Determination of the effect of EDTA and PCMB on purified bromelain activity from pineapple core and in vitro antiplatelet activity

W A Hidayani, S Setiasih and S Hudiyono

Department of Chemistry, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia

Corresponding author’s e-mail: sumi.hudiyono@sci.ui.ac.id

Abstract. Bromelain is an enzyme belongs to the cysteine protease. In this study, bromelain isolated from pineapple core (Ananascomosus [L.] Merr) was purified by fractionation using ammonium sulfate followed by dialysis and then ion exchange chromatography. The fraction of bromelain obtained from each purification step showed an increase in specific activity. The highest specific activity of protease was found in 20-50% ammonium sulfate fraction of 104.018 U/mg with a purity level 3.2-fold compared to crude extract. Further purification by ion exchange chromatography using DEAE-Cellulose, the fraction of bromelain showed an increase in specific activity to 278.33 U/mg with a purity level 8.8-fold compared to crude extract. The determination of kinetics parameter of purified bromelain using Lineweaver-Burk plot gives Km value of 0.15 % (w/v) and Vmax of 0.056 U/min. This bromelain can be strongly inhibited by EDTA and PCMB. The addition of EDTA and PCMB at a concentration of 0.5 mM can decrease the activity of the enzyme up to 88.50% by showing the competitive and mixed-inhibition types of inhibition, respectively. The antiplatelet activity of the bromelain fraction was tested in-vitro based on the Born method, by using plasma (PRP), acetosal as a positive control and ADP as an aggregator. The purified bromelain showed the ability of an antiplatelet agent with percentage of aggregation 29.51% and percentage of inhibition 68.91%.

Keywords: Pineapple core, bromelain, inhibitor, enzyme kinetics, antiplatelet

1. Introduction
Bromelain is a group of cysteine proteases whose catalytic function is to hydrolyse the peptide bonds of proteins [1]. This enzyme is commonly found in various tissues of pineapple plants (Ananas comosus [L.] Merr) and the highest content is in the pineapple core. Bromelain which is an endopeptidase enzyme has a sulfhydryl group (-SH) on its active side that can be inhibited by oxidizing compounds, alkyllators, and heavy metals. In 2012, Marshall and Golden characterized bromelain from Morindacitrifolia (Noni) [2] and observed the non-competitive inhibition of bromelain by HgCl2 [3]. Protease group is inhibited by PCMB (p-Chloromercuriobenzoate), PMSF (Phenylmethylsulphonyl fluoride), EDTA (Ethylenediaminetetraacetic acid), benzamidine, and pepstatin A [4]. Bromelain has been shown to be antiplateletagent [5]. Bromelain will work on the parts that have a high platelet aggregation activity in stroke and infark miokard and it can reduce platelet sensitivity in ADP (Adenosine 5’ diphosphate) induction aggregation on in-vitro test [6].

2. Materials and methods

2.1. Materials
The pineapples were purchased from Pasar Induk Kramat Jati. Ammonium sulfate (Merck), BaCl₂ (Merck), NaCl (Merck), EDTA (Merck), PCMB (Sigma), DEAE Cellulose (Pharmacia Inc.), Platelet Rich Plasma (PRP) was obtained from PMI DKI Jakarta, ADP (Sigma), and a cetosal (Sigma).

2.2. Fractionation with ammonium sulfate
The crude enzyme was slowly added by ammonium sulfate (0-20%, 20-50%, 50-80%) with a constant stirring. It was stored overnight in the refrigerator [7]. Then, the enzyme was tested for the protein content by Lowry method and proteolytic activity test with Kunitz method [8].

2.3. Dialysis
The fraction enzyme was put into a cellophane bag that immersed in 0.05 M phosphate buffer pH 7.0. The buffer was changed every two hours and tested with BaCl₂ 5% and 0.05 M HCl.

2.4. Ion exchange chromatography
The DEAE Cellulose column was eluted with 0.05 M tris-HCl buffer pH 8.0. Then, the subsequent elution with 0.05 M tris-HCl buffer pH 8.0 containing NaCl solution with variation concentration of 0.25 M; 0.50 M; 0.75 M; and 1.0 M [9].

2.5. Kinetics studies
Kinetic parameters such as Km and Vmax were calculated using Lineweaver Burk Plot by plotting 1/V with 1/[S]. Bromelain activity was assayed in the absence and presence of EDTA and PCMB (0.1; 0.3; and 0.5) mM with variation of substrate concentrations (0.25; 0.5; 0.75; 1.0; 1.25; 1.5 %).

2.6. In vitro antiplatelet activity test
The antiplatelet activity test of the enzyme bromelain fraction was carried out by Born method. Calculation of antiplatelet action can be seen from the inhibition percentage of platelet aggregation and percentage of aggregation that occurs [6].

3. Results and discussion

3.1. Bromelain purification from crude extract
Crude enzyme from pineapple core has a specific activity of 31.523 U/mg proteins. Purification by fractionation with ammonium sulfate is based on the principle of salting out. Ammonium sulfate can separate the protein in solution by precipitation without denaturing the protein. Fraction 2 has the highest specific activity than fraction 1 and 3, that is 104.018 U/mg protein with purity level of 3.2-fold to the crude enzyme extract. It means the ammonium sulfate concentration 20-50% can precipitate the bromelain protein at the best.

The next stage to purify enzyme fraction was the dialysis. The principle of dialysis is diffusion process that occurs in small molecules through semi-permeable membrane. The specific activity is 125.365 U/mg protein with purity level of 3.9-fold to the crude enzyme extract.

Bromelain of this fraction has a negative charge and bonded first with positively charged resin and eluted when the salt concentration is high.

3.2. Kinetics studies

3.2.1. Determination of bromelain kinetics parameters. Kinetic parameters (Km and Vmax) are obtained by transforming the Michaelis-Menten equation to the Lineweaver Burk Plot by mapping the function 1/V to 1/S. Based on the Lineweaver Burk plot, the linear equation is y=2.792x+18.057 with slope value of 0.990 was obtained, so the Km and Vmax values were 0.154 % (w/v) and 0.056 U/min.
3.2.2. The Effect of EDTA and PCMB on bromelain activity. Proteolytic activity of bromelain in the presence of EDTA in various concentrations obtained the Km value which increased with addition of EDTA (figure 1). Km with the absence and presence of EDTA was increased to 0.763 % (w/v) at 0.154 % (w/v). However, Vmax remained unchanged of 0.055 U/min implying a competitive type of inhibition for bromelain by EDTA. EDTA can decrease the activity of the bromelain up to 82.29%.

The presence of EDTA on the fraction of bromelain causes the metal ions on the enzyme’s active side is chelated by EDTA, so the structure of enzyme is changed and proteolytic activity is decreased. Based on the mechanism of EDTA inhibiting this protease, it can be said that EDTA is a metalloprotease inhibitor.

Proteolytic activity of bromelain in the presence of PCMB in various concentrations obtained the Km value, which increased with addition of PCMB (figure 2). Km with the absence and presence of PCMB was increased from 0.154 % (w/v) to 0.349 % (w/v). However, Vmax was reduced to 0.045 U/min from 0.056 U/min, implying mix-inhibition type. PCMB can decrease the activity of the bromelain up to 88.50 %.

The effects of bromelain inhibitor PCMB were also analyzed. In this case, bromelain contains thiol group (-SH), which reacts with Hg\(^{2+}\) ions on PCMB. Hg\(^{2+}\) ions will bind to the enzyme-substrate complex to form the enzyme-substrate-Hg complex, so that the bromelain will be inhibited. Thus, the purified bromelain is a cysteine protease group because it can be inhibited by PCMB, which is cysteine inhibitor.

3.3. Antiplatelet activity test in vitro

Antiplatelet activity was assayed by observing percentage of platelet aggregation and inhibition. The percentage of platelet aggregation is the effect of a compound on the process of platelet clot formation in the blood, while the percentage of platelet inhibition is the ability of a compound to inhibit the
aggregation process. If the aggregation percentage was low then the inhibition percentage was high, it shows that the compound was more active as well as the ability of these compounds as antiplatelet agents. The more pure fraction of bromelain has a better ability to inhibit platelet aggregation. The highest antiplatelet activity was found in bromelain from DEAE Cellulose column chromatography (AP4) with aggregation percentage of 29.51 % and inhibition percentage of 68.91 %.

4. Conclusions
Bromelain was successfully purified through several of purification stages, such as extraction of crude enzyme, fractionation with ammonium sulfate, dialysis, and ion exchange chromatography. Bromelain purified by ion exchange chromatography DEAE Cellulose gave the highest specific activity of 278.333 U/mg protein with purity level of 8.8-fold to the crude enzyme extract. The determination of kinetics parameter of purified bromelin using Lineweaver-Burk plot gives Km value of 0.154 % (w/v) and Vmax of 0.056 U/min. Bromelain fraction was inhibited by EDTA and PCMB with inhibition type of competitive and mix-inhibition, respectively. The addition of EDTA and PCMB can decrease the activity of bromelain up to 82 %. The antiplatelet activity test of the purified bromelain showed its ability as an antiplatelet agent. The highest antiplatelet activity was found in bromelain fraction DEAE Cellulose (AP4) with percentage of aggregation 29.51% and percentage of inhibition 68.91 %.

Acknowledgements
This research was funded by Hibah Publikasi Internasional Terindeks untuk Tugas Akhir Mahasiswa Universitas Indonesia (PITTA) 2018 on behalf of Dra. Siswati Setiasih, Apt., M.Si.

References
[1] Borrelli F et al. 2011 *Neurogastroenterol Motil.* 23 745–e331
[2] Golden K D and Smith-Marshall J 2012 *Journal of Scientific Research (JSR)* 4 445–56
[3] Kaur T, Kaur A and Grewal R K 2015 *J. Food Sci. Technol.* 52 5954–60
[4] Walsh G 2002 *Protein Biochemistry and Biotechnology* (New York: John Wiley and Sons)
[5] Manzoor Z, Nawaz A, Mukhtar H and Haq I 2016 *Brazilian Arch. Biol. Technol.* 59 1–16
[6] Moriyama H, Hosoe T, Wakana D, Itabashi T, Kawai K, Iizuka T, Hoshi K, Fukushima K and Lau F C 2009 *Journal of Health Science* 55 103–8
[7] Gautam S S, Mishra S K, Dash V, Goyal A K and Rath G 2010 *Thai J. Pharm. Sci. (TJPS)* 34 67–76
[8] Musfiroh F F, Setiasih S, Handayani S, Hudiyono S and Ilyas N M 2018 *IOP Conf. Ser.: Mater. Sci. Eng.* 299 012017
[9] Setiasih S, Adimas A C D, Dzikria V and Hudiyono S 2018 *IOP Conf. Ser.: Mater. Sci. Eng.* 299 012016