Polycistronic Expression of Human Platelet Factor 4 with Heparin-Neutralizing Activity in Escherichia coli

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Human platelet factor 4 (hPF4) was evaluated as a clinical alternative to protamine for heparin neutralization, a protector against radiation injury and an anti-neoplastic. To achieve high-level expression of hPF4, expression vectors pET-28a(+)-nf PF4 (n = 4, 5, 6) containing n tandem repeats of PF4 were constructed and transformed into the Escherichia coli BL21(DE3) strain. A higher expression level, about 45% of the total proteins (TP), was obtained for E. coli BL21(DE3)/pET28a(+)–nf PF4 (n = 4, 5, 6). The purified His-PF4 protein was further identified by cleavage with enterokinase and MS, and its heparin-neutralizing activity was determined by colony formation assay. This study represents a novel approach to large-scale production of PF4 in E. coli, one that might be applied to large-scale production of PF4 protein for possible clinical applications. It also provides theoretical points for the expression and purification of other small-molecule peptides.

Key words: platelet factor 4; tandem expression; small pharmaceutical proteins; neutralizing heparin; anti-neoplastic

Platelet factor 4 (PF4), a secretory peptide from α-storage granules, binds strongly to heparin and other glycosaminoglycans, as well as to heparan sulfate on storage granules, binds strongly to heparin and other glycosaminoglycans, as well as to heparan sulfate on storage granules, binds strongly to heparin and other glycosaminoglycans, as well as to heparan sulfate on storage granules, binds strongly to heparin and other glycosaminoglycans, as well as to heparan sulfate on storage granules, binds strongly to heparin and other glycosaminoglycans. Currently, the commercial production of recombinant hPF4 is based primarily on the expression of a single copy in E. coli, which achieves expression levels of only about 25% of total proteins (TP). One of the possible reasons is that the relative molecular mass of PF4 is small and hence it can easily be degraded by host proteases. In recent years, tandem expression is widely used for high expression of small molecular peptides, which overcomes low expression level, instability, and other shortcomings. Monocistronic expression of the tandem repeat gene is used mostly in the production of small proteins, which are expressed as concatemers with proteolytic or chemical cleavage sites between the monomeric units. In this method, cleaving the large concatamers is difficult and extra amino acid residues are left at the N- or C-termini of the small proteins following cleavage. However, the application of small pharmaceutical proteins can be negatively affected by the presence of extra amino terminal residues. Polycistronic expression of the tandem repeat gene, a relatively novel method, can prevent these problems. The present study combined this strategy and fusion technology to express hPF4. This is a novel way to achieve highly efficient expression and convenient purification of hPF4 and similar small peptides.

Materials and Methods

Strains, plasmids, and reagents. E. coli strains, DH5α and BL21(DE3), plasmid pET28a(+), and the erythroleukemia cell line (HEL) were maintained at our laboratory. Primer synthesis and DNA sequencing were performed by Beijing AuGCT DNA-SYN Biotechnology (Beijing, China). The oligonucleotides used are listed in Table 1.

Synthesis of the PF4 gene. Several codons of human PF4 genes were replaced to optimize the expression of human PF4 in E. coli. The optimized PF4 genes were amplified by overlapping PCR. Sc., fragment I was amplified by PCR using primers P1/P2, then fragment II was amplified by PCR using fragment I as template and primers P3/P4, and finally the PF4 gene was amplified by PCR using fragment II as template and primers P5/P6. The amplification products were subcloned into pEASY-T3 cloning vector (TransGen Biotech, Beijing, China) to generate pEASY-T3-PF4, and this was confirmed by DNA sequencing.

Construction of pET28a(+)–nf PF4 (n = 1, 2, 3, 4, 5, 6) expression vectors. The series unit (EcoRI Site—XhoI Site—SD Sequence—
ATG—His-Tag—EK Site—hPF4—TAATAA—SalI Site) was amplified by overlapping PCR. Sc., fragment A was amplified by PCR using primers P7/P8 with template pEASY-T3-PF4, then fragment B was amplified by PCR using primers P9/P10, and finally the series unit was amplified by PCR using fragments A and B as primers. The amplification products were subcloned into pEASY-T1 cloning vector (TransGen Biotech, Beijing, China) to generate pEASY-T1-PF4, and this was confirmed by DNA sequencing.

The first series unit (EcoRI Site—ATG—His-Tag—EK Site—hPF4—TAATAA—SalI Site) was amplified by PCR using pEASY-T1-PF4 as template and primers P7 and P11. Compared with the series unit, the first series unit lacked a restriction enzyme site (Xhol) and the SD sequence present in expression vector pET28a(+) was deleted. The amplification products were subcloned into pEASY-T1 cloning vector to generate pEASY-T1-f PF4, and this was confirmed by DNA sequencing.

The large fragment of vector pEASY-T1-PF4 was recycled after it was digested by EcoRI and XhoI (Takara, Dalian, China), and then ligated with T4 DNA ligase (TransGen Biotech, Beijing, China) with a single PF4 gene fragment obtained from pEASY-T1-f PF4 digested with EcoRI and SalI (Takara, Dalian, China) (Fig. 1). The cloning vector, which contained two tandem repeat PF4 genes, was named pEASY-T1-2f PF4. Construction of pEASY-T1-nf PF4 was accomplished as described above.

The large fragments of expression vector pET28a(+) were recycled after they were digested by EcoRI and SalI. An n × PF4 DNA fragment (n = 1, 2, 3, 4, 5, 6) was cloned from vector pEASY-T1-nf PF4 (n = 1, 2, 3, 4, 5, 6) digested by EcoRI and SalI, and then ligated by T4 DNA ligase with the DNA fragments from pET28a(+) to generate expression vectors pET28a(n+)-nf PF4 (n = 1, 2, 3, 4, 5, 6). The confirmed recombinant vectors were transformed into E. coli BL21(DE3) cells to create strains BL21(DE3)/pET28a(n+)-PF4 (n = 1, 2, 3, 4, 5, 6).

**Expression and detection of fusion protein His-PF4.** Strain BL21(DE3)/pET28a(n+)-PF4 (n = 1, 2, 3, 4, 5, 6) was grown at 37 °C in 50 mL of culture medium with 100 mg/L of kanamycin to OD_{600} = 0.6 ± 0.02. IPTG was then added to a final concentration of 0.6 mmol/L. The harvested cells were disrupted by ultrasonication in TN buffer (20 mmol/L of Tris/Cl, 100 mmol/L of NaCl, pH 7.9) and centrifuged at 12,000 × g for 15 min. The culture cells and the soluble and insoluble fractions of the cell extracts were collected for detection of the His-PF4 protein. At 23 °C, various inducing times and IPTG concentrations were tested to optimize the expression of the His-PF4 protein in strain BL21(DE3)/pET28a(n+)-4f PF4. The E. coli BL21(DE3) strain transformed with pET28a(+) vector served as negative control. The pelletted insoluble material, cell extract supernatant, and culture cells were analyzed by SDS-PAGE. Proteins were visualized by Coomassie Blue R-250 staining. The protein concentrations in the samples were determined with a BCA assay kit following the manufacturer’s instructions using bovine serum albumin as standard. The expression levels were assessed by densitometric scanning using Glyco Bandscan 5.0 software.

Western blot analysis. The samples from the SDS-PAGE gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) as described previously. Western blotting analysis was performed using a mouse anti-His monoclonal antibody (Tiangen Biotech, Beijing, China) as primary antibody and goat anti-mouse IgG-HRP (Zhongshan Goldenbridge Biotechnology, Beijing, China) as secondary antibody. His-PF4 fusion proteins were visualized with ECL reagents (Millipore, Bedford, MA, USA) following the manufacturer’s instructions. The image was acquired with imaging system Fusion-SL2 (Vilber Lourmat, Paris, France).

**Purification of fusion protein His-PF4.** E. coli BL21(DE3)/pET28a(n+)-4f PF4 strain in LB medium was cultured under optimal induction conditions. The cells were harvested by centrifugation at 12,000 × g for 10 min at 4 °C, and were disrupted by ultrasonication after resuspension with TN buffer. The His-PF4 fusion protein in the supernatant fraction was collected from the crude cell lysate by centrifugation at 12,000 × g for 20 min, further purified using a 6 × His-tagged affinity chromatograph column (GE Healthcare Biosciences, Piscataway, NJ, USA), and then desorbed with elution buffer (20 mmol/L of sodium phosphate, 0.5 mol/L of NaCl, 0.5 mol/L of imidazole, pH 7.4) through a Hitrap Desalting Column (GE Healthcare Biosciences, Piscataway, NJ, USA), and then stored at −20 °C for further analysis.

**Enterokinase cleavage and mass spectrometric identification of the fusion protein.** His-PF4 fusion proteins were digested with Enterokinase (Shanghai Shengene Molecular Biotechnology, Shanghai, China) following the manufacturer’s instructions. The purified fusion protein was further desalted using a Hitrap Desalting Column and stored in sterile water. Using the desalting protein as sample, MALDI-TOF MS (Autoflex X III TOF/TOF200, Bruker Daltonics, Billerica, MA, USA) was performed to identify the molecular weight of the His-PF4 fusion protein.

**Heparin-neutralizing activity.** HEL cells were cultured to log-phase growth phase using culture medium IMDM with 10% fetal bovine serum. HEL cells were collected and seeded onto a 35-mm dish with semisolid medium (1 mL: 30% FCS, 0.3% Agar, 2 × 10^3 cells, 2,000 ng His-PF4). Meanwhile the negative control was set up. The treatment and negative control groups contained 10 dishes each. After the cells were cultured for 4 d (37 °C, 5% CO_2), colony numbers were counted. Every colony contained more than 50 cells. The inhibition rate of His-PF4 on the HEL cells was calculated as follows: inhibition rate = (N_C − N_T)/N_C. In the above formula, N_C and N_T are the average number of colonies per dish in negative control and treatment group, respectively.

### Table 1. Primers Used in This Study

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<th>Name</th>
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<td>P10</td>
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<td>P11</td>
<td>28</td>
<td>GAATTCgtgccgatcaccacacacacacacacacacacacacacacacacac</td>
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The underlined bases of primers 9 and primer 11 introduce the restriction site XhoI in primer 9 is marked by a wavy line. The restriction site SalI in primer 8 is marked by a dotted line. The lower-case bases at the 5’ end of all the primers indicate overlaps between primers.
Results

Construction of expression vectors pET28a(+)−nf PF4 (n = 1, 2, 3, 4, 5, 6)

Cloning vectors pEASY-T1-PF4 and pEASY-T1-f PF4 were confirmed by DNA sequencing. Expression vectors pET28a(+)−nf PF4 (n = 1, 2, 3, 4, 5, 6) were constructed by the above methods. Then, they were confirmed by restriction enzyme digestion (Fig. 2).

Expression and detection of fusion protein His-PF4 and Western blot analysis

In E. coli BL21(DE3)/pET28a(+)−nf PF4 (n = 1, 2, 3, 4, 5, 6), the His-PF4 fusion protein was detected in the supernatant as a soluble protein and as an insoluble protein inclusion body in the lysate precipitation after ultrasonication. Strain E. coli BL21(DE3)/pET28a(+)−nf PF4 (n = 1, 2, 3, 4, 5, 6) was induced under the same conditions (OD_{600} = 0.6 ± 0.02, 0.6 mmol/L of IPTG, 12 h) and whole bacterial protein expression was detected by SDS–PAGE and Western blot (Fig. 3). Consistent with theoretical expectations, the His-PF4 fusion protein in E. coli BL21(DE3)/pET28a(+)−nf PF4 (n = 1, 2, 3, 4, 5, 6) was expressed at molecular weights, but the apparent molecular weights of the proteins were larger than the theoretical values, at 13.2 kD (the first series unit) and 9.4 kD (the series units). SDS–PAGE and Western blot of whole bacterial proteins indicated...
that the expression of the target protein increased gradually with growing copy number. When the copy number was 4 or more, protein expression did not increase significantly (Fig. 3). Scan analysis showed expression levels of about 28.90%, 33.81%, 38.21%, 44.45%, 44.03%, and 44.28% of TP in E. coli BL21(DE3)/nf PF4 (n = 1, 2, 3, 4, 5, 6). However, the amount of soluble protein did not increase with growing copy number. The increase in total protein was mainly reflected in the inclusion bodies (Fig. 4).

E. coli BL21(DE3)/pET28a(+) -4f PF4 was induced by various IPTG concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 mmol/L), and the expression of recombinant PF4 was not significantly different. An induction time of 10 or 16 h is optimal for the His-PF4 fusion protein expression in E. coli BL21(DE)/pET28a(+) -4f PF4.

Purification of fusion protein His-PF4

E. coli BL21(DE3)/pET28a(+) -4f PF4 was induced by IPTG. The high-purity recombinant protein was obtained once by affinity chromatography (Fig. 5). The first PF4 series unit was scarcely translated in E. coli BL21(DE3)/pET28a(+) -4f PF4, and the protein was partly lost in the purification process, so purified His-PF4 protein was expressed from the PF4 series unit with a molecular weight of 9.4 kD.
Enterokinase cleavage and mass spectrometric identification of the fusion protein

The fusion protein was digested with enterokinase (Fig. 6). Due to the small amount of enzyme and a small His-tag, electrophoresis displayed only two bands (the His-PF4 protein and the PF4 protein). Enterokinase cleavage of the fusion protein further indicated that the target protein was correct, and this laid the foundation for large-scale production of the PF4 protein, but the results indicated that the apparent molecular weights of the His-PF4 and PF4 proteins were larger than their theoretical values. The results of MALDI-TOF MS indicated that the molecular weight of the purified His-PF4 protein was 9.4 kD, which is consistent with its theoretical value (Fig. 7).

Heparin-neutralizing activity

Mathematical expectations of the colony numbers of the experimental group and control group were compared using two sample t-tests. A significant difference was detected ($p < 0.001$). Statistical significance was considered to exist at a $p$ value of less than or equal to 0.001. The purified His-PF4 protein inhibited colony formation of HEL cells at an inhibition rate of about 55.6% (Fig. 8).

Discussion

The expression of PF4 protein in eukaryotic expression systems was studied. Compared with the prokaryotic expression system, the utility model has the advantage of complete post-translational processing, but the recombinant PF4 protein from *E. coli* is capable of inhibiting angiogenesis. Some PF4-derived molecules, such as carboxyl-terminal fragments of recombinant human PF4 and modified and chimeric peptides, which exhibit stronger anti-angiogenic properties than the parent molecule and can serve as leads for further therapeutic developments, have been developed. And the relationship between PF4 structure and function is more complex. Hence, modification after PF4 expression is not necessarily important. The eukaryotic expression system, which has a complex mechanism of expression and regulation, is beset with challenges as for commercial production. Hence the prokaryotic system was used for simple and high expression of the PF4 protein. In addition, monocistronic expression of the tandem repeat gene yielded high-level expression of short peptides, but also causes problems. Polycistronic expression of the tandem repeat gene does not deal with the tendency of small polypeptides with little secondary structure to degrade rapidly in *E. coli*, but this strategy can achieve high yields of small proteins, similarly to the enhancement of gene expression when increasing copy numbers due to mutant plasmid replicon. For small-protein expression, the two expression strategies in tandem should be discussed in depth.

In the present study, SDS–PAGE and Western blot of whole bacterial proteins indicated that expression of target protein increases gradually with increasing copy numbers. When the copy number was 4 or more, protein expression did not increase significantly (Fig. 3). On the one hand, as the copy number increased, more resources in *E. coli* were used for PF4 expression. On the other hand, the reason for the plateau phase of PF4 expression might have to do with suspension of the protein.
maturation process and a low level of mRNA translation accompanied by too many tandem repeats. The series unit after the first is always preferentially translated with increase in copy number. This phenomenon may be ascribed to the secondary structure of mRNA near the SD sequence and the start codon of the first series unit, but the amount of soluble protein did not increase with increasing copy numbers. This may be because the PF4 protein, which has two disulfide bonds, is easily aggregated due to overexpression in E. coli. Optimiza-
tion of expression conditions (such as IPTG concentration, induction time, and temperature) plays a negligible role in the increases in soluble expression. As explained above, the relationship between PF4 structure and function is more complex. Therefore the increase in total protein is found mainly in inclusion bodies, and this is resolved further by denaturation and refolding of inclusion bodies. Sc, the PF4 protein obtained by denaturation and refolding, is probably functional, and this deserves further study. SDS–PAGE showed that the apparent molecular weights of the proteins were larger than their theoretical values. Perhaps the changes in PF4 spatial structure resulted in unusual electrophoretic behavior.

PF4 expression in E. coli was used to obtain the active product. Initial tests showed that purified His-PF4 protein had good heparin-neutralizing activity. A preliminary experiment indicated that no significant difference existed \(* p < 0.01\) between purified His-PF4 and commercially available hPF4 (Sino Biological, Beijing, China). Rigorous tests in vivo must be designed to validate and compare His-PF4 and PF4 activities, but the purified His-PF4 protein can be used as research reagents, e.g., as standard substances and an antigen for the development of the corresponding antibody. Compared with past studies, our results provide a simpler and more effective strategy to obtain active His-PF4 in the E. coli expression system. They might contribute to the development of large-scale, bacterium-based PF4 production.

**Acknowledgment**

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**References**