Expression and Purification of Human Coagulation Factor X in Mammalian CHO-DG44 Cells

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Abstract [**Objectives**] This study was conducted to obtain a Chinese hamster ovary cell line that stably expresses recombinant human coagulation factor X (rhFX), and to induce efficient expression of the target gene with different concentrations of methotrexate (MTX). [**Methods**] PCR was performed to obtain the *rhFX* gene, and a recombinant expression plasmid pOptiVEC-rhFX was constructed and subjected to double restriction endonuclease digestion and sequencing identification. CHO-DG44 (DHFR -) cells were transfected by the liposome method, and the target protein was purified by affinity chromatography and detected by SDS-PAGE electrophoresis and Western blot. A cell line with efficient and stable expression of the target gene was obtained by increasing the concentration of MTX to select positive clones. [**Results**] PCR yielded a 1 509 bp rhFX sequence, and the results of double digestion and sequencing showed that the constructed pOptiVEC-rhFX plasmid was correct. After transfection of cells, MTX significantly increased protein expression. When MTX reached 1.0 μmol/L, the expression efficiency of the target protein was (9 ±0.27) μg/ml. The purity of the target protein purified by affinity chromatography was 93%, which could be used for subsequent experiments. The expression efficiency of rhFX in eukaryotic mammalian cells was improved by increasing MTX concentration, and an affinity chromatography purification process for the target protein was preliminarily established. [**Conclusions**] The results of this study provide data support for the expression and purification of rhFX, and will lay a solid foundation for the development of drugs related to rhFX.

Key words Recombinant human coagulation factor X (rhFX); Eukaryotic expression; MTX; Affinity chromatography

Factor X (FX), also known as Stuart Power factor, is a plasma glycoprotein that participates in the blood coagulation process as one of the key factors[1]. In the body, FX is mainly synthesized by the liver and requires the participation of vitamin K to function normally[2]. Its main function is to convert the precursor of thrombin into active thrombin during the formation of thrombin, thereby promoting blood coagulation. When FX is activated, it can release a peptide segment, forming two peptide chains connected by disulfide bonds, namely heavy chain (HC) and light chain (LC), of which the light chain includes the N-terminal γ-carboxyglutamic acid domain, EGF1 domain, and EGF2 domain, and the heavy chain is the serine protease domain[3-4].

In addition to its role in blood coagulation, FX also plays an important role in other aspects. For example, it can regulate vascular tension and blood flow by activating protease activated receptors (PARs). PAR-1 and PAR-2 activated by FX can promote vascular contraction and platelet aggregation, thereby affecting blood flow[5-6]. Moreover, FX can also participate in physiological processes such as inflammatory response and immune regulation by binding to PAR-1 and PAR-2[7-9]. It also plays an important role of maintaining liver health in the liver by participating in coagulation and clearing fibrinogen activators[10-11].

Due to the multiple physiological functions of FX, including coagulation, mutations in its genes that cannot be synthesized or synthesized normally in the human body can lead to FX deficiency, which can easily lead to symptoms such as subcutaneous congestion, joint bleeding, and gum bleeding[12-13]. FX deficiency is a genetic disease mainly caused by insufficient production or abnormal function of FX. It can be an X-chromosome recessive genetic disease or a sex-linked inheritable disease. Moreover, insufficient production or abnormal function of FX due to liver disease, liver cancer, kidney disease, *etc.*, may also lead to FX deficiency[14]. At present, the main method for treating FX deficiency is realized through infusion of exogenous FX concentrate, which is a protein preparation containing FX extracted from plasma. Such treatment method can usually effectively alleviate bleeding tendencies, but there are also some side effects, such as infusion reactions and infections[15-16]. In addition, due to FX being a plasma protein, its production and preparation costs are relatively high. Therefore, scientists have attempted to synthesize FX through multiple pathways.

Currently, many scholars at home and abroad are paying attention to the construction of stable expression systems for recombinant human coagulation factor X. A good vector needs to have promoters that can promote transcription and translation, while ensuring the stability of the protein sequence. Expression systems commonly used currently include mammalian cells, insect cells, and yeast cells[17-19]. Mammalian cell expression systems have a high degree of biological similarity and expression level. Based on this, in this study, a rhFX eukaryotic expression plasmid was constructed and used to express *rhFX* in CHO-DG44 cells successfully and efficiently under the condition of increasing the concentration of methotrexate (MTX), and an affinity chromatography purification system was established. The experimental results provide data

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support for efficient expression of rhFX and lay a certain foundation for the development of drugs related to rhFX.

Materials and Methods

Experimental materials and equipment

Chinese hamster ovary cells CHO-DG44 (DHFR-), plasmid pET-21a(+)-hFX containing hFX CDS sequence and eukaryotic expression vector pOptiVEC-TOPO, preserved in our laboratory; *Trans*5α chemically competent cells (TransGen Biotechnch Co., Ltd.), SanPrep Enodotoxin-Free Plasmid Mini Kit, EZ-10 Spin Column DNA Gel Extraction Kit, TureColor Pre-stained Protein Marker, DNA marker (250 – 10 000 bp), BCA Protein Assay Kit, methotrexate MTX, Pfu DNA polymerase, T4 DNA ligase, and dNTPs (Sangon Biotechn (Shanghai) Co., Ltd.); Lipofectamine™ 3000 transfection reagent, serum-free medium CD Opti-CHO™ Medium, MEM α (nucleoside), MEM α (nucleoside-free) medium, and unstained protein Marker (Scientific Thermo Fisher Scientific Inc.); vitamin K1 (SIGMA-ALDRICH (SHANGHAI) Trading Co., Ltd.), *Not* I, and *Xba* I restriction enzyme (Takara Biotechnology (Dalian) Co., Ltd.); factor X antibody (sc-101370) (Santa Cruz Biotechnology, Inc.); fetal bovine serum (Biological Industries Israel Beit Haemek Ltd.).

DYY-6C electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd.); H1605 desktop high-speed refrigerated centrifuge (Beckman Coulter Commercial ENTERPRISE (China) Co., Ltd.); TC-96 PCR apparatus (Hangzhou Bioer Technology Co., Ltd.); gel imaging system (Tanon Science&Technology Co., Ltd.); carbon dioxide incubator (Scientific Thermo Fisher Scientific Inc.).

Experimental methods

PCR of *rhFX* gene PCR amplification primers were designed based on the hFX CDS sequence published by NCBI (NCBI Reference Sequence: NP_000495.1), and the forward and reverse restriction endonuclease cutting sites were selected as *Xba* I and *Not* I based on the pOptiVEC-TOPO sequence. The primers were synthesized by Sangon Biotechn (Shanghai) Co., Ltd. With the pET 21a (+) – hFX plasmid as a template, the rhFX expression fragment was obtained through PCR amplification using the aforementioned primers.

Forward primer rhFX-F:

5'-tatctagagccaccatggggcgcccactgc-3', containing *Xba* I restriction enzyme cutting site (tctaga), Kozak sequence (gccaccatgg), signal peptides and protective bases.

Reverse primer rhFX-R:

5'-tagcggccgctcaatgatgatgatgatgctttaatggagag-3', containing *Not* I restriction enzyme cutting site (gcggccgc), 6 × His affinity chromatography purification tag, termination codon, protective bases.

PCR system: Sterile water 38 μl, 10 × PCR buffer 5 μl, DNTP 1 μl, forward and reverse primers 2 μl each, template 1 μl, Pfu DNA polymerase 1 μl.

The PCR amplification program was started with pre-denaturation at 95 ℃ for 3 min, followed by 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 58 ℃ for 10 s and extension at 72 ℃ for 3 min and completed with a final extension at 72 ℃ for 5 min. The PCR products were detected by agarose gel electrophoresis and recovered with gel extraction kit.

Construction of recombinant plasmid rhFX-pOptiVEC The products and the empty vector pOptiVEC-TOPO were amplified by *Xba* I and *Not* I. The double digestion system is shown in Table 1. Electrophoresis detection and gel recovery of double digestion products were performed, and the two were ligated with T4 ligase at 16 ℃ overnight. Next, the ligation products were transformed into *E. coli* Trans5α chemically competent cells, and positive monoclonal colonies were screened using ampicillin.

The positive monoclonal strains were cultured for expansion in LB liquid medium containing ampicillin, and the recombinant plasmid was extracted using a SanPrep Enodotoxin-Free Plasmid Mini Kit. After double digestion and sequencing identification, the correct eukaryotic expression plasmid was named pOptiVEC-rhFX. The corresponding positive monoclonal colony was named Trans5α-POptiVEC-rhFX, which was finally preserved in a refrigerator at a low temperature of – 80 ℃ for a long time after adding glycerol to a final concentration of 20%.

Table 1 DNA double digestion system

Component	Addition amount
Not I	1 μl
Xba I	1 μl
10 × K Buffer	1 μl
0.1% BSA	2 μl
PCR products/empty vector/recombinant plasmid	1 μg
Sterile water	Added to a total volume of 20 μl

Transfection of CHO-DG44 (DHFR-) cells CHO-DG44 (DHFR-) cells were inoculated into MEM α (nucleoside) (containing 10% fetal bovine serum) culture medium, and incubated at 37 ℃ in a 5% CO_2 incubator for later use. According to the Lipofectamine™ 3000 transfection instructions, the CHO-DG44 (DHFR-) cells were transfected with recombinant plasmid pOptiVEC-rhFX. After 48 h, the cells were transferred to MEM α (nucleoside-free) (containing 5% fetal bovine serum) medium for selective culture, and the culture medium was changed once every 72 h. Meanwhile, the untransfected CHO-DG44 (DHFR-) cells and the cells transfected with empty vector were set as the control groups.

Purification of rhFX protein by affinity chromatography The positive cell clone obtained through screening was added with vitamin K1 to a final concentration of 1 μg/ml to promote protein expression. After 2 – 3 d of culture, the supernatant of the culture medium was collected by centrifugation (4 000 r/min, 5 min) and added in a dialysis bag for 4 h of concentration by PEG20000 water absorption. The target protein was then purified by Ni^{2+} affinity chromatography. An appropriate amount of Ni^{2+} beads were loaded into an empty column. After the beads had completely settled, 10 ml of phosphate buffer was added to balance the column.

The concentrated supernatant of the culture medium was poured into the affinity chromatography column and rotated vertically at 4 ℃ for 1 h to fully bind the protein to the beads. Next, the stopper was removed to allow the supernatant to flow out, and the column was washed twice with 10 ml of phosphate buffer to remove impurities. Next, the column was washed with 20 mmol/L imidazole buffer twice to remove non-specific binding proteins. Next, the column was washed 150 mmol/L imidazole buffer to obtain rhFX protein. The protein was added to an ultrafiltration tube for ultrafiltration, which was performed for 5 times using TBS buffer (Tris 3.028 g, NaCl 8.766 g, dissolved in 1 L of ultrapure water) as the ultrafiltration buffer. Finally, inverted ultrafiltration was performed to obtain the target protein. Finally, inverted ultrafiltration was performed to obtain the target protein.

SDS-PAGE electrophoresis and Western Blot detection SDS-PAGE gel electrophoresis was performed on the purified rhFX, while using positive clone cell lysate as the control. Next, the target protein on the gel was electrochemically transferred to a PVDF membrane, sealed overnight at 4 ℃ with 5% skim milk powder, and then washed with PBST (1 × PBS + 0.1% Tween20) for 5 times. Next, the protein on the membrane was incubated with anti-hFX monoclonal antibody, washed and incubated with HRP-labeled goat anti-mouse secondary antibody. Finally, the membrane was exposed and developed using the ECL method.

Acquisition of stably expressed cells by screening with different concentrations of MTX MTX-mediated selection was performed on positive cells cultured to 85% confluence and in good condition. The MTX concentration was set to: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 1.5 μmol/L, and the cells were cultured for 5 generations with each concentration of MTX. When the cells grew to a confluence of 90% under the condition of one MTX concentration, the culture medium was changed to CD Opti-CHO™ Medium, and the cell culture supernatant was collected after 72 h. The target protein was purified by Ni^{2+} affinity chromatography and then detected for concentration by the BCA Protein Assay Kit. The clone with the highest expression level was subcultured and selected with the next MTX concentration.

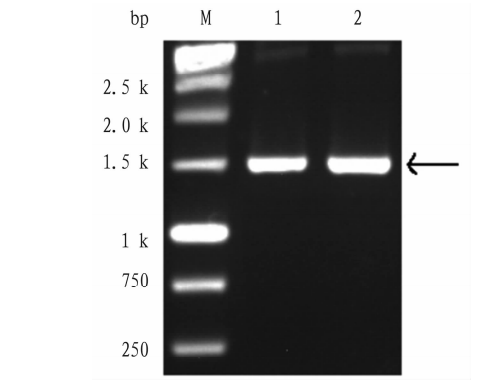
After achieving stable expression cells, the target protein was purified again by Ni^{2+} affinity chromatography, and its concentration was detected. The protein was packaged and frozen in liquid nitrogen and then transferred to a refrigerator at −80 ℃ for storage.

Data analysis The data were expressed using Mean ± SEM, and the differences between groups were compared by *t*-tests using SPSS 22.0, with $P < 0.05$ indicating a statistically significant difference.

Results and Analysis

PCR amplification of *rhFX* gene

After calculation, with the addition of auxiliary fragments such as forward and reverse restriction enzyme cutting sites and kozak sequence, the target gene *rhFX* had a length of 1 509 bp. After PCR amplification with primers rhFX-F and rhFX-R, the product was displayed on agarose gel electrophoresis, and the band size was consistent with the expected results, as shown in Fig. 1. It was preliminarily determined that the target gene was desired for this study.

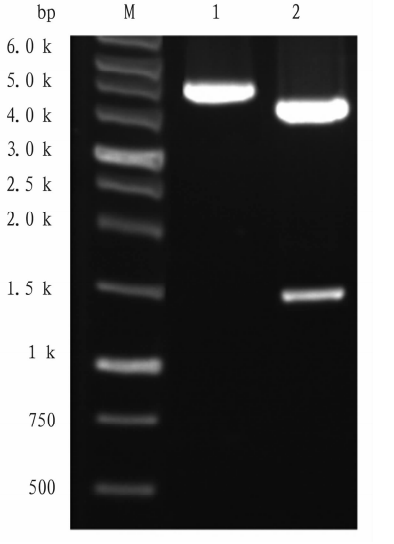


M: DNA Marker; 1 −2: two PCR products.

Fig. 1 Agarose gel electrophoresis pattern of PCR product of rhFX gene

Construction of recombinant plasmid pOptiVEC-rhFX

The obtained recombinant plasmid pOptiVEC-rhFX was digested with both enzymes *Not* I and *Xba* I and detected by agarose electrophoresis. The complete plasmid had a molecular weight of approximately 5 856 bp, and after double digestion, two bands with molecular weights of approximately 1 498 and 4 358 bp should be obtained. The electrophoresis results are shown in Fig. 2, and two bands corresponding to the expected vector and inserted fragment length were obtained, indicating that the target fragment had been successfully inserted into the expression vector. The plasmid identified correctly by double digestion was sent for sequencing, and the results showed that the sequence was correct.



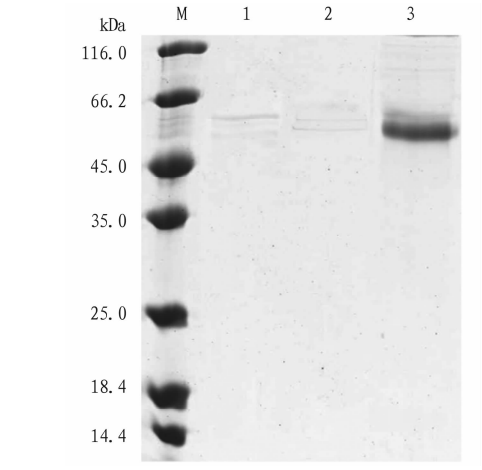
M: DNA Marker; 1: plasmid pOptiVEC-rhFX; 2: double digestion product of plasmid pOptiVEC-rhFX.

Fig. 2 Identification of recombinant plasmid pOptiVEC-rhFX by double enzyme digestion

SDS-PAGE electrophoresis and Western Blot detection of rhFX

The transfected cells were cultured in a serum-free medium for culture. After 72 h, the supernatant of the culture medium was

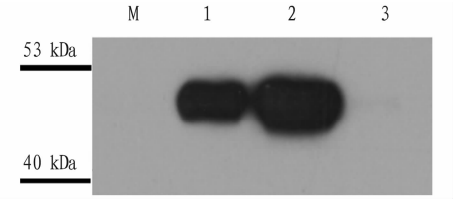
collected by centrifugation, and the rhFX protein with His tag was purified using Ni^{2+} affinity chromatography. Finally, 150 mmol/L imidazole eluent was used to elute the target protein. The rhFX protein purified by affinity chromatography was subjected to SDS-PAGE electrophoresis. Fig. 3 shows that the molecular weight of the target protein was approximately 51 kDa, which was consistent with the expected size. The purity of the purified protein reached 93%, so the purification method could be used for extracting a large number of target proteins.



M: Unstained protein Marker; 1: purified product from untransfected cell culture medium; 2: purified product from the cell culture medium after 4 h of transfection; 3: purified product obtained from the cell culture medium after 72 h of transfection.

Fig. 3 Detection of rhFX protein by SDS-PAGE electrophoresis

Western blot identification was performed on the purified protein using anti-hFX monoclonal antibody. The protein was expressed in the form of secretion and the molecular weight was 51 kDa after the signal peptide was excised by endoplasmic reticula. The Western blot results are shown in Fig. 4, showing a signal at approximately 51 KDa, indicating that the purified protein was the target protein rhFX.



M: Pre-stained protein Marker; 1 – 2: protein purified products of two transfected cell clones; 3: purified product of untransfected cell.

Fig. 4 Western blot detection of purified rhFX protein

MTX pressurization induction for upregulation of target gene expression

The transfected cells were screened with different concentrations of MTX, and the expression level of target protein was analyzed. As the concentration of MTX increased, the expression level of the protein significantly increased (Table 1). When the MTX concentration reached 1.0 and 1.5 μmol/L, the expression level was higher, but there was no significant difference between the two ($P > 0.05$). Therefore, when culturing transfected cells, 1.0 μmol/L MTX could be chosen to induce doubling expression of the gene.

Table 1 Effects of MTX concentration gradient on rhFX expression level

Concentration of MTX//μmol/L	0.05	0.1	0.2	0.4	0.8	1.0	1.5
Protein expression//μg/ml	0.4 ± 0.11 *	1.2 ± 0.13 *	1.93 ± 0.18 *	3.63 ± 0.29 *	6.48 ± 0.33 *	9 ± 0.27	9.13 ± 0.17

* indicates that the differences between the 1.0 μg/ml MTX group and other concentrations of MTX groups were statistically significant ($P < 0.05$).

Conclusions and Discussion

Coagulation factor X is one of the key proteins in the coagulation system, which participates in multiple steps of coagulation, including the formation of thrombin and the synthesis of fibrin. Therefore, studying the expression and function of FX is of great significance for understanding the regulatory mechanisms of the coagulation system. If the expression of FX in the body is insufficient, it can lead to FX deficiency, resulting in bleeding tendencies and sustained bleeding at wound sites. The severity of symptoms may vary depending on the level of FX deficiency. In cases of mild deficiency, the symptoms may not be obvious, but in cases of severe deficiency, the symptoms may be very obvious and severe, and may be life-threatening. At present, injecting exogenous FX is the main way to treat FX deficiency, but the cost of conventional preparations of exogenous FX is relatively high.

In response to the above issues, in this study, we established an efficient expression system for FX in mammalian cell CHO-DG44. In specific, the primer design added a His purification tag while retaining the signal peptide and kozak sequence, forming a secretory expression and facilitating the purification of the target protein by affinity chromatography. The plasmid vector selected was pOptiVEC-TOTO, which contains an IRES sequence. Therefore, the target gene and *DHFR* gene could be ligated together to construct a bicistronic expression vector to express the target gene and DHFR gene in a common efficiency and proportion. Secondly, auxotrophic CHO-DG44 (DHFR-) cells were used as the host. The cells have multiple advantages in eukaryotic expression. Exogenous proteins can be released into the culture medium in the form of secretion in CHO cells, and the proteins can be folded, cleaved, and modified in processing, making their structure and function similar to natural proteins. Meanwhile, the cells are of the dihydrofolate reductase deficient type (DHFR-), and under MTX selection pressure, sequence fragments encoding the exogenous recombinant proteins are also amplified with the amplification of the *DHFR* gene, significantly increasing protein production. In this study, when the MTX concentration was 1.0 μmol/L, the expression level of rhFX protein reached its highest value, and with further increase in MTX concentration, the expression level did not significantly increased.

The above results indicated that the eukaryotic expression system established in this study could efficiently express rhFX, and the corresponding affinity chromatography purification method could extract the target protein with a purity of 93%. The rhFX eukaryotic mammalian cell expression system constructed in this study provides a certain reference basis for the large-scale preparation of rhFX, and also lays a foundation for studying various physiological functions and action mechanisms of FX and the development of drugs for treating FX deficiency.

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