Capture and intermediate purification of human insulin precursor from *Pichia pastoris* culture using cation exchange chromatography

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**Abstract.** Purification has an important role in obtaining protein with a high degree of purity, particularly for human therapeutic purposes. Purification of pharmaceutical proteins requires several unit operations, involving chromatographic separation techniques. To increase purification efficiency and shorten process development, it is necessary to examine the chromatography system in performing a capture and intermediate purification in a single step. We use one of the best *Pichia pastoris* clones obtained from previous studies to produce a human insulin precursor (HIP). To capture and purify HIP from the culture, we clarified the cells through centrifugation and filtration. The supernatant was then loaded into a cation exchange column. Purification was carried on by two-step elution and monitored based on UV absorbance. Effects of loading concentration, flow rate, and pH of samples were evaluated. Fractions of elution were collected and verified by SDS-PAGE. Concentrations of HIP protein were quantified by using ImageJ by incorporating lysozyme as standard and reversed phase HPLC. Loading concentration and pH of the sample have an impact on the recovery. In this study, the best HIP recovery at ~47% resulted from purification with 10% volume of loading concentration and 500 ml loading volume.

1. Introduction

Insulin is a polypeptide hormone that contains 51 amino acids. It has chain A and chain B, which are constituted of 21 and 30 amino acids, respectively. This polypeptide is required for the treatment of diabetes, a serious disease characterized by hyperglycaemia that occurs due to the inability of the pancreas to produce adequate insulin to regulate blood sugar. The WHO report shows a significant increase in the number of adults living with diabetes, at more than 400% between 1980 and 2014 [1]. The economic impact was also projected to account for USD 0.9 trillion increase from 2015 to 2030 [2]. This situation forces the production of insulin at a more reasonable and competitive price.

The advancement of technology on DNA recombinant has shifted insulin production from the extraction of the porcine pancreas into various methods including two major ways. The first route, human insulin precursors (HIP) is produced in the form of an inclusion body by using *Escherichia coli*, followed by solubilization and refolding processes. The second route utilizes yeast as a host which can secrete IP in the culture supernatant, with *Saccharomyces cerevisiae* as the first predominant expression system. Production experiences on a large scale have revealed that both routes were economically viable [3–5]. Production of recombinant protein aims to achieve large amounts of functional protein with a high degree of purity and a reasonable cost. Therefore, purification has an
important role in the whole process in the manufacturing of biopharmaceutical products, particularly for human therapeutic purposes. The proportion of purification cost on the total manufacturing cost of recombinant proteins might account for nearly 50% until up to 90% [6].

Chromatography and electrophoresis are considered the most efficient separation methods based on the theoretical evaluation of separation science. However, for the purpose of preparative use and industrial application, only chromatography is feasible [7]. Purification of pharmaceutical proteins requires unit operations undertaken in sequential, involving chromatographic separation techniques. Chromatography steps play an important role and can be a bottleneck in the purification process of therapeutic proteins. The fact that the composition of culture media is complex creates a huge challenge for chromatography. Separation of target protein must be carried on from various impurities related to the host cell, process, product, and product-related variants [8]. Therefore, a typical purification utilizes multiple chromatography steps with different modes. The steps generally include capture, intermediate separation, and polishing. Several prepacked columns were designed to enable a one-step process of capture and intermediate [9]. The capture step is aimed to concentrate and stabilize target protein by removing critical contaminants such as proteases. Most remaining impurities such as other proteins, nucleic acids, endotoxins, and viruses, are separated at the intermediate step. The polishing step is purposed to remove trace amount impurities of feed to the minimum level.

The capture step, in which speed and capacity are the main focus, generally involves affinity chromatography (AF), immobilized metal ion affinity chromatography (IMAC), and ion-exchange chromatography (IEX) [10]. Ion-exchange chromatography becomes the second common method in the capture step, while affinity chromatography is the most favourable. In the capture of untagged protein, IEX is often selected because the columns have a high binding capacity, allow high flow rates, and are resistant to harsh conditions of column cleaning that may be required after purification of crude samples [10]. In the intermediate step, ion-exchange chromatography is frequently used, where lower load, smaller column, and a higher proportion of target protein are in practice [11]. Cation and anion exchange methods have been applied in the purification of insulin precursor, produced by P. pastoris [4,12–14] and E. coli [15]. Particularly in a purification process, factors that should be optimized include column size, buffer composition, buffer pH, protein-to-resin ratio, buffer additives, flow rate, and ionic strength. [16]. In this study, human insulin precursor is produced on a small scale and purified by using the cation exchange chromatography method. We use one of the best clones of the P. pastoris X-33 strain obtained from the previous study to produce human insulin precursor (HIP) in flasks and bioreactor[17]. Subsequently, purification of the HIP was conducted by incorporating capture and intermediate purification in one step while varying loading concentration, loading flow rate, and pH of loaded samples. The effect of parameters was determined by measuring the recovery of the HIP. The SDS-PAGE method followed by band intensity measurement using ImageJ software and quantification by reversed-phase HPLC was applied.

2. Materials and Methods

2.1. Chemicals, chromatography media, and equipment

Production of HIP in the bioreactor was undertaken by using the Eppendorf BioFlo-120 bioreactor system. An AKTA Avant 150 purification system (GE-Healthcare) was used to develop a purification process. Cation exchange chromatography prepacked columns were purchased from GE-Healthcare. Shimadzu HPLC with autosampler and UV-Vis detector was involved in HIP quantification. Commonly used chemicals for chromatography were purchased from Merck Millipore and Sigma-Aldrich. Production of Human Insulin Precursor in Flasks

Preculture of fermentation was prepared by inoculating a single colony of P. pastoris strain X-33/Mut into YPD medium [yeast extract 10 g/l, peptone 20 g/l, and dextrose 20 g/l], containing 1 µl zeocin per liter YPD. The culture was grown in a shaking incubator at 30°C and 250 rpm for 48 h. The cells were harvested by centrifugation at 3000 × g for 5 minutes at room temperature. The pellets were resuspended in 4 flasks containing 50 ml of BMGY medium [1% (w/v) yeast extract, 2% (w/v)
peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% (w/v) YNB, 4×10⁻⁵ % (w/v) biotin, and 1% (v/v) glycerol] for each. Incubation of culture was carried at 30°C and 250 rpm. After 24 hr, the cells were harvested, centrifuged as above, and the pellets were transferred into two flasks containing 200 ml of BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% (w/v) YNB, 4×10⁻⁵ % (w/v) biotin, 3% (v/v) methanol] for each. A 50% methanol was added every 24h, to the final concentration of 3% (v/v) for induction. The induction period was carried on until 72h. At the end of methanol induction, the culture broth was centrifuged at 10,000 rpm and room temperature for 10 minutes. Subsequently, the supernatant was collected stored at -20°C for purification and assays.

2.2. Production of Human Insulin Precursor in Bioreactor
Preculture of fermentation was prepared by inoculating a single colony of P. pastoris strain X-33/Mut+ into YPD medium [yeast extract 10 g/l, peptone 20 g/l, and dextrose 20 g/l], containing 1 µl zeocin per liter YPD. The culture was grown in a shaking incubator at 30°C and 250 rpm for 24 h. The cells were harvested by centrifugation at 3000 × g for 5 minutes at room temperature. The pellets were resuspended in flasks containing 100 ml of a basal medium [14] at a half concentration. After pH adjustment (pH = 5.0) with 12.5% ammonia, the culture was incubated at 30°C and 250 rpm. After 24 hr, the culture was used to inoculate bioreactor culture containing basal medium [14] at a half concentration with 40 g/l glycerol and 4.35 ml/l trace metal solution, at 1-liter working volume. The growth phase was carried on at a temperature of 28°C. Culture pH was maintained at pH 5.0 by the addition of 12.5% (v/v) liquid ammonia and 1 M H₂PO₄ automatically. The flowrate of dry air was adjusted at 2 liters of air per liter of media (vvm). Dissolved oxygen was set at a minimum of 30% and cascaded with agitation speed at the range of 300-500 rpm to ensure sufficient oxygen in the culture. Methanol induction was started after the growth phase reached 24h. A 100% methanol containing biotin and trace metal solution was added every 24h, to the maximum final concentration of 4% (v/v). Samples were collected at the start point of methanol induction and every period of 24h. The culture broth was centrifuged at 10,000 rpm and 20°C for 10 minutes. Subsequently, the supernatant was stored in separate tubes at -20°C for further assays.

2.3. Cation Exchange Chromatography
Culture supernatant from production in flasks was filtered through a 0.2 µm sterile filter and diluted in equilibrium buffer (buffer A; 20 mM sodium acetate pH 4.0). Subsequently, the second filtration was carried on by using a vacuum pump. The sample was passed through a 0.2 µm diameter of Sartorius filter. The filtrate was drawn using the vacuum pump. The sample was then loaded into a HiTrap SP-HP column (5 ml resin) after pH adjustment at 4.6 ml/min of flow rate. To evaluate the effect of loading concentration on HIP recovery, dilutions (supernatant volume/loading volume; ml) of 20/100 and 25/250 were applied. For loading volume of 100 ml, pH was adjusted to pH~5 and pH~4 by using HCl.

Culture supernatant from production in the bioreactor was filtered through a 0.2 µm sterile filter and diluted in buffer A. To evaluate the effect of loading concentration on HIP recovery, dilutions (supernatant volume/loading volume) of 50/100, 50/167, and 50/500 were applied. The sample was then loaded into a HiTrap SP-HP column (5 ml resin) at 4.6 ml/min. To evaluate the effect of loading flow rate, dilutions of 25/250 was applied, while flow rates were varied at 3.7, 4.6, and 5.5. ml/min.

Purification steps were conducted as follow: equilibrium step (buffer A, 4.6 ml/min, 3 column volume; CV); sample loading step; washing step (buffer A, 4.6 ml/min, 3 CV), elution-1 step [buffer B; 20 mM natrium acetate pH 4.0 containing 50% (v/v) of ethanol; 1.5 ml/min, 3.5 CV], elution-2 step [buffer C; 20 mM natrium acetate pH 4.0 containing 50% (v/v) of ethanol and 500 mM NaCl; 1.5 ml/min, 5 CV]. Column effluent was monitored at 280 nm and eluted fractions were collected for further analysis.

2.4. Analytical method
Purity and semi-quantitative determination of protein was carried on by using SDS-PAGE. The samples from peak containing fraction and unstained polypeptide SDS-PAGE molecular weight standards (BioRad) were diluted with Tricine Sample Buffer (BioRad) containing β-mercaptoethanol (Sigma Aldrich). The separated polypeptides in the gel were stained using Coomassie Brilliant Blue Solution (BioRad). After de-staining of the gel by using methanol and acetic acid solution in distil water, protein concentration was determined semi-quantitatively by using ImageJ [18]. A series concentration of lysozyme was used to establish a standard curve.

Reverse-phase HPLC (RP-HPLC) system, equipped with an autosampler and UV-Vis detector was also used to quantify insulin precursor. The samples were filtered (0.2 µm) and mixed with an equal volume of mobile phase A [0.02 % (v/v) TFA in MilliQ water], then loaded into Jupiter Phenomenex C4 (300 Å, 250 mm L x 4.6 mm ID, 5 µm). Elution was carried on by using mobile phase A and B [0.02 % (v/v) TFA in acetonitrile] with gradient method as follow: 0 - 20%B (0 - 6 min), 20 - 46%B (6 - 32 min). The flow rate and column temperature were maintained at 1 ml/min and 25°C, respectively. Monitoring of column effluent was conducted at 214 nm. Insulin from the bovine pancreas was used to establish a standard curve for quantification.

3. Results and Discussion

Semi-quantitative determination of HIP through SDS-PAGE and ImageJ software on culture supernatant resulted in estimated HIP concentrations of 1,239 and 1,364 mg/l from production in flasks and bioreactor, respectively. Hence, the highest loadings of the HIP, for loading volume of 500 ml was around 13.6 mg/ml resin. Recommended maximum loading capacity of Hi-Trap SP HP 5 ml resin was ~ 55 mg ribonuclease/ml resin. This lower loading capacity of ~25% maximum capacity was applied in this study to ensure that the HIP was below saturation of the column matrix. The fraction collector was set to accumulate eluent from elution-1 and elution-2.

3.1. Purification with different loading concentration of HIP

HIP from production in flasks. To study the effect of loading concentration, the same purification parameters which include purification stage, buffers, and gradient mode was applied. Sample loading concentrations were varied at 10 and 20% (supernatant volume/loading volume). Those corresponded to loading volume of 250 and 100 ml, respectively. Fraction collector was set to accumulate eluent from elution-1 (fraction 1 to 4) and elution-2 (fraction 5 to 10). Two major peaks were detected under UV absorbance at 280 nm wavelength (figure 1). SDS-PAGE analysis of the purified product from the experiment with a sample concentration of 20% (A1 and A2) showed that fraction 8 (A1.8, A2.8) resulted in obvious protein bands at the targeted molecular size (figure 2), while distinct protein bands were observed from fraction 3 (A3.3, A4.3) of experiment with 10% sample concentration (A3 and A4) (figure 3).
Figure 1. Profiles of elution peak from purification with different loading concentrations of 20% (A1, A2) and 10% (A3, A4) monitored by UV absorbance at 280 nm. The HIP samples were produced from flask cultures.

Figure 2. SDS-PAGE result of the purification experiment with loading concentration 20% (loading volume = 100 ml). The HIP samples were taken from flask culture after 72-hour of methanol induction (Feed). A1.3 and A1.8 = fraction 3 and 8 from experiment A1, respectively; A2.3 and A2.8 = fraction 3 and 8 from experiment A2, respectively.

Figure 3. SDS-PAGE result of the purification experiment with loading concentration 10% (loading volume = 250 ml). The HIP samples were taken from culture after 72-hour of methanol induction (Feed). A3.3 and A3.8 = fraction 3 and 8 from experiment A3, respectively; A4.3 and A4.8 = fraction 3 and 8 from experiment A4, respectively.
HIP purification from production in the bioreactor. The effect of loading concentration was also evaluated in the purification of HIP produced from bioreactor which differed mainly in the scale and media composition of culture. The purification involved the same purification stage, buffers, and gradient mode. Sample loading concentrations were varied at 10, 30, and 50% (supernatant volume/loading volume). Those corresponded to loading volume of 500, 167, and 100 ml, respectively. The fraction collector was set to accumulate eluted peak from elution-1 and elution-2. Two major peaks were detected under UV absorbance at 280 nm wavelength. SDS-PAGE results of the purified product of different loading concentrations from experiment A5, A6, and A7 were shown in figure 4. Results from RP-HPLC also showed concentration and peak profile which agreed to the SDS-PAGE results (figure 5).

The result of the semi-quantitative determination of IP concentration using ImageJ was presented in Table 1. It is indicated that higher concentration which corresponded to a higher protein-to-resin ratio resulted in slightly lower recovery of purified IP. Compared to experiment A1 and A2, experiment A3 and A4 employed lower IP loading and concentration. The effect of loading concentration was more obvious from the purification result of HIP produced in the bioreactor. The HIP recovery from experiment A7 with 10% sample concentration was the highest (~ 47%) compared to experiment A5 and A6 which employed higher loading concentration. By using the same flow rate of sample application (4.6 ml/min), a lower loading concentration might improve the binding efficiency of protein onto the resin. This might be due to lower binding competition between feed components which have higher and lower retention. Higher loading capacity could also result in a broader peak (figure 1). Besides, product recovery and purity might be reduced [8]. It is also important to consider that consecutive loading until reaching column saturation is recommended in the presence of more tightly bound impurities. Overloading conditions might limit the binding of protein in this case [15]. Moreover, recoveries of the HIP (table 1) produced from basal medium (A8, A9, A10) were higher than that form rich (more complex) medium (A3, A4) at the same loading concentration and volume. It is predicted that the more complex impurities in the rich medium increased competition to bind onto the resin, especially during the sample loading step.
Figure 5. Result of RP-HPLC of experiment A5, A6, and A7. (a) HIP concentration (mg/l) of loaded sample and peak fraction; Feed = loaded sample; Frac-1 = peak fraction of elution-1; Frac-2 = peak fraction of elution-2. (b) HPLC chromatogram profile of experiment A7 (loaded sample). (c) HPLC chromatogram of experiment A7 (peak fraction of elution-1). (d) HPLC chromatogram of experiment A7 (peak fraction of elution-2).

3.2. Purification with a different loading flow rate
The HIP from bioreactor culture (basal medium) is used to study the effect of loading flow rate. The samples at 10% concentration (v/v) were loaded at 250 ml loading volume with a variable flow rate of 3.7, 4.6, and 5.5 ml/min. The fraction collector was set to accumulate eluted peak from elution-1 and elution-2. The elution peaks were detected under UV absorbance at 280 nm wavelength. SDS-PAGE results of the purified product of different loading flow rates from experiment A8, A9, and A10 were illustrated in Figure 6. Altering loading flow rate by ~ 20% below and above 4.6 ml/min did not result in a significant difference in recovery as shown in table 1. A higher flow rate is favourable to reduce process time. However, further investigation is necessary to balance a higher flow rate for shorter purification time with high HIP recovery.
Figure 6. SDS-PAGE result of purification experiment with loading flow rate 5.5 ml/min (A8), 3.7 ml/min (A9), and 4.6 ml/min (A10). The HIP samples were taken from bioreactor culture. Feed = loaded sample of experiment A8, A9, A10; A8 = elution-2 peak fraction of A8; A9 = elution-2 peak fraction of A9; A10 = elution-2 peak fraction of A10.

Table 1. Recovery of Human Insulin Precursor from different loading concentrations and volumes.

| Experiment | Loading Concentration (sample vol./total loading vol.); in ml | HIP in Loaded Sample (mg) | HIP in fraction (mg) | Recovery |
|------------|-------------------------------------------------------------|---------------------------|----------------------|----------|
| A1<sup>a</sup> | 20/100                                                      | 30.3                      | 3.7<sup>b</sup>      | 12.2%    |
| A2<sup>a</sup> | 20/100                                                      | 30.3                      | 4.8<sup>b</sup>      | 15.8%    |
| A3         | 10/250                                                      | 24.0                      | 4.2<sup>c</sup>      | 17.4%    |
| A4         | 10/250                                                      | 24.0                      | 3.7<sup>c</sup>      | 15.5%    |
| A5         | 50/100                                                      | 68.2                      | 1.67<sup>d</sup>     | 2.46%    |
| A6         | 30/167                                                      | 68.2                      | 5.23<sup>d</sup>     | 7.67%    |
| A7         | 10/500                                                      | 68.2                      | 32.08<sup>d</sup>    | 47.06%   |
| A8         | 10/250                                                      | 34.1                      | 14.35<sup>e</sup>    | 42.08%   |
| A9         | 10/250                                                      | 34.1                      | 14.90<sup>e</sup>    | 43.71%   |
| A10        | 10/250                                                      | 34.1                      | 14.78<sup>e</sup>    | 43.35%   |

<sup>a</sup>pH was adjusted to pH ~5 for experiment A1 and pH ~4 for experiment A2 by using HCl.
<sup>b</sup> fraction 8 of elution-2.
<sup>c</sup> fraction 3 of elution-2.
<sup>d</sup> peak fraction of elution-1 and elution-2.
<sup>e</sup> peak fraction of elution-2.

3.3. Purification with different pH of the loaded sample

The effect of pH of the loaded sample on purification recovery was evaluated at loading volume of 100 ml. pH of diluted sample was adjusted to pH ~5 and pH ~4 by using HCl, for experiment A1 and A2, respectively. The lower pH of the sample (pH ~4) resulted in a slightly higher recovery of the HIP than that of pH ~5 (table 1). Figure 7 illustrates the pH profile during the purification process. The pH of the sample affects the pH profile at the sample application step.

The isoelectric point (pI) value of proteins is often used to predict adsorption conditions for cation-exchange chromatography. The protein net charge is positive at pH lower than its pI value when adsorption on a cation exchanger should occur [19]. Dissociation of a charged group on protein surface is affected by the pH of the sample solution. This might also influence the dissociation of ionic groups on the adsorbent surface. As a result, protein retention and protein-ligand interaction are altered. It is indicated that in cation exchange, electrostatic interaction was a dominant factor for protein binding. Retention factors of proteins might decrease with an increase in buffer pH [20]. Besides, insulin is a relatively hydrophobic peptide with a limited solubility close to the pI of 5.4 [21]. At sample pH closer to its pI, binding of insulin precursor might be lower, resulting in a lower recovery.
Figure 7. Profile of pH during purification of the HIP with a loading volume of 100 ml. pH was adjusted to pH ~5 for experiment A1 and pH ~4 for experiment A2 by using HCl.

4. Conclusions
The result indicates that capture and intermediate purification can be done in one pre-packed cation exchange column. The effect of loading concentration, loading flow rate, and pH of loaded samples was evaluated in this study. Loading concentration below the binding capacity of column resin and pH of the sample have an impact on the recovery of the purified HIP. The recovery increases with the decrease in loading concentration. At higher pH, closest to pI of the HIP, the recovery is likely low. In this study, the best HIP recovery at ~47% resulted from the purification of bioreactor culture with 10% volume of loading concentration and 500 ml loading volume. HIP recovery of bioreactor products that utilize minimal medium tends to be higher than that of flask culture, which contains a complex medium. Provided that HIP recovery is still relatively low (< 50%), further investigation is required to optimize other factors such as salt and ethanol concentration in the buffer to increase the recovery.

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