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COMPARISON OF PURIFICATION METHODS TO PURIFY RECOMBINANT BROMELAIN FROM *Escherichia coli* BL21-A1

(Perbandingan Kaedah Penulenan Rekombinan Bromelain daripada *Escherichia coli* BL21-A1)

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Abstract

Recombinant bromelain is a cysteine protease that can be exploited for its protease activity in food and pharmaceutical applications. The aim of this study was to compare different purification methods aqueous two-phase system (ATPS), ammonium sulphate precipitation, ion exchange, affinity, and gel filtration chromatography) for the recombinant bromelain purification from *Escherichia coli* BL21-A1. From the SDS-PAGE analysis, all methods produced band with molecular weight between 50 to 55 kDa. Among the methods used, the ATPS purification consisting of 13% (w/w) of PEG6000 and 11% (w/w) potassium phosphate at pH 7.0 was chosen as the best purification method. The method produced 16.39 ± 0.03 % of yield, purification fold of 5.35 ± 0.11, and specific activity of 3.47 ± 0.11 unit/mg of recombinant bromelain. This proposed study can be used as a platform for large-scale downstream processing of recombinant bromelain in industry.

Keywords: recombinant bromelain, purification, chromatography, aqueous two-phase system

Abstrak

Rekombinan bromelain adalah sisteina protease yang boleh dieksploitasi dalam aplikasi makanan dan farmaseutikal. Tujuan kajian ini adalah untuk membandingkan kaedah penulenan yang berbeza dengan menggunakan sistem akueus dua fasa (ATPS), pemendakan amonium sulfat, pertukaran ion, afiniti dan gel penapisan kromatografi untuk penulenan rekombinan bromelain daripada *Escherichia coli* BL21-A1. Daripada analisis SDS-PAGE, semua kaedah menghasilkan jalur dengan berat molekul antara 50 hingga 55 kDa. Antara kaedah yang digunakan, sistem akueus dua fasa (ATPS) yang terdiri daripada 13% (w/w) PEG6000 dan 11% (w/w) kalium fosfat pada pH 7.0 telah dipilih sebagai kaedah penulenan yang terbaik. Kaedah ini mendapat 16.39 ± 0.03% hasil, kadar penulenan 5.35 ± 0.11, dan aktiviti rekombinan bromelain sebanyak 3.47 ± 0.11 unit/mg. Kajian ini dicadangkan boleh digunakan sebagai platform untuk pemprosesan hiliran rekombinan bromelain secara besar-besaran dalam industri.

Kata kunci: rekombinan bromelain, penulenan, kromatografi, sistem akueus dua fasa

Introduction

Bromelain is a plant protease that is commercially obtained from stem, fruit, and waste of pineapple plant [1]. Bromelain is notable for being used in multitude industrial applications such as meat tenderization, food, beverages, and pharmaceuticals, hence increases its market demand [2]. However, there is a rising argument whether pineapple

crop should be selected for food or bromelain extraction. Besides, the processing of pineapple crop to produce bromelain generates bulk waste that eventually leads to environmental issues. Considering the significance of economic, efficient production and downstream processing of bromelain, recombinant bromelain was successfully expressed in various hosts, including *Escherichia coli* BL21-A1 [3], *E.coli* BL21 DE3pLysS [4] and *Brassica rapa* [5]. Our research group has successfully cloned the stem bromelain from *Ananas comosus* into the pENTR/TEV/D-TOPO vector and subcloned it into the pDEST17 expression vector. Later, *E. coli* BL21-A1 was transformed with the expression vector [3] and was cultivated in a batch process using auto-induction media [6]. The recombinant bromelain is an intracellular enzyme and remains abundantly in the cytoplasm of the *E. coli* cells. Therefore it is vital to develop a reliable, efficient, and cost-effective purification process to purify the recombinant bromelain after cell disruption process using ultrasonication method [7].

Commonly, recombinant bromelain was purified using affinity chromatography under native condition [3, 8]. An instrument such as AKTA prime plus® (GE Healthcare, USA) provides a quick design to facilitate affinity chromatography purification as the plasmid vector of recombinant bromelain has a unique polyhistidine (His6) tag [3]. It was reported that, the recombinant bromelain purified from *E. coli* BL21-A1 has a molecular weight of 50 – 55 kDa and exhibited optimum proteolytic activity at pH 4.6 [3]. In the majority of food industry, the enzyme is added in bulk quantity. The enzyme purified by Amid et al. [3] was only produced in a small amount (~200 µl) and could not be used for large scale food-related processes. Therefore, there is a need to develop a purification protocol that accompanies higher recovery, lower cost, and shorter processing time. Aqueous two-phase system (ATPS) is one of the alternative purification methods which involves a mixing of two polymers or one polymer and one salt under specific condition to form a phase separation. Due to its low cost and high recovery, ATPS purification method has been addressed previously by other researchers for the purification of recombinant protein [9, 10] and bromelain from pineapple plant [1].

To date, recombinant bromelain has not been extracted by aqueous two-phase technique, which could be promising in the bromelain research. In this paper, we compare the purification efficiency of ammonium sulfate precipitation, aqueous two-phase system, ion exchange chromatography, affinity chromatography, and gel filtration chromatography. Purified recombinant bromelain was further evaluated using SDS-PAGE analysis and enzyme assay with Nα-CBZ-L-lysine *ρ*-nitrophenyl ester (LNPE) as substrate.

Materials and Methods

Media and culture condition

The cultivation of *E. coli* BL21-A1 [3] was conducted in a batch fermentation using auto-induction method [6]. Approximately 10 ml of starter medium supplemented with 100 µg/ml ampicillin was inoculated with single colony of *E. coli* BL21-A1 harbouring the bromelain gene and incubated for 16 hours at 37 °C with shaking at 300 rpm in an incubator shaker (CERTOMAT® IS, Sartorius, Germany). 1 L of ZYM medium [11], supplemented with 100 µg/ml ampicillin, was inoculated with 10 ml of starter culture and incubated in an incubator shaker (CERTOMAT® IS, Sartorius, Germany) for 12 hours at 37 °C with shaking at 250 rpm until the OD_{600nm} reached 0.6-1.0. After 12 hours, the cells were harvested by centrifugation (4 °C, 16000 x g, and 15 min) (Heraeus Multifuge X1R, Thermo Scientific, USA), and the cell pellet was stored at -20 °C until further used. The cell pellet was suspended in lysis buffer at a ratio of 1 g to 5 ml of lysis buffer before cell disruption by sonication technique (20% amplitude and first cycle for 130 ml/min) [7]. The lysate was then centrifuged at 16000 x g for 45 min (Heraeus Multifuge X1R, Thermo Scientific, USA) to obtain a clear supernatant for further analysis.

Enzyme activity assay

The enzymatic assay was based on continuous spectrophotometric rate determination method. The proteolytic activity of recombinant bromelain was measured using Nα-CBZ-L-lysine *ρ*-nitrophenyl ester (LNPE) as a substrate at 44 °C and pH 4.6. Initially, 2.60 ml of LNPE buffer was mixed and incubated with 100 µl of enzyme solution for approximately 3 min. Then, 100 µl of 50 mM LNPE substrate was added and mixed by inversion. The absorbance measurement of enzymatic reaction was measured spectrophotometrically for 5 min at 340 nm using a microplate reader (Thermo Scientific, USA). One unit of enzyme activity corresponds to the release of 1.0 µmole of ρ-nitrophenyl ester from the LNPE substrate per min after reaction with bromelain [12]. The hydrolysis of substrate was monitored at the selected wavelength starting 50 sec after the addition of enzyme in order to allow enough time

for the assay medium to reach 44 °C. Initial velocity measurements were obtained from the data recorded over the next 300 sec (5 min), where linearity was maintained. The results were plotted as ΔAbs per min versus amount of enzyme. The slope of this line, together with the molar absorption coefficient obtained as described in equation 1, allowed us to calculate enzyme activity in terms of units/mg protein as below:

$$\frac{\left(\frac{\Delta A_{340nm}}{\text{min Test}} - \frac{\Delta A_{340nm}}{\text{min Blank}}\right) \times 2.8 \times DF}{6.32 \times 0.1} \quad (1)$$

where the values of 2.8, 6.32, 0.1 and DF denote the assay volume in milliliters, the millimolar extinction coefficient of ρ-nitrophenol at 340 nm, the volume of enzyme used in milliliters, and the dilution factor, respectively. Assays were carried out in triplicate.

Protein assay
Protein content was measured based on the Bradford method using Bio-Rad Protein Assay (Bio Rad, Germany). Absorbance was measured at a wavelength of 595 nm using a microplate reader. (Thermo Scientific, USA). Bovine serum albumin was used as a standard for the protein assay [13]. Assays were carried out in triplicate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Purified samples from ATPS were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [14]. The ATPS samples were diluted two times before applying to 12% resolving gel and 4% stacking gel. After electrophoresis was performed at 200 V, 700 mA for 37 min, the gels were stained using silver stain or Coomassie blue.

Ammonium sulfate precipitation
Protocol for ammonium sulfate precipitation was adopted from Grodzki et al. [15]. The precipitation of the recombinant bromelain was performed by slow addition of ammonium sulfate to the crude cell lysate at 4 °C. Initially, solid ammonium sulfate was slowly added to achieve a final concentration of 30% (w/v). The mixture was incubated for 30 minutes with continuous stirring. After the ammonium sulfate was completely dissolved, the mixture was centrifuged at 4 °C and 16000 x g for 15 minutes (Heraeus Multifuge X1R, Thermo Scientific, USA). Precipitate was discarded, and the recombinant bromelain in supernatant was collected. The supernatant was subjected to further precipitation by slowly adding ammonium sulfate to achieve a final concentration of 60% (w/v). The solution was stirred and incubated overnight at 4 °C to reach equilibrium. The precipitated protein was collected for chromatography analysis after centrifugation at 16000 x g for 15 minutes. The weight of saturated ammonium sulfate in gram that was required for salting out the protein is given in Equation 2.0 [15] as follows:

$$g = Volume\ of\ sample\ (ml) \times 0.515 \frac{g}{ml} \times \frac{(S_2 - S_1)}{1 - S_2} \quad (2)$$

where S_2 and S_1 are final saturation (%) and initial saturation (%), respectively. The value of 0.515 g/ml denotes the solubility of ammonium at 4 °C. The ammonium sulfate has a molecular weight of 131.14 g/mol, and 100% saturation of ammonium salt at 4 °C corresponds to 3.93 M, therefore the solubility of ammonium sulfate at 4 °C is 0.515 g/ml.

Ion-exchange chromatography
Ion exchange chromatography using AKTA Prime Plus® (GE Healthcare, UK) was performed to purify recombinant bromelain. A glass column connected to AKTA Prime Plus® (GE Healthcare, UK) with an inner diameter of 1 cm and the length of 10 cm was packed with DEAE-Sepharose FF® with 5.5 cm^3 column volume and equilibrated with 100 mM of Tris at pH 9.0 followed by 5 ml sample loading from 0.16 g recombinant bromelain produced from 60% ammonium sulfate precipitation process at a flow rate of 1 ml/min. After that, the column was washed with the same buffer. A stepwise elution experiment was performed with elution buffer of 100 mM of Tris and 700 mM NaCl at pH 9.0. Finally, the eluates were analyzed for their enzyme activity, specific activity, total protein content and purification fold.

Affinity chromatography

Affinity purification was conducted using AKTA Prime Plus® (GE Healthcare, UK). A glass column connected to AKTA Prime Plus® (GE Healthcare, UK) with an inner diameter of 1 cm and 10 cm length was packed with 5.5 cm^3 column volume of Ni-NTA agarose and equilibrated with equilibration buffer (50 mM of NaH_2PO_4, 300 mM NaCl and 10 mM of imidazole) at pH 8.0. The protein precipitate (0.16 g) from 60% of ammonium sulfate precipitation process was suspended in 5 ml equilibration buffer and was loaded into the packed column at a flow rate of 1 ml/min. After the unbound protein was washed with washing buffer (50 mM of NaH_2PO_4, 300 mM NaCl and 20 mM of imidazole, pH 8.0), the desired protein was eluted with a stepwise elution using elution buffer (50 mM of NaH_2PO_4, 300 mM NaCI and 250 mM of imidazole, pH 8.0). Collected fractions were analyzed by enzyme activity, specific activity, total protein content, and purification fold.

Gel filtration chromatography

A glass column connected to AKTA Prime Plus® (GE Healthcare, UK) with an inner diameter of 2.5 cm and 100 cm length was packed with 375.57 cm^3 column volume of Sephacryl S-200® and equilibrated with two column volumes of 100 mM Tris buffer, pH 7.0. Recombinant bromelain sample (0.16 g) from 60% of ammonium sulfate precipitation was applied to the packed column at a flow rate of 1 ml/min and washed with two column volumes of the same buffer until no other proteins were eluted. Purified proteins were analyzed for enzyme activity, specific activity, total protein, and purification fold.

Aqueous two-phase system

Stock solution of PEG 1500, 2000, 4000, 6000, 8000 and potassium phosphate was prepared with 50% (w/w) concentration. Potassium phosphate solution was prepared using specified ratios of K_2HPO_4 and KH_2PO_4 to achieve pH 7.0. ATPS was prepared in a graduated tube by adding 20% (w/w) of recombinant bromelain lysate, known weight of the PEG stock solution, potassium phosphate stock solution at definite pH, and deionized water to reach a total of 50% (w/w) concentration of ATPS. The mixture was mixed thoroughly for 3 minutes using a Vortex mixer. Phase separation was achieved by centrifugation at 1000 × g for 10 minutes at 25 ºC. Then, the top and bottom phases were collected for assay analysis. In order to evaluate the purification process, a purification factor (PF) and partition coefficient (K_E) recovered in the bottom and top phases were calculated according to the equation below:

$$PF = \frac{SA_e}{SA_i} \tag{3}$$

where SA_e is a specific activity of each phase and SA_i is an initial specific activity of crude lysate. The partition coefficient (K_E), was defined as follows:

$$K_E = \frac{A_T}{A_B} \tag{4}$$

where A_T and A_B are the enzyme activity in the top and the bottom phase, respectively.

Results and Discussion

Ammonium sulfate precipitation

In many cases, ammonium sulfate precipitation is often used as a preliminary purification of crude cell lysates to remove unwanted proteins prior to use of expensive columns in chromatography technique for its easy operation and economic practicality. Crude lysate of recombinant bromelain obtained after cell lysis was subjected to ammonium sulfate precipitation. It was hypothesized that most of the recombinant bromelain would be susceptible to precipitate from crude cell lysate in the presence of between 30% to 60% ammonium sulfate. The percentages were relative to a saturated solution (100%), which was equal to 3.93 M at 4 °C. Hence, the concentration was between 1.179 M and 2.358 M. The recombinant bromelain was found to precipitate at 60% of saturation with specific activity and purification fold of 0.03 ± 0.03 unit/mg and 0.05-fold, respectively. SDS-PAGE analysis shown in Figure 1 indicates the size of precipitated recombinant bromelain is approximately 50 – 55 kDa [3]. From Figure 1, we could see that most of the unwanted proteins have been removed after ammonium sulfate precipitation process.

This finding was in conformity with the previous works by Devakate et al. [16], stating that the bromelain from the pineapple fruit plant was precipitated at a saturation level of 40 – 60% with 2.81-fold purity. Soares et al. [17] also recovered bromelain at saturation range of 20 to 40% with purification fold of 4.4 using ammonium sulfate and ethanol precipitation. However, the purification fold of precipitated recombinant bromelain in the current work was lower than those of the native precipitated bromelains obtained by Devakate et al. [16] and Soares et al. [17]. The presence of more than one contaminant proteins precipitated with recombinant bromelain at saturation level of 40 – 60% (Figure 1) decrease the purification fold. Therefore, further purification steps such as chromatography purification were needed to obtain a pure protein sample with high purification fold. Besides, there was an absence of carbohydrate moiety in recombinant proteins produced in *E. coli* and folding anomalies might occur due to the inability of *E. coli* to perform post-translational modifications, therefore potentially resulted in modification of enzyme activity [18].

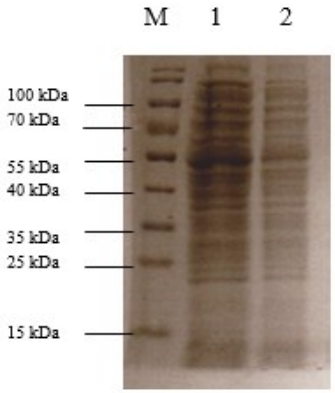
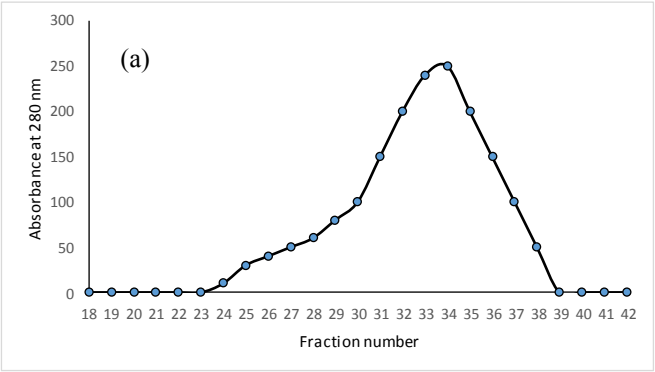


Figure 1. SDS-PAGE of purified recombinant bromelain using ammonium sulfate precipitation. Lane M: the standard molecular weight. Lane 1: crude cell lysate containing recombinant bromelain. Lane 2: 60 % ammonium sulfate precipitation. The position of recombinant bromelain is at estimated size of 50-55 kDa [3].

Ion-exchange chromatography

Ion-exchange chromatography was employed to purify the recombinant bromelain. A stepwise elution experiment was performed using binding buffer containing 100 mM of Tris at pH 9.0 and elution buffer containing 100 mM of Tris with 700 mM NaCl at pH 9.0. A chromatogram of recombinant bromelain purified using DEAE Sepharose is shown in Figure 2. From the elution profile shown in Figure 2, a prominent peak appears at fraction 33. All fractions under the peak were pooled and assayed for protein content and enzyme activity. The pooled fractions exhibited specific activity and purification fold of 0.094 unit/mg and 0.14, respectively. The fractions were analyzed by SDS-PAGE (Figure 2).



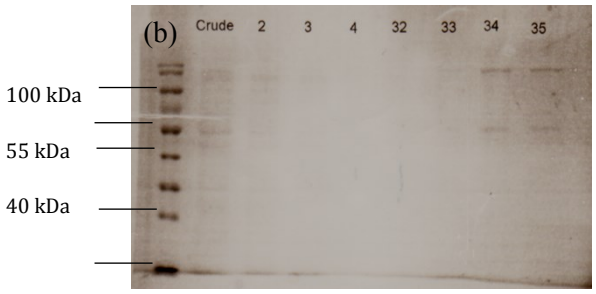
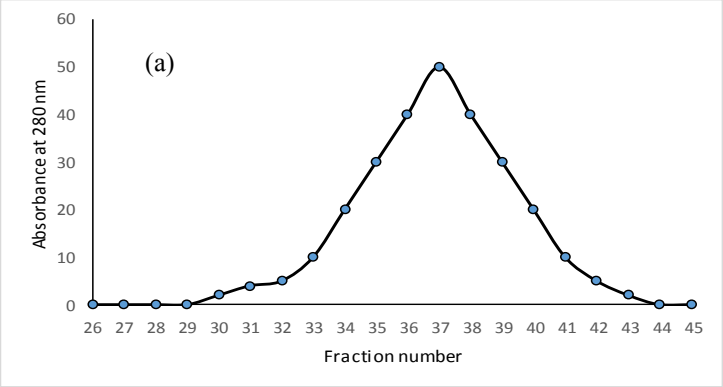


Figure 2. Chromatogram of the purified bromelain from ion exchange chromatography using DEAE Sepharose resin (a). The SDS-PAGE analysis of recombinant bromelain from ion exchange chromatography (b). 1 ml fraction was collected at a flow rate of 1 ml/min. The position of recombinant bromelain is at estimated size of 50-55 kDa [3]

Affinity chromatography

Affinity chromatography was conducted for purification of recombinant bromelain using AKTA prime plus® system. Equilibrium buffer used contained 50 mM of NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole at pH 8.0. Washing buffer contained 50 mM of NaH_2PO_4, 300 mM NaCl, and 20 mM of imidazole buffer, pH 8.0. Elution buffer contained 50 mM of NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole at pH 8.0. A chromatogram of recombinant bromelain purified using Ni-NTA agarose resin is shown in Figure 3. The recombinant bromelain pooled from fractions 33 to 37 exhibited specific activity and purification fold of 1.77 ± 0.02 unit/mg and 2.72-fold, respectively and appeared on the SDS-PAGE at the expected size of 50 – 55 kDa (Figure 3). The purification conditions are in accordance with the research presented by Amid et al. [3] and Bala et al. [8].



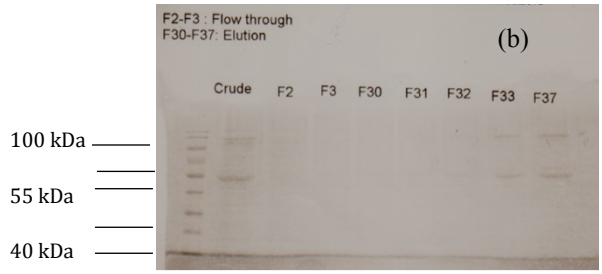
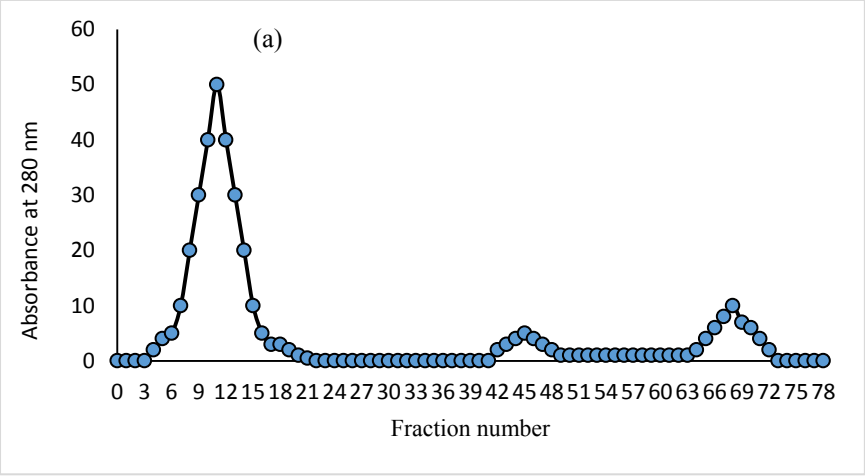


Figure 3. Chromatogram (a) and SDS-PAGE analysis (b) of the purified samples from affinity chromatography using Ni-NTA agarose resin. Equilibrium buffer contained 50 mM of NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole at pH 8.0. Washing buffer contained 50 mM of NaH_2PO_4, 300 mM NaCl, and 20 mM of imidazole buffer, pH 8.0. Elution buffer contained 50 mM of NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole at pH 8.0. 1 ml fraction was collected at a flow rate of 1 ml/min. The position of recombinant bromelain is at estimated size of 50 – 55 kDa [3].

Gel filtration chromatography

Gel filtration chromatography is a separation technique based on the size of the protein. The protein precipitated from the 60% of ammonium sulfate precipitation process was subjected to gel filtration chromatography using Sephacryl S-200® resin and a total of 78 fractions were collected (Figure 4). From the chromatogram, it can be seen that there was a high peak after washing step from fractions 9 to 20, followed by a broad absorbance peak from fractions 42 to 72 (Figure 4). The ammonium sulfate precipitation step removed the majority of protein contaminants and the remaining unwanted proteins were separated based on size by gel filtration beads. Thus, recombinant bromelain with 55 kDa was eluted first. The specific activity and purification fold obtained after gel filtration purification were 2.00 ± 0.51 unit/mg and 3.08 ± 0.79-fold, respectively. The SDS-PAGE analysis from Figure 4 shows that the recombinant bromelain pooled from fractions of 11 to 14 has an apparent molecular weight of 50 – 55 kDa.



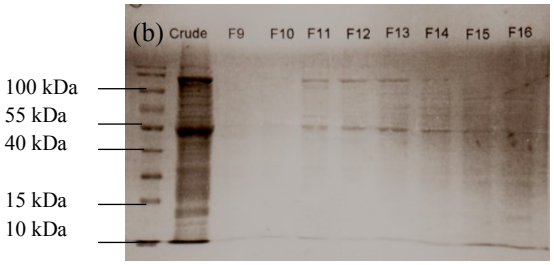


Figure 4. Chromatogram (a) and SDS-PAGE (b) analysis of the purified samples from gel filtration chromatography using Sephacryl S-200® resin. Equilibrium, washing and elution buffers contained 100 mM Tris buffer, pH 7.0. 5 ml fractions were collected at a flow rate of 1 ml/min. The position of recombinant bromelain is at estimated size of 50-55 kDa [3]

The SDS-PAGE analysis was also performed for the remaining peak from fractions 27 to 78, however, no protein band was visible, indicating that no impurities present in the sample. Costa et al. [19] performed ion exchange chromatography prior to gel filtration chromatography to purify bromelain with a molecular weight of 30 kDa from pineapple stem residue. The gel-filtration resin used was Sephadex®G-50. Suh et al. [20] employed gel filtration chromatography to estimate the molecular weight of bromelain as 32.5 and 37 kDa from pineapple fruit and stem, respectively using Sephadex G-200 resin. Hence, it can be concluded that the combination of low-resolution (ammonium sulfate precipitation) and high-resolution (chromatography) methods are most likely provide a higher purity of desired protein.

Aqueous two-phase system

In order to determine the best ATPS extraction conditions for the recovery and purification of recombinant bromelain, different molecular weight of PEG was used. The influence of PEG with different molecular weights (1500, 2000, 4000, 6000, and 8000) at constant volume ratio of 1.0 was investigated for the purification of recombinant bromelain. Most recombinant bromelain partitioned in all two phase systems to PEG-rich top phase and salt rich bottom phase. It is because, in a PEG-salt system, phase separation is caused by the volume exclusion effect of the polymer and salting-out effect of salt [21].

Table 1 shows the partition coefficient (K_E), purification fold (PF), and enzyme activity recovery of recombinant bromelain in the PEG-rich top phase. The PEG1500/phosphate and PEG2000/phosphate systems exhibited relatively small partition coefficient, purification fold and enzyme activity recovery of recombinant bromelain compared to other three systems investigated (PEG4000, PEG6000 and PEG8000). One explanation for this observation is that high molecular weight of PEG has high hydrophobicity and thus increases the salting-out characters that drive the partition of recombinant bromelain to the upper phase [22]. Conversely, low molecular weight of polymer attracts the migration of contaminant proteins to the top phase and causes a decrease in remaining free volume available for the recombinant bromelain to occupy [23]. Thus, the K_E, PF, and enzyme activity recovery values at the top phase were significantly reduced. Bekale et al. [24] had proved by using molecular modelling technique that PEG6000 was strongly adsorbed on bovine serum albumin (BSA) and human serum albumin (HSA) through hydrophobic binding compared with PEG3000. The interaction of polymer-enzyme in ATPS has led to the equilibration between polymer hydrophobicity and salting-out force that explains the preference of recombinant bromelain to migrate to the upper segment [25].

The highest K_E (1.63), PF (5.35), and enzyme activity recovery (53.25%) were obtained in a system consisting of 11% (w/w) of potassium phosphate and 13% (w/w) of PEG6000. Even though the system with the composition of 11% (w/w) PEG8000 and 12% (w/w) potassium phosphate has a high partition coefficient, its purification fold and enzyme activity recovery were found to be low. The purification fold represents the specific activity of purified protein over the specific activity of crude lysate. Thus, high purification fold for the ATPS of 11% (w/w) of potassium phosphate and 13% (w/w) of PEG6000 indicated high specific activity and purity. The molecular weight of partitioned protein may influence the composition of polymer and salt. Ketnawa et al. [1] had successfully

partitioned bromelain from pineapple peels using the system containing 18% (w/w) PEG6000 and 17% (w/w) $MgSO_4$. Ramakrishnan et al. [26] observed that the enzyme recovery of lipase from *Enterococcus faecium* MTCC5695 was increased with the increase of PEG molecular weight from 1000 to 8000. The purified sample from the top phase (PEG-rich) was subjected to SDS-PAGE analysis. All of the purified samples were run on SDS-PAGE under reducing conditions (Figure 5). The protein bands observed at 50-55 kDa represent the molecular weight of recombinant bromelain.

Table 1. The effect of molecular weight to the partition coefficient (K_E) and purification fold (PF). (Note: PP: potassium phosphate, PEG: polyethylene glycol)

MW of PEG	**Composition (PP/PEG) % (w/w) at $V_R = 1$**	**K_E**	**PF**	**Enzyme Activity Recovery (%)**
1500	11/13	0.61 ± 0.12	0.72 ± 0.61	28.31 ± 1.84
	12/15	0.31 ± 0.00	0.12 ± 0.32	16.40 ± 0.45
	13/16	0.04 ± 0.01	0.05 ± 0.05	2.58 ± 0.64
	14/17.5	0.14 ± 0.03	0.14 ± 0.14	7.44 ± 1.05
2000	11/12	1.89 ± 0.09	0.95 ± 0.00	35.36 ± 0.43
	12/14	0.55 ± 0.03	1.00 ± 0.02	38.29 ± 1.02
	14/16	0.35 ± 0.06	0.28 ± 0.01	12.67 ± 0.69
	15/17	0.16 ± 0.02	0.30 ± 0.01	13.36 ± 0.69
4000	10/10	2.15 ± 0.16	1.24 ± 0.03	32.41 ± 1.23
	11/10	0.51 ± 0.03	1.49 ± 0.02	32.96 ± 0.79
	12/12	1.21 ± 0.18	1.31 ± 0.03	28.47 ± 1.10
	13/13	2.42 ± 0.42	3.23 ± 0.02	71.46 ± 0.68
6000	11/13	1.63 ± 0.05	5.35 ± 0.11	53.25 ± 0.91
	12/14	0.64 ± 0.02	3.12 ± 0.06	45.38 ± 0.58
	13/15	0.72 ± 0.02	2.59 ± 0.00	46.95 ± 0.22
	14/16	1.80 ± 0.07	2.01 ± 0.03	48.26 ± 0.94
8000	10/11	22.99 ± 0.14	0.73 ± 0.08	11.56 ± 2.06
	11/12	26.73 ± 0.35	3.00 ± 0.11	34.77 ± 1.89
	12/13	0.70 ± 0.03	2.24 ± 0.05	26.37 ± 0.88
	14/15	1.24 ± 0.09	3.12 ± 0.02	58.72 ± 0.51

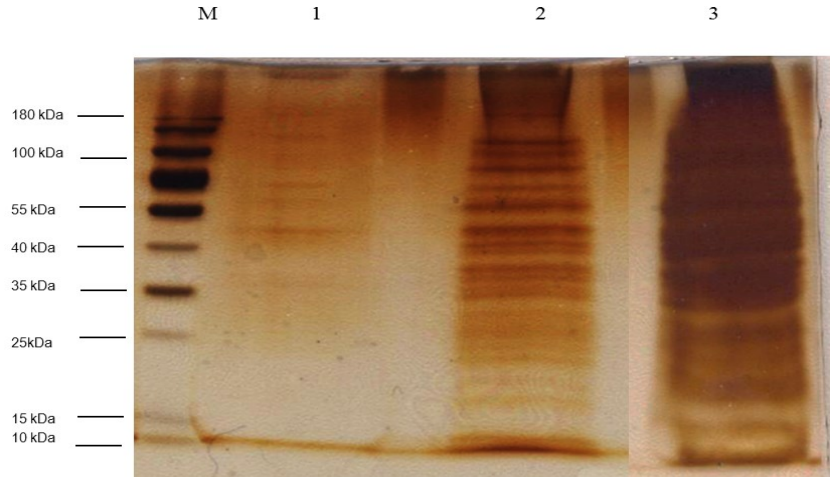


Figure 5. SDS-PAGE analysis for purified recombinant bromelain by ATPS purification techniques. Lane M corresponds to protein marker, lane 1 is the top phase (PEG-rich), lane 2 is the bottom phase (salt-rich), and lane 3 is the crude cell lysate containing recombinant bromelain. The position of recombinant bromelain is at estimated size of 50 – 55 kDa [3].

Comparison of purification by ATPS and chromatographic techniques

The purification efficiency of the recombinant bromelain from various purification methods is summarized in Table 2. The chromatography process for the purification of recombinant bromelain has been well explained by Amid et al. [3]. In chromatography purification, the crude extract from cell lysis process was subjected to ammonium sulfate precipitation to concentrate and precipitate the recombinant bromelain. Later, the precipitated protein was dissolved in equilibrium buffer, depending on the type of chromatography (affinity, ion exchange, and gel filtration) used. In contrast, ATPS purification was performed directly after centrifugation in which the crude extract from cell lysis was applied to the PEG6000-potassium phosphate extraction stage.

Table 2. Purification efficiency of recombinant bromelain by different purification methods.

Purification Methods	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification Folds	Yield (%)
Crude	200	304.96 ± 0.00	197.49 ± 0.00	0.65 ± 0.00	1.00 ± 0.00	100 ± 0.00
Ammonium sulphate precipitation	50	36.05 ± 0.00	1.16 ± 0.00	0.03 ± 0.03	0.05 ± 0.00	0.59 ± 0.02
Affinity (Ni-NTA agarose) chromatography	3	0.13 ± 0.00	0.23 ± 0.00	1.77 ± 0.02	2.72 ± 0.00	0.12 ± 0.00
Ion-exchange (DEAE Sepharose) chromatography	11	5.29 ± 0.01	0.49 ± 0.01	0.09 ± 0.00	0.14 ± 0.00	0.25 ± 0.00

Table 2 (cont'd). Purification efficiency of recombinant bromelain by different purification methods.

Purification Methods	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification Folds	Yield (%)
Gel filtration (Sephacryl S-200®) chromatography	20	0.05 ± 0.00	0.10 ± 0.00	2.00 ± 0.51	3.08 ± 0.79	0.05 ± 0.01
Aqueous two-phase system	20	3.02 ± 0.00	10.50 ± 0.00	3.47 ± 0.11	5.35 ± 0.11	16.39 ± 0.03

In this study, Ni-NTA agarose chromatography (affinity) resulted in purity and specific activity of 2.72-fold and 1.77 ± 0.02 unit/mg, respectively. Meanwhile, DEAE Sepharose chromatography (ion exchange) achieved 0.09 unit/mg and 0.14-fold, respectively. The Sephacryl S-200® chromatography produced specific activity of 2.00 ± 0.51 unit/mg with 3.08 ± 0.79-fold. However, the ATPS purification (13% (w/w) of PEG6000 and 11% (w/w) potassium phosphate, pH 7.0) produced favourable value of purification fold (5.35±0.11-fold) compared with that produced after Ni-NTA agarose chromatography (2.72-fold) and Sephacryl S-200® chromatography (3.08 ± 0.79 fold). With respect to yield percentage, ATPS process exhibited higher yield (16.39 ± 0.03%) compared to ammonium sulphate precipitation (0.59 ± 0.02%), DEAE Sepharose chromatography (0.25%), Ni-NTA agarose chromatography (0.12%), and Sephacryl S-200® chromatography (0.05 ± 0.01%). The yield is defined as the amount of purified protein (total units) divided by the initial amount of protein (defined as 100%).

From economic analysis point of view as stated in Table 3, it can be summarized that ATPS method exhibited the second lowest operating cost with shorter operating time (30 minutes) over chromatographic methods. Among the chromatography methods used, gel filtration chromatography appeared to exhibit higher operating cost due to long separation time (72 hours), causing an increment of buffer usage. Affinity chromatography was positioned as the second most costly method owing to the expensive resin and chemicals used within 1 hour operation time. In terms of reusability, the resin can be re-used up to 100 times only, with an additional cost of using sodium hydroxide, sodium chloride, nickel sulphate, and EDTA for stripping and regenerating the resin after each run. In the case of ATPS, the polymer and salt cannot be recycled as they were part of the final purified enzyme [23]. Such economic and operation analysis provides understanding for further research, and the ATPS process is proven to be a cost-effective, time-saving, and a higher recovery method that may be scaled up for industrial purpose. The purification method should be selected based on the complexity and the application of the recombinant bromelain. In many cases, it is not necessary to have the protease preparation in high purity form, especially for the commercial use such as in food, cosmetics, and textile industries [2]. Therefore, the purification method is aimed to obtain an active protease preparation in high recovery and high yield for industrial purpose. However, for some pharmaceutical uses, the protease must be highly purified. High purity protease is almost invariably expensive due to the purification intricacy to get rid of undesirable contaminating substances. For that reason, this study enables producers and researchers to choose the desired level of protease purity, thereby further reducing the cost of the production process.

Table 3. Direct comparison between chromatography and ATPS methods for the downstream processing of recombinant bromelain

Parameter	**Ammonium Sulfate**	**Ion Exchange**	**Affinity**	**Gel Filtration**	**ATPS**
Operation	Batch	Batch and semi-batch	Batch and semi-batch	Batch and semi-batch	batch
Processing time	24 hours	1 hour	1 hour	72 hours	30 minutes
Cost of the resin	-	DEAE Sepharose: USD 7.84/7ml	Ni-NTA: USD 121.03/7 ml	Sephacryl S-200®: USD 184/200 ml	-
Cost of the column		Flow Adaptor (1.0 cm): USD 191.99 Econo Column (1 cm x 10 cm): USD 69.82	Flow Adaptor (1.0 cm): USD 191.99 Econo Column (1 cm x 10 cm): USD 69.82	Flow Adaptor (1.5cm): USD 191.99 Econo Column (2.5 x 100 cm): USD 71.26	-
Cost of the chemicals (per kg)	Ammonium sulfate: USD 151.24	Tris: USD 197.85 NaCl: USD 118.15	Imidazole: USD 614.55 NaCl: USD 118.15 NaH_2PO4: USD 280.98	Tris: USD 197.85	K_2HPO_4: USD 173.45 KH_2PO_4: USD 210.21 PEG6000: USD 42.42
Cost of the operation per system	USD 151.24	USD 585.65	USD 1396.52	USD 645.10	USD 426.08

Conclusion

The ATPS, ammonium sulfate precipitation, and chromatographic procedures for downstream processing of recombinant bromelain were investigated. Our study clearly demonstrated that ATPS process was proven to be a cost-effective (USD 426.08), time-saving (30 min), and high-recovery method (16.39 ± 0.03%) that may be scaled up for industrial purpose. The optimum conditions of ATPS system comprised of 13% (w/w) of PEG6000 and 11% (w/w) potassium phosphate at pH 7.0 which resulted 3.47 ± 0.11 unit/mg, and 5.35 ± 0.11-fold for the specific activity, and purification fold, respectively. Herein, ATPS can be a potential technique for the purification of single step separation and avoid multistep purification like ammonium sulfate precipitation and chromatography process.

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