Egyptian Journal of Chemistry

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Effects of extraction condition on quality and activity of bromelain enzyme in pineapple's core (Ananas comosus Queen)

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Abstract

The pineapple processing industry provided a large number of beneficial ingredients such as the bromelain enzyme. The study focused on the parameters that influence the efficiency of theextraction process for this ingredient. Total protein content (TPrC) and bromelain enzyme activity (BE activity) were determined after removal with organic solvents in the 1-2-3-4 hours range. The results showed that acetone absolute was suitable for extraction at 1: 2 of material: solvent ratio (w/v) at 3°C reached the highest values 301.82 µg/mL and 0.0886 UI/mL, for TPrC and BE activity, respectively. These findings provide an essential insight for future studies on and application of microencapsulation, storage conditions, and drying methods to futher improve the bromelain enzymes activity.

Keywords: pineapple (*Ananas comosus Queen*), core, protein precipitation, bromelain ezyme, spray drying

1. Introduction

Nowadays, utilizing agricultural by-products and processing industry are becoming more favorable due to the benefits of reducing waste and environmental problems. Studies on other agricultural by-products have been performed previously on cashew apple [1], *Rhodomyrtus tomentosa (Ait.) Hassk* [2], and pomelo peel [3], about 35-40% of which are not properly used [4]. On the other hand, some properties from by-products can be employed to apply in the pharmaceutical field. Typically, the bromelain was found to prevent cloting in the blood vessel, reduce the platelets coagulation rate as well as incorporating with hemoglobulin to hydrolyze molecular link in coagulopathy trousseau's syndrome [5] or anti-inflammatory *in vitro* and *in vivo* [6], [7]. The total production of pineapple around the world was 3.2 million tons in 2019 which was mostly consumed by some countries such as Philippine, China. However, climate change and some plant diseases resulted in the decline of production about 8% [8]. The shell and core of the pineapple contribute to approximately 40-80% which are discarded during the pineapple juice processing [7]. This has led to serious environmental pollution as well as the waste of some bioactive compounds retained in this by-product. Pineapple (*Ananas comosus (L.) Merr*) belongs to Bromeliaceae family, Ananas genus. It was first discovered in South America and domesticated by the Tupi-Guarani Aboriginal people and spread to the Antilles, North Andes and Central America [9]. Considered as a fruit tree, pineapples is an abundant material for fresh consumption, as well as food and pharmaceutical industries. Pineapple contains many nutrients such as dietary fiber, sugar, mineral (e.g. copper and manganese), vitamins (B1, B6, and C) [10] and BE. BE has an important role in human health, which include supporting digestion in the stomach and small intestine, minimizing sinusitis, inhibiting inflammation, reducing edema and hematoma. BE is a protease which belongs to endoprotease group with cysteine-containing active entrails and the disulphuric

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Receive Date: 30 May 2021, Revise Date: 30 July 2021, Accept Date: 11 December 2021
DOI: 10.21608/EJCHEM.2021.78252.3832
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bridges (–S-S–) between two polypeptides that break the protein endothelial peptide bonds into fragments called peptides [11].

Many previous publications used by-products from pineapple to produce bio-fertilizer by using microorganisms or to produce lactic acid by using *Lactobacillus delbrueckii to* ferment pineapple syrup by-product [12]. Many BE extraction techniques were performed previously by R.S. Chaurasiya *et al* (2013) [13]; Benefo and Ofosu (2018) [4].

Results from these studies have shown that BE distributed in most parts of pineapple, especially the fruit pulp, and that operation time could greatly affect the recovery efficiency of the enzyme. However, there are a number of different parameters which also potentially affect the BE extraction eficiency that remained limitedly studied. For this reason, the present study investigates the effects of different material: solvent ratios (from 1:5 to 1:1 v/v) to improve the extraction process efficiency and application of maltodextrin microencapsulation to increase BE's shelf life.

2. Materials and Method

2.1. Preparations of sample

Pineapples of the Queen genus which were not damaged and waterlogged were harvested in Tien Giang province, Vietnam. They were rinsed with water, then had the shell removed to obtain the core of 1.5 of thickness and 15 ± 1cm in lenghth). The cores were then storaged at -20^{o}C and wrapped by a plastic bag to prevent the weight loss. The pineaple core was pressed to collect solution, then centrifuged at 6000 rpm for 20 minutes and filtered with Whatman No.1 paper

2.2. Reagent

$Na_2HPO_4.12H_2O$ (99%), KH_2PO_4 (99%), NaOH (99.8%); HCl (36-38%), TCA (trichloroacetic acid) EDTA (Ethylene diamine tetraacetic acid), ethanol absolute, and H_3PO_4 (85%) were obtained from Xilong, China. Cysteine, Tyrosin, albumin, coomassie brilliant blue G, Casein and maltodextrin was distributed by Himedia; Folinifer Ciocalteu (FCR) was purchased at Merck & Co., Inc., Germany.

2.3. Experimental design

The experiments used single factor investigation to optimize the parameters, presented in table 1

Table 1. The investigation range of the experimental factors

Factor	**Investigation range**
Solvent precipitates	Acetol-Ethanol
Materials:solvents ratio (w/v)	1:1-1:2-1:3-1:4-1:5
Solvent concentration (%)	20-50-70-100
Precipitation time (hour)	1-2-3-4
Treatment temperature (°C)	-15 – 3 – 8 - RT

2.4. Quantification of total protein content (TPrC)

The total protein content was measured by using the Bradford method with a few modifications [14] based on albumin reaction with Coomassie brilliant blue (CBB). A total of 1 mL of sample was placed in a dark-colored test tube, and added with 2 ml of CBB which had beendiluted with 96ºH_3PO_4: 85% ethanol: distilled water at 1: 2: 17 (w/v/v) ratio. The mixture was then centrifuged for 30 min and measured for absorbance at 595 nm of wavelength. TPrC was determined by the equivalent of standard albumin (µg/mL) according to Equation: $y = 0.0073x - 0.0038$ ($R^2 = 0.9993$)

2.5. Determine the BE activity

The BE activity was determined by the tyrosin and FCR color reactions, following the method of López-García *et al.* (2012) with few modifications [15]. A total of 1 ml of sample was added to the tube containing 5 ml of casein 1%, add, agitated at 35.5°C for 35 min. Then, 5ml TCA 10% was added and allowed to react for 25 min before being filtered through Whatman No.1 filter paper. An amount of 1 mL of the filtered solution was collected, added with 2 mL NaOH 0.5N and 0.6 mL of Folin 25% reagent (v/v), vortexed for 2 minutes and placed in the dark for 30 minutes. The absorbance measurement was conducted at 660 nm by using the UV-Vis Spectrometry system (1800 Shimadzu Spectrometer). The protease activity (UI) is calculated by using Equation 1 below and is expressed as the amount of tyrosin (µmol) produced by casein hydrolysis of protease-containing mixture (1 mg) for 1 min under standard conditions (35.5°C, pH= 7.6), according to the tyrosine standard equation: $y= 1.0693x + 0.0275$ (with R^2=0.9952)

$$\text{BE activity} = \frac{x.V.K}{t.v} \quad (1)$$

Where:

x: number of µmol tyrosine deduced from the standard curve

V: total volume of enzyme reaction mixture (11 ml)

v: analytical filtrate volume (1 ml)

k: sample dilution factor

t: response time

1UI (Anson) = 1 µmol tyrosine/mL/min; or 1 µmol/mg/min

2.6. Analysis statistics

The experiment was performed in triplicates. The data is processed by Microsoft Excel software. Analysis of Variances (ANOVA) were carried out using Statgraphics Centurion XV and represented as mean value ± standard deviations. $p < 0.05$ was considered as significant difference.

3. Results and discussion

3.1. The solvent precipitates effective separation of TPrC and BE activity

The results shown Table 1 indicated that as compared to ethanol, a higher amount of wet precipitate was obtained when using acetone as the solvent. This observation can be explained by the fact that acetone possess a greater polarity than ethanol, thereby easily removing the water outside of the protein and causing precipitate formation [16].

Results from ANOVA statistical analysis showed that the type of solvent affect the precipitation, protein content and BE activity at 95% confidence level. The amount of protein per 1 mL when precipitated with ethanol was higher than acetone precipitate while the BE activity in the opposite direction was significantly different ($p < 0.05$, via LSD test). It is possible that the raw material contains some impurities and a high alcohol concentration increases the protein denaturation, leading to a decrease in bromelain activity [17]. On the other hand, Pessoa and Kilikian (2005) assuming that adding ethanol easily leads to enzyme denaturation by forming "pockets" of ethanol in solution [18]. According to Rathnakumar *et al* (2017), a variety of organic solvents such as acetone and ethanol have been used for the precipitation of bromelain [19]. Nguyen Van Thanh *et al* (2013) showed that the specific activity of bromelain when precipitation with acetone (14.15 U/mg) was higher than that of ethanol (13.16 U/mg) [20].

Table 2. TPrC and BE activity by solvent

Solvents	Wet precipitate (g)	TPrC (µg/mL)	BE (UI/mL)
Acetone	1.669 ± 0.010	428.944 ± 6.172	0.027 ± 0.001
Ethanol	1.324 ± 0.006	434.431 ± 6.307	0.018 ± 0.003

3.2. Influence the ratio of raw materials and solvents

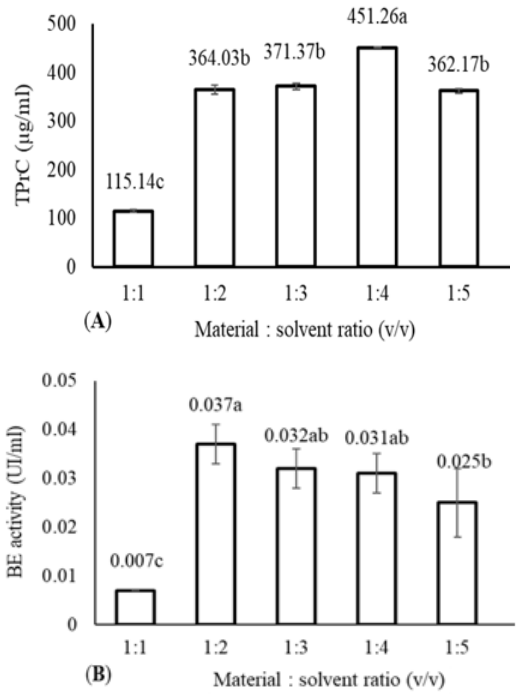

Fig. 1. The effect of material: solvent ratio (v/v) on A) TPrC and B) BE activity. Values showing different letters indicated significant different ($p<0.05$).

The effect of the material: solvent ratio on TPrC and BE activity is shown in Figure 1. Firstly, the TPrC increased from 115.14 µg/mL to 451.26 µg/mL as the material: solvent ratio decreased from 1:1 to 1:4. As the ratio continued to decreased to 1:5, the TPrC declined to 362.17 µg/mL, which was insignificantly different from the TPrC at 1:2 (364.03 µg/mL) and 1:3 (371.37 µg/mL) ratios. Secondly, BE activity reached the highest level at1: 2 of material: solvent ratio (0.037 $UI.mL^{-1}$) and there was no significant difference when increasing the acetone volume. As the volume of acetone increases, it binds to the water molecules that surround the protein, and promotes precipitation, leading to increasing TPrC and BE activity [21].

However, as the acetone volume increased linearly and surpassed 1:4 for TPrC and 1:2 for BE activity, the dielectric constant decreased. The protein molecules

were then able to self-dismantle and became inactive, causing its content and activity to decrease [22], [23]. Specifically, at 1:4 of material: solvent ratio, TPrC is high but has low activity, which can be explained by the presence of protein impurities in the precipitate. Therefore, 1:2 (v/v) of solvent: material ratio was selected for the subsequent experiment.

3.3. Effect of solvent concentration

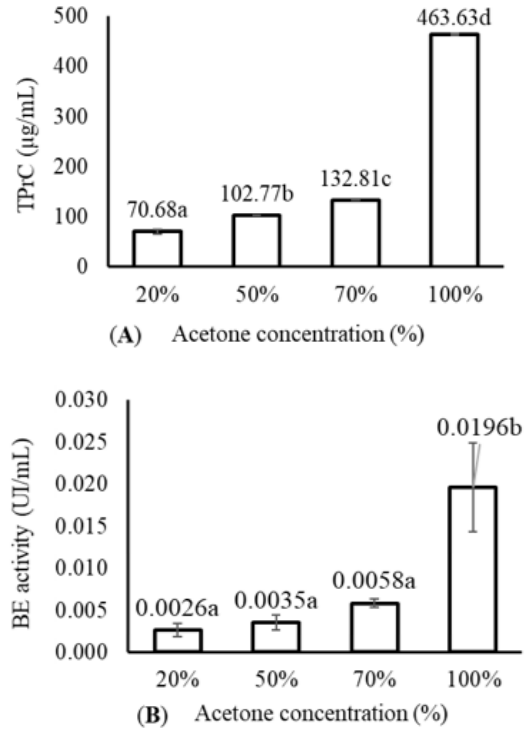

Fig. 2. The effect of acetone concentrations (%) on TPrC and BE activity. Values showing different letters indicated significant different ($p<0.05$)

The results in Fig. 2A and B showed that, the TPrC and BE values were lowest at 20% (70.68 µg/mL; 0.0026 UI/mL) and highest at the absolute solvent concentration (463.63 µg/mL; 0.0196 UI/mL). The LSD statistical test showed that the precipitation solvent concentration had a significant effect on TPrC and BE activity at 95%. TPrC and BE activity have a proportional correlation with acetone concentration. As the addition of organic solvents could reduce the solubility of water surrounding the protein, alarge volume of water can be released, resulting inpartial immobilization of water molecules by hydration of organic solvent molecules. Therefore, when the acetone concentration increases, its repelling effect becomes stronger and promotes precipitation [24].

3.4. *Effect of precipitation temperature and time on TPrC and BE activity.*

Effect of precipitation time

Fig. A and B showed that the precipitation time had a significant effect on the TPrC and BE activity within the LSD test. After 2 hours of treatment, the TPrC and BE activity reached the highest values (320.89 µg/mL and 0.0956 UI/mL, respectively) and decreased gradually. Although prolonged treatment time facilitates the aggregation of protein molecules and increases the amount of protein in precipitateit increases the ability to lose the water layer associated with the protein, causing the denaturation of the protein by the solvent and reducing theefficiency of absorption [21].

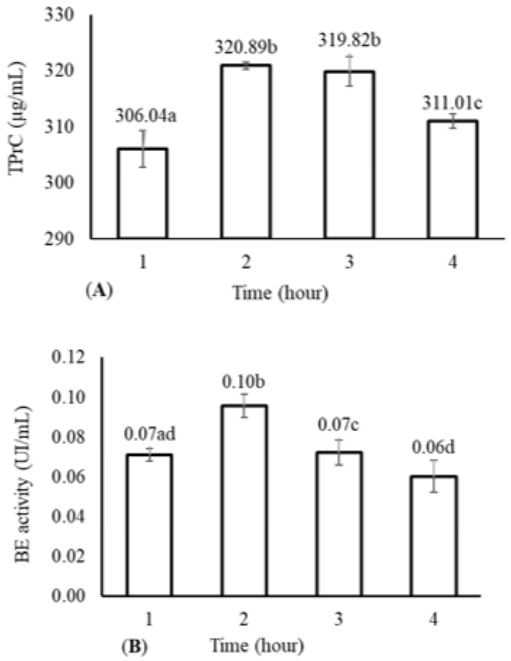

Fig. 3. The effect of precipitation time (hour) on TPrC and BE activity. Values showing different letters indicated significant different ($p<0.05$)

Effect of treatment temperature

The effect on TPrC and BE activity was significantly varied between diferent precipitation temperatures (Figure 4 A and B). The lowest values of TPrC and BE activity were 170.14 µg/mL and 0.0649 UI/mL, achieved at -15 ºC, while the highest values were 301.82 µg / mL and 0.0886 UI/mL, achieved at 3ºC. At low temperature (near or below 0ºC), the enzyme activity is weakened and falls into inactive state [25]. When the temperature raises above 4°C, the precipitation and the ability to limit protein

denaturation are improved. The obtained results are similar to paper of Pereira *et al* (2014), in which acetone used to precipitate bromelain was more effective at low temperatures (about 4°C) than at room ttemperature, as excessively high temperatures increase the ability to break the ligand bonds andthe spatial structure, as well as increase enzyme inactivation [26].

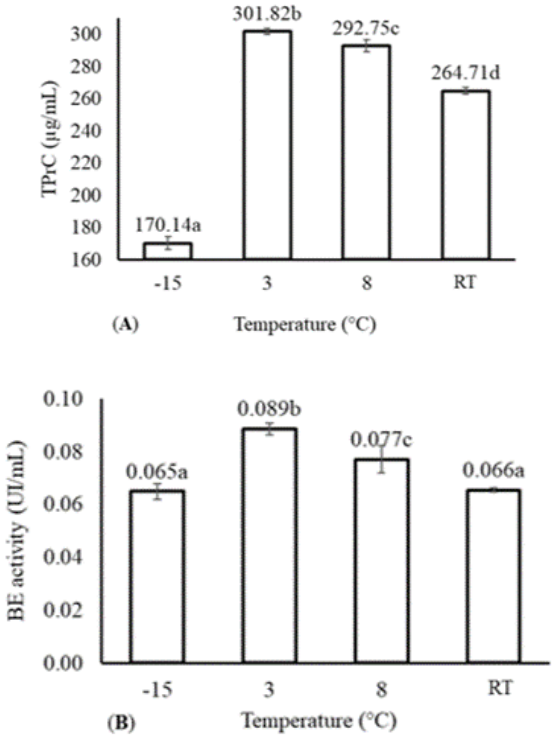

Fig. 4. Precipitation temperature on TPrC and BE activity

The resulted TPrC (278.5 µg/mL) was found to be higher than the previous preport of Ketnawa *et al.*, 2012 (27.1 ± 1.83 mg/100g) [27]. Solvent extraction using DI, DI containing cysteine and ethylenediaminetetraacetic acid (EDTA), sodium phosphate buffer, and PB containing cysteine and EDTA from the study of S. Ketnawa *et al.*, 2011 resulted in 0.215 ± 0.01 mg/mL of the total protein in the shell [28]. The protein content in the crude extract by using ethanol extraction containing a 40% (w/v) of ethylene glycol solution at 0°C was found at 0.23 ± 0.01 mg/mL, reported by Paulo *et al.*, 2012 [29].

4. Results and discussion

Pineapple core is one of the sources for production of bromelain inoculants. TPrC and BE activity are influenced by multiple factors during enzyme extraction by by using organic solvents. The obtained TPrC was significantly affected by the acetone concecntration (the concentration at 100% was 6.61 times higher than at 20%). Similarly, temperature was observed to significaly affect the TPrC at -15°C as compared to the others. This indicated that these factors effectively contribute to the efficacy of the extraction process. Similarly, the BE was least obtained at the material:solvent ratio of 1:1 in which the acetone concentration was 20%. This suggested that the TPrC and BR experienced the same trend in the same extraction condition. As previously discussed, BE activity is dependent on the subtrate.

The high values of TPrC (278.5 µg/mL) and BE activity (0.0103 UI/mL) was achieved when the sample was precipitated with absolute acetone with a solvent: material ratio of 1: 2 (v/w) at 3ºC within two hours. The conditions of storage, purification, and drying methods of product should be studied in the future studies.

5. Conclusions

Pineapple core is one of the sources for production of bromelain inoculants. TPrC and BE activity are influenced by multiple factors during enzyme extraction by by using organic solvents. The high values of TPrC (278.5 µg/mL) and BE activity (0.0103 UI/mL) was achieved when the sample was precipitated with absolute acetone with a solvent: material ratio of 1:2 (v/w) at 3ºC within two hours. The conditions of storage, purification, and drying methods of product should be studied in the future studies.

6. Conflicts of interest

The authors declare no conflict of interest

7. Formatting of funding sources

This research was funded by Nong Lam University, Thu Duc District, Ho Chi Minh City, Vietnam.

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