

Stability Test of Partially Purified Bromelain from Pineapple (*Ananas comosus* (L.) Merr) Core Extract in Artificial Stomach Fluid

S Setiasih¹, A Adimas Ch. D.¹, V Dzikria¹, S Hudiyo¹

¹Departement of Chemistry, FMIPA Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia

E-mail: setiasih@ui.ac.id

Abstract. This study aimed to isolate and purify bromelain from pineapple core (*Ananas comosus* (L.) Merr) accompanied by a stability test of its enzyme activity in artificial gastric juice. Purification steps start with fractionation by a precipitation method were carried out stepwise using several concentration of ammonium sulfate salt, followed by dialysis process and ion exchange chromatography on DEAE-cellulose column. Each step of purification produced an increasing specific activity in enzyme fraction, starting with crude extract, respectively: 0.276 U/mg; 14.591 U/mg; and 16.05 U/mg. Bromelain fraction with the highest level of purity was obtained in 50-80% ammonium sulphate fraction after dialyzed in the amount of 58.15 times compared to the crude extract. Further purification of the enzyme by DEAE-cellulose column produced bromelain which had a purity level 160-fold compared to crude enzyme. The result of bromelain stability test in artificial stomach juice by milk clotting units assay bromelain fraction have proteolytic activity in clotting milk substrate. Exposing bromelain fraction in artificial stomach juice which gave the highest core bromelain proteolytic activity was achieved at estimated volume of 0.4-0.5 mL. Exposure in a period of reaction time to artificial stomach juice that contained pepsin showed relatively stable proteolytic activity in the first 4 hours.

1. Introduction

Bromelain is a protein found in the pineapple plants (*Ananas comosus*) of the family Bromeliaceae belonging to the group of protease enzymes. Bromelain is a protease that can hydrolyze proteins. The presence of proteolytic activity in bromelain is beneficial in the therapeutic field [1].

Proteases are the most well-known among industrial enzymes. The most well-known plant proteases are papain from *Carica papaya*, ficin from *Ficus spp.* and bromelain from pineapple (*Ananas comosus*). Bromelain is one of the protease enzyme found in pineapple [2]. Core bromelain is widely used in industri and medication. Based on literature, bromelain in digestive system can be absorbed without being degraded and without losing its activity [1]. In this research, core part of pineapple was used as source of bromelain. The parameters tested included proteolytic activity and specific activity after fractionation of bromelain using ammonium sulphate followed by dialysis and fractionation with cellulose DEAE column chromatography. After the purification steps were done, the proteolytic activity of the obtained bromelain fraction was tested based on the milk-clotting unit assay after bromelain was incubated in a medium of artificial gastric fluid.



2. Materials and Methods

Instruments used were UV-Vis spectrophotometer, shaker, buchner, refrigerated centrifuge, vortex, ice box, cuvette, pH meter, micropipette and tip, column, test vials, glass equipment (beaker glass etc), waterbath, thermometer, refrigerator, and blender.

Material and chemicals used were pineapple (core part) from Bogor, pepsin, phosphate buffer pH 7.0, Tris-HCl buffer (0.05 M, pH 8.0), casein 1%, HCl, NaOH, NaCl, TCA (trichloroacetate) 5%, BSA standard solution, Follin reagent 1 N, Lowy reagent, ice cubes, aquadest, aquademin, glacial acetic acid, anhydrous sodium phosphate, L-cystein, EDTA, aluminium foil, muslin, ringstand, and filtering paper.

2.1 Sample and Crude Extract Preparation.

Core part of the pineapple were separated, weighed, diced and juiced using blender while kept at 4°C. Then, it was filtered through muslin and centrifuged. Crude extract was stored in refrigerator when not in use. The next steps are testing the protein content and proteolytic activity of the crude extract and core pineapple juice.

2.2 Crude Extract Purification.

Cold filtrate was fractionated using ammonium sulfate at different concentration (0-20%; 20-50%; 50-80%). Protein precipitated produced (in form of pellets) by fractionation were separated through centrifuge, and resuspended in cold phosphate buffer solution pH 7.0. Each fraction, including residual fraction, were tested of its protein content and proteolytic activity [3]. Ammonium sulfate fraction of bromelain with highest proteolytic activity were treated with dialysis to remove the effect of salt on bromelain's purity.

2.3 Purification by Exchange Chromatography on DEAE Cellulose Column

After dialysis, bromelain was purified further with ion exchange chromatography using DEAE-cellulose as a stationary phase. The sample was eluted with Tris-HCl buffer (pH 8.0; 0.05M) containing NaCl with concentration –rise gradually from 0.25 – 1.0 M. Gradient elution at a flow rate of 1.5 mL/min.

2.4 Bromelain Proteolytic Activity.

Proteolytic activity of each bromelain fractions were determined using Kunitz method, using casein as substrate [4,5].

2.5 Bromelain Specific Activity.

Enzyme specific activity was achieved by dividing proteolytic activity with protein content. Protein content was determined using Lowry method.

2.6 Artificial Stomach Juice Preparation.

Artificial stomach fluid was prepared by dissolving 2.0 g of NaCl and 3.2 g of purified pepsin (derived from porcine stomach mucosa, with an activity of 800 to 2500 units per mg of protein) in 7.0 mL of hydrochloric acid and water up to 1000 mL [6].

2.7 Stability Test of Bromelain in Artificial Stomach Fluid.

Bromelain fraction with highest activity were tested its stability in artificial stomach juice. Substrate used was buffered milk substrate based milk clotting units assay [7].

3. Results and Discussions

3.1 Crude Extract and Results of Fractionation with Ammonium Sulfate

From the pineapple core sample, core juice gave specific activity of 0.006 U/mg protein, while crude extract gave specific activity of 0.276 U/mg. Table 1 show the highest specific activity in bromelain fraction can be found in 3rd fraction (50-80%), which is 14.591 U/mg with degree of purity 52.8 fold in comparison with crude extract, and yield of 0.97%.

Table 1 Results of Bromelain Activity Evaluation at Steps of Isolation and Fractionation using Ammonium Sulfate

Fraksi	V	Total		SA	SA	DoP	Y (%)
		PA	PC				
Core Juice	409,6	0,267	42,9	1,272	0,006	-	100
Crude Extract	359	6,85	24,74	7,318	0,276	46	57,67
F 0-20%	14,2	0,2	1,44	0,985	0,138	0,5	5,82
F 20-50%	17	1,9	1,42	11,323	1,331	4,8	5,73
F 50-80%	7,1	3,6	0,24	51,873	14,591	52,8	0,97
Residual Fraction	256	0,116	8,04	0,014	0,014	0,05	32,49

V –Volume (mL); PC –Protein Content (mg); PA –Proteolytic Activity (Unit); SA –Specific Activity (Unit/mg); DoP –Degree of Purity; Y(%) –Yield (%)

The result of fractionations is not fully purified, because the protein precipitated by ammonium sulfate is not only bromelain protein. Enzyme fraction with highest specific activity need to purified further, and to achieve higher degree of purity the fraction will be treated with dialysis.

3.2 Result of Dialyzed Fraction

After treating the ammonium sulfate fraction with dialysis, specific activity of the bromelain fraction is increased from 14.591 U/mg to 16.05 U/mg, with degree of purity of 58.15 fold in comparison with crude extract and yield of 0.6%. Specific activity of bromelain fraction before and after dialysis is shown at Table 2.

Table 2 Result of Dialyzed Bromelain Fraction

Fraksi 3	V	Total		SA	SA	DoP	Y (%)
		PA	PC				
Before dialysis	5	3,6	0,24	51,873	14,591	52,8	0,97
After dialysis	7	2,5	0,15	55,555	16,05	58,15	0,60

V –Volume (mL); PC –Protein Content (mg); PA –Proteolytic Activity (Unit); SA –Specific Activity (Unit/mg); DoP –Degree of Purity; Y(%) –Yield (%)

3.3 Result of Ion Exchange Chromatography

After treating the dialysis fraction with Ion exchange chromatography, specific activity of the bromelain fraction is increased from 55.55 U/mg to 157.61 U/mg, with degree of purity of 160-fold in comparison with crude extract. The ion exchange chromatogram is shown at Figure 1.

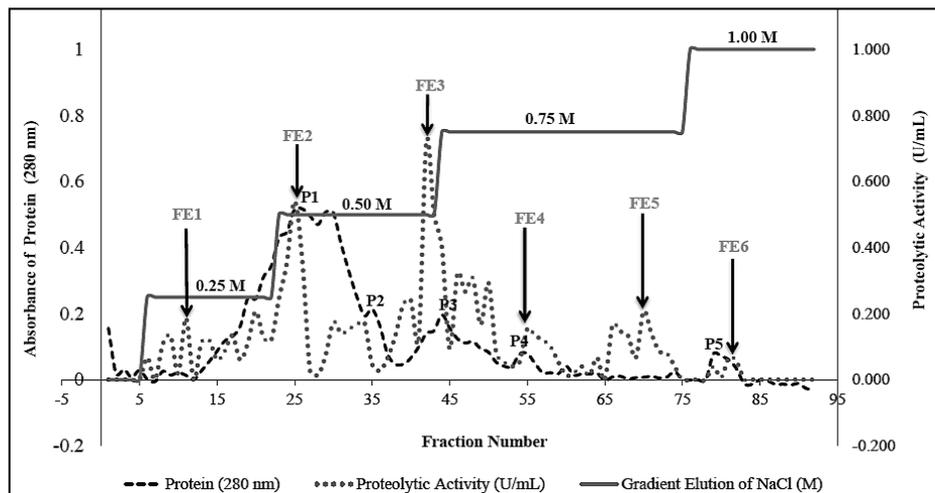


Figure 1. Chromatogram for 50-80% Ammonium Sulfat Fraction Using DEAE-CelluloseMatrix. Pr: The Peak of Protein at 280 nm, FE: The Peak of Protein with Proteolytic Activity

Separation conditions: column size 1.6 x 20 cm, matrix 135 mL volume flow rate was set at 1.5 mL/min and fraction were collected every 5 mL. The elution sycore using a combined method of linear gradient and step wise elution. The initial stage of the column was eluted with Tris-HCl buffer 0.05M pH 8.0; subsequent elution using a Tris-HCl buffer 0.05M pH 8.0 containing 0.25 M to 1.0 M NaCl.

In this process, the enzyme solution were diluted that the proteolytic activity was reduced, but the protein content was also reduced, resulting in an increase of specific activity. Bromelain fractions achieved at each step of purification gave an increasing factor of purity, as shown in Figure 2.

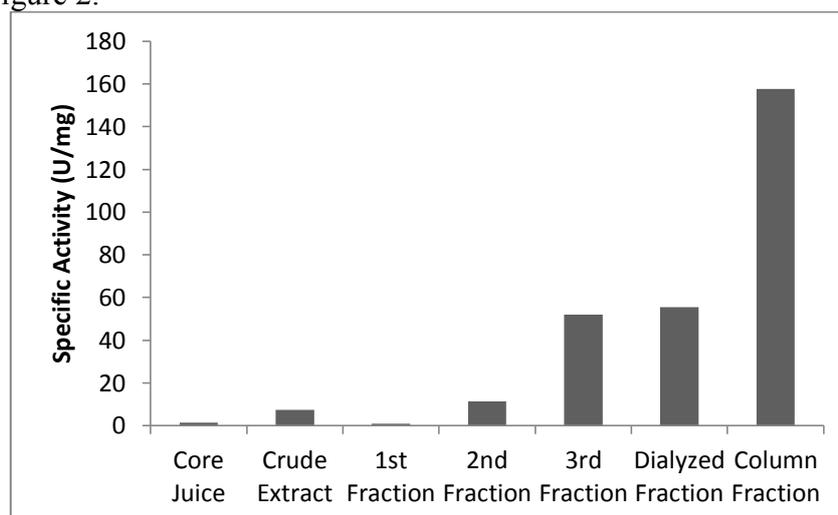


Figure 2. Specific activity of bromelain fraction with each purification step.

3.4. Results of Bromelain Stability Test in Artificial Stomach Fluid.

In this research, proteolytic activity of bromelain was tested in vitro in artificial stomach fluid. Bromelain will be 4hydrolyzin by stomach acid and other protease after consumption through oral pathway [6]. Simulated gastric fluid test solution is an artificial dissolution medium used to represent stomach acid. Stomach state simulated by this solution is fasted state. After the determined time for reaction with arificial stomach fluid has passed, the bromelain activity was

tested using milk clotting unit assay. The assay was conducted using milk substrate that has been equilibrated to 40°C, where enzymatic activity in clotting milk substrate is at maximum capacity [7]. Due to artificial stomach fluid containing other protease (pepsin) which is capable of hydrolyzing milk protein, in this research a negative control containing only buffer is used. The stability test is conducted in three steps: (1) stability test of different bromelain fractions, (2) stability test of a bromelain fraction at different concentration, and (3) stability test of bromelain fraction at prolonged reaction time.

First, each bromelain fraction's stability was tested as a comparison, in this case the crude extract, 3rd ammonium sulfate fraction, and the dialyzed fraction. As a further comparison to the purified bromelain fraction, commercial bromelain's stability was also tested. Result of the test is shown in Figure 3.

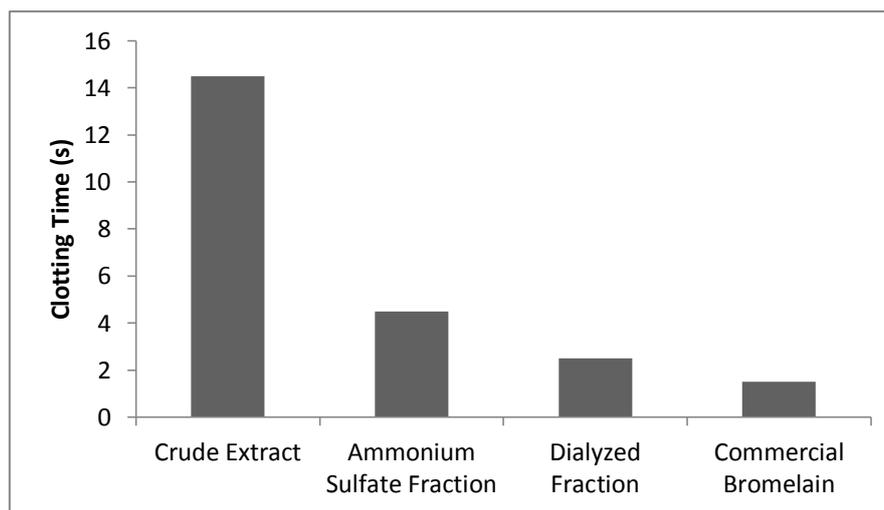


Figure 3. Clotting time of milk substrate by bromelain fractions after reaction with artificial stomach fluid.

Based on the clotting time of each sample, the bromelain fractions showed an existing clotting activity, with an increase of activity based on reduced clotting time needed by purer (dialyzed) fraction. The activity of dialyzed fraction is still relatively low when compared with commercial bromelain, which had higher degree of purity.

Second, a bromelain fraction's stability was tested at different concentration. The fraction tested was the dialyzed fraction. Result of this test is shown in Figure 4.

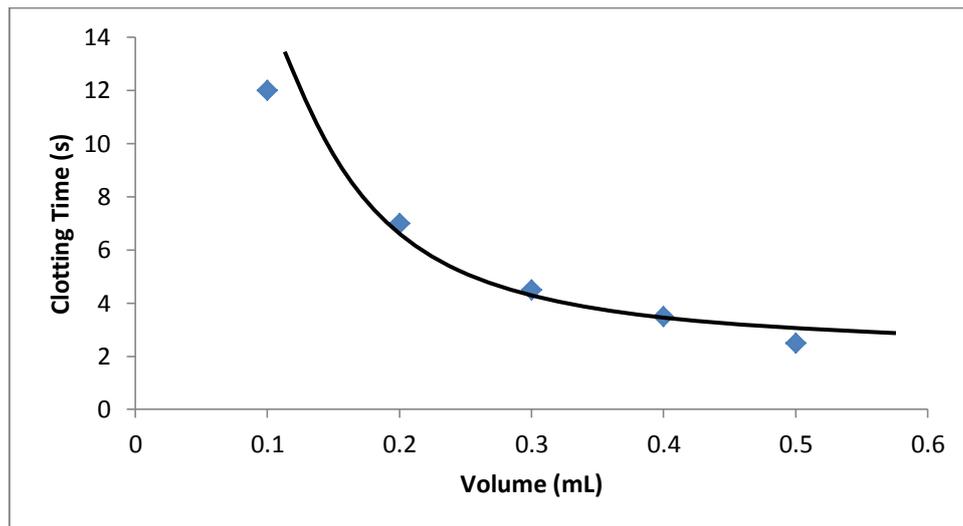


Figure 4. Milk clotting time by bromelain fraction at different concentration.

The time needed by higher volume of bromelain fraction to clot milk substrate is reduced. Higher bromelain concentration is capable of coagulating casein in milk at faster rate than lower concentration [2]. At increased concentration, as the volume approach 0.4 mL, the increase in proteolytic activity became insignificant.

Third, a bromelain fraction's stability was tested at a prolonged reaction time with artificial stomach juice. The volume of dialyzed fraction used was 0.5 mL. Result of this test is shown in Figure 5.

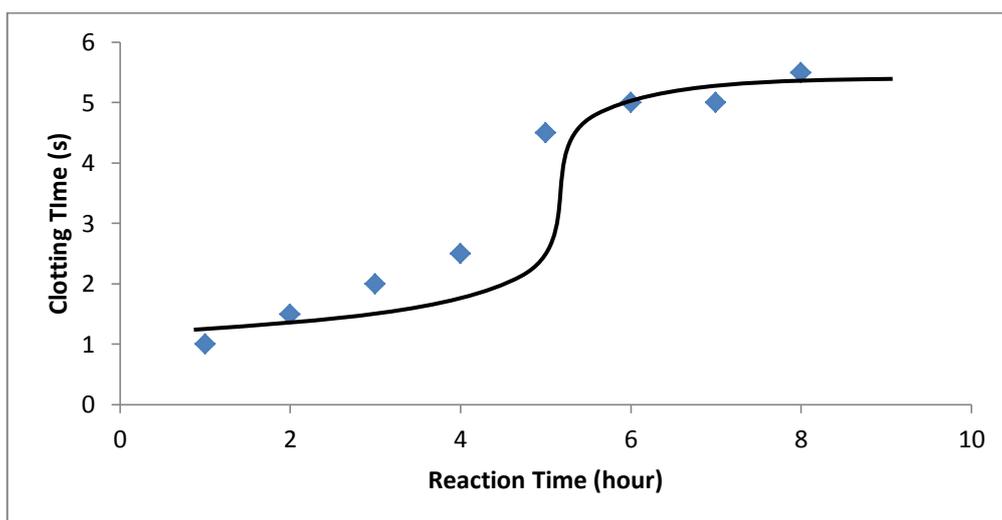


Figure 5. Stability of bromelain fraction in artificial stomach fluid after 8 hours of reaction.

Bromelain proteolytic activity at the first 4 hours is relatively stable, although slowly decrease as the reaction time goes on. After 4th reaction hour, the activity was reduced significantly. The reduced activity was due to deactivation and degradation of bromelain by gastric enzyme (pepsin) and low pH of stomach acid [2]. At 5th hour and onward, the reduction of bromelain activity became insignificant.

4. Conclusions

Bromelain was successfully purified through a series of purification steps, starting from isolation of crude extract, fractionation using ammonium sulfate, and dialysis. Further step of purification yielded purer bromelain fraction, as shown by ion exchange chromatography fraction gave higher specific activity. Bromelain activity in artificial stomach fluid showed that at higher degree of purity, bromelain fraction gave higher proteolytic activity. Bromelain also showed higher activity when higher concentration of bromelain fraction reacted with artificial stomach fluid, and relatively stable for a period of time. However, prolonged reaction time with artificial stomach fluids showed that bromelain would have significantly reduced activity, giving an insight on a relation between digestive system and bromelain (pineapple). For future study of bromelain stability in artificial stomach fluids, it's suggested to add another purification step to achieve bromelain fraction with higher degree of purity, like gel filtration column chromatography. It's also suggested to prepare another artificial stomach juice simulating stomach on fed state.

Acknowledgement

This research was funded by Biaya Operasional Perguruan Tinggi Negeri (BOPTN) DIKTI 2016.

References

- [1] Bala M, Ismail N A, Mel M, Jami M S, Salleh M and Amid A 2012 Bromelain Production : Current Trends and Perspective. *Archives Des Sciences* **65** (11) 369–399
- [2] Neta JLV, DaSilva LA, Lima AA, Santana JC, Leite NS, Ruzene DS, SilvaDP, DeSouza R R 2012 Bromelain Enzyme from Pineapple: In Vitro Activity Study under Different Micropropagation Conditions. *Appl Biochem Biotechnol* **168** (2) 234-246
- [3] Setiasih S, Ilyas N M, Handayani S and Hudiyono S 2016 Proceeding of International Seminar: Collaborative Seminar of Chemistry and Industry (COSCI), Surabaya, 50-54
- [4] Febriani K, Wahyuni I, Setiasih S and Hudiyono 2017 AIP Conference Proceedings 1862, 030095; doi: 10.1063/1.4991199
- [5] Setiasih S, Wahyuni I and Hudiyono S 2016 Proceeding of International Conference on Biosciences & Medical Engineering (ICBME)s UTM-Johor Malaysia 163-170
- [6] Shiew P S, Fang Y L, Adibah F and Majid A 2010 in Vitro Study of Bromelain Activity in Artificial Stomach Juice and Blood October
- [7] Dash P, Maharana L and Ghosh G 2015 Proteolytic and Milk Clotting Activity of Fractionated Protein of *Lagenaria siceraria*. *Journal of Applied Pharmaceutical Science* **5** (12) 086–091