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COMPARISON OF DIFFERENT CELL DISRUPTION METHODS AND CELL EXTRACTANT BUFFERS FOR RECOMBINANT BROMELAIN EXPRESSED IN *E.COLI* BL21-A1

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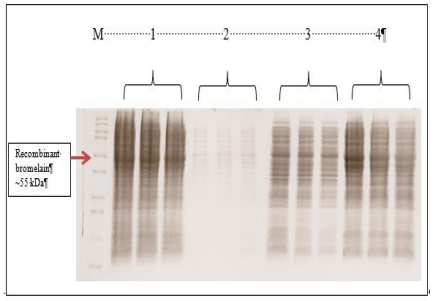
Graphical abstract



Abstract

The often-encountered problem such as protein degradation has driven various methods of cell lysis in obtaining recombinant protein post fermentation. In this paper, we compare methods such as homogenization, sonication, sonication with lysozyme and chemical lysis using B-PER reagent with lysozyme to extract the recombinant bromelain from *E. coli* BL21-AI. The sonication process is found to be the most effective in releasing recombinant bromelain without any pre-treatment. To obtain the high quality of protein from sonication method, the influence of different extractant buffer was investigated including phosphate buffer saline (PBS), PBS containing cysteine and EDTA (PBS-CE), and sodium phosphate buffer containing cysteine and EDTA (EB-CE). The highest specific enzyme activity was obtained when it was extracted with EB-CE buffer. Under sodium dodecyl sulfate polyacrylamide gel electrophoresis, the recombinant bromelain showed protein band at 55kDa. In conclusion, the sonication method with extractant buffer containing 100mM phosphate buffer pH7.0 with 15 mM cysteine and 2 mM EDTA (EB-CE) was shown to give high specific activity of recombinant bromelain.

Keywords: Recombinant bromelain, cell disruption, sonication buffer

1.0 INTRODUCTION

Bromelain is a proteolytic enzyme present in stem, fruit, peel, core, crown, and leaves of pineapple plants [1]. Bromelain is well known in multifaceted industrial applications such as food, textile, brewing, cosmetic, dairy and organic synthesis due to its ability to digest protein [2]. Bromelain gene was successfully cloned in various hosts including *E.coli* BL21-A1 [3], *E.coli* BL21 DE3pLysS [4] and *Brassica rapa* [5]. Previously, our research group had successfully cloned the stem bromelain from *Ananas comosus* into the pENTR/TEV/D-TOPO vector and subcloned into the pDEST17 expression vector. Later, the expression vector was transformed into *E.coli* BL21-A1 [3]. The positive transformants were cultivated in a batch process using an auto-induction media [6].

The recombinant bromelain is an intracellular enzyme and remains abundantly in the cytoplasm. Hence, the bacterial cell wall needs to be disrupted to release the target proteins in the soluble forms. However, there is lack of information on the cell disruption methods for *E.coli* host that expressed recombinant bromelain. Current findings by Othman, *et al*. [7] and George, *et al*. [4] only focused on the ultrasonication and repeated freeze-thawing in liquid nitrogen methods to disrupt *E.coli* BL21-A1 and *E.coli* BL21 DE3pLysS cells harboring recombinant bromelain respectively. Various cell disruption methods including sonication with lysozyme, homogenization, chemical lysis and freeze-thawing have been used to recover recombinant intracellular proteins [8-10]. The selection of the cell disruption method is dependent on the cost, the ease of operation and the ability of recombinant protein to retain its activity after disruption process. Besides, the presence of protein released after cell disruption process can be confirmed by SDS-PAGE electrophoresis and quantified via enzyme activity and total protein assays as quality control procedures [11].

Besides, some of the issues related to cell disruption protocol including the choice of buffer, presence of chelating agents, reducing agents and protease inhibitor must be taken into consideration because it depends on the properties of the cell type and biological activity of the target protein. Reducing agents such as cysteine and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) function to prevent oxidation of the protein [12]. Chelating agents such as ethylenediaminetetraacetic acid (EDTA), cyclohexane-1,2- diaminoetetraacetic acid (CDTA) and N hydroxyethylethylenediamine triacetic acid (HDTA) can inactivate the protease in the cell by sequestering the heavy metal such as Ca^{2+}, Fe^{3+} and Mg^{2+}. This paper describe the effectiveness of different cell disruption methods including homogenization, sonication, sonication with lysozyme and chemical lysis using B-PER reagent with lysozyme in the process of extraction recombinant bromelain from *E. coli* BL21-AI. The suitability of the extractant buffers employed in the cell disruption process were also delineated by comparing the specific enzyme activity in buffer saline (PBS), PBS containing cysteine and EDTA (PBS-CE), and sodium phosphate buffer containing cysteine and EDTA (EB-CE).

2.0 EXPERIMENTAL

Cultivation of Recombinant Bromelain

Recombinant bromelain clone was obtained with courtesy of Assoc. Prof Dr. Azura Amid, Kuliyyah of Engineering, IIUM Gombak. The cultivation of recombinant *Escherichia coli* BL21-A1 [3] was conducted in a batch process using an auto-induction media [6]. Approximately 10 mL of starter culture supplemented with 100 µg/ml ampicillin was inoculated with five single colonies of recombinant *E. coli* BL21-A1 harboring the bromelain gene. After growth for 16 hours at 37˚C and 300 rpm, 10 mL of starter culture was grown in 1 L of ZYM medium broth [13] supplemented with 100 µg/ml ampicillin. The culture was incubated in an incubator shaker for 12 hours at 37˚C and 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 minutes). The cell pellets were collected and stored at -20˚C until needed.

Cell Disruption

i) Homogenization

10 g of cell pellets were resuspended in 300 ml of phosphate buffer saline, pH7.4 and homogenized at pressure of 20 MPa and pump speed of 18 psi using laboratory pilot homogenizer (NS2006L, England) [14].

ii) Sonication

10 g of cell pellets were resuspended at a ratio of 1 g:5 ml of phosphate buffer saline, pH7.4 and undergo sonication process using a lab scale ultrasonic homogenizer (Sartorius, Germany). It was operated at 30 kHz frequency with 20 % amplitude. This equipment equipped with a 10mm diameter titanium needle probe to disrupt the cells. The disruption period was 1 minute with 60s intervals that repeated three times. The bursting cycle (pulse operation) was constant at 0.5s.

iii) Sonication with lysozyme

10 g of cell pellets were resuspended at a ratio of 1 g:5 ml of phosphate buffer saline, pH7.4 and treated primarily with lysozyme, 0.50 mg/ml in 10 minutes incubation at 4˚C [15-17]. Then, the samples will undergo sonication using lab scale ultrasonic homogenizer (Sartorius, Germany) at 20 % amplitude, 0.5s per cycle for 1 min. The process repeated three times with 60s off for each interval.

iv) Chemical lysis with B-PER reagent and lysozyme

10 g of cell pellets were resuspended at a ratio of 1 g:5 ml of phosphate buffer saline, pH7.4 and treated with lysozyme, 0.10 mg/ml (equivalent to1 ml of B-PER reagent) for 10 minutes on ice. Then, for every 1 g of cell pellet, 4 ml of B-PER reagent was added [18].

Preparation of Extractant Buffer

Three extractants were used for selected cell disruption method from the previous experiment. The extractants were as follows: i) phosphate buffer saline (PBS) pH7.4, ii) phosphate buffer saline with 15 mM cysteine and 2 mM EDTA (PBS-CE) pH7.4, iii)100 mM phosphate buffer with 15 mM cysteine and 2 mM EDTA (EB-CE) pH7.0. All samples were kept on ice during lysis process to avoid overheating and protein denaturation. The lysed cells were then centrifuged at 16000 x g for 45 minutes to obtain a clear supernatant and remove the cell debris. The lysate was analyzed

for total protein, enzyme assay, and SDS-PAGE electrophoresis.

Enzymatic Assay

The enzymatic assay was based on continuous spectrophotometric rate determination method. The proteolytic activity of recombinant bromelain was measured using Na-CBZ-L-lysine p-nitrophenyl ester (LNPE) as a substrate at 44˚C and pH 4.6. Initially, 260 µl of LNPE buffer consisting of 30 mM acetate buffer, 100 mM potassium chloride, and 1 mM L-cysteine, was mixed with 100 µl of enzyme solution. Then, 100 µl of 50 mM LNPE substrate was mixed using inversion, and the increase of the absorbance reading at 340 nm was measured for 5 minutes using a macro plate reader (Multiskan™ Go, USA). One unit of enzyme activity corresponds to the release of 1.0 µl of a p-nitrophenyl ester from the LNPE substrate per minute after reaction with bromelain [19]. The bromelain activity can be calculated using the equation below:

$$\frac{\left(\frac{\Delta A_{340nm\ test}}{min} - \frac{\Delta A_{340nm\ blank}}{min}\right) x\ 2.8\ xDF}{6.32\ x\ 0.1}$$

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

Protein Assay

The protein content of the cell lysate was measured based on the Bradford method using Bio-Rad Protein Assay (USA) at 595 nm with the macro plate reader (Multiskan™ Go, USA). Bovine serum albumin was used as standard protein assay [20]. All sample assays were carried out in triplicate.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The lysate from every lysis methods were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [21]. The lysate was separated on a 12% resolving and 4% stacking gel. 20 µl of lysate with sample buffer was loaded on the gel. After electrophoresis had been performed at 200 V, 700 mA for 37 minutes, the gels were stained with Coomassie blue.

Table 1 Comparison of different methods of cell lysis on enzyme activity(unit/ml), total protein (mg/ml) and specific activity of recombinant bromelain lysate. For each cell disruption method, 10 g of cell pellets were resuspended in phosphate buffer saline, pH7.4

	Homogenization	Sonication	Sonication with lysozyme	B-PER reagent with lysozyme
Enzyme activity (unit/ml)	0.008±0.001	0.11±0.012	0.11±0.008	0.08±0.007
Total protein (mg/ml)	1.00±0.032	5.75±0.023	7.31±0.354	4.99±0.828
Specific activity (unit/mg)	0.008±0.004	0.019±0.002	0.015±0.001	0.016±0.004

Data points represent mean value ± standard deviation (n = 3)

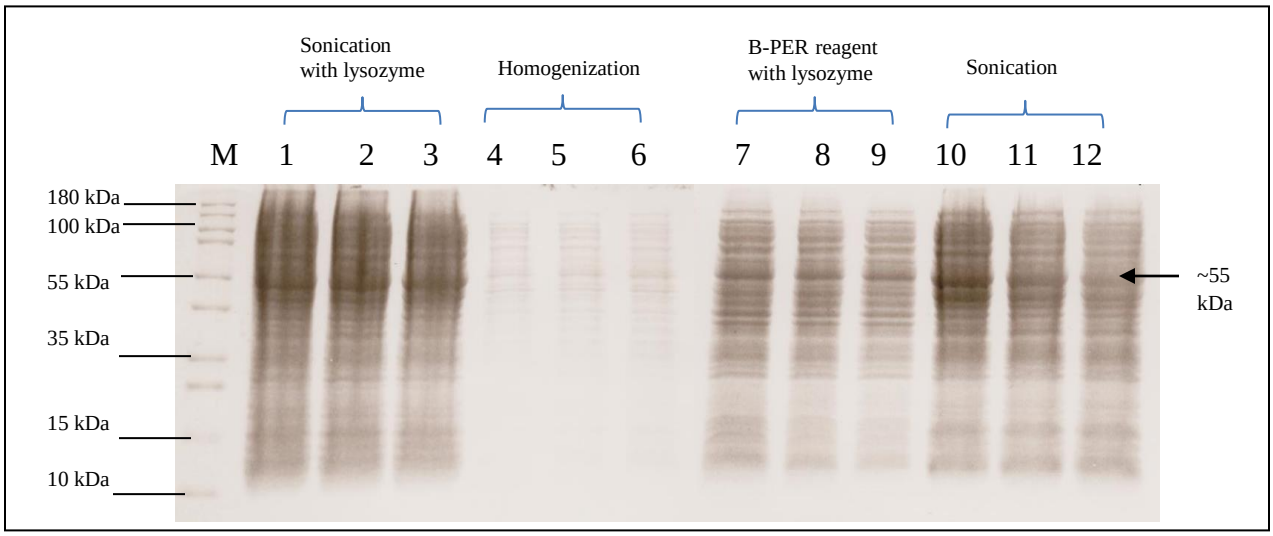


Figure 1 SDS-PAGE of recombinant bromelain lysate after cell disruption process. For each cell disruption method, 10 g of cell pellets were resuspended in phosphate buffer saline, pH7.4. The lane M, molecular mass markers in kDa; lane 1-3, replicates from sonication with lysozyme; lane 4-6, replicates from homogenization; lane 7-9, replicates from chemical lysis using B-PER reagent with lysozyme and lane 10-12, replicates from sonication process. For each replicate, 20 µl of lysate with sample buffer was loaded on a 12% resolving and 4% stacking gel. The electrophoresis had been performed at 200 V, 700 mA for 37 minutes. The gel was stained with Coomassie blue after electrophoresis. The arrow shows the position of the 55 kDa recombinant bromelain

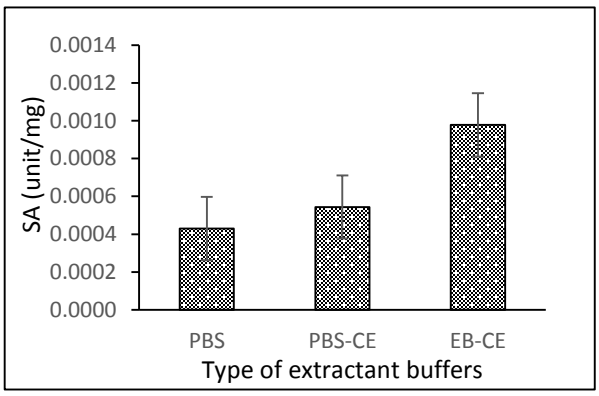


Figure 2 Influence of different extractant buffers for sonication method on the specific activity of recombinant bromelain lysates. The type of extractant buffer studied was as follows: PBS (phosphate buffer saline pH7.4); PBS-CE (phosphate buffer saline pH7.4 with 15 mM cysteine and 2 mM EDTA) and EB-CE (100mM phosphate buffer pH7.0 with 15 mM cysteine and 2 mM EDTA). Error bars shows standard deviation (n = 3)

3.0 RESULTS AND DISCUSSION

Among the cell disruption methods studied, the bromelain highest specific activity was obtained when the cell was disrupted by sonication in the absence of any pretreatment (Table 1). The sonication process widely used for different cell models such as recombinant β-galactosidase [22], *Agrobacterium tumefaciens* [23] and yeast [24]. On the contrary, the total protein content from sonication with lysozyme was high but resulted in low specific activity compared to the lysate from sonication and chemical approach using B-PER reagent with lysozyme. It is noted that, the specific activity is a ratio of enzymatic reaction to the total amount of protein. Therefore, when the lysozyme treatment was applied with sonication process, the total protein was increased due to the increase of proteins released from *E.coli* host cells including the endogenous proteases, resulting the decrease of specific activity of recombinant bromelain [8]. With respect to the specific activity, the chemical lysis using B-PER reagent with lysozyme is comparable to sonication method. Chemical lysis using B-PER reagent as a non-ionic detergent is a milder and an easier alternative to eradicate the need for harsh mechanical disruption procedure like homogenization and sonication [16]. This method can break the lipid barrier by hydrophobic binding to the cell wall surface and subsequently allowing the lysozyme to penetrate the peptidoglycan layers and released the periplasmic protein [11]. Even though the chemical lysis using B-PER reagent with lysozyme is attractive and specificity to the bacterial cell wall structure, however, it is restricted by the cost of the detergent and enzyme that lost into the extract. It is also not reusable and for large scale cell disruption process may not possible to be executed [8,25]. The homogenization method was shown to have the lowest total protein content and specific activity in comparison to the other cell disruption methods. This method is suitable for large-scale microbial cell disruption and involves harsh physical conditions [8]. The temperature rise about 2°C per 10 MPa is due to the adiabatic compression and inadequate cooling between multiple passes during homogenization can result to the recombinant bromelain denaturation [25]. To check the accuracy of quantitative method conducted, the supernatant of recombinant bromelain after lysis process was applied to SDS-PAGE as shown in Figure 1. The amount of recombinant bromelain released after lysis was comparable to the total enzyme present in the sample. The band intensity for sonication and sonication with lysozyme were higher than other cell disruption methods using homogenization and B-PER reagent with lysozyme. It was notable that the least recombinant bromelain released was from the homogenization process.

The result indicated that the most suitable method for recombinant bromelain extraction was the sonication technique. Further study was done to investigate the suitability of a range of lysis buffer during the sonication process. As presented in Figure 2, it was found that the highest specific activity was observed when the lysate was sonicated in the EB-CE buffer. The EB-CE has a high concentration of phosphate buffer that promote the maximum activity and maintain the pH. Kalisz, *et al*. [26] had reported that, the increasing phosphate buffer concentration from 50 mM to 1 M caused an increase of activity and thermostability of *P. amagasakiense* glucose oxidase. On the other hand, the more concentrated of buffer system, the higher its capability to stabilize the pH of the protein [27].

Both PBS-CE and EB-CE buffer caused high specific activity of recombinant bromelain when cysteine and EDTA were added as an additional extractants in contrast to PBS buffer alone. Proteases from papaya and pineapple peels showed maximum casein hydrolysis after the addition of reducing and chelating agents like cysteine and EDTA respectively [28,29]. These reducing agent reversibly alter the inactive form of the enzyme to the active form that provide protection against oxidation process [28]. L-cysteine is an amino acid that associated with the stability structure of various proteins. It acts as an antioxidant that prevent the disulfide bonds of protein reduced [30]. According to Murachi and Neurath [31], the presence of ethylenediaminetetraacetic acid (EDTA) besides cysteine, causes increased enzyme activity of stem bromelain. The highest bromelain activity was found in the pineapple peel extract using the extractant buffer containing EDTA and cysteine [29].

4.0 CONCLUSION

Sonication technique provides fast, effective, high activity retention of recombinant bromelain extraction. The EB-CE lysis buffer containing 100mM phosphate buffer pH7.0 with 15 mM cysteine and 2 mM EDTA showed the high specific activity of recombinant bromelain. The presence of phosphate buffer, cysteine, and EDTA maintain the pH system and biological activity of recombinant bromelain during the sonication process.

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