A Rapid Method for Determining the Concentration of Recombinant Protein Secreted from Pichia pastoris.

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Abstract Pichia secretive expression system is one of powerful eukaryotic expression systems in genetic engineering, which is especially suitable for industrial utilization. Because of the low concentration of the target protein in initial experiment, the methods and conditions for expression of the target protein should be optimized according to the protein yield repetitively. It is necessary to set up a rapid, simple and convenient analysis method for protein expression levels instead of the generally used method such as ultrafiltration, purification, dialysis, lyophilization and so on. In this paper, acetone precipitation method was chosen to concentrate the recombinant protein firstly after comparing with four different protein precipitation methods systematically, and then the protein was analyzed by SDS-Polyacrylamide Gel Electrophoresis. The recombinant protein was determined with the feature of protein band by the Automated Image Capture and 1-D Analysis Software directly. With this method, the optimized expression conditions of basic fibroblast growth factor secreted from pichia were obtained, which is as the same as using traditional methods. Hence, a convenient tool to determine the optimized conditions for the expression of recombinant proteins in Pichia was established.

1. Introduction
Pichia expression system is a kind of widely used eukaryotic expression system for recombinant proteins[1,2].It is of advantages of higher eukaryotic expression system such as protein processing, protein folding, and posttranslational modification, higher expression levels, higher density fermentation and suitable for secretive protein expressions [3]. Because of few native proteins secreted, it is also easy to separate and purify the heterologous proteins.As the secreted heterologous protein comprises the vast majority of the total protein in the medium, it will reduce the procedures of purification therefore reduce the loss of the target protein during purification. Therefore, P. pastoris was widely used as a tool for the secretive expression of recombinant proteins [4-6].
However, protein expression level in P. pastoris is generally low in the initial expression experiments. Only after the expression conditions were optimized, protein yields will reach the peak level. Moreover, usually protein amount should be determined through a series of protocols such as ultrafiltration, concentration, separation and purification by chromatography, dialysis, lyophilization, and quantitation analysis. So it is necessary to set up a rapid analysis method for the secretive heterologous protein during the optimizing process of protein expression.

Hence, combining with the Automated Image Capture and 1-D Analysis Software using bovine serum albumin (BSA) as a control, an optimized method of protein precipitation and concentration was set up after systematically comparing with other four protein precipitation methods. It is a rapid, convenient and effective method for low concentration protein assay. Then the supernatant protein assay of basic fibroblast growth factor (bFGF, FGF2) which was secretively expressed in Pichia pastoris, was determined by this optimized assay method. The results were compared with the traditional method. bFGF has a lot of biological effect in different cells and organs, and has been applied in the therapeutics of wound healing[7-8]. So it is important to improve the recombinant bFGF yield for both clinical application and mechanistic investigations. The optimized assay method in this paper has solved a difficult analysis problem and may be also suitable for other secretive expression system of heterologous protein expression.

2. Materials and Methods

2.1. Materials

Peptone and yeast extract was purchased from OXOID. Yeast Nitrogen Base, Amino acid Mix, D-Biotin, and Dextrose (D-Glucose) were obtained from Invitrogen. Standard hbFGF was purchased from national institute for the control of pharmaceutical and biological products. BSA was got from BBI. Tris base, Tricine, and SDS were purchased from BioRad. Acrylamide, N,N’-Methylenebisacrylamide, Glycine, Ammonium persulfate, and TEMED were purchased from Amresco. Methanol, ethanol, and acetone were all obtained from Beijin Chemistry Reagent Factory. Pichia pastoris strain which expressed bFGF was constructed by our lab. Heparin affinity column was purchased from GE healthcare. All other chemicals and solvents used in the study were analytical grade.

2.2. Methods

2.2.1. Protein precipitation Methods.

2.2.1.1. 10% TCA/acetone precipitation.

10% TCA/acetone precipitation was based on the method described before Lu [9].1ml of sample protein (0.5mg/ml BSA) was added into 8-10 volume of ice-cold acetone containing 10% (w/v) TCA. After vortexed the mixture was incubated at -20°C for 2 h. The sample was centrifuged at 15,000 rpm at 4°C for 15min. The protein pellet was washed three times with 3-4 volume of ice-cold acetone, dried at room temperature for about 30min, and dissolved in 100ul double distilled water.

2.1.2.2. TCA precipitation.
TCA protein concentration was carried out accordingly Sambrook J et al [10]. 100% (w/v) TCA was added to 1ml of sample (0.5mg/ml BSA) and the final TCA concentration was 20%. The mixture was stored at -20°C for 2 h. The protein pellet was collected by centrifugation at 15,000 rpm at 4°C for 15min and washed with 3-4 volume of ice-cold acetone for three times, dried at room temperature for about 30min and then dissolved in 100ul double distilled water.

2.1.2.3. Alcohol precipitation.
Alcohol protein concentration method was derived from Xu et al [11]. 1ml of sample protein (0.5mg/ml BSA) was mixed with 8-10 volume of ice-cold alcohol, proteins was left to precipitate at -20°C for 2 h, then centrifuged at 15,000 rpm at 4°C for 15min. Pellet was washed three times with 3-4 volume of ice-cold alcohol, dried at room temperature for about 30min and then dissolved in 100ul double distilled water.

2.1.4. Acetone precipitation.
Acetone precipitation method was based on the method described before Lodish H et al [12]. 1ml of sample protein (0.5mg/ml BSA) was added in 8-10 volume of ice-cold acetone, after vortexed the mixture was stored for at -20°C 2 h. It was then centrifuged at 15,000 rpm at 4°C for 15min. The final protein pellet was washed with 3-4 volume of ice-cold acetone three times, dried at room temperature for about 30min and then dissolved in 100ul double distilled water.

2.2.1.5. Determination of protein concentration.
Protein concentrations of all samples were determined according to Bradford assay using a commercial dye reagent with BSA as a standard and MiliiQ water as blank and each assay repeated three times. Protein yield was calculated using the followed formula: Protein yield=Protein concentration × Dilution multiples × Sample volumes.

2.2.1.6. SDS-PAGE and Tricine-SDS-PAGE.
SDS-PAGE analysis was performed using 12.5% gel according to the method of Laemmli[13], and the method of Simpson R et al[14]. Proteins obtained with different precipitation methods were separated by polyacrylamide gel electrophoresis (SDS-PAGE) or tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE), and stained with Coomassie Blue G-250.

2.2.1.7. Image scan and analysis.
The gels were scanned by Image Master Scanner 6.0 and the image was analyzed with BIO-RAD Quantity One 4.3.1 software.

2.2.2. Verification of the bFGF amount in the fermentation supernatant of P.pastoris.
The fermentation supernatant was harvested by centrifugation at 15000 r/min for 20 min at 4 °C. 500mL of supernatant was precipitated by the above methods (2.2.1.2, 2.2.1.3, 2.2.1.4) respectively. Then protein pellets were resuspended and analyzed by Tricine-SDS-PAGE. The gels were scanned and analyzed according to 2.2.1.7.

2.2.3. Comparison with affinity chromatography and acetone precipitation methods on the effects of bFGF yields induced by methanol at different concentration.
Fermentation supernatants of bFGF which was induced by 0.4% and 0.8% methanol were purified with heparin-Sepharose Fast Flow column by Fast Purification Lipid Chromatography (FPLC). The column was washed with 50 ml of buffer A (20mM Tris-HCl, pH 7.4, 1mM EDTA) and then with buffer A containing 0.5 M NaCl. The recombinant 18-kDa human bFGF was eluted with buffer A containing 3 M NaCl. The elution was dialyzed against 4 liters of buffer B (20mM Tris-HCl, pH 7.4, 150 mM NaCl), and lyophilized. The protein concentration was determined with Bradford protein assay (2.2.1.5). The same sample was precipitated by acetone and the protein pellet was resuspended and analyzed by Tricine-SDS-PAGE. The gels were scanned and analyzed according to 2.2.1.7.

3. Results and Discussion

3.1 Screen and verification of the protein concentration methods

![Figure 1](image1.png)  ![Figure 2](image2.png)

**Figure 1.** Tricine-SDS-PAGE of four protein concentration methods. 1. 10% TCA/acetone precipitation method; 2. TCA precipitation method; 3. Ethanol precipitation method; 4. Acetone precipitation method; 5. BSA; 6. Marker.

**Figure 2.** Tricine-SDS-PAGE of three kinds of protein concentration methods. 1. TCA precipitation method; 2. Ethanol precipitation method; 3. Acetone precipitation method; 4. Pure bFGF obtained by HPLC; 5. Marker.

| Table 1. Protein yields from four protein concentration methods (n=3). |
|---------------------------------------------------------------|
| Concentration methods | 10% TCA/acetone precipitation | TCA precipitation | Ethanol precipitation | Acetone precipitation |
|------------------------|--------------------------------|-------------------|----------------------|----------------------|
| Protein yield (mg)     | 242 ± 2.18                     | 368 ± 3.45        | 393 ± 2.94           | 440 ± 5.89           |

| Table 2. Protein yields from different protein concentration methods (n=3). |
|---------------------------------------------------------------|
| Concentration methods | TCA precipitation | Ethanol precipitation | Acetone precipitation |
|------------------------|-------------------|----------------------|----------------------|
| Protein yield (mg)     | 26 ± 0.33         | 40 ± 0.83            | 78 ± 1.41            |
Protein bands using four protein concentration methods were obtained by SDS-PAGE. The densities of protein bands were assayed using BIO-RAD Quantity One 4.3.1 software (Figure1, Table1). The protein band from acetone precipitation was the thickest and its yield is always the highest. This indicated that protein could be precipitated completely by acetone. Although the protein yield from alcohol precipitation was also higher, it still has significant difference compared with acetone precipitation. As a superior polarity organic solvent, alcohol has better water-solubility and it could not completely precipitate proteins, which resulted in certain amount loss of protein. The protein band and yield obtained using 10% TCA/acetone was the weakest and lowest. There are two reasons for this result: Firstly, 10% TCA/acetone may not completely precipitate all the proteins, and secondly 10% TCA/acetone destroyed the protein structure and reduced the resolution. Although the concentration of TCA in TCA precipitation was increased to 20%, no obviously improvement was obtained according to Figure1 and Table1. Hence, acetone precipitation method got the highest protein yield and followed with the alcohol, and TCA precipitation. However, 10% TCA/acetone revealed the lowest protein yield. The conclusion was verified on the supernatant of bFGF which was secreted from P. pastoris, in which higher protein yield methods (acetone precipitation, alcohol precipitation and TCA precipitation method) were applied. Data were shown in Figure2 and Table2. The protein band obtained from acetone precipitation was the thickest, which further illustrated that as a low polarity dissolvent, acetone can precipitate the water-solubility protein commendably. In addition, this method needs less reagents and the operation is simple with better stability. It is an adaptive protein precipitation method for the low concentration protein, especially for secretive recombinant proteins.

3.2 Effects of concentration methods on bFGF yields from different methanol induction

| Methanol Concentration (%) | Protein yield (mg) |
|---------------------------|--------------------|
|                           | affinity chromatography | acetone precipitation |
| 0.4                       | 440±4.55            | 418±5.23              |
| 0.8                       | 780±5.82            | 753±2.04              |

The fermentation supernatants of bFGF which was induced by different concentration of methanol, were purified by traditional affinity chromatography, followed by dialysis and lyophilization. Proteins were assayed, with yields at 440±4.55mg and 780±5.82 mg, respectively (Data shown in Table 3). Likewise, the samples were analyzed by acetone precipitation and image analysis, resulting in protein yields at 418±5.23mg and 753±2.04 mg, respectively. These data indicated that 0.8% methanol is better than 0.4% during inducing expression process, which means that the optimized assay (acetone precipitation and image analysis) can reach the same analysis result with the traditional one. In addition, the combination of acetone precipitation and image analysis is a rapid, time saving and less consuming assay.

4. Conclusion

The levels of majority target proteins at initial period in secretive expression systems were low, so how to determine the target protein level rapidly and accurately remains a difficult
problem. Acetone precipitation combined with image analysis was used in this study which revealed a rapid analysis of protein amount. Moreover, the conclusion that acetone precipitation assay has the same results as the traditional chromatography assay on the analysis of bFGF yields under different inducing conditions in P. pastoris indicated that optimized assay is a rapid, convenient, accurate, and less consuming method, which will provide a powerful tool for all secretive expression systems including P. pastoris.

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