Inhibition Study of EDTA and PCMB on Purified Bromelain Activity from Pineapple Core [*Ananas comosus* (L.) Merr.] Using Ion Exchange Chromatography Column and Antiplatelet Activity Test

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Keywords: Pineapple core, bromelain, purification, chromatography, kinetic study, antiplatelet.

Abstract: The aim of this research was to isolate and purify bromelain from pineapple core (*Ananas comosus*) through fractionation using ammonium sulfate followed by dialysis and ion exchange chromatography. Fractionation of bromelain with ammonium sulfate produces highest specific activity on fraction 2 (20-50%) of 260.042 U/mg with purity level 2.548 fold compared to crude extract. Further purification by ion exchange chromatography DEAE-sepharose and CM-Sephadex C-50 showed an increase in specific activity, sequentially 500 U/mg with purity level 4.901 fold compared to crude extract and 729.167 U/mg with purity level 7.150 fold compared to crude extract. The determination of kinetics parameter of purified bromelain using LM plot gives Km and Vmax value for casein and azocasein substrate of 0.94% (w/v); 0.07 U/min and 0.87% (w/v); 0.05 U/min respectively. Bromelain fraction was inhibited competitively with EDTA and mix-inhibition type was observed in the presence of PCMB. The addition of EDTA and PCMB at a concentration of 0.5 ppm can decrease the activity of the enzyme up to 70%. *In vitro* study of antiplatelet agent activity using human Platelet Rich Plasma (PRP) revealed that the purified bromelain show activity as an antiplatelet agent with percentage of aggregation 20.892% and percentage of inhibition 77.994%.

1 INTRODUCTION

The fifth largest pineapple producer in the world is Indonesia (Rugayah, 2012). Bromelain is a group of cysteine proteases found in all plant tissues of pineapple [*Ananas comosus* (L.) Merr.] (Babagana, 2016). This enzyme break down a protein by breaking a peptide bond and produce a more simple peptide. Bromelain can be found in all parts of pineapple plants. Stem bromelain (EC 3.4.22.32) is a glycoprotein with isoelectric pH 9.5 and Fruit Bromelain (E.C. 3.4.22.33) lacks carbohydrate moiety with isoelectric pH 4.6 (Kaur, 2015).

This enzyme is used in many therapeutic applications and was known has antithrombic effect as antiplatelet agent that useful in cardiovascular diseases treatment (Bhattacharya, 2008). The benefits of bromelain is quite extensive in the field of pharmacology and the food industry, stimulate many researchers to learn more. Bromelain research in pineapple has been started for a long time until now. Various methods of isolation and purification have been done to get the best bromelain activity from pineapple (Neta, et al, 2012).

Various heavy metals such as mercury, cobalt, and zinc can inhibit bromelain enzyme. In 2012, Marshall and Golden characterized bromelain from *Morinda citrifolia* (Noni) and observed that inhibition of bromelain by HgCl2 is the non-competitive inhibition type. (Kaur, 2015). Protease group is inhibited by PCMB (p-Chloromercuriobenzoate), PMSF (Phenylmethylsulphonyl fluoride), EDTA (Ethylenediaminetetraacetic acid), benzamidine, and pepstatin A (Walsh, 2002). Several factors that can influence enzyme kinetics are the enzymes source and conditions such as pH or temperature environment (Kaur, 2015).

In this research the crude bromelain was extracted from pineapple core and purified through several stages. We also study the kinetics behavior in the presence of inhibitor and antiplatelet activity test by *in vitro* method.

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2 EXPERIMENTAL

2.1 Enzyme Extract from Pineapple Core

The pineapples that were used in this research were obtained from Palembang, Kramat Jati Market. Crude enzyme was prepared by making pineapple core puree using 0.2 M phosphate buffer, pH 7.0 at 4°C and then filtered. The filtrate had been centrifuged at 6000 rpm for 20 minutes at 4°C. The supernatant obtained from each sample was crude bromelain extract. The crude bromelain was stored in a refrigerator to be used as a source of enzyme.

2.2 Fractionation of Enzyme with Ammonium Sulfate

Fractionation of crude bromelain was done by using ammonium sulfate at different range of concentration (0-20%, 20-50%, 50-80%). About 150 ml of crude enzyme extract was put in a beaker glass which was placed in a salt ice bath. The crude enzyme was slowly added by ammonium sulfate (0-20%) with constant stirring using a magnetic stirrer. After the addition of salt was completed, the stirring process had been continued for 20 minutes. The solution was then allowed to settle overnight in the refrigerator. The solution was centrifuged (6000 rpm) for 20 minutes at 4^{o}C and the precipitate was dissolved in 0.2 M phosphate buffer, pH 7.0.

2.3 Dialysis

The enzyme solution was put into a cellophane bag. The cellophane bag was then immersed in a solution of 0.05 M phosphate buffer, pH 7.0. The dialysis was taken place at 4°C using ice salt bath by constant stirring. The buffer was changed for every 2 hours.

2.4 Protein and Enzyme Activity Assay

Lowry method is used to determine the protein concentration. The enzymatic activity assay was perfomed by Kunitz method using casein as substrate at 37°C for 30 minutes. The enzyme was inactivated by adding 3 ml of 5% TCA. The solution then had been incubated in ice water bath for 30 minutes.

2.5 Kinetics Studies

2.5.1 Determination of Kinetics Parameter

Kinetic parameters (Km: Michaelis-Menten constant and Vmax: maximum velocity) were determined from Lineweaver Burk Plot between the enzyme activity at optimum pH and temperature.

2.5.2 The Effect of EDTA and PCMB on Bromelain Activity

The absence and presence of EDTA (0,1; 0,3 and 0,5 mM) and PCMB (0,1; 0,3 and 0,5 mM) were used to assay the bromelain activity. Km and Vmax were calculated using Lineweaver Burk plot.

2.6 *In vitro* Antiplatelet Activity Test

The antiplatelet activity test of the enzyme bromelain fraction was carried by Born method. The absorbance of the sample solution was measured before and after the addition of aggregators. Aggregators that used was ADP (Adenosine Diphosphate 5'). Samples that tested were crude enzymes and enzyme fractions obtained from each stage of purification with the highest specific activity. Aspirin was used as a positive control and distilled water is used as a negative control.

In the sample tubes containing enzyme sample (70 ml), was added PRP (Platelet Rich Plasma) (560 ml) and saline water and then shaken with a vortex (low speed). The solution was incubated for 2 min at 37 °C. The absorbance of the solution was measured by a spectrometer at a wavelength of 600 nm. Once measured, the solution was added by 70 ml of ADP and incubated for 10 min at 37 °C, subsequently re-measured the absorbance. For the control solution is done the same way, but the sample solution is replaced with distilled water (negative control) and asetosal 1 mg / ml (positive control). Calculation of antiplatelet action can be seen from the percentage inhibition of platelet aggregation and presentation of aggregation that occurs. Calculation of percentage inhibition were also performed to determine how large the inhibition of platelet aggregation in each solution (Moriyama, 2009).

3 RESULTS AND DISCUSSION

3.1 Purification of Bromelain from Crude Extract by Ammonium Sulfate Precipitation

Crude bromelain extract from pineapple core has been purified by fractionation using ammonium sulfate, followed by dialysis. Crude enzyme obtained from pineapple core have a specific activity of 102.018 U/mg protein. The next stage to purify crude enzyme was the fractionation method using ammonium sulfate with different of range concentrations produced enzyme fractions that have different proteolytic activity and protein content (Table 1). Fraction 2 of pineapple core has higher proteolytic activity than fraction 1 and 3.

Table 1: Purification of Crude Enzyme by using Ammonium Sulfate

Fraction	Volume (mL)	Total		Specific Activity (U/mg)	Purity Level (Fold)
		Activity (U)	Protein (mg)		
Fraction 1 (0-20%)	7.1	122.356	0.818	149.465	1.465
Fraction 2 (20-50%)	11	223.116	0.858	260.042	2.548
Fraction 3 (50-80%)	4	32.4	0.532	60.902	0.596
Leftover	186	306.9	8.556	35.869	0.351

Table 2 shows that the specific activity before and after dialysis. The fraction 2 from the pineapple core after dialysis has the highest specific activity. This is because in dialysis some proteins that has molecular weight less than bromelain can pass through the cellophane membrane. The specific activity of bromelain from pineapple core is 381.287 U/mg protein with a purity level of 3.737 fold to the crude enzyme extract.

Table 2: The Result of Dialysis on Bromelain Enzyme

Fraction	Volume (mL)	Total		Specific Activity (U/mg)	Purity Level (Fold)
		Activity (U)	Protein (mg)		
Fraction 2 Before Dialysis	8	223.116	0.858	260.042	2.548
Fraction 2 After Dialysis	11	208.450	0.546	381.287	3.737

3.2 Bromelain Fractionation with Ion Exchange Chromatography Columns

Ion exchange chromatography column is an advanced stage to purified bromelain. In this research, the reversible interaction between charged molecules and a resin which has the opposite charge of the protein molecules will be affected by the eluent through the column. This method is called stepwise gradient elution (gradient method and elution rise). If the ion charge of protein molecules was same with resin's ion charge, then the molecules weren't bound and eluted out first. The molecules that have different ion charge interacted with the resin in different strengths, then it required eluent that has a gradually concentration to release protein molecules bound from the resin. The resin that used as the stationary phase in the column is a DEAE (diethylaminoethyl) Sepharose (anion exchange resin). Here are the values of proteolytic activity against fraction number can be mapped in the form of chromatograms and had 5 peak proteins (Figure 1).

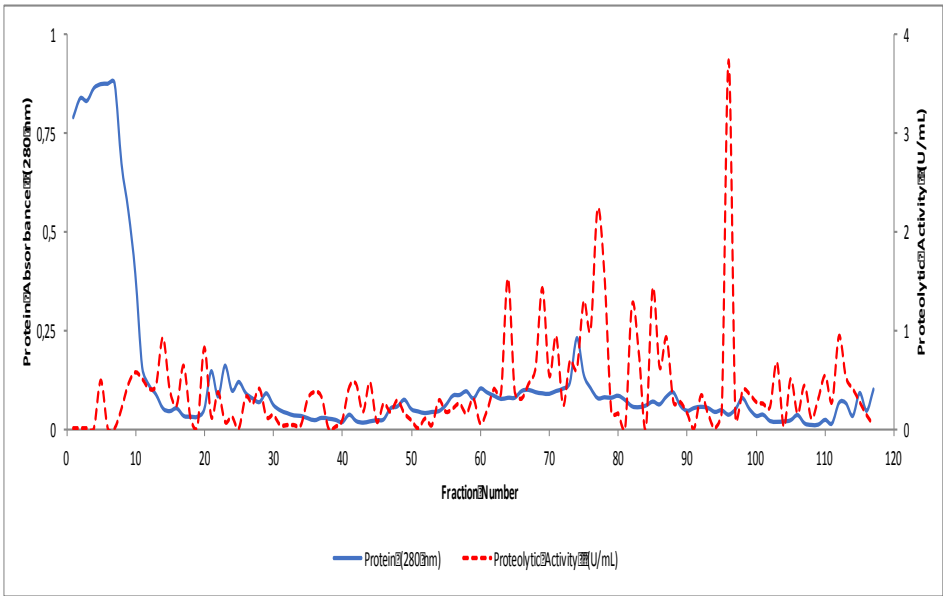

Figure 1: Anion exchange chromatography DEAE – Sepharose Chromatogram. SP: protein absorption peak (280 nm); AP: proteolytic activity peak.

The value of proteolytic activity, protein content and specific activity at this stage of purification by ion-exchange chromatography column in Table 3.

Table 3: Results of Purification Phase by Ion Exchange Column Chromatography with DEAE-Sepharose

Fraction	Volume (mL)	Total		Specific Activity (U/mg)	Purity Level (Fold)
		Activity (U)	Protein (mg)		
AP1 (13-20)	40	13.320	0.612	21.764	0.213
AP2 (36-55)	100	28.300	0.110	257.272	2.521
AP3 (59-80)	110	143	0.286	500	4.901
AP4 (82-89)	40	44	0.272	161.764	1.585
AP5 (92-115)	70	56	0.224	250	2.450

Further purification, the bromelain fraction was purified by ion exchange chromatography CM (carboxymethyl)-Sephadex C-50 (cation exchange resin). Here are the values of proteolytic activity against fraction number can be mapped in the form of chromatograms and had 4 peak proteins (Figure 2).

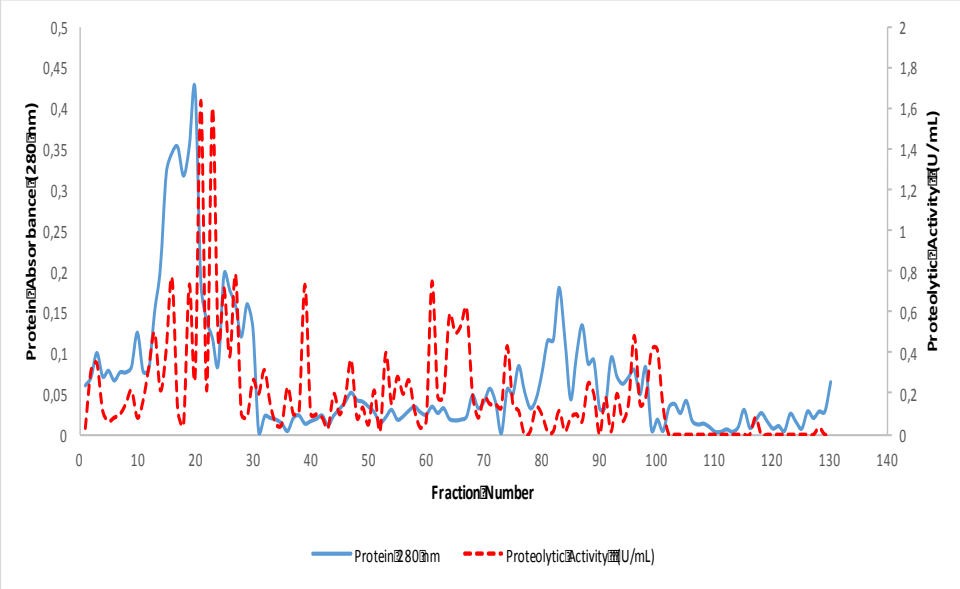

Figure 2: Cation exchange chromatography CM–Sephadex C-50 Chromatogram. SP: protein absorption peak (280 nm); AP: proteolytic activity peak.

The value of proteolytic activity, protein content and specific activity at this stage of purification by ion-exchange chromatography column in Table 4.

Table 4: Results of Purification Phase by Ion Exchange Column Chromatography with CM–Sephadex C-50

Fraction	Volume (mL)	Total		Specific Activity (U/mg)	Purity Level (Fold)
		Activity (U)	Protein (mg)		
AP1 (16-27)	60	35	0.048	729.167	7.150
AP2 (32-47)	80	10.667	0.240	44.444	0.435
AP3 (53-67)	75	7.500	0.450	16.67	0.163
AP4 (88-100)	65	1.083	0.104	10.417	0.102

3.3 Kinetics Studies

3.3.1 Determination of Kinetics Parameter

Kinetic parameters (Km and Vmax) were determined from Lineweaver Burk Plot between the enzyme activity at optimum pH and temperature in Figure 3 and 4.

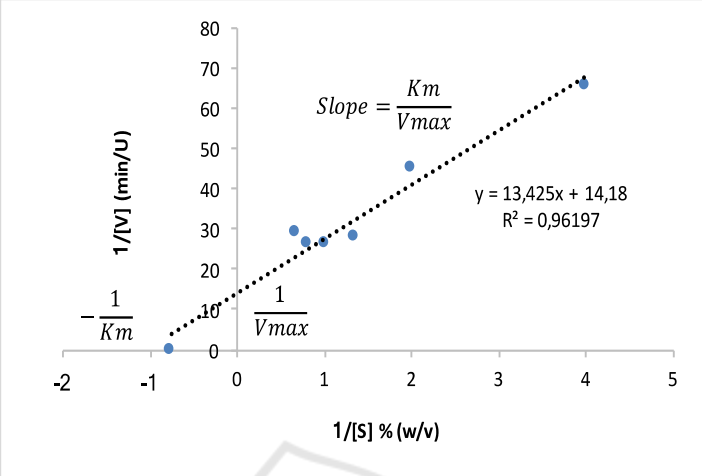

Figure 3: Lineweaver Burk Plot for the Hydrolysis of Casein by Purified Bromelain.

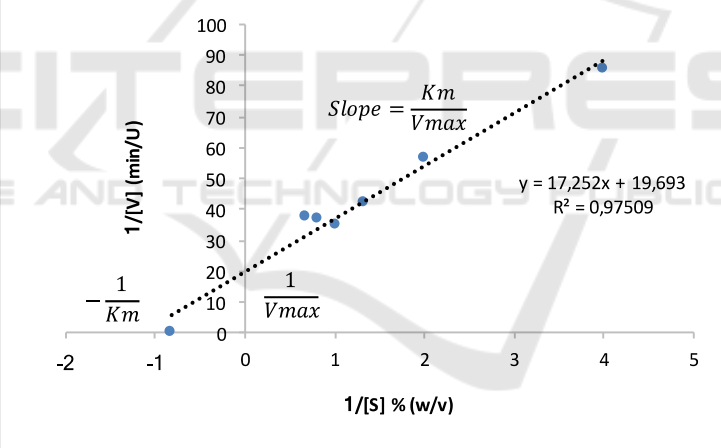

Figure 4: Lineweaver Burk Plot for the Hydrolysis of Azocasein by Purified Bromelain.

The value of Kinetics Parameter and Proteolytic Activity Bromelain on Casein and Azocasein in Table 5.

Table 5: Kinetics Parameter and Proteolytic Activity Bromelain on Casein and Azocasein.

Substrate	Proteolytic Activity (U/mL)	Km (%)	Vmax (U/min)
Casein	1.133	0.94	0.07
Azocasein	0.866	0.87	0.05

Based on these data, the value of Km fraction of bromelain on casein and azocasein is relatively same and low. A low Km bromelain on casein and azocasein indicate that the Enzyme-Substrate

complex is good with a high affinity between bromelain to both substrates. Several factors that can influence enzyme kinetics are the enzymes source and conditions such as pH or temperature environment (Kaur, 2015).

3.3.2 The Effect of EDTA and PCMB on Bromelain Activity

The absence and presence of EDTA (0,1; 0,3 and 0,5 mM) and PCMB (0,1; 0,3 and 0,5 mM) were used to assay the bromelain activity. Lineweaver Burk plot in the presence of EDTA and PCMB were used to calculate Km and Vmax (Figure 5 and 6). Bromelain fraction was inhibited competitively with EDTA and mix-inhibition type was observed in the presence of PCMB.

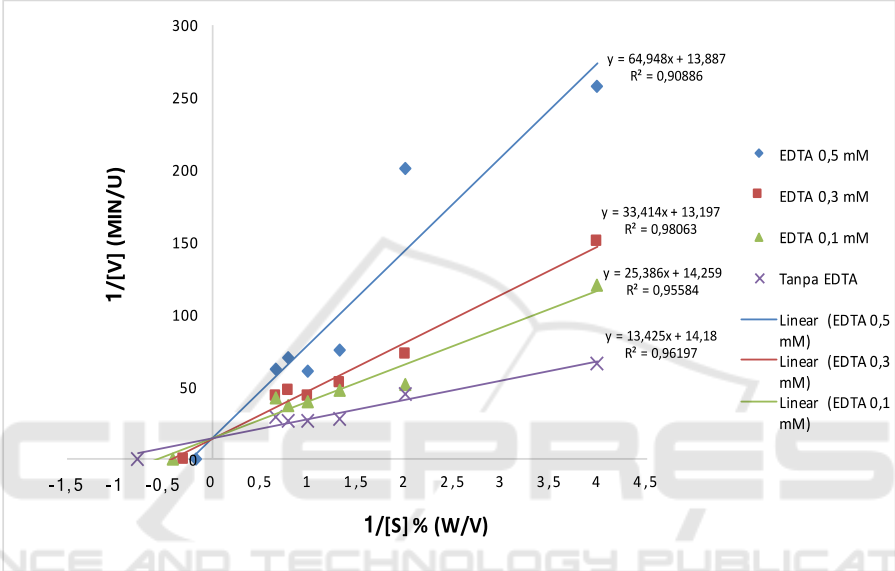

Figure 5: Lineweaver Burk Plot for the Hydrolysis of Casein in the Presence of EDTA (0,1; 0,3; 0,5 mM).

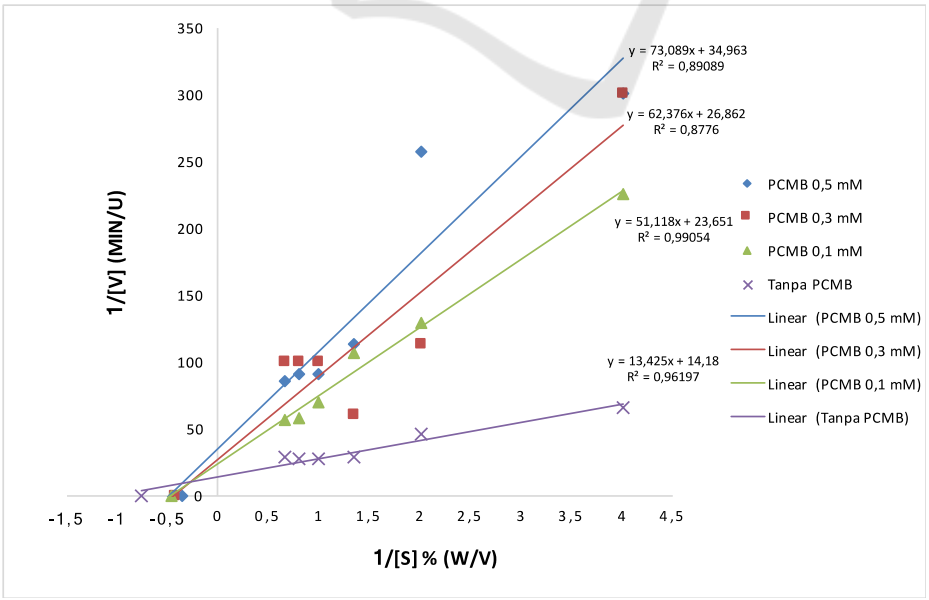

Figure 6: Lineweaver Burk Plot for the Hydrolysis of Casein in the Presence of PCMB (0,1; 0,3; 0,5 mM).

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The value of Kinetics Parameter and Inhibition Percentage Bromelain by EDTA and PCMB in Table 6.

Table 6: Kinetics Parameter and Inhibition Percentage Bromelain by EDTA and PCMB

[I] mM	Inhibitor (I)					
	EDTA			PCMB		
	Km (%)	Vmax (U/min)	%Inhibition	Km (%)	Vmax (U/min)	%Inhibition
0	0.940	0.070	-	0.940	0.070	-
0.1	1.780	0.070	33.824	2.000	0.040	22.059
0.3	2.532	0.075	39.706	2.322	0.030	61.765
0.5	4.676	0.072	55.882	2.900	0.020	70.588

The addition of EDTA and PCMB at a concentration of 0.5 ppm can decrease the activity of the enzyme up to 70%. Bromelain belongs to the cysteine protease containing thiol groups. PCMB is an organic compound that reacts through a mercury-sulfur affinity with a sulfhydryl group in peptides, proteins and other molecules. PCMB interacts and reacts with thiol groups in bromelain and therefore PCMB is an inhibitor that can inhibit the reaction between bromelain and casein substrate. The interaction between cysteine protease (bromelain) and PCMB is highly dependent on the reactivity of the thiol (-SH) group.

3.4 Antiplatelet Activity Test *In Vitro*

Antiplatelet agent activity test (in vitro) was used to observe the value of the platelet aggregation percentage and inhibition of platelet aggregation percentage. Platelet aggregation (thrombosis) is the ability of platelets to form a clot in the blood. The percentage of platelet aggregation showed the effect from a compound on the process of platelet clot formation, while the percentage of inhibition of platelet aggregation showed the ability from a compound to inhibit the aggregation process. If the inhibition percentage was high, it shows that the compound was more active as well as the ability of these compounds as antiplatelet agents. An in vitro study using Platelet Rich Plasma (PRP) with turbidimetric method (Sathyapriya, 2012). The platelet inhibition percentage by bromelain fraction shown in Figure 7 and 8.

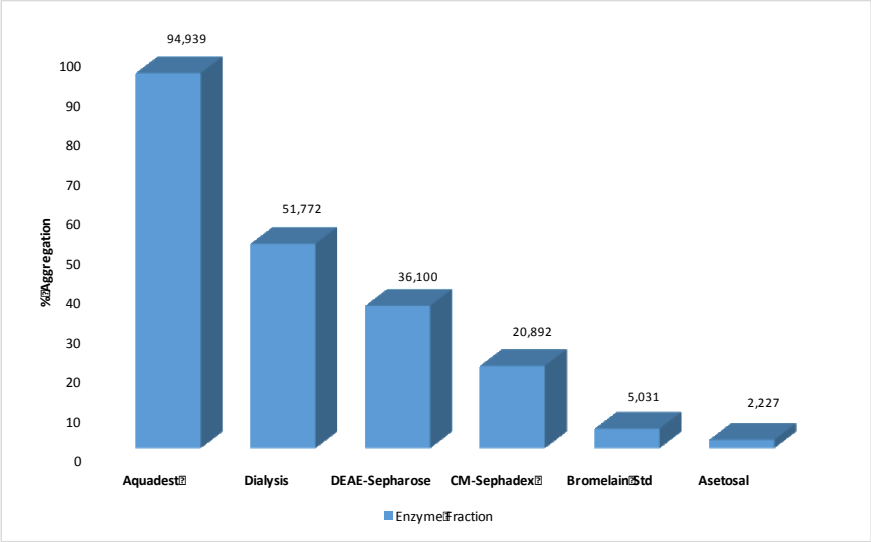

Figure 7: Percentage of platelet inhibition by bromelain fraction (Aggregation Percentage)

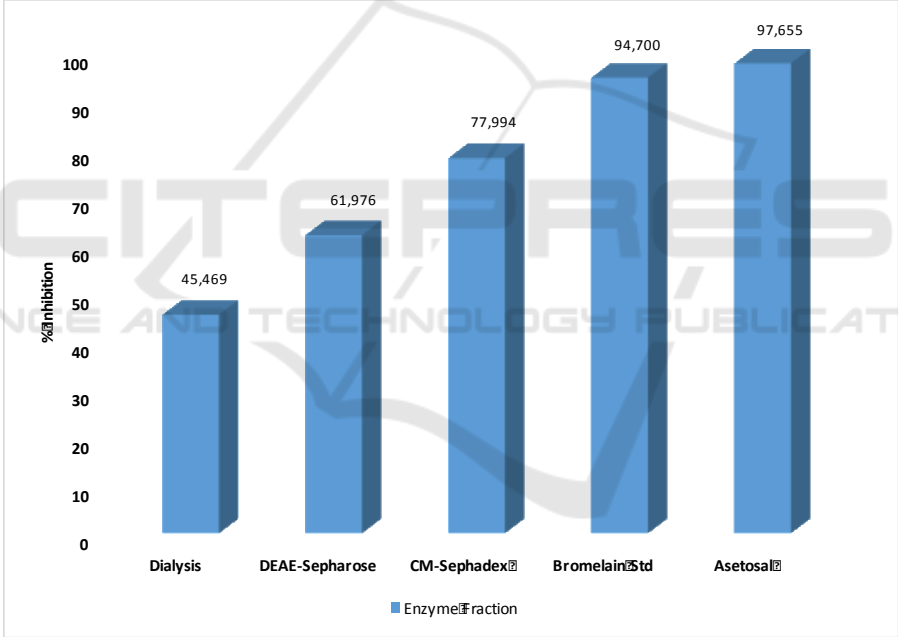

Figure 8: Percentage of platelet inhibition by bromelain fraction (Inhibition Percentage)

4 CONCLUSIONS

Bromelain was successfully purified through several purification methods. The result of bromelain after purified by ion chromatography exchange CM-Sephadex C-50 gave the highest specific activity. Bromelain fraction was inhibited competitively with EDTA and mix-inhibition type was observed in the presence of PCMB. The addition of EDTA and PCMB at a concentration of 0.5 ppm can decrease the activity of the enzyme up to 70%. All samples with the highest specific activity of each stage of purification has the ability as an antiplatelet agent. The highest value of antiplatelet activity was from the purest enzyme fraction that purify using ion-exchange chromatography column with percentage of aggregation 20.892% and percentage of inhibition 77.994%.

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