

IN VITRO ANTI-PLATELET AGGREGATION ACTIVITY OF HYDROXYPROPYL CELLULOSE-CYSTEAMINE BASED NANOPARTICLES CONTAINING *CRUDE BROMELAIN*

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ABSTRACT

Objective: The aim of the present study was to formulate bromelain into nanoparticles in order to improve its stability and activity.

Methods: *Crude bromelain* was prepared by protein precipitation from the pineapple stem juice using ammonium sulphate at the concentration of 60% (w/v). Nanoparticles containing *crude bromelain* were generated using the ionic gelation method with hydroxypropyl cellulose-cysteamine (HPC-cysteamine) conjugate as a matrix. *Crude bromelain* was then added to the HPC-cysteamine solution for ionic interaction to construct the nanoparticles, which were then analyzed for their particle size and zeta potential. The resulting nanoparticles were mixed with adenosine diphosphate (ADP) to perform anti-platelet aggregation.

Results: The nanoparticle had 928.3 nm in particle size and -7.25 mV in zeta potential. Anti-platelet activity of *crude bromelain* and the nanoparticles were determined with modification of light transmission aggregometry (LTA), in which ADP was used to induce an aggregation while a spectrophotometer UV-Vis was used to measure the absorbance at the wavelength of 600 nm. The result showed that *crude bromelain* and the nanoparticles rendered percentage inhibition of $8.00 \pm 1.17\%$ and $48.56 \pm 11.19\%$, respectively.

Conclusion: Based on the result of a one-way analysis of variance (ANOVA), it was concluded that there was a significant difference in percentage inhibition between the two samples. The nanoparticles demonstrated a better anti-platelet aggregation activity compared to *crude bromelain*.

Keywords: Crude bromelain, Anti-platelet aggregation, HPC-cysteamine, Nanoparticles

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INTRODUCTION

Cardiovascular disease (CD) is caused by impaired heart function and blood vessels, one of which is coronary heart disease (CHD). Heart attacks occur when the damage is experienced by the heart muscle (myocardium) due to reduced blood supply to the heart muscle. A sudden drop in blood supply to the heart can occur when one of the coronary arteries is blocked due to blood clots and platelet aggregation. Many studies report that blockage of the brain and heart arteries often results from platelet hyperactivity that can increase platelet aggregation [1, 2].

In most cases, antiplatelets can cause aggregation inhibition. One of which is aspirin, but there have been some reports of the resistance to the use of aspirin as an antiplatelet. Aspirin, which belongs to the group of NSAIDs has side effects of irritation and even bleeding in the stomach [3-5]. One of the natural products found in Indonesia is pineapple (*Ananas comosus* (L.) Merr.) that contains vitamin A, vitamin C, beta-carotene, calcium, phosphorus, magnesium, iron, sodium, potassium and proteins in the form of bromelain enzymes. The health benefits of bromelain are to facilitate digestion, accelerate wound healing, treat burns and prevent tumor. The fiber content can facilitate defecation in patients with constipation [6].

Bromelain enzymes are mostly found in ripe pineapples compared to in immature green pineapples [7]. Pineapple stems are often thrown away because of the unpleasant taste, but they have the highest content of bromelain enzymes compared to the other parts [8]. Some types of pineapple which are widely available in Indonesia are Palembang, Bogor, and Subang pineapple. In a study conducted in 2010, the levels and protein activity of Palembang, Bogor, and Subang pineapple were compared to each other. The highest bromelain content with the highest activity was found in Palembang pineapple stems [9]. It was, therefore, the aim of this research to study the anti-platelet aggregation of *crude bromelain* entrapped within the nanoparticles based on HPC-cysteamine to reach the optimum activity and stability of bromelain in the biological system.

MATERIALS AND METHODS

Materials

Palembang pineapple stem (*Ananas comosus* (L.) Merr.) (the voucher specimen number is 2/DR/HLF/UP and the authentic number given by Research Center for Biology-LIPI is 2618/IPH.1.01/11.07/XII/2016), hydroxypropyl cellulose (HPC), cysteamine, HCl (Sigma Aldrich), MES (2-(N-Morpholino) ethanesulfonic acid hydrate) (Sigma Aldrich), ammonium sulphate (Emsure), sodium periodate (Sigma Aldrich), sodium cyanoborohidride (Sigma Aldrich), Bradford reagent (Sigma Aldrich), adenosine diphosphate (ADP) (Helena Laboratories), sodium alginate (Sigma Aldrich), bovine serum albumin (BSA) and NaCl (Ecosol).

Methods

Synthesis of HPC-cysteamine

1.5 g of HPC was dissolved in 20 ml of 96% ethanol and 120 ml of distilled water in erlenmeyer using a magnetic stirrer. The erlenmeyer was wrapped with aluminum foil and added to 800 mg of sodium periodate. The mixture was stirred for 3 h and added with 200 μ l of ethylene glycol to stop the reaction. The final mixture was purified in a dialysis tube for 3 d, and demineralized water for dialysis was replaced every 12 h. 1 g of oxidized HPC was dissolved in 40 ml aqua dest, 1 g MES buffer and 0.7 g cysteamine. HCl was added. pH value of the solution was adjusted to pH 5 and distilled water was added up to 50 ml. The mixture was stirred for 3 h and 4 g of sodium cyanoborohydride was added and a permanent stirring was continued for 3 d. Afterward, the final mixture was purified with a dialysis tube for 3 d [10].

Preparation of pineapple juice and *crude bromelain*

Small pieces of pineapple stem were added with 0.1 M phosphate buffer pH 7 and milled using a blender. The suspension was filtrated and centrifuged at 3500 rpm for 15 min. The final filtrate was collected and added with a saturated ammonium sulphate solution to a final concentration of 60% (w/v) and stored overnight in the

refrigerator to precipitate *crude bromelain*. The centrifugation at 2500 rpm for 20 min was carried out to obtain the precipitate, which was then dissolved in 0.1 M phosphate buffer pH 7. The solution was purified by a dialysis process in distilled water for 24 h.

Determination of total protein content

A standard protein solution was prepared by dissolving 0.2 g BSA in 100 ml to obtain the stock solution in the concentration of 2000 ppm. The solution was diluted to prepare a series of standard protein solutions with concentrations of 100, 200, 400, 600, 800, 1000, and 1200 ppm. Afterward, 0.1 ml of each standard solution was added to 3.0 ml Bradford reagent, homogenized with a vortex, and incubated at room temperature for 45 min. The resulting solutions were measured using a UV-Vis spectrophotometer at a wavelength of 595 nm. 1 g of samples were dissolved in 10 ml of 0.1 M phosphate buffer to reach a final concentration of 100 000 ppm. A sample solution was treated in the same manner as the standard solutions to assay the total protein content [11].

Preparation of nanoparticles containing *crude bromelain* based on HPC-cysteamine

The stock solution of HPC-cysteamine 2% (w/v) was diluted with distilled water to obtain a concentration of 0.2% (w/v). 50 ml 0.2% of the solution was stirred with a magnetic stirrer. Afterwards, 20 ml *crude bromelain* solution was added dropwise. The formed nanosuspension was dried in a freeze dryer. Determination of particle size and polydispersion index was carried out by diluting the nanoparticles into distilled water with a ratio of 1:15 (v/v) at a temperature of 25 °C using particle size analyzer. The zeta potential test was performed by a zeta potential analyzer at a temperature of 25 °C. The nanoparticles were diluted using distilled water before analysis and the conductivity of the solution was increased up to 50 µS/cm using a sodium chloride solution. The morphology of nanoparticles was performed using a transmission electron microscope (TEM).

In vitro anti-platelet aggregation activity of the nanoparticles

1 ml of platelet-rich plasma (PRP) was added to 4 ml of 0.9% NaCl (tube 1). 1 ml PRP was added to 1 ml of *crude bromelain* solution which was equivalent to 10 µg/ml bromelain (5000 ppm) and 3 ml of 0.9% NaCl (tube 2). 1 ml PRP was added to 1 ml of the nanoparticles suspension, which was equivalent to 10 µg/ml bromelain (6415 ppm) and 3 ml of 0.9% (w/v) NaCl (tube 3). 1 ml of platelet-poor plasma (PPP) was added to 1 ml of 2% (w/v) HPC-cysteamine which was equivalent to the amount of HPC-cysteamine in tube 3 (2475 ml) and 1525 ml of 0.9% (w/v) NaCl (tube 4). 1 ml PPP was added to 4 ml 0.9% NaCl (tube 5). Tube 1, 2, 3, 4, and 5 were incubated at 37 °C for 30 min, then 20 µl 10 µM ADP was added, then incubated for 20 min at 37 °C. Tube 1 served as a negative control and tube 5 as a blank for tube 1 and 2, while tube 4 as a blank for tube 3. The absorbance of the solution in tubes 1, 2 and 3 were measured by UV-Vis spectrophotometer at a wavelength

of 600 nm, then the percentage of anti-platelet aggregation activity was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Abs PRP} - \text{Abs (PRP+sample)}}{\text{Abs PRP}} \times 100\% \quad (1)$$

where Abs is absorbance [12, 13].

RESULTS AND DISCUSSION

Synthesis of HPC-cysteamine

After the oxidation, the generated aldehyde could be further oxidized to a carboxylic acid group; thus, ethylene glycol was added to end the reaction. The low viscosity was examined to presume the presence of the aldehyde. The conjugation cysteamine to the aldehyde was stabilized by sodium cyanoborohydride (NaCNBH₃) as a reducing agent because of the stability in relatively strong acidic conditions (~ pH 3) and different selectivity at different pHs. In this study, the reaction was in the acid condition to immobilize a high amount of free thiol groups [14]. Based on the previous studies, the conjugate displayed the amount of free thiol groups of 1063.03±64.27 µmol/g using Ellman's reagent [15]. At pH 8 the reaction did not occur at all. By using the same procedure, the amount of free thiol groups of chitosan-mercaptopethylamine was 1878±70 µmol per gram of polymer [16]. Thereby, the procedure demonstrated a high coupling rate for both HPC and chitosan.

Preparation of pineapple juice and *crude bromelain*

Bromelain in the small cut of pineapple stem was stabilized in 0.1 M phosphate buffer pH 7 at a temperature of 4 °C to preserve its activity. The optimum pH of the bromelain enzymes was maintained to avoid the change in groups with ions in the active site of bromelain. The precipitation of bromelain with a saturated solution of ammonium sulphate was based on the process of salting-out. In order to take out bromelain from the stem juice, the choice of buffer systems used was crucial. The components in the stem should be compatible with those in the buffer to keep the biological activity of bromelain. Therefore, ion concentration and ionic strength in the buffer played an important role. Since bromelain has a molecular weight of 33.5 kDa, bromelain was trapped in the membrane tube while phosphate buffer and ammonium sulphate were displaced from it [17-19].

Determination of total protein content

The absorbance for the standard curve was obtained after the reaction based on the Bradford method. The resulting absorbance can be seen in table 1. The reaction caused the red form of the dye Coomassie Brilliant Blue G-250 was converted into its blue form under acidic conditions. The *crude bromelain* obtained after freeze-drying was rather moist, which indicated the water content was quite high whereas its protein content was very small (0.2% w/w). The dye can bind to the amino acid residues in the protein present in the sample. In addition, hydrophobic and ionic interaction can play an important role in the reaction. They stabilize the reaction to display a visible color change which can be spectrophotometrically measured [20, 21].

Table 1: Absorbance of BSA

BSA concentration (ppm)	Absorbance
100	0.1258
200	0.1788
400	0.3746
500	0.4297
700	0.5608
800	0.5927

Preparation of nanoparticles containing *crude bromelain* based on HPC-cysteamine

In most cases of the synthesis methods of polymeric nanoparticles, a crosslinker is acquired. The buffer containing negatively charges of phosphate ions could act as a crosslinker. The buffer was originated from *crude bromelain* solution and was still present after the freeze-drying.

The formation of nanoparticles occurred when the turbidity was visually observed without any sedimentation. The turbidity

describes the number of nanoparticles, while the sedimentation shows the unstability of nanoparticles. The mixture of HPC-cysteamine and *crude bromelain* was already turbid; thus, there was no need to add a crosslinker to generate the nanoparticles [15].

The nanoparticles was a translucent liquid that demonstrated the presence of colloid system. Since in the preparation of *crude bromelain*, phosphate buffer had been included, the resulting *crude bromelain* contained phosphate ions which have negatively charged

ions like those in sodium tripolyphosphate. Hence, they could act as a crosslinker in the preparation of the nanoparticles.

Particle size and zeta potential evaluation are necessary to determine the characterization and to predict the stability of nanoparticles. The nanoparticles demonstrated that their particles size and zeta potential were 928.3 ± 41.40 nm and -7.25 ± 3.95 mV, respectively. The measurement of particle size of nanoparticles was divided into 3 types, which were based on number, intensity, and volume. Measurement-based on quantities is intended to group and counts the number of particles based on the differences in size. Measurement-based on volume is aimed to investigate the volume occupied by each group with different particle sizes. Measurement-based on intensity is performed to collect the light refracted by each group with different particle sizes.

The presence of crude bromelain, which has a large molecular weight in the nanoparticles caused the particles size were large and almost micro scale. In addition, the particle size could be interfered by the presence of phosphate buffer in crude bromelain, which is not a polyanion. Molecules that have polyanions such as sodium tripolyphosphate could form the smaller size of nanoparticles because one molecule of which has many anions thus has a greater opportunity to associate with more amine groups in HPC-cysteamine as a cationic thiomers.

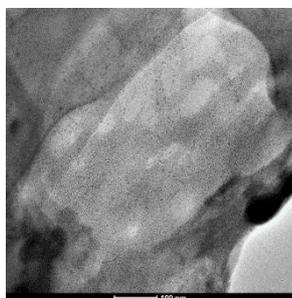


Fig. 1: Morphology of the nanoparticles using TEM

The small polydispersity index shows a narrow particle size distribution. The nanoparticles formed had a polydispersity index of 0.480. This shows that the nanoparticles had a uniform size. The uniformity is very important because it will affect the uniformity of the biological activity of the nanoparticles. The higher zeta potential will result in a greater repulsive force between nanoparticles, which means that the nanoparticles are more stable. The nanoparticles had a negatively charge of zeta potential because *crude bromelain* was attached by negatively charged phosphate ions, which were derived from the buffer used during the isolation.

In vitro anti-platelet aggregation activity of the nanoparticles

The study of anti-platelet aggregation activity used LTA (light transmission aggregometry) method with a slight modification. The method still refers to the principle of turbidimetry, and the tool used to measure the turbidity was UV-Vis spectrophotometer at a wavelength of 600 nm instead of an aggregometer. The blank solution for the test contained PPP, which was added to HPC-cysteamine as a thiomers. Blood plasma contains albumin, globulin, and fibrinogen. To obtain the plasma, a centrifugation technique was required at 3000 rpm for 15 min. Platelets could be present in the plasma components because of their small size. Hence, PPP served as a blank to compensate for the turbidity from plasma components. The anti-platelet aggregation activity of *crude bromelain* and the nanoparticles were described by percentage inhibition calculated from the decrease in the absorbance of negative controls, which showed a decrease in platelet aggregation. PRP was incubated with the samples and its aggregation was induced by ADP. The inhibition percentage of platelet aggregation produced by each sample against ADP can be seen in table 2 and 3.

In normal circumstances, platelet aggregation prevents bleeding in the body. However, aggregates can be formed even though there are no vascular injuries; such aggregates are called a pathological thrombus. The bromelain enzymes belongs to the sulfhydryl group which is able to hydrolyze proteins into amino acids and can reduce platelet sensitivity to ADP [22-24].

Table 2: Crude bromelain activity in inhibition of platelet aggregation

Sample	PRP*	PRP+Crude bromelain*	
	Absorbance	Absorbance	% Inhibition
I	0.4137	0.3815	7.78%
II	0.3628	0.3282	9.54%
III	0.3422	0.3193	6.69%
Average			$8.00 \pm 1.17\%$

Notes: *: Data represented as mean \pm SD (n=3) and PPP as a blank

Table 3: The nanoparticles activity in inhibition of platelet aggregation

Sample	PRP	PRP+The nanoparticles*	
	Absorbance	Absorbance	% Inhibition
I	0.2273	0.0895	60.62%
II	0.2140	0.1040	51.40%
III	0.2220	0.1473	33.65%
Average			48.56 ± 11.19

Notes: *: Data represented as mean \pm SD (n=3) and the mixture of PPP and the thiomers as a blank

The results showed that *crude bromelain* could inhibit platelet aggregation as the decrease in the absorbance of PRP occurred. Bromelain can inhibit platelet aggregation because it can cut off the fibrin bridge formed when platelets aggregate [4]. The nanoparticles could increase the anti-platelet aggregation activity of *crude bromelain* as the decrease in the absorbance of PRP incubated with the nanoparticles, was greater compared to those incubated with *crude bromelain*.

Polymeric nanoparticles with free thiol groups can improve the attachment of a loaded drug on the gastrointestinal mucus consisting of mucin glycoproteins through the formation of disulfide

bonds [10, 25]. In the case of the platelets, the sulfhydryl groups on the surface of nanoparticles support the attachment of the nanoparticles on the platelet glycoprotein receptors through the formation of disulfide bonds. Hence, ADP could not reach the receptors; thus, platelet activation was inhibited. In addition, bromelain could cut off fibrin bridges between aggregated platelets. Hence, the anti-platelet aggregation activity of the nanoparticles was greater than that of *crude bromelain*. The activity of bromelain within the nanoparticles was protected due to the presence of strong disulfide bonds. Moreover, the disulfide bonds could control the release of bromelain to prolong its activity [10, 26-29].

CONCLUSION

Crude bromelain could be entrapped within the nanoparticles, which was prepared by ionic gelation method. The nanoparticles were based on HPC-cysteamine as a thiomers, which has free thiol groups. The properties of the thiomers might influence the increase in the activity of anti-platelet aggregation of bromelain within the nanoparticles. Thereby, nanoparticles based on HPC-cysteamine could be a potential platform for therapeutic proteins delivery.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

The authors report no conflicts of interest

REFERENCES

- Anderson KM, Odell PM, Wilson PWF, Kannel WB. Cardiovascular disease risk profiles. *Am Heart J* 2006;121:2293-8.
- Putri RRRF, Ulfa EU, Riyanti R. Antiplatelet activity of red cabbage ethanolic extract (*Brassica oleracea* var. *capitata* L.). *Pustaka Kesehatan* 2013;2:111-4.
- Hankey GJ, Eikelboom JW. Aspirin resistance. *Lancet* 2006;367:606-17.
- Marmitt DJ, Bitencourt S, Silva ADCE, Goettert MI, Rempel C. Medicinal plants of RENISUS with analgesic activity. *Crit Rev* 2016;3:1-4.
- Thiagarajan P, Jankowski JA. Aspirin and NSAIDs; benefits and harms for the gut. *Best Prac Res Clin* 2012;26:199-206.
- Septiatin A. Fruits as medicinal plants. Bandung: CV Yrama Widya; 2009.
- Murniati E. Sweet, scaly pineapple on the tongue. Surabaya: Surabaya Intellectual Club; 2012.
- Heinicke RM, Gortner WA. Stem bromelain-a new protease preparation from pineapple plants. *Econ Bot* 1987;1:225-34.
- Mulyono M, Rosmeilia E, Moi GJP, Valentine BO, Suhartono MT. Quantity and quality of bromelain in some Indonesian pineapple fruits. *Int J Appl Bio-Pharm Tech* 2013;4:235-9.
- Rahmat D, Sakloetsakun D, Perera G, Schnürch AB. Design and synthesis of a novel cationic thiolated polymer. *Int J Pharm* 2011;411:10-7.
- Bradford M. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle dye binding. *Anal Biochem* 1976;72:248-54.
- Hall R, Mazer CD. Antiplatelet drugs: a review of their pharmacology and management in the perioperative period. *Anesh Analg* 2010;112:292-318.
- Sathyapriya E, Velpandian V, Anbu J, Anjana A. *In vitro* antiplatelet aggregation activity and thrombolytic activity of *Cheenalina chendhuram*. *Int J Life Sci* 2012;4:51-5.
- Ghoshal K, Bhattacharyya M. Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. *Sci World J* 2004;1-16. <https://doi.org/10.1155/2014/781857>
- Rahmat D, Rahman FA, Nurhidayati L, Laksmiawati DR. Synthesis and characterization of hydroxypropyl cellulose-cysteamine conjugate as a novel cationic thiomers with lipophilic properties. *Int J Appl Pharm* 2019;11:222-6.
- Muller C, Rahmat D, Sarti F, Leithner K, Schnürch AB. Immobilization of 2-mercaptoethylamine on oxidized chitosan: a substantially mucoadhesive and permeation enhancing polymer. *J Mater Chem* 2012;22:3899-908.
- Ketnawa S, Chaiwut P, Rawdkuen S. Extraction of bromelain from pineapple peels. *Food Sci Technol Int* 2011;17:395-402.
- Wu WC, Ng HS, Sun IM, Lan JCW. Single-step purification of bromelain from *Ananas comosus* pulp using a polymer/salt aqueous biphasic system. *J Taiwan Inst Chem E* 2017;79:158-62.
- Abreu DCA, Figueiredo KCDS. Bromelain separation and purification processes from pineapple extract. *Braz J Chem Eng* 2019;36:1029-39.
- Nouroozi RV, Noroozi MV, Ahmadiyadeh M. Determination of protein concentration using Bradford microplate protein quantification assay. *Int Electron J Med* 2015;4:11-7.
- Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem* 1996;236:302-8.
- Gautam SS, Mishra SK, Dash V, Goyal AK, Rath G. Comparative study of extraction, purification and estimation of bromelain from stem and fruit of the pineapple. *Plant Thai J Pharm Sci* 2010;34:67-76.
- Vellini M, Desideri D, Milanese A, Omini C, Daffonchio L, Hernandez A, et al. Possible involvement of eicosanoids in the pharmacological action of bromelain. *Arzneimittel Forschung* 1986;36:110-2.
- Menon A, Priya VV, Gayathri R. Preliminary phytochemical analysis and cytotoxicity potential of pineapple extract on oral cancer cell lines. *Asian J Pharm Clin Res* 2016;9:140-3.
- Hanif M, Zaman M, Qureshi S. Tiomers: a blessing to evaluating era of pharmaceuticals. *Int J Polym Sci* 2015;2015:1-9.
- Dai Q, Zhu X, Yu J, Karangwa E, Xia S, Zhang X, et al. Mechanism of formation and stabilization of nanoparticles produced by heating electrostatic complexes of WPI-dextran conjugate and chondroitin sulfate. *J Agric Food Chem* 2016;64:5539-48.
- Mishra R, Mir SR, Amin S. Polymeric nanoparticles for improved bioavailability of cilnidipine. *Int J Pharm Pharm Sci* 2017;9:120-39.
- Arafat M. Approaches to achieve an oral controlled release drug delivery system using polymers. *Int J Pharm Pharm Sci* 2015;7:16-21.
- Deore S, Shahi SR, Dabir P. Nanoparticle: as a targeted drug delivery system for depression. *Int J Curr Pharm Res* 2016;8:7-11.