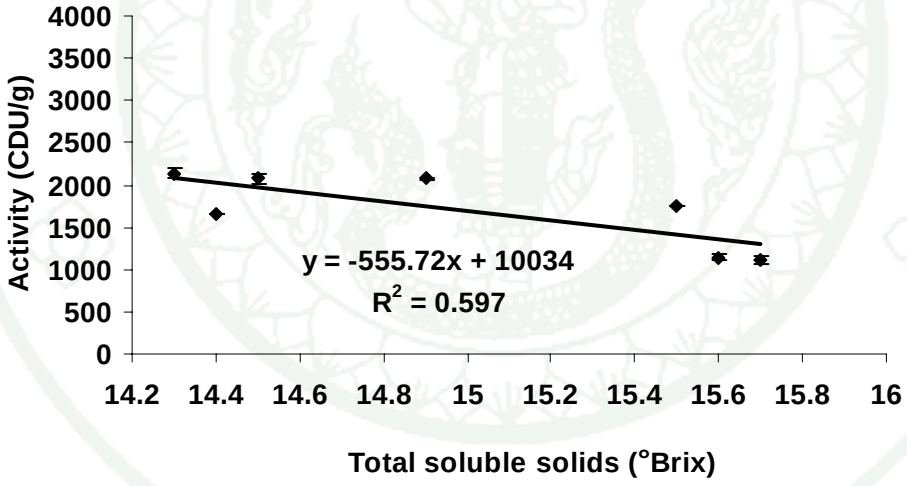

Figure 15 The relationship between total soluble solids 14.1–16.0 °Brix and bromelain activity.

4. The Relationship between Bromelain Activity and Total Acidity

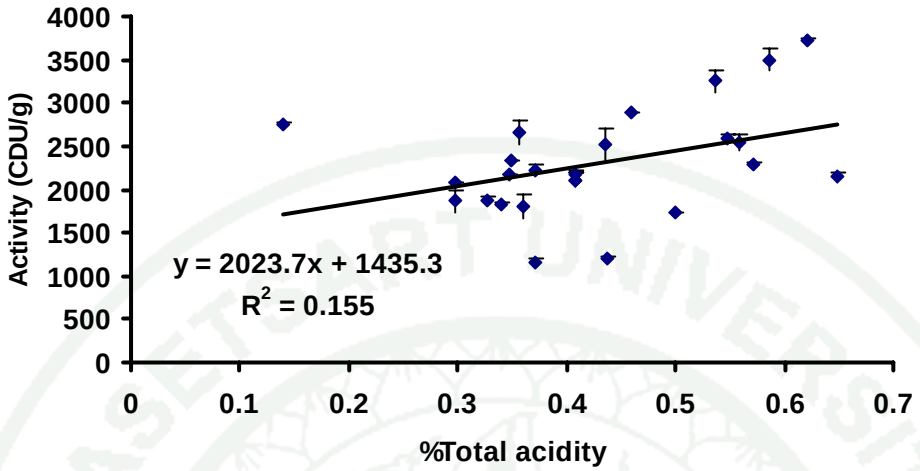

Figure 16 The relationship between total acidity and bromelain activity.

Figure 16 and Appendix Table B2 were as undertaken to determine the relationship between bromelain activity and total acidity in Smooth Cayenne type pineapple. The plot of bromelain activity and total acidity showed a positive slope, and the value of coefficient of determination (R^2) for bromelain activity at different total acidity was 0.155.

There was no relationship between total activity of pineapple and bromelain activity.

5. Optimal pH of Bromelain

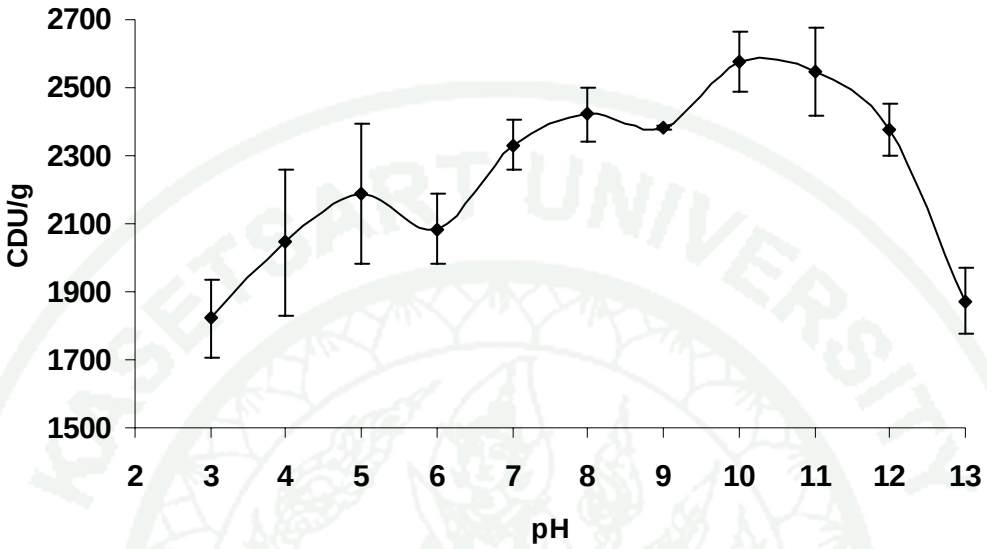

Figure 17 Effect of pH on bromelain activity.

As you can see in Figure 17 and Appendix Table B3, pH range (3 – 5) had high standard deviation. Because our method for this buffer range could not control capacity of buffer fluctuation. Therefore, we suggested that the suitable pH buffer was to decrease the high standard deviation, the suitable pH buffer should be used such as sodium acetate (p*K*a = 4.96) for pH 3.0 – 5.0 or glycine-NaOH 0.1 M pH 8.7 buffer instead of sodium phosphate buffer 0.1 M pH 8.0. Therefore, we would like to consider only pH 6 – 13 that had lower standard deviation.

Pineapple juice exhibited high bromelain activity (higher than 2,000 CDU/g) in a broad pH range (6 –10), with two maximum: at pH 8.0 and 10.0. Bromelain activity was 2,083.91 ± 103.53 CDU/g at pH 6, 2,421.71 ± 79.55 CDU/g at pH 8

and 2,577.13 ± 87.58 CDU/g at pH 10.0. Activity of fruit bromelain from pineapple juice was less than activity of stem bromelain extracted by sedimentation with ammonium sulphate and freeze dryer that bromelain activity had 5,750.77 CDU/g (Supaniuda *et al.,* 2008), acetone extraction that bromelain activity had 7,256 CDU/g (Orwin, 1984) and vacuum evaporation and ultrafiltration that bromelain activity had 18,030 CDU/g (Theerakulkait, 1985).

The activity of pineapple juice sharply decreased at very alkaline pH, probably due to the denaturation of enzymes. Under alkaline pHs, the changes in enzyme conformation was possibly caused by charge repulsion, which is associated with a decrease in electrostatic bonds (Vojdani, 1996).

A broad pH optimum between pH 6.0-10 and their activity appears to be dependent on prototropic groups of active site with p*K*a values near 5.5 and 8.5 that sulfhydryl group in cysteinyl residue had p*K*a = 8.0 – 8.5 and Imidazolium (of histidinyl residue) had p*K*a = 5.5 – 7.0 (Whitaker, 1994). While, Valles *et al*.(2007) reported that pineapple fruit juice exhibited high caseinolytic activity (higher than 80%) in a broad pH range (5 – 9), with two maximum: at pH 6.0 and 9.0 .Whereas, Liang *et al.* (1999) found that pineapple fruit juice have optimum pH 6.8 – 9. The optimal pH of bromelain from commercial (stem bromelain) for casein is 6 – 8 (Helmut, 1998). But our study on proteolytic activity of bromelain also had maximum activity at pH 10.0 (2,577.13 ± 87.58 CDU/g). It could be explained that the conformation of bromelain at pH 6.0-10.0 was no changes (Murachi and Yamazaki, 1970). When the pH was increased from pH 10.1 to 12.0, a large change in

conformation is indicated by unfolding of the molecule. Such unfolding is what was from the titration data that seven additional tyrosine hydroxyl groups per mole of protein became ionized only after the protein had been exposed to alkali, with an abnormal p*K* value of 11.8. The conformational changes of bromelain occurred through at least two distinct stages (pH 10 –12 and pH higher than 12) with a concomitant loss of activity because of abnormality of phenolic hydroxyl ionization with increasing pH values (Murachi and Yamazaki, 1970). Therefore, when bromelain exposed to alkali, loosens gradually the internal folding of the molecule between pH 10 and 12, and undergoes further change to an extensively denatured form above pH 12 (2,376.45 ± 76.16 at pH 12 and 1,872.44 ± 97.64 CDU/g at pH 13).

6. Determination of Michaelis Constants (K_m) and Maximum Reaction Velocity (V_{max})

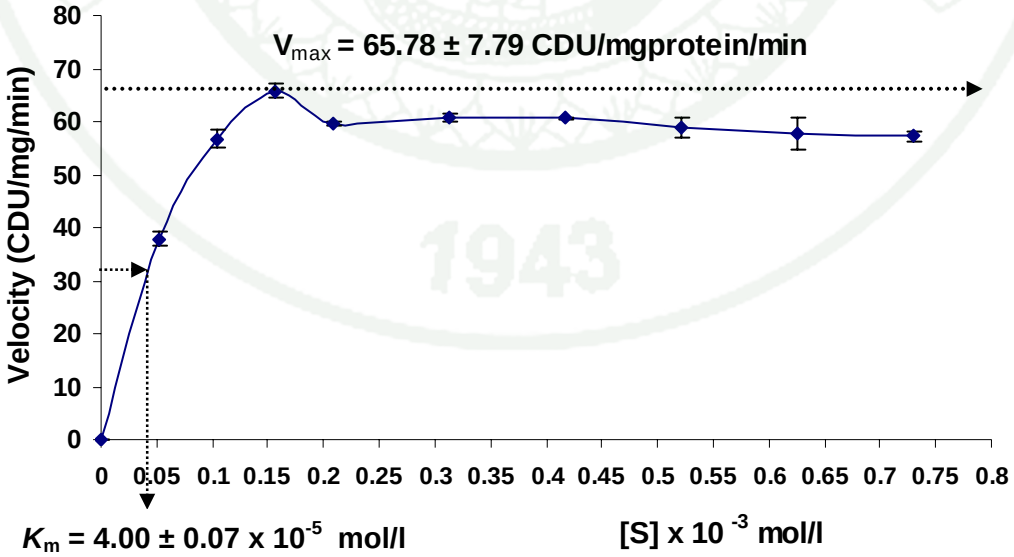

Figure 18 Effect of substrate concentration on bromelain activity

As the substrate concentration is increased, more and more enzyme molecules are combined with substrate until finally, at high concentration, all enzyme molecules are so combined. Once all the enzyme molecules have combined with substrate (enzyme saturated with substrate), further increases in substrate concentration will not increase the rate of reaction, The velocity at this point is the maximum velocity, V_{max} (Whitaker, 1994). The velocity (µmoles of substrate changed per minute) is the effectiveness of a enzyme that is described by its molecular activity (turnover number), which is defined as the number of molecules of substrate transformed per minute per molecule of enzyme (Aurund *et al*., 1987). But, if the reaction rate continuosly decreases in region in spite of an excess of substrate. The decrease in the reaction rate can be considered to be a result of enzyme denaturation, or the products formed increasingly inhibits enzyme activity or, after the concentration of the product increases the reverse reaction takes place, converting the product back into the initial reactant (Belitz and Grosch, 1987).

K_m is equal to the initial substrate concentration at which $V_O = 0.5\ V_{max}$. A small K_m value indicates a high affinity of the enzyme for its substrate, while a large K_m value indicates a low affinity (Aurund *et al*., 1987).

As you can see in Figure 19 and Appendix TableB4, Michaelis constants (K_m) and maximum reaction velocity (V_{max}) were $4.00 \pm 0.07 \times 10^{-5}$ mol/l and 65.78 ± 7.79 CDU/mg protein/min respectively. While, Liang *et al*. (1999) reported that K_m of bromelain in concentrated fruit pineapple juice by ultrafiltrator that casein as substrate was 8.06×10^{-6} mol/l. Whereas, Bhattacharya and Bhattacharya (2009)

reported that K_m of bromelain in purified stem pineapple that azocasein as substrate was 11.2 x 10^{-6} mol/l ,or benzoyl-L-arginine ethyl ester and benzoyl-L-argininamide as substrate were 1.7 x 10^{-2} mol/l and 1.2 x 10^{-3} mol/l respectively (Inagami and Murachi, 1963). Our study had higher K_m value than Liang *et al*. report because our study had lower purity of bromelain. The higher purified bromelain had lower value of K_m. Therefore, the lower value of K_m has higher affinity to casein than higher value of K_m.

Higher maximum reaction velocity value (V_{max}) could interact to substrate faster than lower V_{max}.

7. Thermal Stability of Bromelain

As shown in Figure 19 and Appendix Table B5, no bromelain activity loss was observed when bromelain was incubated at 40 °C during a period up to 60 min, whereas almost 83% of activity remained at 50 °C 60 min. Bromelain activity was retained at a 44 % level at 60 °C after 8 min. However, the enzyme was almost completely inactivated by heating at 80 °C for 8 min.

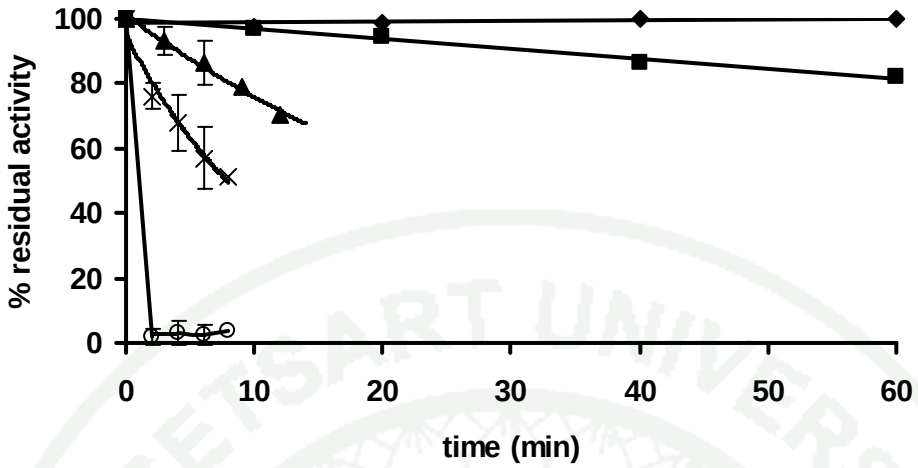

Figure 19 Effect of incubation temperature on bromelain activity: 40(♦), 50(■), 55(▲), 60 (×) and 80 °C (O). Bromelain activity was assayed at 37 °C with casein as substrate.

From the log-linear plots of residual bromelain activity against inactivation time at constant temperature, typical plots of data are shown in Figure 20 and Appendix Table B6. The enzyme was stable at 40 °C, but above 40 °C there was loss of activity; and the higher the temperature, the greater the rate of activity loss.

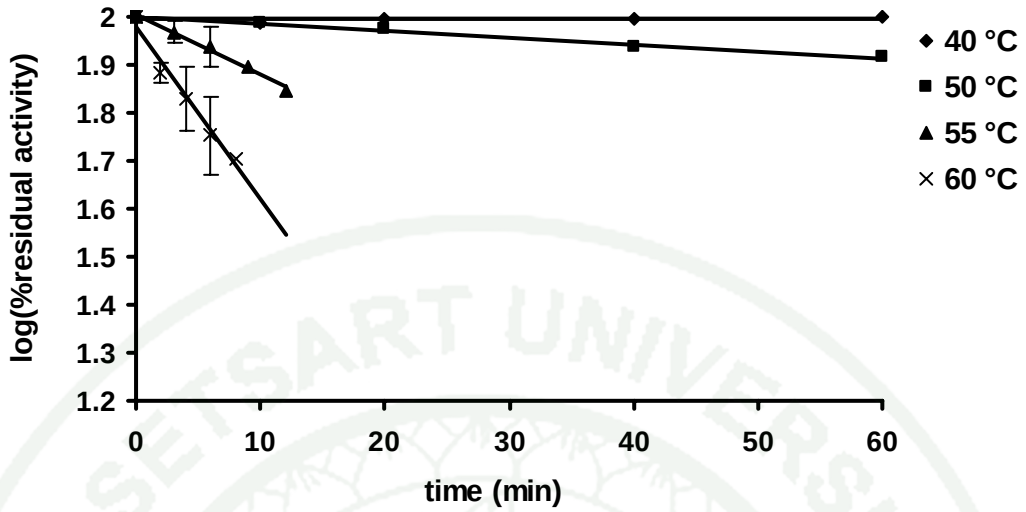

Figure 20 Heat inactivation plots of bromelain at different temperatures.

The rate constants (k) for inactivation were determined from the slopes of the logarithmic plot of activity against time: log (%residual activity) = - (k/2.303) t. To determine Ea, two plots are required. The first is a plot of experimentally determined product concentration versus time at various temperatures (Figure 20 and Appendix Table B6) and the second is a plot of log k, the reaction rate constant versus 1/T (Whitaker, 1996), as shown in Figure 21 and Appendix Table B7. Ea was calculated from the slope of the Arrhenius plot according to the equation log k = -Ea/(2.303RT), where k is the rate of inactivation at T, R is the gas constant (8.314 J mol^{-1} K^{-1}), and T is the temperature in Kelvin. Our calculated Ea for bromelain inactivation was 313.18 ± 57.44 kJ/mol

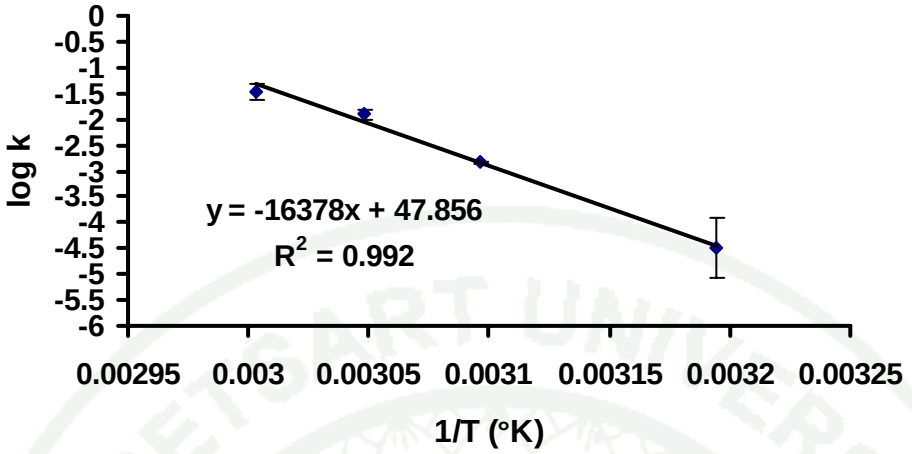

Figure 21 Arrhenius plot for the thermal denaturation of bromelain.

From this study and a previous study by Liang *et al*. (1999), it seems that bromelain from pineapple juice or fruit is more stable when undergoing heating than commercial bromelain obtained from pineapple stems. Our study indicated that bromelain from pineapple fruit retained approximately a 51 % activity level after 8 min at 60 °C; and Liang *et al*. (1999) found that bromelain from pineapple juice by ultrafiltrator remained at 50% activity after 60 min at 60 °C. The difference in these results might be due to the purity of pineapples used in these experiments. Yoshioka *et al*. (1991), however, found that commercial bromelain from pineapple stems was completely inactivated by heating for 30 minutes at 60 °C; while Gupta *et al*. (2007) found that commercial bromelain from pineapple stems retained a 50% activity level after 20 min at 60 °C.

Table 6 Comparison of activation energy (*E*a) for bromelain inactivation

Samples	*Ea* (kJ/mol)	References
Fruit pineapple	313.18 ± 57.44	Our study
Fruit pineapple	326	Sriwatanaponse *et al*. 2000
Stem pineapple	174.47	Yoshioka *et al*., 1991
Stem pineapple	181± 35	Arroyo-Reyna and Hernandez-Arana, 1995

Table 6 showed the calculated *E*a for bromelain inactivation in this study was 313.18 ± 57.44 kJ/mol. This result correlated with the experiment by Sriwatanaponse *et al*. (2000) that the *E*a of bromelain in pineapple juice was 326 kJ/mol. The difference of our study from Sriwatanaponse *et al*. (2000) was proteolytic activity assay (Kunitz, 1947) and part of fruit pineapple that pineapple juice was from flesh and core parts of pineapples which flesh and core parts were put in a press-cloth bag and expressed under a pressure of 50,000 kPa in a wine or basket press. Comparable activation energies of commercial pure bromelain extracted from pineapple stems were 174.47 kJ/mol (Yoshioka *et al*., 1991) and 181 ± 35 kJ/mol (Arroyo-Reyna and Hernandez-Arana, 1995). *E*a was activation energy for denaturation of enzyme. The higher *E*a value of bromelain from pineapple juice was 313.18 ± 57.44 kJ/mol. It is more stable to high temperature than bromelain from pineapple stem that *E*a had 174.47 kJ/mol.

8. Effects of Solutes on Thermal Stability of Bromelain

From Figure 19 and Appendix Table B8, activity of the enzyme kept in 40 °C does not change so much even after 60 min, while in 80 °C the enzyme's activity drops to 3.60% after 10 min; therefore, a gradual change in enzyme's activity that could be followed accurately occurred only in 60 °C (Figure 19). This temperature was chosen for further analysis of enzyme's activity and stability in the presence and absence of solutes. (sucrose, trehalose and sorbitol at 1.3 M concentration in pineapple juice and sucrose 0.6 M concentration).

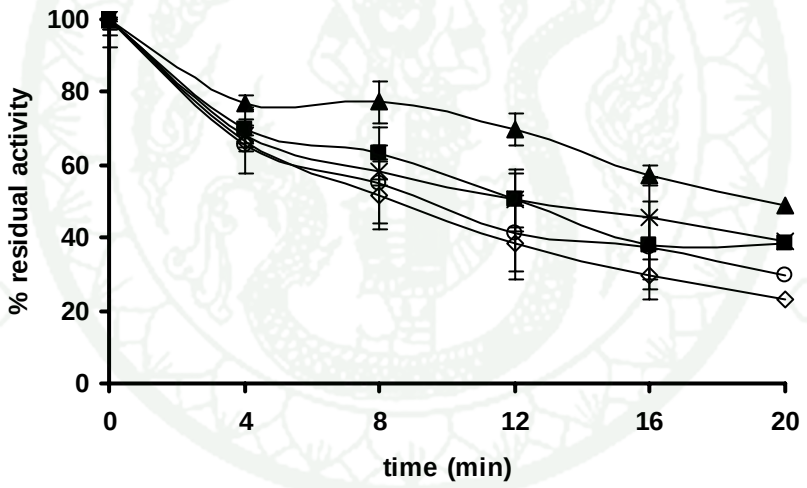

Figure 22 The effect of different solutes on %residual activity of bromelain in pineapple juice at 60 °C. (◇) control (it was replaced solute with distilled water), (■)sorbitol, (▲) trehalose, (×)sucrose,(O) sucrose 0.6 M.

The effect of different solutes on the thermal stability of bromelain was measured through measurements of the enzyme activity. The enzyme with and without solutes was heated at 60 °C for different time intervals.

Figure 22 and Appendix Table B8 showed the effect of different solutes at 1.3 M concentration on the thermal inactivation of bromelain. For sucrose, 0.6 M concentration was included to verify the dilution effect. All the solutes used were able to retain activity of the enzyme. The native enzyme without any solutes retained only 51.72% and 23.16% activity after incubation at 60 °C for 8 and 20 min respectively. In presence of sorbitol the enzyme retained 63.07% of the enzyme activity even after storage at 60 °C for 8 min and it retained almost 38.57% of its activity even after incubation for 20 min. Similar to the effect had seen in case of sorbitol, the thermal stability of bromelain also increased in presence of other solutes. In the case of sucrose 1.3 M, the enzyme retained 57.98% and 38.95% of its activity after heating at 60 °C for 8 min and 20 min respectively. Whereas, sucrose at 0.6 M concentration the enzyme retained 54.81% and 29.81% of its activity after heating at 60 °C for 8 min and 20 min respectively, indicating the decreased thermal stability of bromelain with decreased concentration of the solute. The maximum effect was seen with the application of trehalose, where the enzyme retained 77.28% and 48.70% of its activity even after heating at 60 °C for 8 and 20 min, respectively, an indication for the increased thermal stability of bromelain in presence of trehalose. Devaraj *et al*. (2008) studied the presence of trehalose in ficin, the enzyme retained 85% of its activity after 20 min and 53% even after 60 min of incubation. As for sorbitol and sucrose the enzyme retained the activity of 82% and 80% respectively, after 20 min of incubation. In addition, trehalose was the best stabilizer of porcine pancreatic lipase residual specific activity, followed by sorbitol (Gangadhara *et al*., 2009).

Sucrose stabilizes enzyme by prevention of unfolding without involvement of any specific interactions of sucrose with enzyme (Devi and Rao, 1998). Trehalose and sorbitol retained a higher activity than sucrose. The best stabilizer was trehalose. There are some hypothesis that explain the better stabilizer of trehalose than the other solutes. The first hypothesis is that trehalose and sorbitol bind weakly to enzymes. Trehalose binds to enzyme better than sorbitol (Kaushik and Bhat, 2003). Therefore, trehalose provided a more compatible environment and protection from heat inactivation The structural and kinetic stabilities of enzyme increased in the presence of sorbitol and trehalose (Nasiripourdoria *et al*., 2009). The second hypothesis is that the protection of enzymes at higher temperature is due to the ability of the solutes to replace water molecules in the medium. (Sathish *et al.,* 2007; Sola-Penna and Meyer-Fernandes, 1998). Trehalose has a larger hydrated volume than other related sugars. Trehalose occupies at least a 2.5 times larger volume than sucrose, maltose, glucose and fructose. This property correlated with the ability to protect the structure and function of enzymes against thermal inactivation. Larger hydrated volume, trehalose can substitute more water molecules in the solution; the higher size exclusion effect is responsible for the difference in efficiency of protection against thermal inactivation of enzymes (Sola-Penna and Meyer-Fernandes, 1998). The third hypothesis is that the protection effects are related to length of solute carbon chains and the number of hydroxyl groups, indication for the increased length of carbon chains and number of hydroxyl groups with aggravated protection against thermal inactivation of enzymes. Trehalose has 12 carbon atoms and 8 hydroxyl groups. Sorbitol has 6 carbon atoms and 6 hydroxyl groups. Therefore, trehalose protected the enzyme activity better than sorbitol (Devi and Rao, 1998; Gangadhara *et al*.,

2009). Hydroxyl group interactions exert a key role in the maintenance of the active structure of the protein (Obon *et al*., 1996).

In contrast, Habib *et al.,* (2007) studied bromelain from stem and found that the decrease in activity of bromelain at 60 °C as a function of time was more in the presence of 1 M sucrose/trehalose than for the control sample. Trehalose inactivated bromelain more than sucrose. Because sucrose/trehalose lead to preferential hydration of the denatured bromelain as compared to the native one, hence stabilizing more the denatured conformation.

From Appendeix Table B9, background of pineapple juice with sorbitol, sucrose, trehalose addition and control (without solutes) were significant difference, but sorbitol and trehalose were not significant difference.

From Appendix Table B10, total soluble solids of pineapple juice with sorbitol, sucrose, trehalose addition and control (without solutes), the highest total soluble solids was trehalose.

9. Enzyme Denaturation Studies Using Differential Scanning Calorimetry

Table 7 Thermal denaturation temperature of bromelain in presence of different solutes at 1.3 M concentration.

Solutes	*Td* onset (°C)[2/]	*Td* peak (°C)[2/]	*Td* conclusion (°C)[2/]
Control[1/]	75.0 ± 1.1[a]	83.1 ± 0.7[a]	90.0 ± 0.0[a]
Sucrose	78.1 ± 0.1[a]	86.8 ± 0.0[bc]	92.4 ± 2.5[a]
Sorbitol	77.3 ± 0.8[a]	85.7 ± 0.5[b]	94.1 ± 0.3[a]
Trehalose	77.9 ± 2.0[a]	87.7 ± 0.1[c]	95.0 ± 0.3[a]

[1/]Control was bromelain without solute

[2/]Different letters in the same column indicate statistical differences ($P < 0.05$).

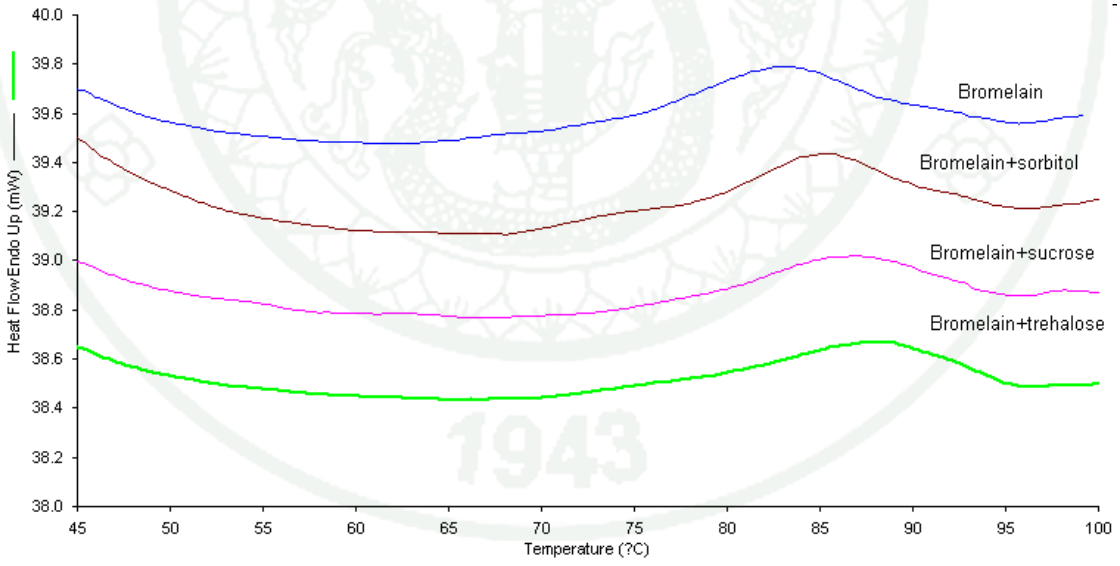

Figure 23 Endotherms of commercial bromelain added sorbitol, sucrose, trehalose and control (without solutes).

DSC was also used to verify and elucidate the effect of different solutes on the thermal stability of bromelain. Figure 23 showed the DSC thermogram of bromelain in presence of different solutes in the range 45-100 °C. Maximum stabilization was seen in bromelain with trehalose where *Td* peak increased from 83.05 °C in control to 87.65 °C (Table 7). In case of sucrose *Td* peak increased to 86.80 °C (Table 7). Similarly for sorbitol, *Td* peak increased to 85.66 °C (Table 7). Calorimetric studied clearly indicate the increased thermal stability of the molecule in presence of these solutes. Comparison of *Td* peak between sucrose and trehalose were not significant difference ($P< 0.05$) (Table 7). However, trehalose had much higher *Td* peak than control. *Td* onset and *Td* conclusion of control (without solute), sucrose, sorbitol and trehalose were not significant difference. Because standard deviation of *Td* onset and *Td* conclusion were high. There were no changes in protein conformation at *Td* onset and protein denatured at *Td* conclusion. Completed profile of DSC was Appendix Figure B1, B2, B3 and B4.

Fruit bromelain from pineapple juice of our study had denaturation peak (83.05 °C) higher than denaturation peak of stem bromelain from commercial (59.3 °C) (Arroyo-Reyna and Hernandez-Arana, 1995). Bromelain in pineapple juice is a native enzyme which native enzyme is more stable to temperature in an intact tissue or in a homogenate, where its structure is protected by the presence of other colloidal material (proteins, carbohydrates, pectins, etc.), than it is in a purified form (Whitaker, 1994).

CONCLUSION

This research was undertaken to determine the relationship of total soluble solids, total acidity and bromelain activity in Smooth Cayenne type pineapple. The plot of bromelain activity and total soluble solids showed a negative slope, and the value of coefficient of determination (R^2) for bromelain activity at different total soluble solids levels was 0.482. The value of coefficient of determination (R^2) at three total soluble solids ranges (8.0 – 11.0, 11.1 –14.0 and 14.1 – 16.0 °Brix) were 0.338, 0.095 and 0.597, respectively. An inverse relationship appeared to exist between the bromelain activity and total soluble solids in pineapples, especially ($R^2 = 0.597$) for 14.1 – 16.0 °Brix . In other words, within this °Brix range, more mature pineapple exhibited low bromelain activity. There were no relationship between total acidity and bromelain activity.

Pineapple juice exhibited high bromelain activity (higher than 2,000 CDU/g) in a broad pH range (6 –10) with two maximum: at pH 8.0 and 10.0. Michaelis constants (K_m) and maximum reaction velocity (V_{max}) were $4.00 \pm 0.07 \times 10^{-5}$ mol/l and 65.78 ± 7.79 CDU/mg protein/min, respectively.

The temperature stability profiles as a function of different time intervals showed higher retention of enzyme activity at low temperature. Incubation at 40 °C showed no bromelain activity lost up to 60 min, whereas at 50 °C almost 83% of activity remained. Incubation at 60 °C for 8 min caused almost completely activity lost. Thermal inactivation of bromelain in the temperature range of 40 – 60 °C, the

calculated activation energy (*Ea*) for bromelain was 313.18 ± 57.44 kJ/ mol. Nevertheless, it should be studied the effect of residual bromelain activity on quality of these processed pineapple juice. Denaturation temperature peak of bromelain with sucrose, sorbitol, trehalose and without solute using differential scanning calorimetry (DSC) were 86.8 ± 0.0 °C, 85.7 ± 0.5, 87.7 ± 0.1 °C, °C and 83.1 ± 0.7 °C, respectively. Denaturation temperature peak of bromelain used to elucidate the results. The enzyme activity measurements indicated the increased in the thermal stability of the enzyme in all the solutes used. The thermal stability of bromelain increased with incremented concentrations of sucrose. Trehalose was the best stabilizer of bromelain, followed by sorbitol and sucrose, respectively. Therefore**,** if we want to preserve bromelain activity in pineapple juice from heat treatment, trehalose should be added to pineapple juice before heat treatment.

RECOMMENDED FUTURE RESEARCH

1. Thermal stability and sensory evaluation of added sugar or polyol pineapple juice should study together.

2. Other polyol can be added to pineapple juice such as xylitol (sweetness equal to sucrose, prevention of dental caries, used as a sweetener in diabetic diet and less calories).

3. Trehalose was suitable for enzyme industry because the cost was higher than other sugars.

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APPENDICES

APPENDIX A

Enzyme Activity Analysis

A1. Crude Bromelain Extract Extraction Method (According to Pardo *et al*., (2000))

1.1 Apparatus

1.1.1 Juice extractor (Hitachi Ltd., Tokyo, Japan)

1.1.2 Refrigerated centrifuge (Sorvall RC 5C Plus, U.S.A.)

1.2 Reagents

1.2.1 EDTA disodium salt (ethylenediaminetetra acetic acid disodium salt) (Analytical grade, Ajax)

1.2.2 L-Cysteine (Analytical grade , Fluka)

1.2.3 Sodium dihydrogen orthophosphate (Analytical grade, Ajax)

1.2.4 Disodium hydrogen orthophosphate dodecahydrate (Analytical grade, Ajax)

1.3 Procedure

Pineapple juice 4 g was mixed with cold 0.1 M sodium phosphate buffer (pH 8.0, 5 °C) comprised of 5 mM EDTA and 25 mM cysteine 50 ml. The mixture of pineapple juice and buffer had 10°C and the mixture was centrifuged for 30 min at 16,000 *g* 4 °C. The supernatant – "crude bromelain extract" – was then collected.

A2. Proteolytic Activity Assay (According to Pardo *et al.*, (2000))

2.1 Apparatus

2.1.1 UV-visible Spectrophotometer(Genesys 10 UV Biomate 3)

2.1.2 pH-meter (Jenco 6173 ,China)

2.1.3 Water bath (Tempest HB25, Thailand)

2.2 Reagents

3.2.1 Casein from bovine milk (Analytical grade , Fluka)

3.2.2 EDTA disodium salt (ethylenediaminetetra acetic acid disodium salt) (Analytical grade , Ajax)

3.2.3 L-Cysteine (Analytical grade , Fluka)

3.2.4 Trichloroacetic acid (TCA) (Analytical grade, Merck)

3.2.6 Glycine (Analytical grade, Fisher)

3.2.7 Sodium hydroxide (Analytical grade, Merck)

2.3 Procedure

The reaction mixture contained 1.1 ml of 1% (w/v) casein solution in 0.1 M glycine sodium hydroxide buffer (pH 8.7) containing 25 mM cysteine and 0.1 ml of crude bromelain extract. The mixture was incubated for 10 min at 37 °C, and the reaction stopped by the addition of 1.8 ml of 5% (w/v) trichloroacetic acid (TCA).

Blanks were prepared by adding TCA to the crude enzyme extract and then adding the substrate. The mixture was filtered through filter paper (Whatman No. 1). The absorbance of the filtrate was measured at 280 nm. Casein digestive units (CDU) were used to express proteolytic activity. One CDU was defined as the amount of enzymes that liberated the equivalent of 1 μg of tyrosine in 1 min at 37 °C.

2.4 Standard Curve

2.4.1 Weigh tyrosine 0.0204 g into volumetric flash and adjust volume to 100 ml. with 0.1 N hydrochloric acid in order to get 204 μg/ml.

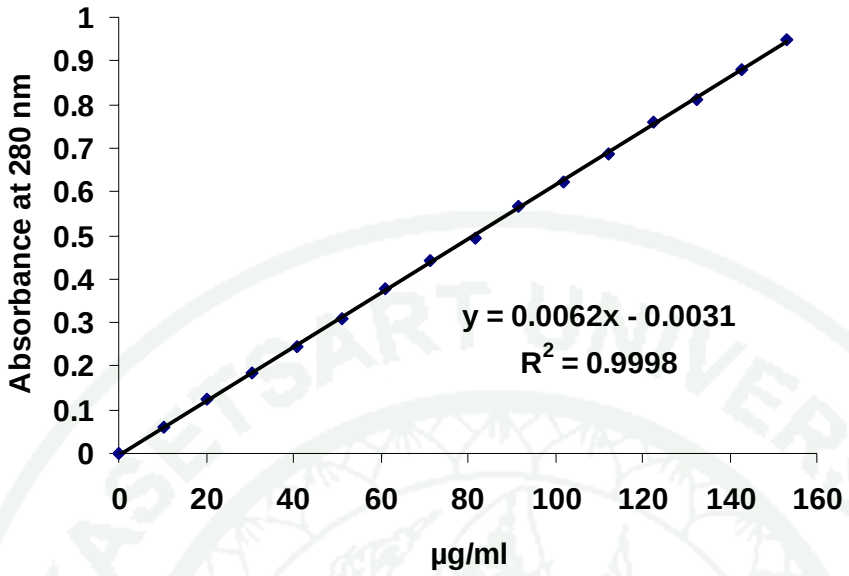
2.4.2 Blanks are prepared by 0.1 N hydrochloric acid.

2.4.3 Prepare each tyrosine solution as follows;

Tyrosine Concentration (µg/ml)	204 µg/ml tyrosine solution (ml)	0.1 N hydrochloric acid (ml)
0	0	1.0
10	0.5	9.5
20	1.0	9.0
30	1.5	8.5
40	2.0	8.0
50	2.5	7.5
60	3.0	7.0
70	3.5	6.5
80	4.0	6.0
90	4.5	5.5
100	5.0	5.0
110	5.5	4.5
120	6.0	4.0
130	6.5	3.5
140	7.0	3.0
150	7.5	2.5

2.4.4 Read absorbance at 280 nm, using the blank to zero the spectrometer.

2.4.5 Plot absorbance at 280 nm against tyrosine concentration for a standard curve.



Appendix Figure A1 Standard curve of tyrosine

A3. Protein Determination According to (Bradford, 1976)

3.1 Apparatus

3.1.1 Spectrophotometer (Genesys 10 UV Biomate 3)

3.1.2 Cuvette

3.1.3 Volumetric flash 10 ml

3.2 Reagents

3.2.1 Bovine serum albumin (Analytical grade, Fluka)

3.2.2 Coomassie Brilliant Blue G-250 (Analytical grade, Fluka)

3.2.3 Ethanol 95% (Analytical grade, Merck)

3.2.4 85% phosphoric acid (Analytical grade, Ajax)

3.2.5 Sodium phosphate buffer 0.1 M

3.3 Procedure

3.3.1 Preparation of 0.01% (w/v) protein reagent. Coomassie Brilliant Blue G-250(100 mg) is dissolved in 50 ml 95% ethanol. To this solution 100 ml 85%(w/v) phosphoric acid is added. The resulting solution is diluted to a final volume of 1 liter.

3.3.2 Pipette 1 ml. of sample (crude bromelain extract) into the test tube.

3.3.3 Add 1 ml of protein reagent and mix by vortex mixer.

3.3.4 Measure absorbance at 595 nm.

3.4 Standard curve

3.4.1 Weigh bovine serum albumin (BSA) 0.1012 g into volumetric flash and adjust volume to 10 ml with sodium phosphate buffer 0.1 M in order to get 0.0101 g/ml.

3.4.2 Pipette 1 ml of 0.0101 g/ml BSA solution into volumetric flash and adjust volume to 9 ml. in order to get 0.0010 g/ml.

3.4.3 Pipette 1 ml of 0.0010 g/ml BSA solution into volumetric flash and adjust volume to 9 ml. in order to get 0.0001 g/ml.

3.4.4 Blank are prepared by substituting phosphate buffer for the sample solution..

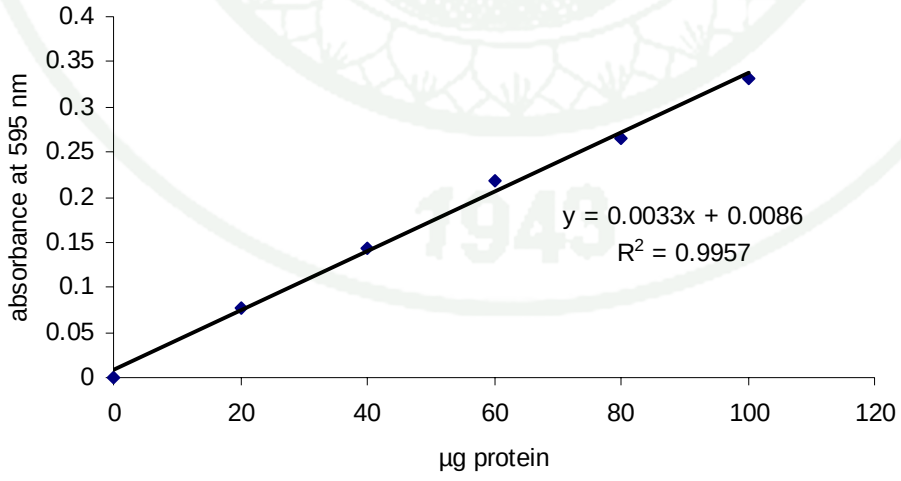
3.4.5 Prepare each bovine serum albumin solution as follows;

BSA (µg/ml)	0.0001 g/ml BSA(ml)	phosphate buffer (ml)
0	0.0	1.0
20	0.2	0.8
40	0.4	0.6
60	0.6	0.4
80	0.8	0.2
100	1.0	0.0

3.4.6 Add 1 ml of protein reagent and mix.

3.4.7 Read color absorbance at 595 nm, using the blank to zero the spectrometer.

3.4.8 Plot absorbance at 595 nm against BSA concentration for a standard curve.



Appendix Figure A2 Standard curve of bovine serum albumin

A4. Specific Activity of Bromelain

Specific activity of bromelain obtained from total activity (CDU) divided total protein (mg protein)

$$\text{Specific activity (CDU/mg)} = \frac{\text{Bromelain activity (CDU/g pineapple juice)}}{\text{Protein content (mg/g pineapple juice)}}$$

APPENDIX B

Results

Appendix Table B1 The relationship between enzyme bromelain activity and total soluble solids (8.0-16.0 °Brix) of slices pineapples.

Number	Total soluble solids (°Brix)	Activity* (CDU/g)
1	8.3	3,351.64 ±136.42
2	8.8	2,429.67 ±101.80
3	10.0	3,113.22 ±143.52
4	10.3	2,481.10 ±36.25
5	10.8	2,241.38 ±137.65
6	11.4	1,847.15 ±34.95
7	11.4	2,194.23 ±28.34
8	11.4	2,411.02 ±183.91
9	11.4	2,629.84 ±28.18
10	11.6	3,559.62 ±28.03
11	12.2	2,762.65 ±12.41
12	12.3	1,725.54 ±0.62
13	12.5	1,726.40 ±141.06
14	12.8	1,981.55 ±136.47
15	12.8	1,782.24 ±21.44
16	12.9	2,052.93 ±61.21
17	13.5	2,546.51 ±138.82
18	13.7	2,022.05 ±82.25
19	14.3	2,134.72 ±57.54
20	14.4	1,654.99 ± 0.94
21	14.5	2,078.55 ±55.56
22	14.9	2,076.44 ±11.36
23	15.5	1,751.97 ±10.82
24	15.6	1,141.95 ±36.46
25	15.7	1,107.24 ±49.48

*Means and standard deviations of duplicate samples based on two measurements for each sample

Appendix Table B2 The relationship between enzyme bromelain activity and total acidity of slices pineapples.

Number	%Total acidity*	Activity (CDU/g)*
1	0.140	2,746.72 ± 26.08
2	0.297	1,861.45 ± 126.28
3	0.297	2,069.62 ± 19.83
4	0.327	1,882.25 ± 32.34
5	0.340	1,829.84 ± 10.01
6	0.348	2,168.74 ± 10.51
7	0.349	2,341.00 ± 0.01
8	0.356	2,659.69 ± 128.46
9	0.360	1,803.13 ± 130.54
10	0.372	2,229.60 ± 53.25
11	0.372	1,156.45 ± 45.79
12	0.408	2,111.91 ± 76.11
13	0.408	2,111.91 ± 76.11
14	0.408	2,170.93 ± 51.41
15	0.436	2,518.18 ± 180.99
16	0.438	1,192.70 ± 33.74
17	0.459	2,885.44 ± 11.48
18	0.500	1,728.55 ± 0.87
19	0.536	3,251.59 ± 132.81
20	0.547	2,591.37 ± 33.54
21	0.558	2,537.66 ± 94.20
22	0.571	2,291.76 ± 26.23
23	0.586	3,500.60 ± 126.24
24	0.621	3,717.82 ± 25.94
25	0.649	2,144.17 ± 56.64

*Means and standard deviations of duplicate samples based on two measurements for each sample

Appendix Table B3 Effect of substrate concentration on bromelain activity velocity

Substrate concentration X 10^{-3} (mol/l)	Velocity(CDU/mg protein/ min)*
0.000	0.00
0.052	37.79±1.31
0.104	56.78±1.87
0.156	65.78±1.39
0.208	59.76±0.38
0.313	60.92±0.75
0.417	60.59±0.04
0.521	58.85±1.95
0.625	57.74±3.16
0.729	57.17±1.05

*Means and standard deviations of duplicate samples based on two measurements for each sample

Appendix Table B4 Effect of pH on bromelain activity

pH	Bromelain activity (CDU/g)*
3	1,821.03 ± 116.24
4	2,045.69 ± 216.01
5	2,187.92 ± 207.44
6	2,083.91 ± 103.53
7	2,330.77 ± 73.74
8	2,421.71 ± 79.55
9	2,380.38 ± 6.71
10	2,577.13 ± 87.58
11	2,546.65 ± 128.76
12	2,376.45 ± 76.16
13	1,872.44 ± 97.64

*Means and standard deviations of duplicate samples based on two measurements for each sample

Appendix Table B5 Effect of incubation temperature on bromelain activity that was assayed at 37 °C with casein as substrate.

Time(min)	% residual activity*				
	Temperature (°C)				
	40	50	55	60	80
2	-	-	-	76.2 ± 4.2	1.7 ± 2.4
3	-	-	93.1 ± 1.4	-	-
4	-	-	-	67.8 ± 4.9	3.0 ± 0.6
6	-	-	86.3 ± 4.3	56.9 ± 8.7	2.5 ± 3.5
8	-	-	-	51.2 ± 9.5	4.0 ± 3.2
9	-	-	78.9 ± 1.4	-	-
10	97.6 ± 3.0	97.1 ± 0.1	-	-	-
12	-	-	70.6 ± 6.7	-	-
20	98.7 ± 1.8	94.7 ± 1.4	-	-	-
40	99.8 ± 0.3	86.3 ± 0.0	-	-	-
60	100.0 ± 0.0	82.3 ± 0.0	-	-	-

*Means and standard deviations of duplicate samples based on two measurements for each sample

Appendix Table B6 Heat inactivation plots of bromelain at different temperatures

Time(min)	Log (%residual activity)*			
	Temperature (°C)			
	40	50	55	60
2	-	-	-	1.88 ± 0.02
3	-	-	1.97 ± 0.01	-
4	-	-	-	1.83 ± 0.03
6	-	-	1.94 ± 0.01	1.71 ± 0.07
8	-	-	-	1.65 ± 0.08
9	-	-	1.90 ± 0.01	-
10	1.99 ± 0.01	1.99 ± 0.01	-	-
12	-	-	1.85 ± 0.04	-
20	1.99 ± 0.01	1.98 ± 0.01	-	-
40	1.99 ± 0.01	1.94 ± 0.01	-	-
60	2.00 ± 0.01	1.92 ± 0.01	-	-

*Means and standard deviations of duplicate samples based on two measurements for each sample.

Appendix Table B7 Arrhenius plot for the thermal denaturation of bromelain.

Temperature(°C)	1/ Temperature (°K)	k*	Log k*
40	0.003194	0.00001	-4.50±5.77
50	0.003096	0.00145	-2.84±0.02
55	0.003048	0.01255	-1.91±0.10
60	0.003003	0.03610	-1.46±0.15

*Means and standard deviations of duplicate samples based on two measurements for each sample.

Appendix Table B8 The effect of different solutes on the thermal stability of bromelain in pineapple juice at 60 °C

Solutes	%Residual activity of bromelain*				
	4 (min)	8 (min)	12 (min)	16 (min)	20 (min)
Control*	66.49±0.76^{a}	51.72±1.59^{ab}	38.62±9.40^{a}	29.86±10.26^{a}	23.16±6.72^{a}
Sorbitol 1.3 M	69.84±4.40^{a}	63.07±6.01^{b}	50.32±7.30^{a}	37.88± 7.37^{ab}	38.57±11.92^{bc}
Sucrose 1.3 M	68.37±2.82^{a}	57.98±2.29^{ab}	50.29± 3.72^{a}	45.54± 8.72^{bc}	38.95±11.28^{bc}
Trehalose1.3 M	77.18±2.61^{a}	77.28±1.90^{c}	69.80± 5.59^{b}	57.10± 4.34^{c}	48.70± 2.58^{c}
Sucrose 0.6 M	65.18±7.49^{b}	54.81±7.29^{a}	41.30±10.59^{a}	37.19±10.27^{ab}	29.81± 8.37^{ab}

*Control (without solute)

*Means and standard deviations of duplicate samples based on two measurements for each sample.

Different letters in the same column indicate statistical differences (P< 0.05).

Appendix Table B9 The effect of different solutes on background absorbance of denatured bromelain in fruit pineapple juice

Solutes	Background absorbance (280 nm)*
Sorbitol 1.3 M	0.131±0.001[c]
Sucrose 1.3 M	0.121±0.000[a]
Trehalose 1.3 M	0.131±0.001[c]
Sucrose 0.6 M	0.146±0.000[d]
Control	0.124±0.000[b]

*Means and standard deviations of duplicate samples based on two measurements for each sample.

Different letters in the same column indicate statistical differences ($P< 0.05$).

Appendix Table B10 The effect of different solutes on total soluble solids of pineapple juice

Solutes	Total soluble solids (°Brix)*
Sorbitol 1.3 M	20.06
Sucrose 1.3 M	30.47
Trehalose 1.3 M	31.25
Sucrose 0.6 M	21.87
Pineapple juice	14.50
Control (added distilled water)	8.21

APPENDIX C

Statistical Analysis

Appendix Table C1 Statistical analysis by Analysis of Variance (ANOVA) of the effect of different solutes on the thermal stability of bromelain in pineapple juice at 60 °C

Time	Source	Degree of Freedom (df)	Type III Sum of Square (SS)	Mean Square (MS)	F-value	P-value
4	Treatment	4	351.862	87.966	4.845	.010
	Error	15	272.342	18.156		
	Total	19	624.204			
8	Treatment	4	1611.601	402.900	20.024	.000
	Error	15	301.818	20.121		
	Total	19	1913.419			
12	Treatment	4	2403.851	600.963	10.190	.000
	Error	15	884.604	58.974		
	Total	19	3288.455			
16	Treatment	4	1707.258	426.814	5.921	.005
	Error	15	1081.271	72.085		
	Total	19	2788.529			
20	Treatment	4	1518.701	379.675	4.852	.010
	Error	15	1173.864	78.258		
	Total	19	2692.565			

Appendix Table C2 Statistical analysis by Analysis of Variance (ANOVA) of the thermal denaturation temperature of bromelain in presence of different solutes at 1.3 M concentration.

Temperature	Source	Degree of Freedom (df)	Type III Sum of Square (SS)	Mean Square (MS)	F-value	P-value
Onset	Treatment	3	11.930	3.977	2.738	.178
	Error	4	5.810	1.452		
	Total	7	17.740			
Peak	Treatment	3	24.565	8.188	43.671	.002
	Error	4	0.750	0.188		
	Total	7	25.315			
End	Treatment	3	29.015	9.672	9.672	.149
	Error	4	12.340	3.085		
	Total	7	41.355			

Appendix Table C3 Statistical analysis by Analysis of Variance (ANOVA) of the effect of different solutes on background absorbance of denatured bromelain in fruit pineapple juice

Varieties	Source	Degree of Freedom (df)	Type III Sum of Square (SS)	Mean Square (MS)	F-value	P-value
Solutes	Treatment	4	0.001	0.000	233.25	.000
	Error	5	0.000004	0.0000008		
	Total	9	.001			

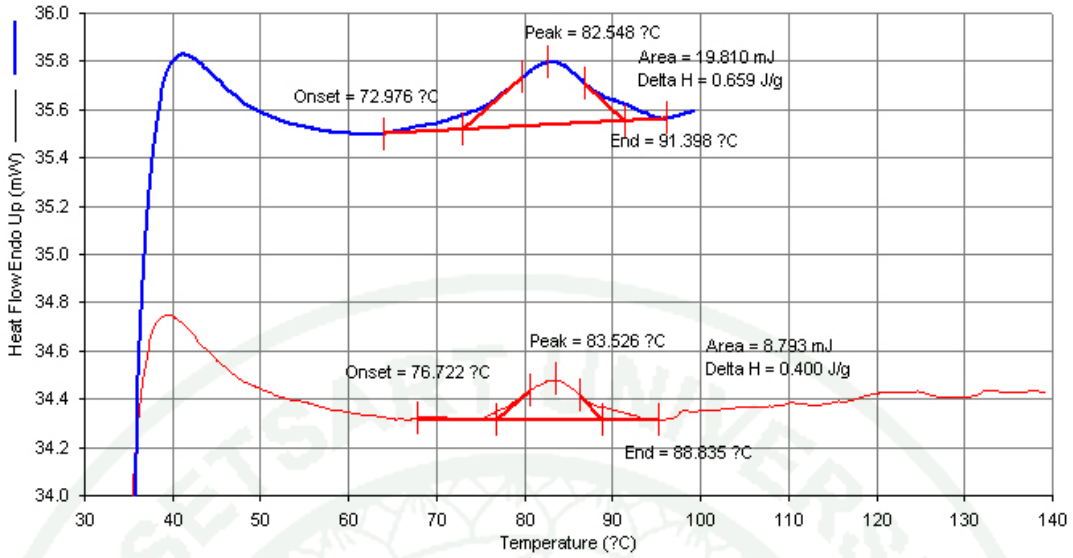


Figure Appendix B1 Completed profile of DSC from commercial bromelain(control)

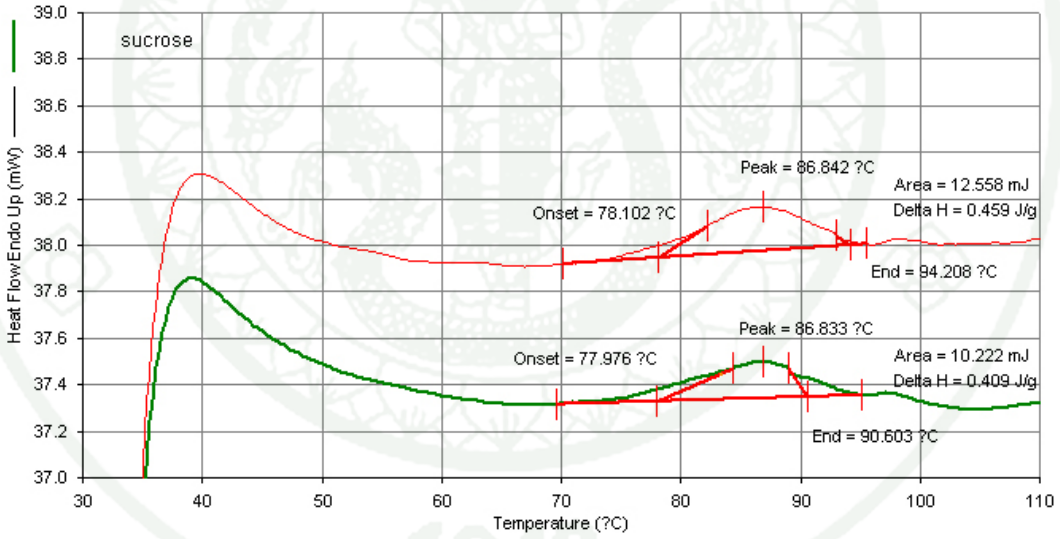


Figure Appendix B2 Completed profile of DSC from commercial bromelain added sucrose

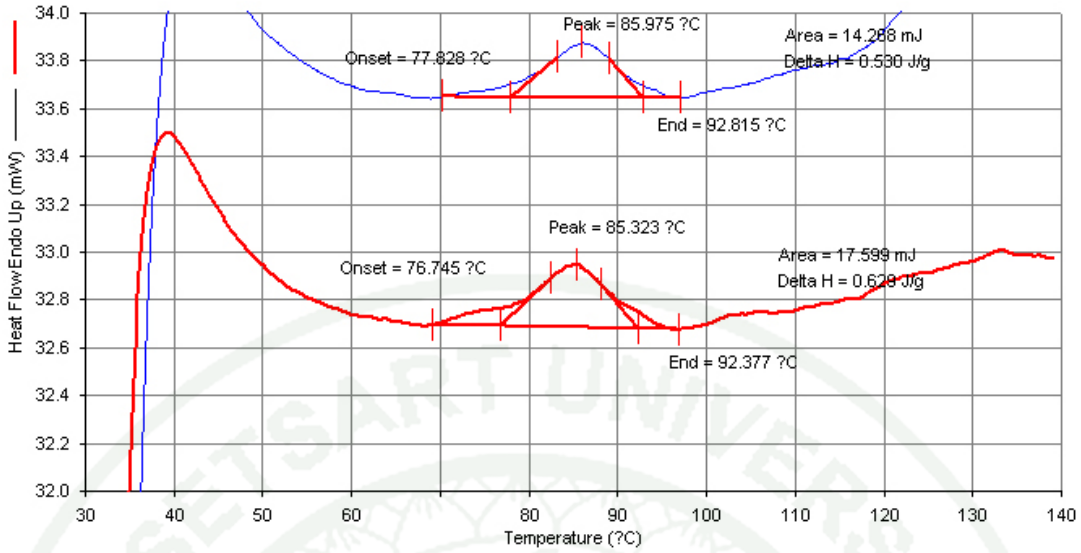


Figure Appendix B3 Completed profile of DSC from commercial bromelain added sorbitol

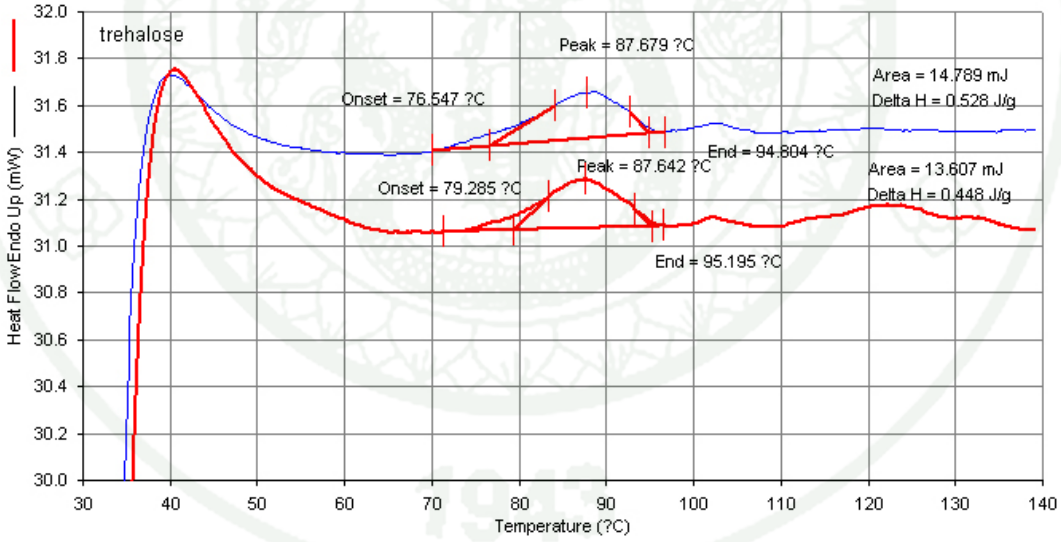


Figure Appendix B4 Completed profile of DSC from commercial bromelain added trehalose

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1. Jutamongkon, R. and S. Charoenrein. 2008. The relationship between bromelain activity and total soluble solids in Smooth Cayenne type pineapple. **Poster presentation in 46 th Kasetsart University Annual Conference 2008**. Bangkok. Thailand.

2. Jutamongkon, R. and S. Charoenrein. 2008. The Effect of Temperature on Stability of Bromelain from Smooth Cayenne Pineapple. **Poster presentatioin The 10th Agro-Industrial Conference Food Innovation Asia 2008.** Healthy Food for All 12th-13 th June**.** Bangkok. Thailand.

3. Jutamongkon R. and S. Charoenrein. 2009. Effect of Temperature on the Stability of Bromelain from Smooth Cayenne Pineapple. **Kasetsart Journal (Natural Science)** (Accepted).

4. Jutamongkon R. and S. Charoenrein. 2009. Effects induced by solutes on the thermal stability of bromelain from Smooth Cayenne pineapple. **International Journal of Food Science and Technology**. (submitted).