

# In Vitro Dissolution Test of Bromelain Isolated from Pineapple Core Encapsulated in Hydrogel Semi-IPN Methyl Cellulose-Chitosan

Ahmad Aly Irfan<sup>1</sup>, Adinda Azkia<sup>1</sup>, Achmad Buhori<sup>1</sup>, Hegi Adi Prabowo<sup>1</sup>, Elmira Vania<sup>1</sup>, Nathasya Humaira Adriani<sup>1</sup>, and Siswati Setiasih<sup>1</sup>

<sup>1</sup>Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 1642, West Java, Indonesia

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**Abstract:** One of many sources of bromelain is pineapple core. However, in its application as an enzyme-based oral drug, its activity can be reduced due to interactions with gastric fluids. The isolated bromelain from the pineapple core is encapsulated with a hydrogel semi-IPN methyl cellulose-chitosan in order to control its release and to maintain its activity. Isolation of bromelain was conducted with several stages of fractionation with ammonium sulfate salt and dialysis. The bromelain in semi-IPN methyl cellulose-chitosan dissolution capability is evaluated in vitro at artificial gastric fluid pH and artificial intestine environment. The specific activity of bromelain obtained from several purification steps shows an increment. The crude enzymes, the ammonium sulfate fraction and the dialysis fractions have specific activity value of 22.39 U/mg, 76.73 U/mg, and 111.72 U/mg, respectively. Hydrogel semi-IPN methyl cellulose-chitosan was used for encapsulation which has the value of crosslinking degree of 46.63% and swelling ratio of 397.59%, respectively. Meanwhile, post loading encapsulation efficiency is 89.47%. The dissolution test results shows proteolytic activity of bromelain can be maintained up to 2.83 U/mL in artificial pH environment. The release rate of bromelain is relatively larger in artificial intestinal environment than artificial gastric fluid.

## 1 INTRODUCTION

Indonesia occupied third place in ASEAN as an exporting country of canned pineapple with an average contribution of 16.42% in 2009-2013 (Respati, 2016). Pineapple core is the solid waste from pineapple canning industrial process. Meanwhile, pineapple core is one of many source of bromelain that can be utilized in the medical application such as anti-platelet aggregation agents and other therapeutic effect (Musfiroh et al., 2018, Manzoor et al., 2016).

Bromelain is a protein-digesting enzyme, thus the structure can be destabilized due to the pH change. Destabilization of enzyme structure leads to decreasing proteolytic activity of bromelain. Based on studies with milk clotting assay method in artificial gastric fluid, proteolytic activity of bromelain was reduced slowly and relatively stable only in the first 4 hours (Setiasih et al., 2018). Castell et al. (1997) states that bromelain can be absorbed in the human intestine without losing its

biological activity. However, in the human digestive system, before reaching the intestine, oral consumption of bromelain passes through the stomach, which has very acidic environment. Therefore, bromelain needs to be encapsulated in order to obtain a controlled delivery release of the bromelain enzyme.

Encapsulation slows down the release of bromelain, stabilizes bromelain enzyme in gastric fluid and maintains the proteolytic activity until it reaches intestine environment. One of the materials used for encapsulation is semi-interpenetrating polymer network (semi-IPN) hydrogel. The dissolution of bromelain was performed in vitro on artificial gastric fluid (pH 1.2) for 2 hours and in an artificial intestinal environment (pH 7.4) in the next 10 hours (Farooq et al., 2017).

## 2 METHODS

### 2.1 Isolation, Ammonium Sulfate Precipitation and Dialysis of Bromelain

In the cold condition, pineapple core was crushed into juice using blender. The juice was filtered and centrifuged at  $\pm 4$  °C for 45 min with rotor spinning at 6000 rpm (rotation per minute), the supernatant obtained was called crude enzyme. Then, protein in the crude enzyme was precipitated using  $(\text{NH}_4)_2\text{SO}_4$  with interval concentration variation of 0-20%; 20-50%; and 50-80%. The mixture was centrifuged at  $\pm 4$  °C for 25 min and 6000 rpm. The precipitate obtained (pellet) from each fraction was dissolved in 0.20 M phosphate buffer pH 7 solution which had been cooled. Then, dialysis with cellophane sac in a 0.05 M phosphate buffer pH 7 with continuous stirring at  $\pm 4$  °C was done in the fraction with highest specific activity. The dialysis buffer solution was replaced every 2 hours and tested with  $\text{BaCl}_2$  in acidic condition. If there is no a white precipitate of  $\text{BaSO}_4$ , thus dialysis process has been completed.

### 2.2 Determination of Enzyme Specific Activity

The specific activity of the enzyme was obtained by dividing total number of the units of enzyme activity (U) from Kunitz method with total protein (mg) of Lowry method for each enzyme fraction (Setiasih, 2018).

$$\text{Specific activity} \left( \frac{\text{U}}{\text{mg}} \right) = \frac{\text{A (U)}}{\text{B (mg)}} \quad (2.1)$$

A and B are the total proteolytic and total protein, respectively.

### 2.3 Synthesis of Chitosan and Semi-IPN Methyl Cellulose-Chitosan Hydrogel

The synthesis of non-covalent chitosan hydrogel was carried out by dissolving 2 g chitosan into 2% acetic acid as much as 50 mL until homogenous. Then, the mixture was molded and dried at 60 °C with an oven until the hydrogel is completely dry. Furthermore, synthesis of semi-IPN methyl cellulose-chitosan was carried out by adding 1.33 g methyl cellulose into the homogenous mixture of non-covalent chitosan and stirred for 24 hours at room temperature until

homogeneous. Then the crosslinking agent as much as 10% (w/w) to chitosan weight of glutaraldehyde 0.10 M was added to the mixture and stirred for 2 hours. Hydrogels were shaped in a molding container and dried at 60 °C using an oven for 24 hours. Hydrogels were stored in desiccators for further testing and characterization (Rokhade et al., 2007).

### 2.4 Swelling Ratio and Degree of Crosslinking Determination

Hydrogels were immersed in 0.20 M phosphate buffer pH 7 at room temperature for 60 minutes, and removal of water remains on the hydrogel surface was conducted using filter paper. The percentage of swelling ratio was determined by the equation (Katime and Mendizábal, 2010):

$$\text{Swelling Ratio (\%)} = \frac{m - m_0}{m} \times 100\% \quad (2.2)$$

Where,  $m_0$  and  $m$  are the weights of dry hydrogel and swollen hydrogel, respectively. Dry hydrogel ( $m_0$ ) weight was measured and then the hydrogel was immersed into 2% acetic acid for 24 hours. Then, the hydrogel was grounded and stirred at 60 °C to a fixed weight ( $W_g$ ). The degree of crosslinking was determined by the following equation (Abdel-Mohzen et al., 2011):

$$\text{Degree of Crosslinking (\%)} = \frac{W_g}{W_0} \times 100\% \quad (2.3)$$

### 2.5 Bromelain Encapsulation into Hydrogel

Dried semi-IPN methyl cellulose-chitosan hydrogel was spilled with bromelain and incubated for up to 24 hours. Then, the hydrogel was rinsed with cold phosphate buffer pH 7. The amount of encapsulated bromelain and encapsulated activity can be determined by the following formula (Croisfelt et al., 2015):

$$\text{Encapsulated Bromelain} = [\text{C}]_1 - [\text{C}]_0 \quad (2.4)$$

$$\text{Activity Loading} = A_1 - A_0 \quad (2.5)$$

$$\text{Efficiency} = \frac{[\text{Bromelain loading}]}{[\text{protein of F2D}]} \times 100\% \quad (2.6)$$

$[C]_1$  and  $[C]_0$  are the bromelain before encapsulation and bromelain rinsed.  $A_1$  and  $A_0$  are Activity before encapsulation and activity rinsed.

## 2.6 In Vitro Release Study of Bromelain

In vitro release study was carried out by adding a bromelain encapsulated in semi-IPN hydrogel into a container containing 10 mL buffer solution of pH 1.2 at a temperature of 37 °C, stirred at a rotational speed of 100 rpm. After 2 hours, a hydrogel was transferred into 10 mL phosphate buffer of pH 7.4 to a total of 12 hours. Then the release solution was tested for activity by Kunitz method and protein content by Lowry method.

## 2.7 Hydrogel Characterization

Chitosan hydrogel, semi-IPN chitosan-methyl cellulose and encapsulated bromelain in semi-IPN methyl cellulose-chitosan were characterized using FT-IR instrumentation, and optical microscope.

## 3 RESULTS

### 3.1 Isolation, Ammonium Sulfate Precipitation and Dialysis of Bromelain

Proteolytic activity of isolation product of bromelain was determined by Kunitz method with modification

at its optimal condition (pH 8; 37 °C; 30 min). In this method casein was used as substrate, where casein will be dispart into tyrosine and measured by UV-Vis. Meanwhile, the total protein content was determined by the Lowry method. The principle of this method is to sharpen the colors produced on the Biuret method. After reacting with the Biuret reagent, the system was added with phosphomolibdate-phosphotungstate reagent. Reduction-oxidation reactions will occur at the tyrosine and tryptophan groups of proteins.

Bromelain is further purified to enhance its specific activity by ammonium sulfate precipitation. Ammonium sulfate precipitation method works by using the salting out principle, in which the salt will be solvated by the solvent so that the interaction between proteins is stronger and forming aggregates (pellets) with low solubility. After precipitation, the dialysis process was done to remove the content of excess salt. Dialysis works by the principle of diffusion, in which smaller molecules of the membrane pores will go out to a buffer whose concentration is lower than the buffer to dissolve the bromelain pellets. Data of total proteolytic activity and total protein content of bromelain can be seen in Table 1.

Based on these data, it can be seen that from each stage of purification causes an increase in the specific activity of bromelain. The highest specific activity was found in dialysis fraction of bromelain (F2D) with value of 111.72 U/mg, with purity level 5 times its crude enzyme, and yield percentage of 39.81%.

Table 1: Bromelain Activity from each Purification Process

Sample	Volume (mL)	Total		Specific Activity (U/mg)	Purity (times)	% Yield
		Proteolytic Activity (U)	Protein (mg)			
Isolation Process						
Pineapple Core Juice	150	275.00	23.28	11.82	-	-
Crude Enzyme	65	256.75	11.47	22.39	1	100
Ammonium Sulfate Precipitation						
F1 (0-20%)	5.4	59.31	1.05	56.34	2.52	23.10
F2 (20-50%)	11.1	137.09	1.79	76.73	3.43	53.39
F3 (50-80%)	3.4	9.01	0.59	15.38	0.69	15.19
F4 (remaining)	55	2.75	4.94	0.56	0.03	2.01
Dialysis						
F2D	12	102.20	0.92	111.72	5.00	39.81

### 3.2 Semi-IPN Methyl Cellulose-Chitosan Hydrogel

Visually, semi-IPN methyl cellulose-chitosan hydrogel has brown color, in contrast to its control (chitosan non-covalent) that has yellow color. FTIR characterization of bromelain, chitosan powder and hydrogel were shown in Figure 1. FTIR spectrum of powder chitosan can be seen in Figure 1a, where there is a wide band of uptake at  $3568\text{-}3003\text{ cm}^{-1}$  for N-H and O-H which overlap each other. Furthermore, at the wave number of  $1653\text{ cm}^{-1}$  there is an absorption for C=O amide stretch of residual acetyl in chitosan. Furthermore, the N-H stretch of the primary amine was identified at  $1592\text{ cm}^{-1}$  and the C-O-C bridge at  $1037\text{ cm}^{-1}$ . FTIR spectrum from bromelain showed several absorption bands for C-N stretch bands at wave number  $1540\text{ cm}^{-1}$ , absorption band with strong intensity for C=O stretch (amide)

at  $1653\text{ cm}^{-1}$ , at  $2933\text{ cm}^{-1}$  for C-H stretch and at wave number  $3317\text{ cm}^{-1}$  for N-H stretch (Figure 1b). The difference between the two hydrogels (Fig. 1c and d) lies in the intensity of the C = N imine band uptake at the wave number  $1596\text{ cm}^{-1}$ . The imine-absorbing bands arise because of a Schiff base occurs from a crosslink reaction.

The physical parameters of semi-IPN methyl cellulose-chitosan hydrogel were compared with non-covalent hydrogels (as control) to ensure that crosslinking with glutaraldehyde have been successfully performed. Physical parameters test including the determination of swelling ratio and degree of crosslinking. The test results of hydrogel physical parameters are presented in Table 2. The semi-IPN hydrogel has a higher crosslinking degree and relatively smaller swelling. This indicates that the crosslink reaction has reacted well. Visual appearance of hydrogel has been shown at Figure 2.

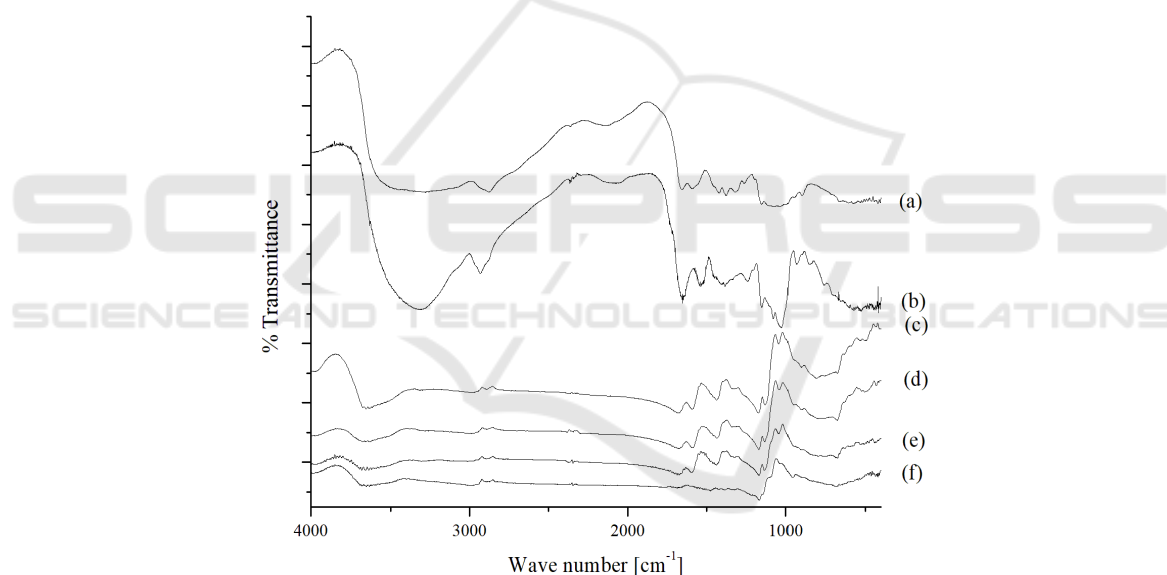


Figure 1: FTIR Spectra a) chitosan powder, b) bromelain, c) chitosan non-covalent hydrogel, d) semi-IPN methyl-cellulose chitosan hydrogel, e) semi-IPN methyl cellulose-chitosan hydrogel after encapsulation, f) semi-IPN methyl-cellulose chitosan hydrogel after in vitro release study

Table 2: Result of Swelling Ratio & Degree of Crosslinking Determination (n=3)

Hydrogel	Degree of Crosslinking (%)	Swelling Ratio (%)
Chitosan non-covalent	$0.50 \pm 0.03$	$2627.58 \pm 89.75$
Semi-IPN Methyl cellulose-chitosan	$46.63 \pm 2.92$	$397.59 \pm 8.66$

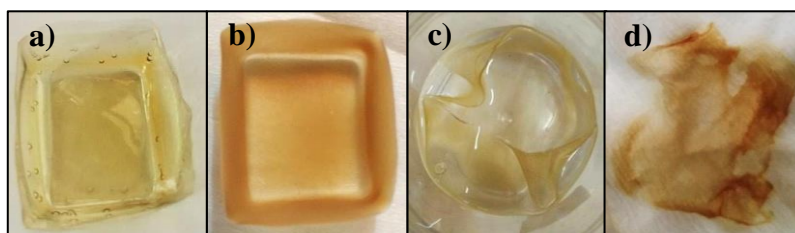


Figure 2: Visual appearance of a) chitosan non-covalent, b) semi-IPN methyl cellulose-chitosan, c) semi-IPN hydrogel swollen, d) semi-IPN after crosslinking degree test.

### 3.3 Bromelain Encapsulation into the Hydrogel

Post loading encapsulation method is chosen to make the encapsulation process more efficient and fewer bromelain of dialysis fraction are being used. The post loading efficiency can be determined indirectly by washing the encapsulated hydrogel with phosphate buffer pH 7, the results was tabulated in Table 3.

Furthermore, the hydrogel after encapsulation is characterized by FTIR and the spectra was presented in Figure 1e. From the comparison of hydrogel absorption spectra before and after encapsulation, bromelain does not resulting a new absorption band. This indicates that the interaction that occurs is only physical interactions in the form of hydrogen bonds which is characterized by changes in intensity at  $3568-3003\text{ cm}^{-1}$ . In addition, there was also a shift of absorption bands from  $1683\text{ cm}^{-1}$  to  $1672\text{ cm}^{-1}$ .

Table 3: Post Loading Efficiency of Bromelain into the Hydrogel

Material	Protein Content of Encapsulated Bromelain ( $\mu\text{g/mL}$ )	Proteolytic Activity of Encapsulated Bromelain (U/mL)	Post Loading Efficiency (%)
Semi-IPN methyl cellulose-chitosan	$68.00 \pm 3.46$	$8.30 \pm 0.03$	$89.47 \pm 4.56$

### 3.4 In Vitro Release Study of Bromelain

Dissolution is also referred as a release process that aims to determine the protein content of bromelain coming out of the hydrogel matrix and monitored its proteolytic activity. The visual appearance of the hydrogels before and after the dissolution test is shown in Figure 3a-d.

Hydrogel after dissolution test are also characterized using the FTIR presented in Figure 1f.

In the FTIR results, it appears that there are some absorption bands that missing from the matrix, such as C=N and C=O. This is due to damage to the matrix network due to dissolution process. The damage is also morphologically identified using the optical microscope presented in Figure 4a and b. Meanwhile, the encapsulated bromelain release profile and its proteolytic activity are shown in Figure 5a and b.

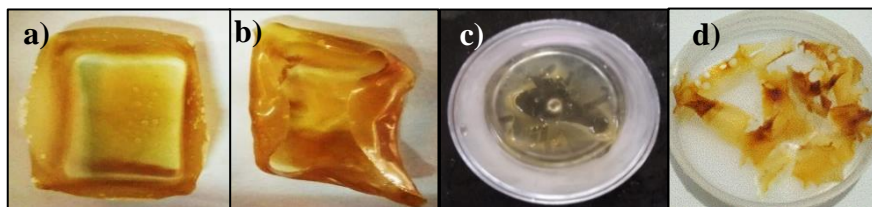


Figure 3: Visual appearance of semi-IPN methyl cellulose-chitosan hydrogels a) before and b) after encapsulation, c) dissolution process, d) after dissolution test.

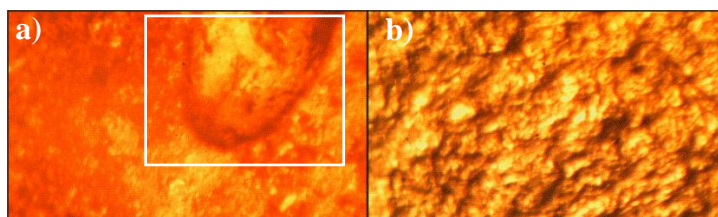


Figure 4: Morphological structure of semi-IPN methyl cellulose-chitosan hydrogels characterized by *Boeco Germany* optical microscope with 45 times magnification a) before and b) after dissolution

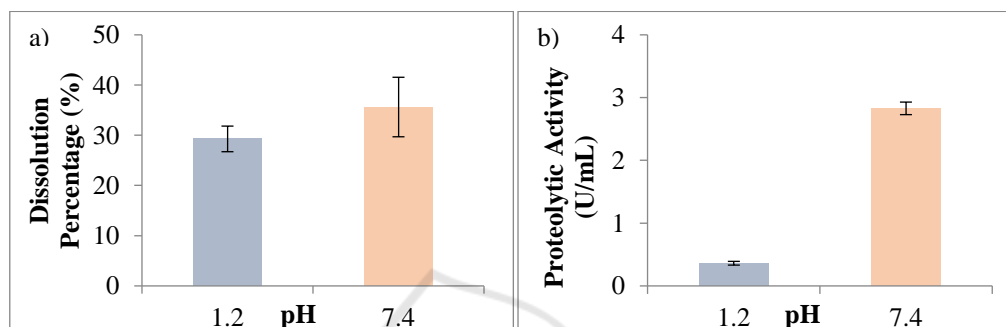


Figure 5: In Vitro release study of bromelain, a) dissolution percentage of bromelain and b) proteolytic activity of dissolution result.

The apparent release in the artificial gastric fluid ( $29.27 \pm 2.53$ ) was smaller than in the artificial intestinal environment ( $35.61 \pm 5.91$ ). In line with this, the proteolytic activity of bromelain in the artificial intestinal environment ( $2.83 \pm 0.10$ ) is also greater than in the artificial gastric fluid ( $0.36 \pm 0.03$ ).

## 4 CONCLUSIONS

Bromelain is successfully encapsulated in semi-IPN methyl cellulose-chitosan hydrogel. The release rate of bromelain in the artificial intestinal environment is greater than in the artificial gastric fluid. Furthermore, the activity of dissolved bromelain in the artificial intestinal environment is greater than in the intestinal environment. This suggests that the activity of bromelain can be maintained up to the intestine.

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