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# PROTEASE ACTIVITY AND CHARACTERIZATION OF BROMELAIN EXTRACT OF PINEAPPLE (*Ananas comusus* (L.) Merr) CROWN FROM SUBANG, INDONESIA

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**ABSTRACT**

Pineapple (*Ananas comusus* (L.) Merr) has a high bromelain content with protease activity which hydrolyzes proteins. Bromelain is distributed to all parts of pineapple, including the pineapple crown, which is agricultural waste that has not been utilized properly. The purpose of this study was to determine the protease activity and the character of bromelain extract from the Indonesian pineapple crown. The pineapple crown was collected from Subang district, West Java, Indonesia. The bromelain extract was obtained through the precipitation process with ethanol, then a protein qualitative test was performed. Solubility test was conducted to determine the physicochemical property. UV spectrophotometer was used to determine protease activity by measuring the tyrosine concentration which produced from hydrolysis of casein as a substrate. The bromelain character was determined by protease activity in various pH, temperature, and substrate concentration. The bromelain extract was protein with specific solubility in various solvents and the protease activity was 7.72 ± 0.45 IU/mg. The optimal activity was reached at pH 5 and 55 °C, $K_M$ as 1.00 mg/mL, $V_{max}$ as 8.11 IU/mg.min, and $K_{cat}$ as 0.03/sec for casein as substrate. Thermal inactivation of bromelain extract can be described by a first-order model and the calculated *E*a value was 642.20 ± 10.60 kJ/mol. The bromelain extract of pineapple crown has protease activity with the specific character and first-order thermal inactivation.

**Keywords:** Casein, First-Order Thermal Inactivation, Precipitation, Specific Character



## INTRODUCTION

Bromelain is widely used in the food, health, pharmaceutical, and cosmetic industries. The food industries use bromelain as meat softening, beer purifier, grain protein dissolving, and protein hydrolyzate production.[1] The health and pharmaceutical fields use bromelain as modulate the tumor growth, third-degree burns, digestion, antibacterial, anti-inflammatory, anticoagulant, and anticancer.[2] The cosmetic industries use bromelain to eliminate stratum corneum cells (peeling).[3]

Bromelain is obtained from pineapple (*Ananas comosus* (L.) Merr) which grow in several subtropical and tropical countries.[4] Bromelain is a pineapple crude extract with the main composition is a proteolytic fraction of sulfhydryl, with escarase (a non-proteolytic component which important for the bromelain topical action), peroxidase, acid phosphatase, some protease inhibitors, and calcium.[5] Bromelain is a glycoprotein with an active site of -Cys-Gly-Ala-Cys-Trp-Asn-Gly-Asp-Pro-Cys-Gly-Ala-Cys-Cys-Trp-.[6] Pineapple production always produces waste, including the fruit core, peel, and crown. Bromelain is known to spread throughout pineapple. The difference in the growth place and the pineapple part affects the bromelain character.[4] The main pineapple producers are Brazil, China, Philippines, and Thailand, followed by India, Nigeria, Kenya, Indonesia, México and Costa Rica.[7] This study was used pineapple which collected from Subang district, West Java, Indonesia, one of the pineapple production center in Indonesia.[8] The novelty of this research was the bromelain extraction from pineapple crown along with its



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characterization. The reason for choosing a pineapple crown, because in Indonesia this part has not been utilized properly and becomes agricultural waste.

## EXPERIMENTAL

### Materials

The pineapple crowns were collected for 12 months old fruits which planted in Subang district, West Java, Indonesia. The plant was identified by Plant Taxonomy Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia with No. 519/HB/02/2018. All chemical reagents, i.e. Millon reagent, ninhydrin, copper sulfate, tyrosine, casein, trichloroacetic acid (TCA), and Bradford reagent, were analytical grade and purchased from Merck.

### Bromelain Extraction

The pineapple crown was mashed with aquadest, then filtered. Ethanol was added to the filtrate (1: 4) and stored for 8 h at 4 °C, then centrifuged at 15,000 rpm for 15 min. The sediment was dried at 30 °C.[9]

### Protein Qualitative Test

**i. Millon Test**
The extract solution was added to Millon reagent to form a white precipitate. The mixture is heated to form a reddish-brown color.[10]

**ii. Ninhydrin Test**
The extract solution was added to ninhydrin. The mixture is heated to form a purple-blue color.[11]

**iii. Biuret Test**
The extract solution was added to biuret reagent to form a blue-purple color.[12]

### Solubility Test

Bromelain extract was mixed to aquadest, acetone, ethanol, 0.1 N HCl, and 0.1 N NaOH. The solubility was observed.

### Determination of Total Protein Content

The casein standard, extract, and blank, each were added to Bradford reagent. The absorbance was measured at 595 nm after 5 min incubation.[13]

### Determination of Protease Activity

The tyrosine standard, as a product of protease, was measured at 275 nm. Bromelain extract (1 mg/mL) and casein solution (1 mg/mL) are incubated for 30 min. The TCA solution was added and the solution was incubated at 90 °C for 5 min, then cooled to room temperature. Absorbance was measured at 275 nm.[14] Protease activity is calculated by eq.-1.

$$\text{IU/mg} = \frac{\mu\text{mol produced tyrosine}}{\text{mg extract x time}} \quad (1)$$

### Partial Characterization

Determination of the optimum temperature was carried out at various temperature (30, 40, 50, 60, and 70 °C). Determination of optimum pH was carried out at various pH (3, 4, 5, 6, 7, 8, 9, 10, and 11).[14]

### Determination of Kinetic Parameters

The kinetic parameters were determined by protease activity at various casein concentrations (0.25, 0.5, 1, 2, 4, 8 and 12 mg/mL). Michaelis-Menten graph was used to determine the $V_{max}$, $K_M$, and $K_{cat}$ of bromelain extract.[14]

### Determination of Thermal Stability

Bromelain extract was stored at two different conditions, i.e. 25 °C RH 90% and 40 °C RH 75% for 42 days for thermal stability determination. At certain times, bromelain extracts (3 mg/mL) were prepared

and reacted with the same method to determine the protease activity. Residual bromelain extract activity was measured by the same method.[14] The kinetics of thermal inactivation of bromelain extract was determined. The inactivation rate constants ($k$) were calculated from a semilogarithmic plot of residual activity as a function of time. The activation energy ($E$a) for thermal inactivation was calculated from the slope of the Arrhenius plot by eq.-2.[15]

$$\text{Log } k = -E\text{a}/(2.303RT) \quad (2)$$

Where $k$ = rate of inactivation at $T$
$R$ = gas constant (8.314 J/mol.K)
$T$ = absolute temperature in Kelvin

**Statistical Analysis**

Data were shown as mean and standard deviation (SD). Data were statistically analyzed using one-way analysis of variance.

## RESULTS AND DISCUSSION

**Bromelain Extraction**

Subang is one of the pineapple production centers in Indonesia with 1,633 tons produced in 2017. Subang district altitude is 0-1500 m above sea level[8] which suitable for pineapple growth.[17] Bromelain was isolated by ethanol precipitation, due to globular protein which dissolves in water and precipitate in ethanol as organic solvent. Organic solvents interfere the hydrophobic interactions that form a stable nucleus of globular proteins but do not damage the covalent bonds in the peptide chain.[16] The precipitation method will concentrate the target protein. As much 20.3 g of bromelain extract was produced from 9.08 kg of pineapple crown, the yield was 0.23%. Bromelain extract was light brown powder, tasteless, and slightly pineapple fragrance. The yield was 0.23% showed a low protein content in the pineapple crown. This result was similar to the literature, i.e. 0.25%.[17]

**Protein Qualitative Tests**

Bromelain main composition is protein,[6] so bromelain identifications were conducted by a protein qualitative test based on color reaction. Qualitative tests showed that bromelain extract was a protein, due in accordance with the literature. The qualitative test was detected the tyrosine residues,[10] peptide bond,[11] and free amine groups.[12] Millon reagent detects soluble proteins by reacting with tyrosine residues to form a reddish-brown solution or sediment.[10] Millon test is not a specific reaction, because it also detects all phenol compounds, and need confirmation with other tests. Ninhydrin test is used to detect amino acids and proteins with free amine groups to form the purple-blue complex.[11] Biuret test (Piotrowski test) is used to detect peptide bonds in proteins which form a purple-blue coordinating complex.[12]

**Solubility Test**

Solubility test was used to determine the physicochemistry property of the protein. Globular protein is soluble in water, insoluble in organic solvents, dilute acid and base.[16] Bromelain extract was forming a colloidal solution in water, and insoluble in ethanol, acetone, 0.1 N HCl and 0.1 N NaOH. These results were accorded to protein solubility in water, organic solvents, dilute acid and base.[15] Bromelain extract was forming a colloidal solution in water, which can be penetrated by light because of the Tyndall effect.[19] The bromelain extract was insoluble in ethanol and acetone, as an organic solvent, because of hydrophobic interactions intervention.[16] The bromelain extract was insoluble in 0.1 N HCl and 0.1 N NaOH solution. Acidic solutions provide hydrogen cations and alkaline solutions provide hydroxide anions which change the total charge of the protein, which causes electrostatic repulsion and interference with hydrogen bonds.[16]

**Determination of Total Protein Content**

The total protein content in bromelain extract was determined through a reaction between protein and Bradford reagent (Coomassie Brilliant Blue G-250 dye) which form colored product and measured at 595

nm. The electrostatic and van der Waals interactions are formed between this dye by the amine and carboxyl groups of protein. There is no reaction between non-protein compounds and the dye.[20]

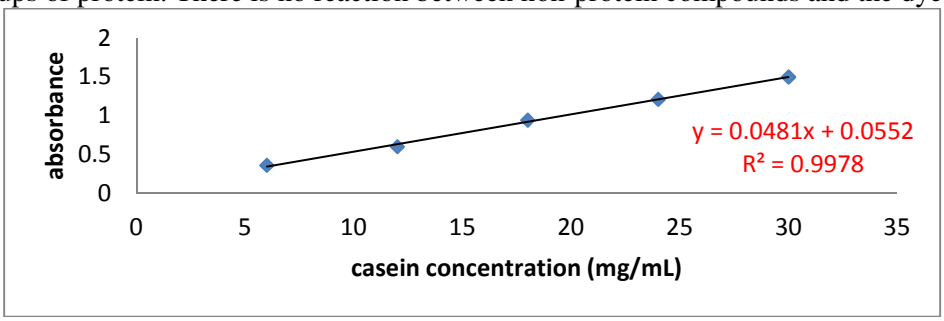



Fig.-1: Casein Calibration Curve

The correlation coefficient was 0.9978 (Fig.-1) which indicate that the instrument response is proportional to the concentration.[14] The absorbance of bromelain extract (30 mg/mL) was 0.682 which equivalent to 13.03 mg/mL of casein as standard protein. This value was shown that bromelain extract contains non-protein compounds.[5] Bromelain extract contains 43.44% of protein and 56.56% of other compounds, i.e. ethanol-insoluble compounds. This result was higher than pineapple pulp (25-30%).[21] Total protein content was used for determining the ratio of bromelain activity of total protein.

**Determination of Protease Activity**

The protease activity of bromelain was determined by tyrosine as the product from degradation of casein as substrate. The optimum incubation time was 30 min, the tyrosine number was increased with the increased incubation time.[22] The correlation coefficient was 0.9999 (Fig.-2) showed that the instrument response and the concentration are proportional.[21] Casein degradation by bromelain extract (30 mg/mL) was observed from tyrosine, which can be measured by UV spectrophotometry due to aromatic residue.[15] The absorbance of tyrosine from casein degradation was 0.425 ± 0.026 which equivalent with 57.88 ± 3.37 µg/mL of tyrosine, so protease activity was 7.72 ± 0.45 IU/mg. This result was higher than bromelain from fruit (4.71 IU/mg) and stem (4.52 IU/mg).[23] So, pineapple crown is potential to be developed as a bromelain source. The protease activity difference was caused by difference in parts of the plant as a bromelain source. The water content of crown is less than fruit and stem, so wet weight produces a different dry weight of different plant parts. This result was accorded to Mondal *et al*.[4] that the difference in the growth place (India and Indonesia) and the pineapple part (crown, fruit, and stem) affects the bromelain character.

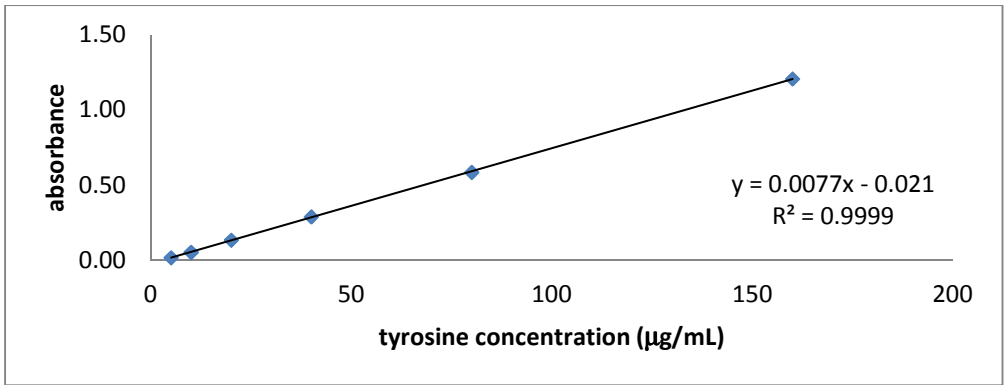


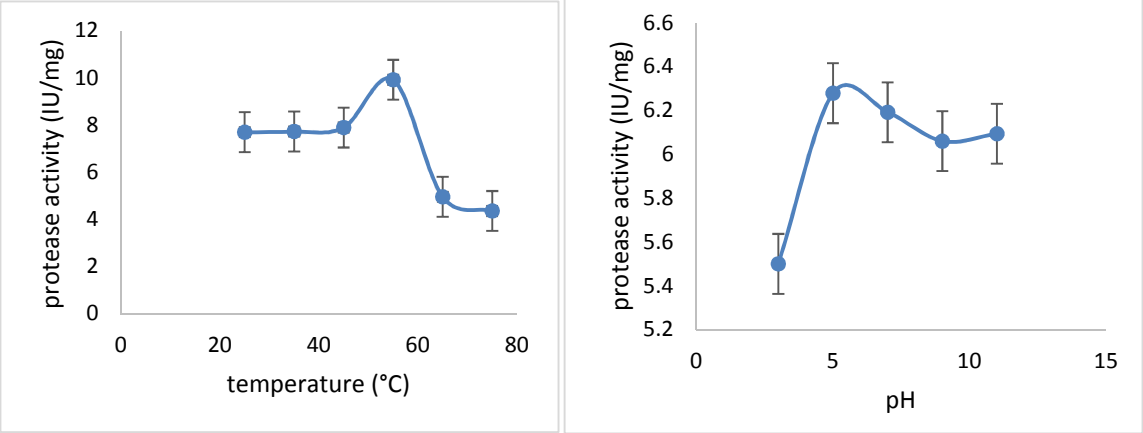

Fig.-2: Tyrosine Calibration Curve

**Partial Characterization**

The optimum temperature of crown bromelain was 55 °C (Fig.-3a) which statistically significant ($p$ = 2.33 x $10^{-8}$). This result is in the optimum temperature range of bromelain stem, i.e. 50-60 °C[6,23-25] and fruits, i.e. 37-70 °C.[6,26,27] The wide optimum temperature range is due to the various molecular weight of

bromelain. The higher the molecular weight, the higher the optimum temperature, because of bromelain structure is more stable, due to chemical bonds. Various molecular weight is due to different isolation methods. This study used ethanol precipitation, followed by centrifugation to accelerated sedimentation. Increased temperature will increase kinetic energy. After optimum temperature, higher energy will be inactivated the enzymes due to disrupted the peptide bonds and disulfide bonds.[16]

The optimum pH of crown bromelain was pH 5 (Fig.-3b) which statistically not significant ($p$ = 0.08). This result was consistent with the pH range of fruit bromelain, i.e. 3-8.[6,26,27] The optimum pH is influenced by the nature of the substrate, concentration and type of buffer, and the presence of reducing agents.[30]




(a) (b)

Fig.-3: Graph of Protease Activity on (a) Various Temperature and (b) Various pH (n = 3)

**Determination of Kinetic Parameters**

The rate of bromelain extract to degrade casein at various concentrations was used to determine the kinetic parameter (Table-1). When bromelain extract was saturated with casein, so the degradation rate was maximized. Increased casein concentration will not increase the degradation rate, and form the plateau on the Michaelis-Menten graph (Fig.-4). Extrapolating the plateau to the y-axis produces $V_{max}$ and extrapolating a half of $V_{max}$ on the x-axis produce $K_M$.[16] Maximum velocity ($V_{max}$) was obtained from the plateau-like $V_0$ region (Fig.-4), i.e. 8.11 IU/mg.min. This $V_{max}$ was used to calculate $K_M$ from Michaelis-Menten equation, i.e. 1.00 mg/mL. These two values were used to calculate $K_{cat}$, i.e. 0.03/sec. This kinetic parameter was specific to casein as substrate, because this parameter was specific for each substrate. This result was higher than bromelain fruit and stem,[24-27] due to different character of bromelain extracts from different part of the pineapple.

Table-1: Protease Activity of Bromelain Extract at Various Casein Concentration

| Casein concentration (mg/mL) | Absorbance | Tyrosine concentration (µg/mL) | Protease activity (IU/mg) |
|---|---|---|---|
| 1.25 | 0.255 | 35.887 ± 2.087 | 4.785 ± 0.278 |
| 2.5 | 0.280 | 39.091 ± 2.138 | 5.212 ± 0.285 |
| 5 | 0.321 | 44.459 ± 1.879 | 5.928 ± 0.251 |
| 10 | 0.432 | 58.398 ± 2.220 | 7.786 ± 0.296 |
| 20 | 0.474 | 64.329 ± 2.873 | 8.577 ± 0.383 |
| 40 | 0.465 | 63.160 ± 1.105 | 8.421 ± 0.147 |

**Determination of Thermal Stability**

The protease activity of bromelain extract was decreased during storage for 42 days. The remaining % residual activity of bromelain extract was only 0.36% for 25 °C RH 90% and 0.04% for 40 °C RH 75% (Fig.-5). Statistical analysis showed that there were significant difference in protease activity during

storage ($p$ = 1.22 x $10^{-5}$). The current study indicated that bromelain extracts retained approximately 50% activity after 21 days at 25 °C and RH 90%, but only 4 days at 40 °C and RH 75% (Fig.-5). This result was better than Hale *et al.*[5], which found that the proteolytic activity of concentrated bromelain solutions was 7 days at 25 °C. The difference in these results might have been due to differences in the proteolytic method, the concentration of bromelain extract, part of pineapple fruit as bromelain sources, and fruit varieties. Hale *et al.*[5] used pineapple fruit as bromelain source, while the current study used pineapple crown as bromelain source.

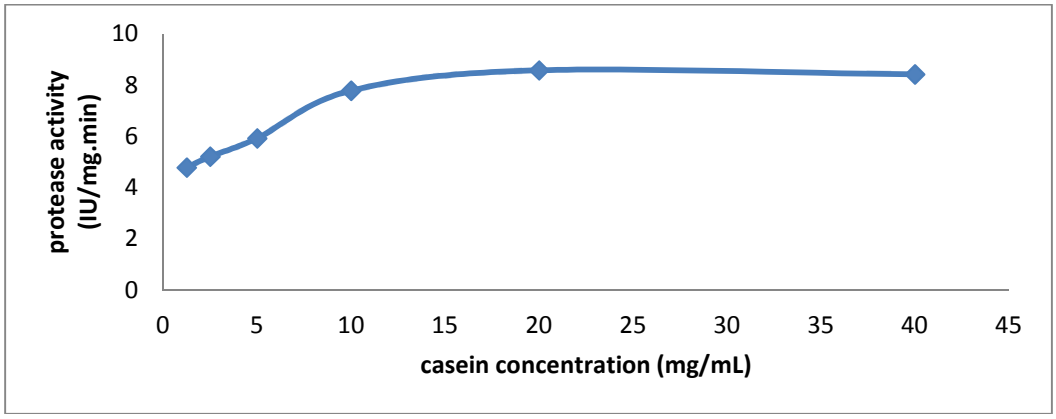



Fig.-4: Michaelis Menten Graph of Bromelain Extract

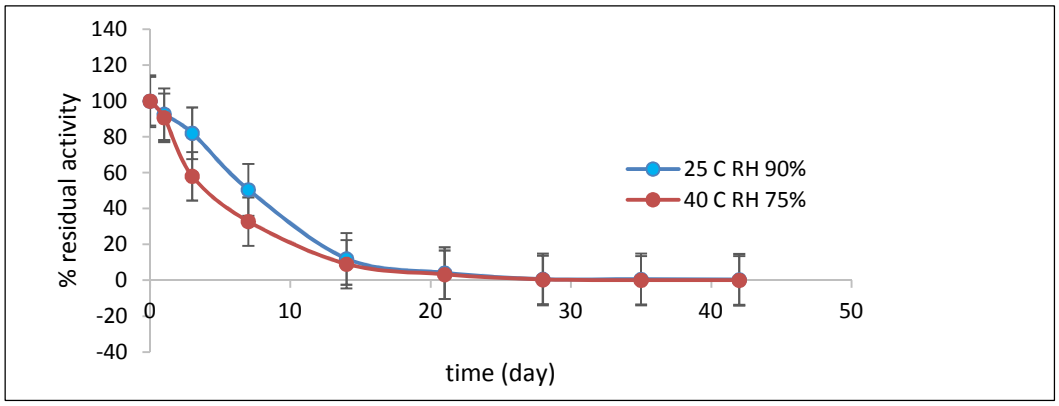



Fig.-5: Effect of Storage Temperature and Humidity Relative to Bromelain Activity with Casein as Substrate (n = 3)

The log-linear plots of residual activity of bromelain extract against inactivation time at constant temperature for 21 days were shown the first-order model (Fig.-6). The first-order rate constant of the denaturation of bromelain extract at 25 and 40 °C was 0.077 and 0.082 $min^{-1}$, respectively. The rate constant was increased with the increased temperature, which in accordance with a study by Paramita *et al.*[29] The calculated *E*a value for bromelain extract inactivation was 642.20 ± 10.60 kJ/mol. The activation energies for enzyme denaturation are within the range 209.2-627.5 kJ/mol,[15] but our result was higher than the range (642.20 ± 10.60 kJ/mol). High calculated *E*a indicated that the crown bromelain extract was more stable than fruit bromelain (313.18 ± 57.44 kJ/mol)[31] or commercial pure bromelain from pineapple stems, i.e. 174.47 kJ/mol[32] and 181 ± 35 kJ/mol.[33] The difference between these values was due to differences in fruit varieties, part of pineapple fruit as bromelain sources, the concentration of bromelain extract, and the proteolytic activity assay. Jutamongkon and Charoenrein[31] used fruit bromelain from Cayenne pineapple, while the current study used crown bromelain from Subang pineapple. These results indicated the pineapple crown was a potential bromelain source to be developed due to good stability and high activation energy. It can be used as an alternative to the utilization of agricultural waste.

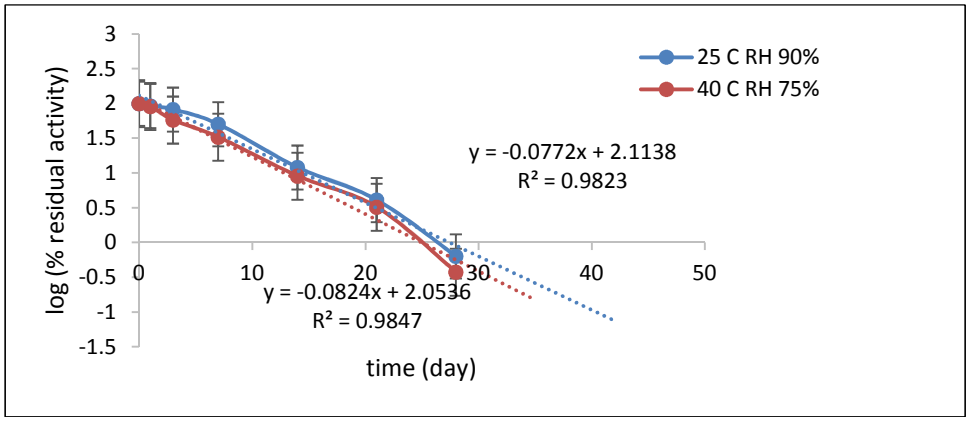



Fig.-6: Thermal Inactivation Plots of Bromelain Extract at Different Condition (n = 3). The Rate Constants (*k*) for Inactivation were Determined from the Slopes of the Logarithmic Plot of Activity Against Time: log (% Residual Activity) = (*k*/2.303)t.

## CONCLUSION

The bromelain extract of pineapple crown has protease activity with the specific character and first-order thermal inactivation.

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