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M Nurjayadi[1]*, R Afrizal[1], D Hardianto[2] and K Agustini[2]

[1] Department of Chemistry, Mathematics and Science Faculty, Universitas Negeri Jakarta, Jakarta, Indonesia
[2] Laboratory of Agro and Biomedical Industry Technology Development, Agency for the Assessment and Application of Technology (BPPT), Indonesia

*muktiningsih@unj.ac.id

Abstract. Typhoid fever is an endemic disease in Indonesia. Prevention of typhoid fever can be done by administering vaccines. It is known that one of the raw materials for vaccines is a recombinant protein. This study aims to obtain information on the optimum conditions for purification of Salmonella typhi Fim-C recombinant protein with Ni-NTA resin as vaccine raw material. The three main stages of the purification process in this study were binding, washing, and elution of S. typhi Fim-C recombinant proteins. The binding and washing variations of recombinant proteins were carried out twice, four times and six times, while the elution process was carried out at imidazole concentrations of 200 mM, 250 mM, and 300 mM. Purification with a binding process four and six times gave almost the same intensity of S. typhi Fim-C protein bands. Whereas protein was elution at an imidazole concentration of 300 mM showed higher band instability. The results of characterization using SDS-PAGE and analysis using software ImageJ gel analysis showed that the longer the incubation time and the repetition of the binding process, the more protein bound to the resin. Furthermore, the more washing processes are obtained the purer proteins. Based on the data obtained it can be concluded that the purification of the S. typhi Fim-C recombinant protein was optimum at a four-time binding process, six times washing and a 300 mM imidazole concentration. These results are expected to be the basis for recombinant protein refining on a pilot scale and industry scale in better vaccine preparation.

1. Introduction

Typhoid fever occurs throughout the world, especially in developing countries with poor sanitation. Eighty percent of typhoid cases in the world come from Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan. Typhoid fever infects every year 21.6 million people (3.6/1,000 population) with a mortality rate of 200,000 / year [1]. One effort to prevent typhoid fever is through vaccination, one of which is a vaccine from recombinant proteins. Recombinant protein is a clinically relevant protein and can be produced on a large scale. Genes for specific proteins are cloned in vectors and expressed as proteins in certain organisms [2]. Recombinant vaccine is one of the alternatives offered to prevent typhoid fever. Recombinant vaccines are produced from a particular gene or protein that is potential in the pathogenesis mechanism and generated with genetic engineering technology. Those vaccines have several advantages, including: better protection for patients, higher purity, more specific immune

response, and can be produced on a large scale. In a previous study, it was successful in over expression the fim-C gene *Salmonella typhi* in host cells of *E. coli* BL21DE3 producing recombinant Fim-C Protein *S. typhi*. Immunogenicity test and toxicity test of *S. typhi* Fim-C protein as a result of over expression in mice showed that the protein could raise antibody titters, and was safe for these animals [3]. Based on the results obtained by Fim-C-*S. typhi* recombinant protein has the opportunity to be an alternative vaccine that is expected to have a better potential.

The essential stage that must also be considered in preparing recombinant proteins as candidate vaccines is the purification stage. Previous research has moreover carried out purification of Fim-C-*S. typhi* protein with standard procedures. However, it has not provided optimal yields [4-7]. Based on those studies, the optimization of Fim-C *S. typhi* recombinant protein using Ni-NTA resin with binding variations, washing (washing), and the concentration of imidazole for the elution process, to obtain pure protein with a higher yield is needed [8]. The results of this study are expected to be used as a basis for purification on a larger scale in the provision of vaccine.

2. Research method

The steps following in this study consist: (1) Production of *Salmonella typhi* Fim-C recombinant protein according to the pET system procedure [9-11]; (2) Characterization of *S. typhi* Fim-C recombinant proteins with SDS PAGE according to Bio-Rad Laboratories [12]; (3) Optimization of purification of *Salmonella typhi* Fim-C recombinant protein according to Thermo Scientific [13] (5) Measurement of *S. typhi* Fim-C recombinant protein concentration with Thermo Scientific BCA Kit reagent [14] and (6) Analysis of protein band density using *Image*J software gel analysis [15].

3. Results and discussion

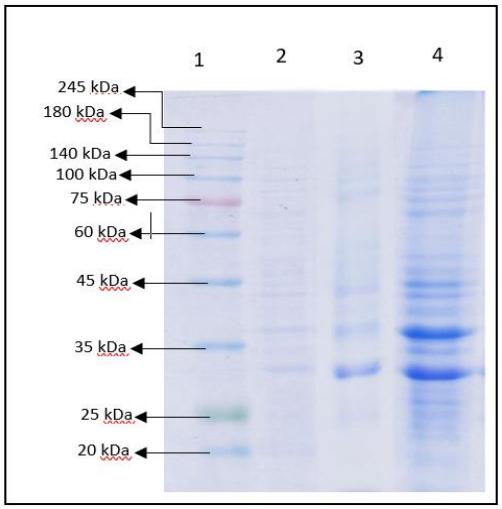
3.1. Production of recombinant protein Fim-C S.typhi

The production process of *S. typhi* Fim-C protein in the volume of 100 mL LBK (Luria Bertani-Kanamycin) media in 500 mL Erlenmeyer flask by following the pET-System procedure as carried out in previous studies resulted in cell pellets of 1.355grams [9-11]. The cell pellets obtained were dissolved with two mL of native equilibration buffer, and the color of the solution became ivory white. The next stage is the sonication process using a sonicator which functions to physically break down cells with waves at a frequency of 4 Hertz, so that cells can be lysed and protein extracts are obtained. During the cell breaking process, the suspension is placed in a container filled with ice water to prevent overheating, which can damage the protein. After sonication, the recombinant protein extracts dissolved in the Native Equilibration Buffer was centrifuged at a speed of 12,000 rpm for 15 minutes 4^{o}C. The supernatant produced is a Fim-C protein which is soluble in the cytoplasm. Whereas the resulting pellets are Fim-C proteins that form aggregates (inclusion bodies). The formation of the inclusion bodies protein is known because of the interaction between molecules in the hydrophobic regions of proteins during the folding process, and this is often found in the process of expression of proteins using *E. coli* as host cells [9,16]. In addition, inclusion bodies can as well as be formed because the amount of Fim-C protein produced from overexpression process is very large, resulting in the solubility of the Fim-C protein becoming smaller and forming aggregates. The formation of inclusion bodies is moreover, influenced by the type of protein, host cell type, expression level, a condition of cell growth and induction conditions [10,16-18].

3.2. Characterization of Fim-C S. typhi recombinant proteins with SDS PAGE

The isolation protein in the production phase was characterized using SDS PAGE 12%, and the results are presented in Figure 1. The results of SDS-PAGE in Figure 1 lane 2 has not seen any protein bands that have different thickness as target proteins due to the absence of IPTG, so lac repressor (*Lac* I) can stick to the promoter part of DNA and inhibit the expression process so that *S. typhi* Fim-C recombinant protein has not been formed. In Figure 1 lanes 3 and 4 there is a protein band with a higher band density at a molecular weight of ± 31 kDa according to the size of the target protein. This means that the over

expression process has occurred with the addition of IPTG as an inducer. The molecular weight of *S. typhi* Fim-C protein can be measured by comparing the migration distance of the protein in the gel with the logarithm of the curve of the Marker protein molecule as standard using ImageJ software [19]. The distance of gel migration from the marker protein electrophoresis as standard is presented in table 1. The relationship curve of Log BM (Molecular Weight) with the migration distance of protein markers is shown in Figure 2.



Lane 1: Marker protein 5 µL; Lane 2: 20 µL of *S. typhi* Fim-C protein before induction; Lane 3: 20 µL of Fim-C protein *S. typhi* after induction; Lane 4: 20 µL protein Fim-C Inclusion of Bodies of *S. typhi*.

Figure 1. The results of protein characterization with SDS PAGE.

Table 1. Data on the results of measurements of migration of protein markers.

Migration Distance (mm)	Molecular Weight (kDa)	Log Molecular Weight
5,34	245	2,39
7,07	180	2,26
9,07	140	2,15
11,60	100	2,00
14,95	75	1,88
19,20	60	1,78
25,60	45	1,65
34,29	35	1,54
43,80	25	1,40
48,41	20	1,30
55,74	15	1,18

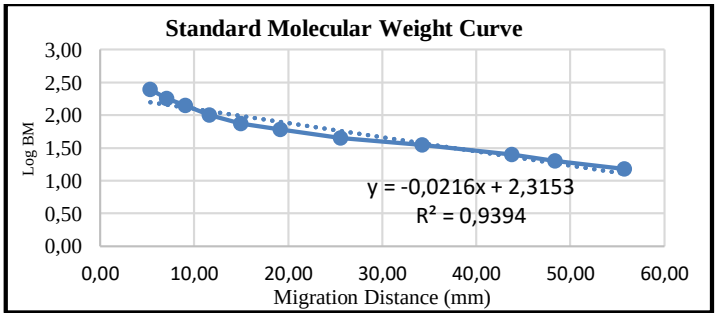


Figure 2. Migration distance curve of marker protein with Log molecular weight as standard.

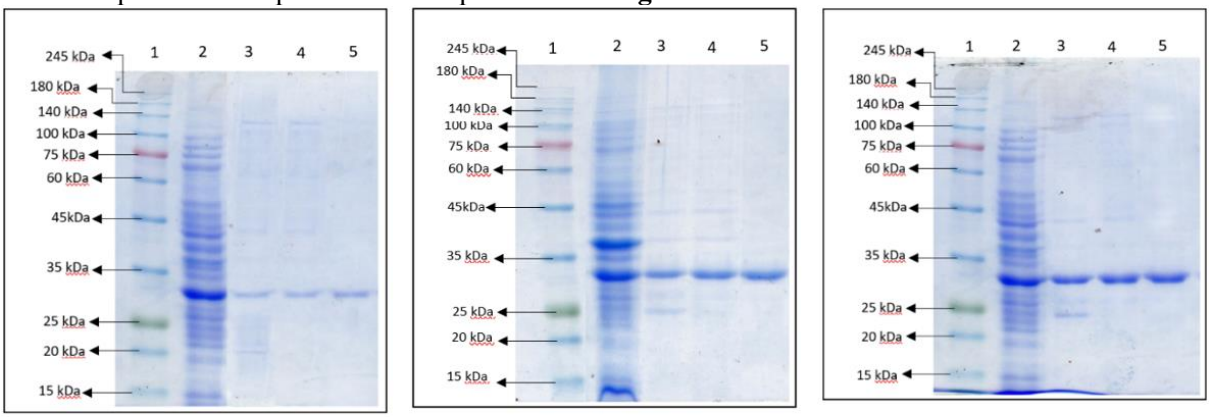
Based on these curves can be known $R^2 = 0.9394$ which mean it had good regression. The line equation y = -0.0216x + 2, 3153. The migration distance of *S. typhi* Fim-C protein is 38.14 mm, if it is entered into the value of x, get the value y = 1.49. The weight of protein molecules is measured by the formula

BM = 10^{y}. From this formula, it is proven that the molecular weight of the *S. typhi* Fim-C protein is 31.01 ≈ 31 kDa.

3.3. Optimization of purification using Ni-NTA resin

The protein optimization process is carried out at the stage of binding, washing and elution. Variations in the binding process are repeated twice, three times and four times. The repetition of the binding process is expected to have more interaction between Histidine amino acids contained in Recombinant proteins bound to Ni-NTA resin. After the protein is bound, non-target protein (impurity) which is not a *Salmonella typhi* Fim-C protein is washed using wash buffer with repeated variations twice, four times, six times. Repetition is done in the hope that more and more impurities will be washed away. In general, the level of contaminants in refining results is higher for samples from the eukaryotic expression system, because more and more endogenous proteins contain histidine consecutively or metal binders. In many cases, contaminants that participate in purification can be eliminated by optimization under stricter binding and washing conditions, for example, increasing the concentration of imidazole, increasing the salt concentration to 2 M NaCl, or adding detergent at low concentrations. However, the parameter with a very high impact on protein purity is the ratio of Ni-NTA resin to protein given tagging [16].

The last step is the elution of S. typhi Fim-C recombinant protein. Elution conditions for mild and flexible protein purification (100-500 mM imidazole, pH 5.9-4.5, or EDTA) [20]. Depending on specific proteins, elution buffer solutions require some optimization [19]. In this study elution buffer was optimized with imidazole with concentrations of 200 mM, 250 mM, and 300 mM respectively. The results of purification optimization are presented in **Figure 3**.



Lane 1: 5 µL marker protein (Smobio); Lane 2: 20 µL of 25 µg Fim-C protein; Lane 3: 20 µL of binding and washing of Fim-C S. typhi Protein two times; Lanes 4: 20 µL and washing of Fim-C S. typhi Protein four times; Line 5: 20 µL of binding and washing of Fim-C *S. typhi* Protein six times.

Figure 3. Characterization of protein purification with difference Imidazole concentration (A) 200mM, (B) 250 mM and (C) 300 mM.

Based on Figure 3, it can be seen that the more leaching there will be produced proteins that have fewer impurities. In washing twice (figure 3 A) there are still many impurities in the presence of non-target protein bands, if compared to four times washing (figure 3B) and six times washing (figure 3C).

Washing six times has fewer impurities than washing four times. This is because the more the washing process then the impurities will be lost carried by the wash buffer solution containing 25 mM imidazole. Low concentration imidazole in wash buffer can minimize nonspecific binding of resin [11,17].

In the binding process, it can be seen that the level of the thickness of the protein band increases with the number of binding processes. This is because the more binding is done, the binding of His-Tag possessed by *S. typhi* Fim-C recombinant protein is more optimally bound with Ni-NTA resin. In the elution process, it was seen that the thickness of the protein band increased with the increase in the

concentration of imidazole. Imidazole is competitive against histidine to bind to resin. Increasing the concentration of imidazole to more than 200 mM causes proteins that have histidine residues to dissociate because they are unable to compete to bind to the resin binding site. Imidazole has a structure similar to histidine amino acid so that it can shift the position of histidine binding to Ni-NTA resin and recombinant protein *S. typhi* Fim-C inclusion bodies as target proteins can be eluted [11]. The tags bind immobilized metal through the histidine imidazole ring so that the bound protein can be easily eluted using elution buffer with imidazole (100–250 mM) or low pH (4,5–6) [18].

3.4. The profile of protein band density using ImageJ

The profile of protein bands from electrophoresis with SDS-PAGE was analysed using ImageJ Gel Analysis. Measurements using ImageJ allow us to get a density profile, peak height and peak or volume intensity [15]. In this study the profile of Fim-C *S.typhi* recombinant protein before being purified was standardized and its relative density was considered to be the same as **1** (maximum value) [14], then compared with the protein profile after purification. The results of ImageJ Gel analysis are presented in tables 2-5:

Table 2. Data on the thickness measurement of *S. typhi* Fim-C protein bands prior to purification.

Area	Percentage	Relative density
11091,48	15,37%	1

Table 3. Measurement data for the thickness of purified Protein band with elution concentration of Imidazole 200 mM.

Binding	Area	Percentage	Relative density
Two times	1902,447	2,636%	0,171503
Four times	2114,326	2,93%	0,190631
Six times	2685,276	3,721%	0,242095

Table 4. Measurement data for the thickness of purified Protein band with elution concentration of Imidazole 250 mM.

Binding	Area	Percentage	Relative density
Two times	7270,095	10,074%	0,655433
Four times	8407,459	11,65%	0,75797
Six times	8747,874	12,122%	0,788679

Table 5. Measurement data for the thickness of purified Protein band with elution concentration of Imidazole 300 mM.

Binding	Area	Percentage	Relative density
Two times	9028,803	12,511%	0,813988
Four times	10285,581	14,253%	0,927326
Six times	10632,116	14,733%	0,958556

Area numeric data generated from analysis using ImageJ is basically an arbitrary number; this number can only be generated on quantifying a single gel peak at the time of measurement. This number does not have units like µg or other unit units [15]. From the analysis, using ImageJ can be calculated the relative density of the results of SDS-PAGE protein purification. It is seen that the density is relatively increasing along with the length of time and repetition of binding. Likewise, in the elution process, the higher concentration of imidazole used, the high its density will be. From these data, it can be seen that in the binding and washing process four and six times the thickness of the protein band and the impurity intensity were not much different. However, for efficiency, binding and washing 4 times can be chosen to give good results. While the imidazole concentration provides optimal results in elution conditions with an imidazole concentration of 300 mM.

3.5. Concentration measurement of recombinant Fim-C S. typhi extract protein

The recombinant Fim-C S. typhi extract protein was measured by the BCA method (bicinchoninic acid) using the Thermo ScientificTM BCA Kit Assay. Based on the law of Lambert Beer, the amount of absorbance is directly proportional to concentration. Standard BSA curve (bovine serum albumin) is made to find out the equation of the line obtained, so that the sample concentration can be known. The standard concentrations used include, 25 µg/mL, 125µg/mL, 250 µg/mL, 500 µg/mL, 750 µg/mL, 1000 µg / mL, 1500 µg/mL and 2000 µg/mL. Concentration measurements were carried out at a wavelength of 590 nm using an ELISA reader (curves not shown). The known line equation based on BSA standard solution curve is y = 0,0006x with a regression of 0, 9946. The protein before purification obtained ABS value of 0.203 so that the concentration of 3383.33 µg/mL was obtained. Data on absorbance, concentration, and yield of protein after purification are presented in table 6-8.

Table 6. Data measurement of protein absorbance values after purification.

Imidazole Concentration		Binding and Washing		
		Two times	Four times	Six Times
200 mM	Elution 1	0,075	0,080	0,101
	Elution 2	0,039	0,045	0,056
250 mM	Elution 1	0,182	0,193	0,205
	Elution 2	0,125	0,138	0,140
300 mM	Elution 1	0,210	0,229	0,235
	Elution 2	0,149	0,168	0,176

Table 7. Data measurement of protein absorbance values after purification.

Imidazole Concentration		Binding and Washing		
		Two times (µg/mL)	Four times (µg/mL)	Six Times (µg/mL)
200 mM	Elution 1	125	133,33	168,33
	Elution 2	65	75	93,33
250 mM	Elution 1	303,33	321,66	341,66
	Elution 2	208,33	230	233,33
300 mM	Elution 1	350	381,66	391,67
	Elution 2	248,33	280	293,33

Table 8. Protein yield data after purification.

Imidazole Concentration	Binding and Washing		
	Two times	Four times	Six Times
200 mM	11,23%	12,30%	15,33%
250 mM	30,25%	32,61%	33,98%
300 mM	35,37%	39,11%	40,49%

Based on the table 8 data, it can be seen that yield of S. typhi Fim-C Protein in the binding and washing process six times, and elution of imidazole concentration of 300 mM increased compared to previous Salmonella team studies [4-7] which yielded a percentage of each protein -as 22.8%, 24.03%, 25.44%, 29.75%.

4. Conclusion

The results of this study conclude that the Recombinant Fim-C *S.typhi* protein with molecular weight 31 kDa give higher results and pure in the variation of binding and washing as much as six times. The washing process effects to protein purity, washing six times shows purer protein results. The stronger the concentration of imidazole in the protein elution process, the more Fim-C *S. typhi* recombinant proteins are released from the resin so that the yield obtained is bigger. Elution at a concentration of 300 mM gives maximum results.

Acknowledgments
Thank you to the LPPM UNJ for providing research grants through the University's Featured Research Scheme 2018-2019. We also express our gratitude to the BPPT, Serpong, Agro and Biomedical Industry Technology Development Laboratory Team for the cooperation that has been carried out. To the entire Salmonella team for their hard work and contribution.

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