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Thermal stability of free bromelain and bromelain-polyphenol complex in pineapple juice

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Abstract: Pineapple (*Ananas comosus*), *Bromeliaceae* family, is a fruit grows in tropical countries including Malaysia. This fruit has several pharmacological benefits due to the presence of high concentration of bromelain (cysteine proteases). Condition of elevated temperature will induce deformation of enzyme and result in loss of activity. Sulfhydryl groups in cysteine proteases are readily to be oxidized and might account for the denaturation of bromelain at elevated temperature. Polyphenol from ethanolic cashew leave extract could be complexed with bromelain to stabilize the enzymatic activity. In thermal stability test, the heat damage effect on bromelain was ten times reduced after complexing with cashew extract. The enzymatic activity of free bromelain decreased gradually from 25°C to 95°C. Complexed bromelain was stable in activity to heating up to 85°C. Bromelain-polyphenol complex showed a good heat resistance. The result revealed that polyphenol could protect bromelain in pineapple juice from heat denaturation.

Keywords: Stabilization, enzymatic activity, cysteine proteases, cashew leave, heat resistance

Introduction

Bromelain (EC 3.4.22.32) is a mixture of cysteine proteases and non proteases components, extracted from pineapple plant (*Ananas comosus*) especially pineapple stem (Heinecke and Gortner, 1957; Rowan *et al*., 1990). This proteolytic enzyme is used as a phytomedical compound and demonstrates, *in vitro* and *in vivo*, anti-inflammatory (Hale *et al*., 2005), anti-edemateous (Seltzer, 1964), absorption of antibiotic drugs (Neubauer, 1961), anti-thrombotic (Metzig *et al*., 1999), inhibition of tumor cells proliferation (Batkin *et al*., 1988), debridement action (Klaue *et al*., 1979) and exhibition of strong immunogenicity (Hale *et al*., 2002).

Bromelain proteases are usually unstable and sensitive under stress conditions, for example elevated temperature, acidity and gastric proteases in stomach juice, organic solvents and chemicals (Xue *et al*., 2010). Its instability will result in decrease in enzymatic activity of this health beneficial enzyme in pineapple and also limit its pharmacological, industrial and biotechnological applications. Therefore, the enhancement of enzyme stability and activity is necessary to broad its application. In addition to our consumption of proteolytic active enzyme in pineapple juice, bromelain can be applied as enzyme in biotechnological, food and pharmacological industry for synthesis of biologically active compounds or drugs.

Numerous chemical modifications of proteins and enzyme have been performed to maintain its native conformation and stabilize the enzymatic activity. For example, stem bromelain modified with anhydride groups exhibited good heat resistance, remarkable thermal stability and maintained high activity over a wider pH range (Xue *et al*., 2010). There was a study demonstrated an improvement of thermal stability after complexion of bromelain with tea polyphenol (Liang *et al*., 1999), instead of using chemical methods such as cross linking with chemicals as mentioned above. The authors hypothesized that the improved thermal stability of tea-polyphenol-complexed bromelain might be attributed to the antioxidant properties and protein binding characteristics of polyphenol. Polyphenol could interact with free thiol groups of cysteine residues in bromelain for prevention of oxidation and to stabilize and retain the enzymatic activity from heat denaturation.

In this study, polyphenol from ethanolic cashew leave extract is used to complex with bromelain in pineapple fruit juice. Heating in sterilization process is an unavoidable stress conditions applied in food production and processing. The interaction of polyphenol and bromelain provides an alternative method for giving enzyme some protective characteristics related to thermal stability and enzymatic activity.

The objectives of this work are to investigate the effect of elevated temperature on free bromelain and bromelain-polyphenol complex in pineapple and also determine the thermal stability. These enzymes are inactivated and the extent of protection effect exerted

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by cashew leave polyphenol could be determined by comparison of the residual enzymatic activity between free and complexed bromelain after heat deactivation. Bromelain complexed with polyphenol is hypothesized to have good heat resistance, which is not achievable by using free bromelain.

Materials and Methods

Cashew leave extract preparation

Cashew leaves were collected from cashew tree planted in Universiti Teknologi Malaysia campus. The leaves were air dried at room temperature and powdered in a mill. The leave powder was extracted with ethanol at room temperature for 48 h. Following the filtration, the extract was concentrated to dryness on rotary evaporator. The resultant extract was kept in dark at 4°C for further analysis. The ethanolic extract was tested for antioxidant assays and total phenolic content.

Pineapple juice processing

Josa pineapples (Josapine) were collected at Ladang Nenas MPIB, Alor Bukit, Pekan Nanas located at 80 km from UTM campus. Pineapples were peeled, sliced and crushed into juice using laboratory juicer. The crude juice was centrifuged for 30 min at 4°C. The supernatant was passed through two stages of vacuum filtration and media filtration, stored at 4°C until use. Bromelain activity and protein content of clarified josa pineapple juice were analyzed.

Complexing cashew polyphenol with pineapple bromelain

Ethanolic cashew leave extract was added into the clarified pineapple juice. The mixture was held in water bath at 25°C under constant stirring. The mixture was subsequently cooled at 4°C overnight. It was followed by centrifugation and bromelain-polyphenol complex precipitate was separated from supernatant. The concentrations of cashew leave in pineapple juice used in this study were 1.5%, 1.0% and 0.5%. If cashew leave extract was added into josapine juice, the complexed bromelain was called cashew-josapine complexed bromelain. Positive control complexed bromelain was made by dissolving cashew leave extract in 0.4% bromelain solution and it was named as cashew-0.4% bromelain complexed bromelain. 1.5% cashew-josapine complexed bromelain was named if 1.5 g cashew leaved extract mixed with 100 ml josapine juice.

Thermal stability of free bromelain and complexed bromelain

The thermal stability for both free bromelain and bromelain-polyphenol complexed bromelain was evaluated by incubating enzyme in test tube at 60°C for 150 min, using a water bath. Clarified josa pineapple juice was used for free bromelain test. Complexed bromelain was prepared in 0.1 M sodium phosphate buffer, pH 4.5. The buffer solution was preheated before adding the enzyme. During incubation time, the tubes were slightly shaked for achieving designated temperature. Every 30 minutes, heat treated sample was withdrawn onto ice water. The sample was assayed for enzymatic activity at 30 min intervals and the activity was expressed relatively. The residual activity could be calculated as logarithm value. Logarithm of residual enzyme activity versus incubation time was plotted and denaturation rate constant and half life were then calculated from the slope of linear portion. The plot gives the first order reaction which the equation is described as $\ln [v]/[v_o] = -kt$, where $[v]$ and $[v_o]$ are enzyme activities at times t=t and t=0. k is denaturation rate constant at the temperature studied. Half life, $t_{1/2}$, is defined as the time required to loss half of the initial concentration, where $t_{1/2}$ is calculated from equation of $1/k \ln 2$.

Effect of temperature on free bromelain and complexed bromelain

To investigate the effect of temperature on enzyme, the deactivation of free bromelain and complexed bromelain was carried out by incubating the sample at various temperatures of 25°C, 35°C, 45°C, 55°C, 65°C, 75°C, 85°C, 95°C and 105°C for 30 min, using standard assay as described above. The heated sample was taken out and cooled immediately in ice water. The residual activity was measured. The temperature of sample solutions was adjusted to the standard enzymatic assays condition after heat deactivation.

The thermal stability analysis was assayed at optimum temperature obtained from the above analysis, by incubating sample solution of free bromelain and complexed bromelain for 1 to 5 h. Analysis of sample was assayed every hour interval.

Determination of antioxidant activity

Antioxidant activity was evaluated by DPPH radical scavenging activity (Liyana-Pathiranan and Shahidi, 2005). The DPPH radical scavenging effect was calculated using the equation of scavenging effect % = [1- (absorbance of sample at 517nm/ absorbance of control at 517 nm)] x 100%. EC50 is defined as concentration of substrate that causes 50%

DPPH activity loss.

Determination of total phenolic content

Total phenolic content was determined colorimetrically using modified Folin-Ciocalteu method (Singleton and Rossi, 1965). Result was expressed as mg gallic acid equivalent per g dry weight extract (mg GAE/g).

Determination of total protein content

Bicinchoninic acid (BCA) assay was used for colorimetric detection and quantitation of total protein (Smith *et al*., 1985). BCA was used as a standard.

Measurement of bromelain activity

Bromelain activity was assessed by method modified by Takahashi *et al*. (1973). One unit of bromelain activity is defined as 1 µg tyrosine produced in 1 minute per ml of sample when casein is hydrolyzed under standard condition of 37°C and pH 7.0 for 10 minutes. Bromelain activity was expressed as units per ml (U/ml). Tyrosine was used as a standard.

Results

Total phenolic content of ethanolic cashew leave extract

Polyphenol used to be complexed with bromelain in the present study was ethanolic extracted from cashew leave. The total phenol in ethanolic cashew leave extract was evaluated at 62.87 mg GAE/g through Folin-Ciocalteu method. Besides, this cashew extract had EC50 of 0.03 mg/ml obtained from the DPPH scavenging assay. Cashew leave extract exhibited a marked antioxidant activity and considerable amount of total phenols. Ethanolic cashew leave extract could be a potential source of polyphenols and natural antioxidant.

Bromelain enzymatic activity of josapine juice

After processing and clarification of josapine, the bromelain activity was found to be 666.41 U/ml. The total protein content was 14.41 mg/ml and the specific activity became 46.25 U/mg.

Thermal stability of free bromelain and complexed bromelain at incubation temperature of 60°C

A study from Xue *et al*. (2010) showed the maximum activity of native bromelain and modified bromelain with anhydride was obtained at 60°C and high activity was observed at incubation temperature between 50°C to 70°C. Besides, result obtained from the effect of temperature on clarified bromelain and tea-polyphenol-complexed bromelain from pineapple showed that the optimum temperature for bromelain activity was 55°C (Liang *et al*., 1999). Therefore, the thermal stability test of free and complexed bromelain in the present study was conducted by incubation at temperature of 60°C.

The concentrations of cashew leave extract in josa pineapple juice used were 1.5%, 1.0% and 0.5%. The thermal stability test of free bromelain from josapine juice, 0.4% bromelain solution, cashew-josapine complexed bromelain and cashew-0.4% bromelain complexed bromelain (positive control complex) was investigated after 150 min incubation at 60°C (Figure 1 and Figure 2). The activities of 1.5% and 1.0% cashew-josapine complexed bromelain did not vary significantly with increasing incubation duration while the free bromelain's activity decreased continuously. After 150 min incubation, the activities of these two complexed bromelain were above 98% while that of free bromelain from pineapple juice was about 60%. The stability of polyphenol-complexed bromelain with 1.5% and 1.0% cashew concentration was significantly higher than that of free bromelain. However, the activities of 0.5% cashew-josapine complexed bromelain decreased sharply and only about 55% activity remained after the incubation. Complexion of bromelain with cashew leave extract polyphenol improved its thermostability and high concentration of cashew extract (1.5% and 1.0%) gave a significant thermal stabilization.

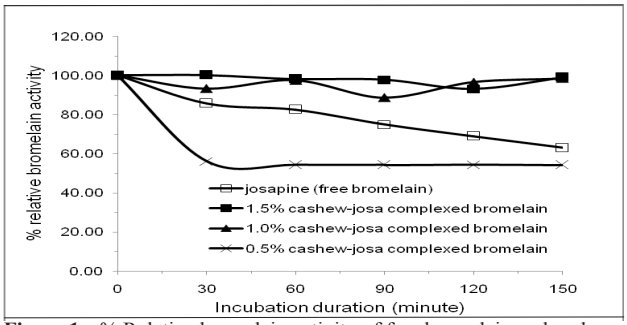

Figure 1. % Relative bromelain activity of free bromelain and cashew-josapine complexed bromelain after incubation at 60°C, 150 min. Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

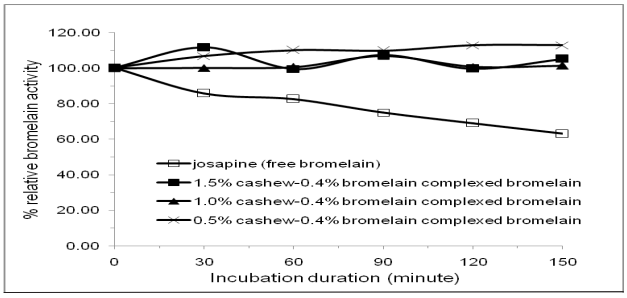

Figure 2. % Relative bromelain activity of free bromelain and cashew-0.4% bromelain complexed bromelain (positive control complex) after incubation at 60°C, 150 min. Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

Plot of logarithm of residual bromelain activity versus incubation time at 60°C was made (data not shown). The straight line plot for both free and complexed bromelain exhibited first order reaction characteristic with slope of line equal to -k. The equation can be arranged as ln [v]/[v_o] = -kt, where [v] is residual activity, k is rate constant. Since bromelain activity decreased with incubation time, the rate constant was interpreted as the rate of bromelain denaturation. Denaturation rate constant and half life could be calculated from the linear slope of the plot of ln[v] versus incubation time. The extent of heat denaturation was measured by denaturation rate constant. As shown in Table 1, 1.5% and 1.0% cashew-josapine complexed bromelain exhibited high stability by having low calculated denaturation rate constants which were 3.0×10^{-4} min^{-1} and 2.0×10^{-4} min^{-1} respectively. Whereas free bromelain and 0.5% cashew-josapine complexed bromelain had 10-fold higher in denaturation rate constants (2.9×10^{-3} min^{-1} and 3.0×10^{-3} min^{-1} respectively). It could be noted that the heat damage effect on bromelain was ten times reduced after complexing with cashew extract of 1.5% and 1.0% concentration. Great thermal stability was observed notably in positive control complex. There was no decline in enzymatic activity along the 150 min incubation time for 1.5% cashew-0.4% bromelain complexed bromelain. Rate constant values for 1.0% and 0.5% positive control complexed bromelain were 50% and 76% decrease compared with their corresponding sample complex respectively.

Table 1. Denaturation rate constants of free bromelain, cashew-josapine complexed bromelain, cashew-0.4% bromelain complexed bromelain (positive control complex), 0.4% bromelain solution obtained from thermal stability test at 60°C, 150 minutes. Indicated values are expressed as mean (n=3)

Sample	Sample	Rate constant, k (min^{-1})
Free bromelain	Josapine juice	2.9×10^{-3}
Sample complex	1.5% cashew-josapine	3.0×10^{-4}
	1.0% cashew-josapine	2.0×10^{-4}
	0.5% cashew-josapine	3.0×10^{-3}
Positive control complex	1.5% cashew-bromelain	-
	1.0% cashew-bromelain	1.0×10^{-4}
	0.5% cashew-bromelain	7.0×10^{-4}
Solution	0.4% bromelain solution	5.7×10^{-3}

Effect of temperature on free bromelain and complexed bromelain

Effect of temperature is an important parameter to be investigated. The tertiary or quaternary structure of the enzyme will be influenced by temperature and the enzyme can be denatured by altering the structure at extremes of temperature.

The variation of relative activity of free bromelain, complexed bromelain at different temperatures was showed in Figures 3-5. As shown in Figure 3, the enzymatic activity of free bromelain in josapine juice decreased gradually with increasing temperature from 25°C to 85°C and there was a sharp drop between 85°C to 95°C. At 85°C, free bromelain showed 60% of maximum activity. A significant reduction in activity was measured between 85°C and 95°C, which occupied half of the total activity loss (254.36 U/ml activity loss out of the total 512.64 U/ml). There was practically no difference in activity between 95°C and 105°C. Free bromelain retained about 20% of its original activity after incubation at 105°C.

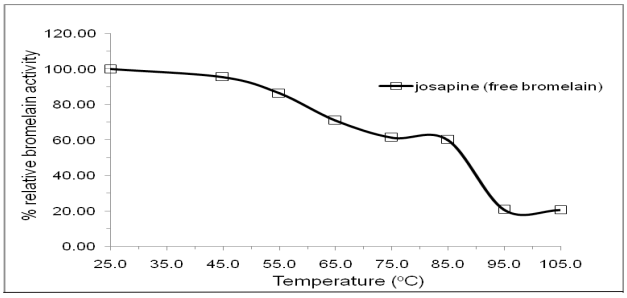

Figure 3. Effect of temperature on bromelain activity of free bromelain in josapine juice. Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

In the view of sample complexed bromelain's activity (Figure 4), in comparison with free bromelain, the relative activities of 1.5%, 1.0% and 0.5% cashew-josapine complexed bromelain did not vary significantly with increasing temperature up to 85°C. All complexed bromelain were able to adapt to a wide temperature range comparing to free bromelain, displaying high activity in the range of 25°C - 85°C. Bromelain complexed with polyphenol produced an enhancement of its thermal stability. Complexed bromelain retained its activity, even there was a slight increase observed in 1.0% and 0.5% cashew-josapine complexed bromelain's activity after exposure at 25°C - 85°C, whereas free bromelain exhibited about 40% activity loss under the same condition. While at temperatures above 85°C, the activity for all the complexed bromelain decreased sharply with the increase of temperature, especially within the range of 85°C - 95°C. A significant drop in activity was observed in 1.5% cashew-josapine complexed bromelain at 85°C - 95°C where there was 92% activity reduction out of the total loss. The activity loss after incubation at 105°C for 1.5%, 1.0% and 0.5% cashew-josapine complexed bromelain were about 65%, 39% and 48% respectively. At 95°C - 105°C, the activity did not differ appreciably and about 100-130 U/ml of enzymatic activity remained.

In the case of positive control complexed bromelain (Figure 5), a higher stability was measured especially in activity of 1.5% cashew-0.4% bromelain complex, which preserved its original activity as

the temperature increased up to 105°C. Likewise in activity of sample complexed bromelain, as temperature was increased to 85°C above, ~60% and 43% activity was remained for 1.0% and 0.5% positive control complexed bromelain respectively. Activity loss could be the result of the effect of temperature on secondary or above structure of enzyme.

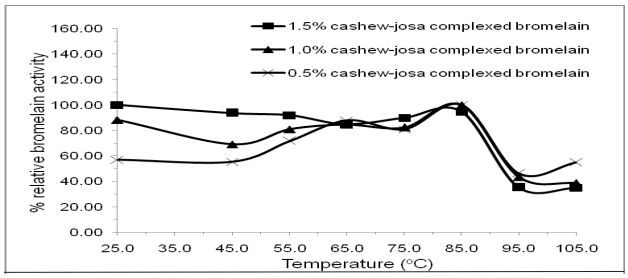

Figure 4. Effect of temperature on bromelain activity of cashew-josapine complexed bromelain. Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

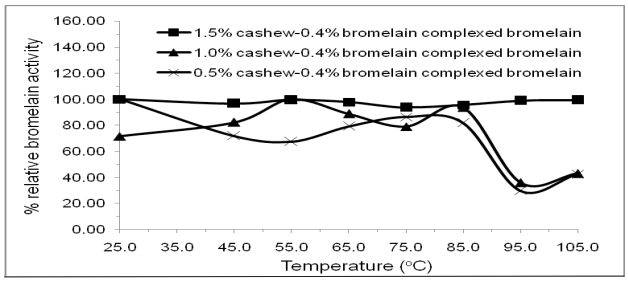

Figure 5. Effect of temperature on bromelain activity of cashew-0.4% bromelain complexed bromelain (positive control complex). Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

It would be interesting to notice that as incubation temperature was risen to 85°C above, the enzymatic activity decreased sharply for either complexed bromelain or free bromelain. The gradient of activity reduction in three parts which were located at 25°C - 85°C, 85°C - 95°C and 95°C - 105°C was measured. A plot of logarithm of bromelain activity versus incubation temperature was made and the calculated gradient was presented in Figure 6. For every sample of free bromelain and complexed bromelain, three different first order kinetics were modeled and rate constant (k) could be calculated from the equation which was described as $\ln [v] = -kt + \ln [v_o]$, v is enzymatic activity. Between 25°C and 85°C, the rate constants for free bromelain, 1.5% cashew-josapine complexed bromelain and 1.5% cashew-0.4% bromelain complexed bromelain were -0.0098, -0.0013 and -0.0009 respectively. The rate constants with minus symbol represented denaturation rate constants, indicating activity decrease in the measured temperature range. Therefore, this data signified that free bromelain was more readily to be denatured as the temperature increased, while complexed bromelain exhibited great resistance against thermal denaturation. Again, the denaturation rate constant of free bromelain (k=0.1063) was higher than cashew-josapine complexed bromelain and positive control complexed bromelain at 85°C - 95°C. These observations indicated that complexed bromelain had a tendency for increased thermal stability, as compared to free bromelain.

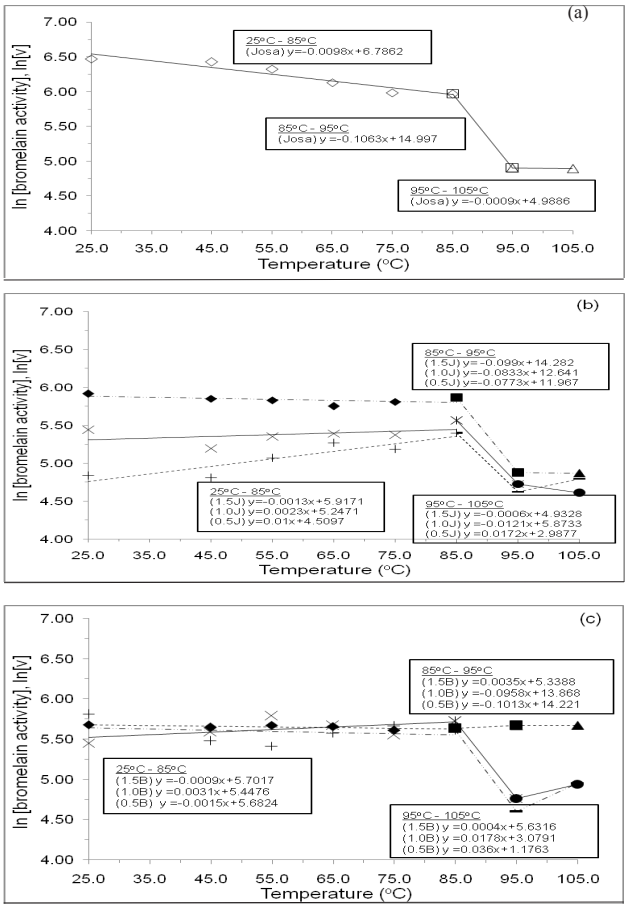

Figure 6. ln [bromelain activity] vs incubation temperature after the test of effect of temperature on activity of (a) free bromelain (b) cashew-josapine complexed bromelain (c) cashew-0.4% bromelain complexed bromelain (positive control complex). Three different first order kinetics are obtained for each sample. Indicated values are expressed as mean (n=3). Josapine activity (free bromelain): ◊ 25°C - 85°C, □ 85°C - 95°C, Δ 95°C - 105°C; 1.5% complexed bromelain activity: ◆ 25°C - 85°C, ■ 85°C - 95°C, ▲ 95°C - 105°C; 1.0% complexed bromelain activity: × 25°C - 85°C, * 85°C - 95°C, ● 95°C - 105°C; 0.5% complexed bromelain: + 25°C - 85°C, - 85°C - 95°C, ━ 95°C - 105°C.

Our result was extended by comparing the activity of free bromelain with that of each complexed bromelain, relatively. Result shown in Figure 7 and Figure 8 reported that either sample complexed bromelain or positive control complexed bromelain gave a higher overall relative activity than free bromelain. The denaturation rate constant of complexed bromelain was less than that of free bromelain within the temperature range of 25°C - 85°C and 85°C - 95°C. Complexed bromelain showed a better tolerance to heat at elevated temperature and its increased stability might be due to the stabilization effect associated with polyphenol complexing.

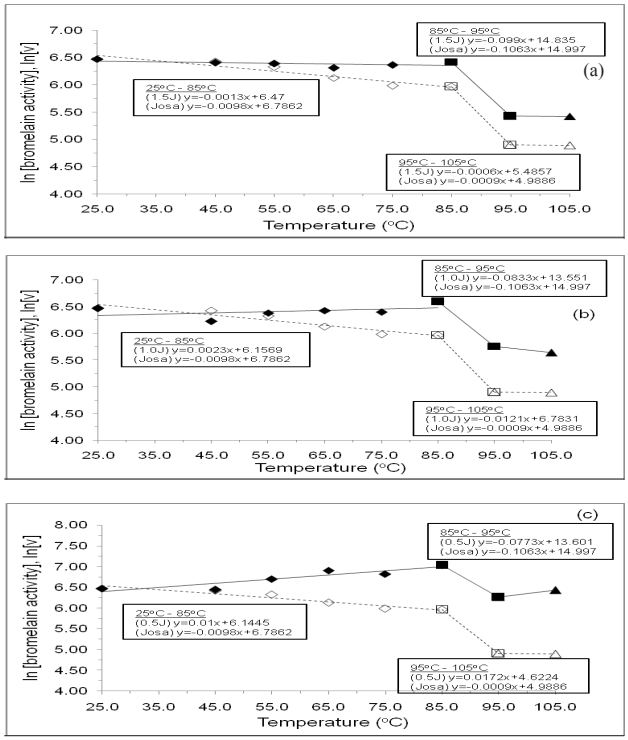

Figure 7. ln [bromelain activity] vs incubation temperature after the test of effect of temperature between free bromelain and (a) 1.5% (b) 1.0% (c) 0.5% cashew-josapine complexed bromelain. Three different first order kinetics are obtained for each sample. Indicated values are expressed as mean (n=3). Josapine activity (free bromelain): ◊ 25°C - 85°C, □ 85°C - 95°C, Δ 95°C - 105°C; complexed bromelain activity: ◆25°C - 85°C, ■ 85°C - 95°C, ▲ 95°C - 105°C.

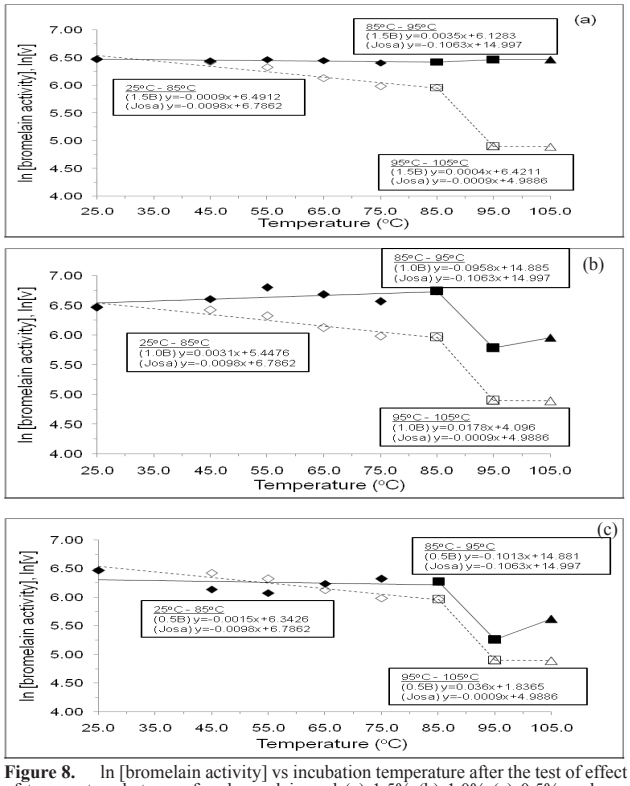

Figure 8. ln [bromelain activity] vs incubation temperature after the test of effect of temperature between free bromelain and (a) 1.5% (b) 1.0% (c) 0.5% cashew-0.4% bromelain complexed bromelain (positive control complex). Three different first order kinetics are obtained for each sample. Indicated values are expressed as mean (n=3). Josapine activity (free bromelain): ◊ 25°C - 85°C, □ 85°C - 95°C, Δ 95°C - 105°C; complexed bromelain activity: ◆25°C - 85°C, ■ 85°C - 95°C, ▲ 95°C - 105°C.

Thermal stability of free bromelain and complexed bromelain at incubation temperature of 85°C

The effect of temperature on enzymatic activity was evaluated and the highest temperature of maximum activity was 85°C for all complexed bromelain, except 1.5% cashew-0.4% bromelain complex's activity. Basically, continuous loss of enzymatic activity with increasing temperature is expected due to the protein denaturation by effect of heat. Complexed enzyme maintaining its activity at this high temperature could possess another beneficial property, which is low microbial activity. High temperature is required for sterilization process for eliminating microbe. Therefore, it would be interesting to know about the incubation duration that enzyme could deserve and at the same time, retain its activity at this elevated temperature.

Thermal stability test assayed at 85°C was carried out by incubating sample for 5 h. Although 1.5% cashew-0.4% bromelain complex was stable to heating up to 105°C, incubation temperature at 85°C was set for thermal stability analysis in this case. As shown in Figure 9, the activity decreased 11.32% and 47.18% when 1.5% and 0.5% cashew-josapine complexed bromelain were incubated for 5 hours at 85°C respectively. There was no practically declination observed in activity of 1.0%. In the case of positive control complex (Figure 10), the reduction was ~4.47%, 28% and 20% for 1.5%, 1.0% and 0.5% cashew-0.4% bromelain complex respectively under the same conditions. Free bromelain retained only 42% of its original activity. Very high percent of activity decrease (especially observed in free bromelain and 0.5% cashew-josapine complexed bromelain) occurred in the first h incubation. The remaining activity was followed by a stable line after 1 h incubation for either free or complexed bromelain. In overall view, complexed bromelain (except 0.5% cashew-josapine complexed bromelain) showed a less activity decrease (<30%) after 5 h incubation, when compared to those obtained for free bromelain (58%), collaborating a higher thermal stability. Figure 11 indicated that activities of 0.5% cashew-josapine complexed bromelain and free bromelain only showed double kinetic with turning point at 1 h. The denaturation rate constant of free bromelain (k=0.802) was higher than that of 0.5% cashew-josapine complexed bromelain (k=0.642). Single kinetic with very low declination was detected in activity of other complexes at 85°C for 5 h.

Discussion

Cashew (*Anacardium occidentale* L.) is a plant native to Brazil and well cultivated in many tropical countries including Malaysia. Many parts of the cashew plant (cashew leave, cashew apple, cashew

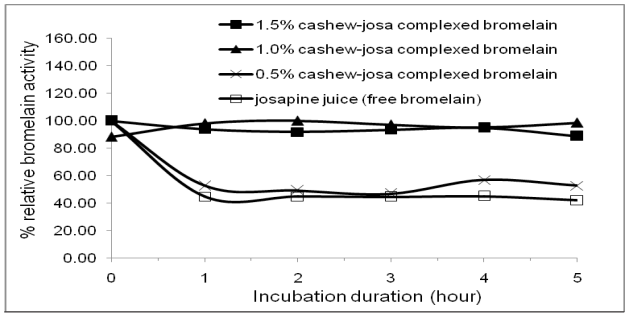

Figure 9. % Relative bromelain activity of free bromelain and cashew-josapine complexed bromelain after incubation at 85°C, 5 h. Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

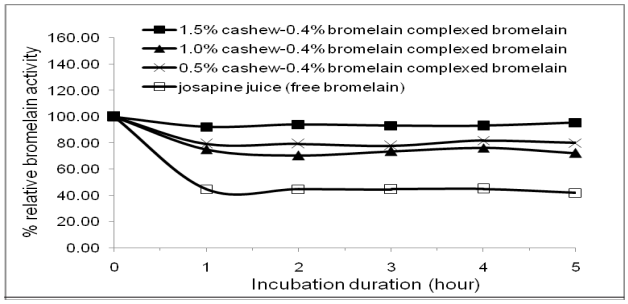

Figure 10. % Relative bromelain activity of free bromelain and cashew-0.4% bromelain complexed bromelain (positive control complex) after incubation at 85°C, 5 h. Relative activity is the ratio of the activity to the initial activity, expressed as percentage. Indicated values are expressed as mean (n=3).

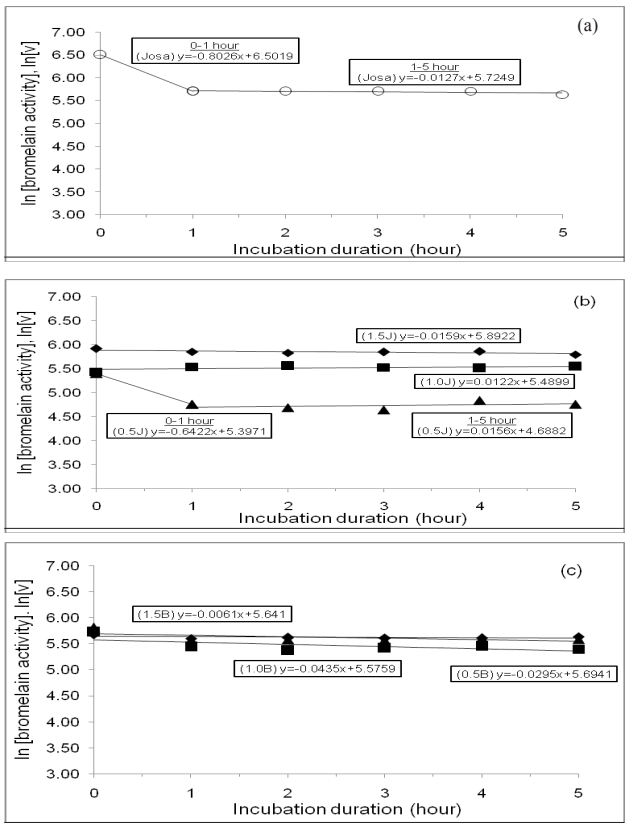

Figure 11. ln [bromelain activity] vs incubation temperature after thermal stability test at 85°C for (a) free bromelain (b) cashew-josapine complexed bromelain (c) cashew-0.4% bromelain complexed bromelain (positive control complex), 5 h. First order kinetics are obtained for each sample. Indicated values are expressed as mean (n=3). o Josapine activity (free bromelain); complexed bromelain activity: ◆ 1.5% cashew extract, ■ 1.0% cashew extract, ▲ 0.5% cashew extract.

nut and cashew nut shell liquid) have been subjected to toxicological test, phytochemical substances screening and analysis of its relationship with antioxidant activity (Trevisan *et al*., 2006; Kamath and Rajini, 2007; Konan *et al*., 2007; Konan and Bacchi, 2007). Cashew leave was detected with several polyphenol components including flavonoids, tannins, proanthocyanidins and glycosylated quercetin (Arya *et al*., 1989; Konan and Bacchi, 2007). Earlier studies suggested that polyphenol contents correlated significantly with antioxidant activity (Trevisan *et al*., 2006). Result obtained in the antioxidant activity analysis in this study showed that ethanolic cashew leave extract contained considerable amount of phenolic compounds and this indicated that this extract could be a potential source of polyphenol and natural antioxidants.

Bromelain belongs to thiol proteases which the catalytic nucleophile is sulfhydryl groups of cysteine residues. Stem bromelain is constituted of a single polypeptide chain consisting of 212 amino acids (Yon-Kahn and Herve, 2010). The polypeptide chain is folded into two structure domains and the structure is stabilized by disulphide bridges. The active site is located on the surface molecules between domains, with two catalytic residues Cys25 and His159 for hydrolysis of bond cleaved and substrate specificity (Ishihara *et al*., 1979). The first N-terminal domain contains mainly antiparallel β segments while the C-terminal domain is composed of α helices. In addition to disulphide bonds, there are numerous hydrogen bonds involved in the structural establishment of two domains (Yon-Kahn and Herve, 2010).

Bromelain is applied in food processing and beverage industry, therefore the effect of temperature and thermal stability are very essential to be investigated. Nucleophile and other catalytic residues which are important for catalysis and structural maintaining are located in active site of enzyme (Rawlings *et al*., 2007). Heating process will alter the secondary, tertiary or quaternary structure of enzyme and distort the binding sites of amino acid residues that arrange along the groove on catalytic site (Joly, 1965). These structural changes in active sites of enzyme which are involved in substrate binding, catalysis and structural maintenance, will affect the function of enzyme, leading to the denaturation and loss in catalytic activity (Rawlings *et al*., 2007).

As shown in the result obtained from thermal stability analysis conducted at 60°C. Free bromelain in pineapple juice was highly susceptible to thermal deactivation. It was found that complexion of bromelain with 1.5% and 1.0% cashew extract notably reserved the enzymatic activity from losing by heat denaturation whereas loss stability was observed with 0.5% cashew-josapine complexed

bromelain. Cashew-0.4% bromelain complex used as positive control in this study exhibited high stability. This might be explained due to the formation of rigid structure by covalent and noncovalent interaction between pure bromelain and polyphenol and it will efficiently protect the bromelain from exposure to external heat stress conditions.

It could be concluded that bromelain in pineapple juice complexed with ethanolic cashew leaved extract had a stronger thermal stability than free bromelain. The cashew leave polyphenol might have the potential to reduce the heat denaturation effect on bromelain enzymatic activity by protein complexion.

Takahashi *et al*. (1973) reported that bromelain is proline rich protein and the mole percent of proline in polypeptide is essential in haze formation with polyphenol (Asano *et al*., 1982). Polyphenol could bind reversibly at the proline sites of open extended polypeptide. Polyphenol-protein binding interacts further by multivalent and noncovalent cross linking and precipitates eventually (Hagerman and Butler, 1981). Polyphenol components in ethanolic cashew leave extract were previously detected with flavonoids, tannins (Arya *et al*.,1989) and proanthocyanidins (polymer of catechin and epicatechin) (Konan and Bacchi, 2007), whereas tannic acid (Siebert *et al*., 1996), catechin, epicatechin and proanthocyanidin (Spanos and Wrolstad, 1990; McMurrough *et al*., 1992) were showed to be haze-active polyphenol. Cross linking of polyphenol and polypeptide chain would alter the influence of external environmental conditions on enzyme structurally or functionally to an extent. Variation in stability in response to heat noticed for free bromelain and polyphenol-complexed bromelain might probably be due to the difference in enzyme's tertiary and above structure (Sode *et al*., 1996).

In the study of the effect of temperature on enzyme, the result indicated that complexed bromelain showed good heat resistance by displaying high activity over a wide elevated temperature range. A stable thermal stability was observed with complexed bromelain from 25°C to 85°C. This observation was in the agreement with the hypothesis that bromelain complexion with polyphenol would improve the stability of enzyme in extreme environmental conditions especially in terms of heat. It could be seen from the data, the highest temperature at which the maximum activity complexed bromelain exhibited was 85°C. High tolerance to heat denaturation under this high temperature could become an advantage for complexed bromelain. In food and beverage production industry, high temperature is one of the conditions required for industrial process including juicing, extraction, sterilization and compressing to improve productivity. It would signify that bromelain complexed with polyphenol would be more stable by retaining this enzymatic activity under the condition employed for food and beverage processing, manufacturing for drugs and pharmaceutics and other industrial application.

Thermal stability describes the ability of enzyme to exhibit resistance against thermal unfolding at elevated temperature (Georis *et al*., 2000). Complexed bromelain showed an increased thermal stability after complexing with polyphenol. Large number of cysteine residues in the polypeptide chain of native and modified bromelain would be one of the reasons for showing an enhanced thermal stability comparing to other enzymes (Xue *et al*., 2010). It might be due to the presence of disulphide bonding in the protein structure. A further thermal stabilization could be made by addition of noncovalent bonds including hydrogen bonds, hydrophobic bonds and salt bridge to stabilize the native enzyme (Daniel, 1996). The involvement of hydrogen and hydrophobic binding in the interaction between protein and polyphenol was evidenced by several studies. For example, condensed tannins (polyphenol) were interacted with bovine serum albumin (protein) by hydrogen binding (Hagerman *et al*., 1998). Haze forming activity was inhibited by nonpolar solvent suggested hydrophobic binding interaction between protein and polyphenol (Asano *et al*., 1982). From the examples stated above, the interaction between bromelain and cashew polyphenol might involve hydrogen or hydrophobic binding and this additional putting of noncovalent bonds could stabilize the free bromelain.

Conclusion

In conclusion, ethanolic cashew leave extract polyphenol was used to complex with josapine juice bromelain to form bromelain-polyphenol complex. After the test of effect of temperature on enzyme and thermal stability, bromelain-polyphenol complexed bromelain showed good heat resistance by displaying high activity over a wide elevated temperature range, as compared to free bromelain in josapine juice. Further thermal stability test will be performed by incubating the sample at five different increasing temperatures at 15-30 min intervals for 2 h to determine the thermodynamic parameters and estimate the activation energy. The thermodynamic parameters such as enthalpy of activation (ΔH^*), entropy of activation (ΔS^*) and free energy of thermal denaturation (ΔG^*) will be determined to help the understanding of probable mechanism of thermal

denaturation.

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