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Fast preparation of recombinant human stem cell factor from inclusion bodies using different hydrophobic interaction chromatographic columns

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Abstract：A method was developed to increase the recovery of recombinant human stem cell factor (rhSCF) from inclusion bodies using high performance hydrophobic interaction chromatography (HPHIC). The target protein was first solubilized in 8.0 mol/L urea solution, and was purified and refolded simultaneously by HPHIC with different chromatographic cakes. Experimental conditions, such as the ligand structures of stationary phase and the composition of mobile phase, were optimized. Under the optimal conditions, high mass recoveries and specific activities of rhSCF were acquired, the purities of rhSCF were above 95.5%, and the mass recoveries of rhSCF were above 49.6%. The final product was also verified as monomer by size exclusion chromatography and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). These results provided further evidence that HPHIC is an effective tool in the refolding and purification of recombinant proteins.

Key words：high performance hydrophobic interaction chromatography (HPHIC)；recombinant human stem cell factor；inclusion bodies

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采用不同疏水相互作用色谱柱从包涵体中快速制备重组人干细胞因子

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摘要：为了提高重组人干细胞因子(rhSCF)的复性效率，改进了高效疏水相互作用色谱(HPHIC)纯化和复性rhSCF的方法。首先将目标蛋白溶解于 8.0 mol/L 脲中，然后将 rhSCF 包涵体的提取液直接进样到不同规格的 HPHIC 柱进行纯化和复性。优化了固定相配基结构和流动相组成等实验条件，结果表明，本方法可以快速地获得高质量回收率和高生物活性的 rhSCF，rhSCF 在 40 min 内即可完成复性与纯化，目标蛋白的纯度在 95.5%以上，质量回收率高于 49.6%。通过体积排阻色谱和基质辅助激光解吸离子化飞行时间质谱(MALDI-TOF-MS)的分析，确认 rhSCF 以单体存在。结果进一步证明 HPHIC 法是同时复性和纯化重组蛋白的有效工具。

关键词：高效疏水相互作用色谱；重组人干细胞因子；包涵体

The refolding and purification of recombinant proteins is the main obstacle [1] for most eukaryotic proteins expressed in *Escherichia coli* (*E. coli*) form inclusion bodies (IBs). In view of the products for high yields and high biological activities, considerable effort is being expanded into new refolding and purification procedures. Protein folding liquid chromatography (PFLC) for recom-

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binant protein refolding has undergone a remarkable development in recent years [2,3]. Currently, it has been developed to four branches, including high performance hydrophobic interaction chromatography (HPHIC), size exclusion chromatography (SEC), affinity chromatography (AC) and ion exchange chromatography (IEC). PFLC has several advantages in recombinant protein purification and refolding, for example, it is easy to carry out protein refolding and purification simultaneously, easy to amplification, time saving, and highly automated. HPHIC has been used for refolding of recombinant proteins in large scales in recent years [4]. However, remarkable precipitates still form during PFLC, resulting in low mass and bioactivity recovery of product, hence, the product is hard to scale up. Thus, the main way to enhance refolding yields of denatured proteins is to reduce aggregates and misfolded state. It is very exciting that PFLC has already been widely used for refolding of various proteins. Geng et al [5] reported a series of specially designed units for the simultaneous renaturation and purification of proteins (USRPP). Because their diameters are much larger than their lengths, these columns are also named as chromatographic "cake". It has some advantages over conventional chromatographic columns, such as higher column loading capacity, better resolution, and higher allowable flow rate. Different experimental approaches and strategies were applied to increase protein refolding efficiency and yield on columns [6]. Optimization of factors in PFLC for protein refolding with simultaneous purification is thus much more important than that in usual LC. One solution is to involve some suitable concentration of urea, or guanidine hydrochloride (GuHCl), or arginine and so on, in the refolding buffer to inhibit protein aggregation during protein refolding [7 – 9]. On the other hand, the efficiency of column refolding for target proteins is influenced by several factors, including the concentration of urea in the mobile phase, pH, flow rate, sample loading volume, and ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) for disulfide-bridged protein [10,11].

The soluble recombinant human human stem cell factor (rhSCF) has multi-lineage hematopoiesis-stimulating activities, and has been considered as a potential therapeutic for various diseases [12,13]. High-level expression of rhSCF in *E. coli* accumulates as insoluble aggregates *in vivo*, i. e. IBs. In this work, rhSCF was selected as a target protein to find an efficient HPHIC protocol for the refolding with simultaneous purification of rhSCF IBs. The experimental conditions were optimized, including the kinds of stationary phase, elution modes and mobile phase composition by laboratory and preparative scale columns.

1 Experimental

1.1 Apparatus and chemicals

HPHIC was carried out on an LC-10ATvp high performance liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of two pumps (LC-10A), a variable wavelength UV-Vis detector (SPD-10AV), and a system controller (SCL-10B). Stainless steel columns (150 mm × 4. 6 mm i. d.) and stainless steel chromatographic cakes (USRPP) (10 mm × 20 mm i. d., 10 mm × 50 mm i. d.) were packed. The HPHIC packing materials were based on silica (particle size, 5 μm; pore size, 21 nm) with various kinds of ligands including polyethylene (PEG)-200, 400, 600, 800 and tetrahydrofurfuryl alcohol (THFA). USRPP were designed for both laboratory and preparative scales. The thickness (depth) is only 10 mm, but the internal diameter ranges from 10 mm to 500 mm [14].

Acrylamide, bis-acrylamide, bovine serum albumin (BSA), the standard relative molecular mass markers were obtained from Sigma (USA). Tris, glycine and sodium dodecyl sulfate (SDS) were obtained from Amresco (USA). Coomassie Brilliant Blue G-250 was purchased from Fluka (MO, USA). Control standard rhSCF and erythropoietin (EPO) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China. All other chemicals were of analytical grade.

1.2 Preparation of rhSCF extract

The strain used was recombinant *E. coli* DH5α harboring the plasmid pBV220 [15]. rhSCF was expressed in *E. coli* in the form of IB, and then the IB were recovered by washing and centrifugation and finally solubilized in 8.0 mol/L urea solution. The protein concentration of the supernatant was adjusted to a final concentration using 8.0 mol/L urea solutions.

1.3 Chromatographic procedure

Chromatography run was carried out at room temperature on a HPHIC column (150 mm × 4.6 mm i.d.) or USRPP (10 mm × 20 mm i.d., 10 mm × 50 mm i.d.) packed with various stationary phases. The columns were equilibrated with 100% mobile phase A (3.0 mol/L $(NH_4)_2SO_4$, 50 mmol/L phosphate buffer solution (PBS), pH 7.0) or mobile phase A containing different concentrations of urea. After equilibration in this manner, 8.0 mol/L urea of crude rhSCF solution was directly injected into the column through the sample valve, respectively. rhSCF was eluted with a linear gradient or non-linear gradient from 100% mobile phase A to 100% mobile phase B (50 mmol/L PBS, pH 7.0), or mobile phase B containing different concentrations of urea at a selected flow rate. All chromatograms were obtained using UV absorbance at 280 nm.

1.4 Analytical procedures

The total protein concentration was determined using the Bradford method, and mass recovery was evaluated by reference [16]. The purity of rhSCF was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15% acrylamide, and the density of each band after staining with Coomassie Brilliant Blue was quantified by scanning the gel using a thin-layer gel scanner (Cs-930, Shimadzu, Kyoto, Japan). Monomers and aggregate forms of rhSCF were analyzed by SEC using a Superdex-75 column (200 mm × 16 mm i.d., Amersham Pharmacia). The relative molecular mass of rhSCF was determined by matrix assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF-MS) (Axima CFR plus, Shimadzu, Japan).

1.5 Assay for bioactivity of hSCF

The bioactivity of rhSCF was measured using the hSCF-dependent cell line UT-7 [17]. Briefly, cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum and maintained in the presence of Erythropoietin (EPO). Cells were washed with the culture medium and cultured in the presence of the purified rhSCF at different concentrations. Proliferation of the cells was determined using the methylthiazol tetrazolium (MTT) method [17].

2 Results and discussion

2.1 Stationary phase

Silica-based packing materials were reported to have bi-functions of purification and renaturation for proteins. The hydrophobicity and structure of the ligand used in the stationary phase of hydrophobic interaction chromatography (STHIC) were found to be the most important factor influencing protein mass recovery. Solubilized/denatured proteins interact with the surface of the STHIC tightly, which not only prevents aggregation of unfolded proteins, but also dominates the formation of steric structures in proteins and thus assists the refolding of the denatured proteins [18]. Moreover, the refolded proteins can be simultaneously purified during the HPHIC process.

The effect of different stationary phases on the refolding of rhSCF was investigated at first. Five kinds of stationary phases with different end groups were evaluated, the hydrophobicity of their end groups is in this sequence, PEG200 < PEG400 < PEG600 < PEG800 < THFA. Solubilized rhSCF in 8.0 mol/L urea solution was directly loaded onto five HPHIC columns (or USRPP) packed with the above mentioned stationary phases. Proteins were eluted by linear gradients in 40 min. The mass recoveries of rhSCF obtained with the five types of columns are shown in Fig. 1. As can be seen from Fig. 1, the mass recoveries of the collected rhSCF from the five STHIC are different, indicating that the ligand PEG-400 is more favorable for rhSCF refolding using different col-

umns. In terms of hydrophobic strength, the ligand PEG-400 is not very high, also not very low. It is concluded that an STHIC with a suitable hydrophobic strength and ligand structure to the hydrophobic of the unfolded protein molecules would greatly facilitate protein refolding. It further demonstrates that a suitable hydrophobic surface with good hydrophobic strength and a ligand structure plays a key role in protein refolding [19].

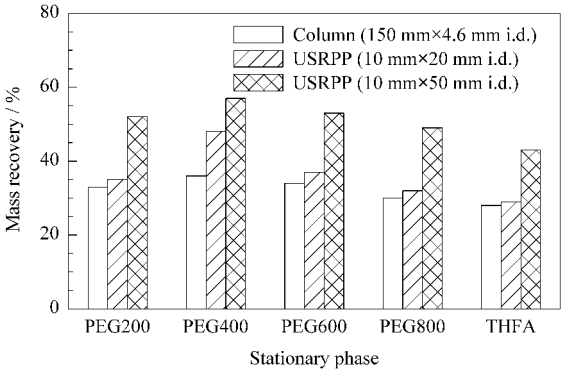

Fig. 1　Mass recoveries of refolded rhSCF from different ligands

Sample：8.0 mol/L urea solutions. Injection volumes：100 μL (150 mm×4.6 mm i.d.), 500 μL(10 mm×20 mm i.d.), 1000 μL (10 mm×50 mm i.d.). Flow rates：1.0 mL/min (150 mm×4.6 mm i.d.), 2.0 mL/min (10 mm×20 mm i.d.) and 5.0 mL/min (10 mm×50 mm i.d.), respectively. Linear gradient elution：100% mobile phase A (3.0 mol/L $(NH_4)_2SO_4$, 50 mmol/L PBS, 4.0 mol/L urea, pH 7.0) to 100% mobile phase B (50 mmol/L PBS, 4.0 mol/L urea, pH 7.0) in 40 min with 5 min delay.

2.2　Mobile phase

It is well known that the secondary structures of proteins are destroyed when they are dissolved in high concentration denaturant solutions, such as urea and guanidine hydrochloride. But if these denaturants solution are removed from environment, the ternary or quaternary structure of the proteins can be spontaneously refolded. During this process, the protein molecules in unfolded states may aggregate with each other. The same circumstance also occurs in column refolding, a published result [4] indicates that the continuously changing environment of the mobile phase and a broad concentration range of salt during gradient elution is beneficial for protein refolding and is helpful to obtain high mass recovery [3]. Wu et al [20] investigated those effects of mobile phase composition, including gradient mode, and flow rate on the mass recovery and bioactivity of recombinant human interferon-γ (rhIFN-γ). It was found that these factors were very important for the refolding with simultaneous purification of rhIFN-γ on column.

Fig. 2 shows the results of refolding with simultaneous purification of rhSCF by HPHIC with two types of salts-buffer solution；different concentrations of urea were supplemented in the mobile phases. It can be seen from Fig. 2 that there are increases in the specific activity of rhSCF when the urea concentration increased from 0 to 4.0 mol/L, with a maximum value at a urea concentration of 4.0 mol/L. But when the urea concentrations are over 4.0 mol/L, the specific activity of rhSCF shows a significant decrease. The results from two types of salts show PBS buffer solution (Fig. 2b) performs better than Tris-HCl (Fig. 2a). In practice, it is predictable that the

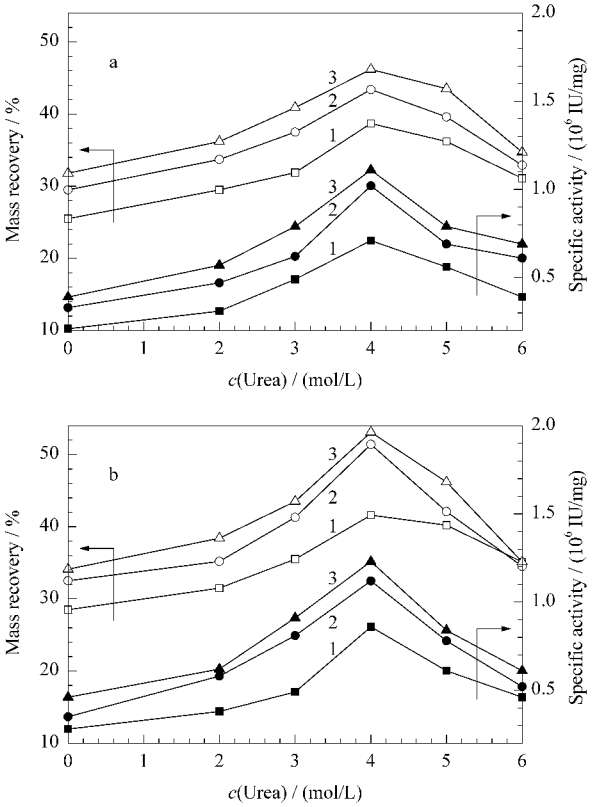

Fig. 2　Effect of urea concentration on refolding of rhSCF with different columns

a. Tris-HCl buffer；b. PBS buffer.
Conditions are the same as in Fig. 1.
1. column (150 mm×4.6 mm i.d.)；2. USRPP (10 mm×20 mm i.d.)；3. USRPP (10 mm×50 mm i.d.).

composition of the mobile phase should also play an important role in rhSCF refolding by HPHIC.

2.3 Fast refolding with simultaneous purification of rhSCF

After recovered and solubilized in 8.0 mol/L urea solution, the rhSCF was refolded with simultaneous purification by HPHIC columns with dimensions of 10 mm × 20 mm i. d., and 10 mm × 50 mm i. d., respectively. Fig. 3 shows the chromatograms of refolding with simultaneous purification of rhSCF using two types of hydrophobic columns. As can be seen from Fig. 3a, one-step refolding with simultaneous purification process allowed us to run 1 mL and 2 mL of rhSCF sample solution each time, then a linear gradient was performed for about 40 min. rhSCF fractions were collected, and the purity of rhSCF was determined by Coomassie Brilliant Blue stained SDS-PAGE and thin-layer gel scanning (data not shown). The rhSCF directly refolded from 1 L of E. coli culture by the USRPP (10 mm × 20 mm i. d.) and USRPP (10 mm × 50 mm i. d.) showed the average purities of 95.5% and 95.8%; the average mass recoveries of 49.6% and 59.1%. The protocol we developed here provides new evidence that HPHIC is a reliable and fast tool for refolding with simultaneous purification of rhSCF.

2.4 Analysis of the final bulk

In PFLC, HIC has mild chromatographic conditions which have advantages such as close to the body 's physiological conditions; they are beneficial to maintain biological activity of proteins. The specific activity of renatured and purified rhSCF was measured by UT-7 (human megakaryoblastic leukemia cell) dependent cell line. According to dose-response curve of SCF on UT-7 cell proliferation, the refolded rhSCF possesses a higher bioactivity in supporting the growth of an SCF-dependent cell line UT-7, meaning that the purified rhSCF protein has comparable activity as natural product. The refolded rhSCF had an average specific bioactivity of 1.26×10^6 IU/mg by USRPP. SEC was also employed for the analysis of the purified rhSCF under native conditions for rhSCF to verify its monomeric state. In the MALDI-TOF-MS analysis of the lyophilized refolded and purified rhSCF, as shown in Fig. 3c, the data obtained suggest that its relative molecular mass is close to the natural hSCF (the relative molecular mass of natural hSCF is 18 589).

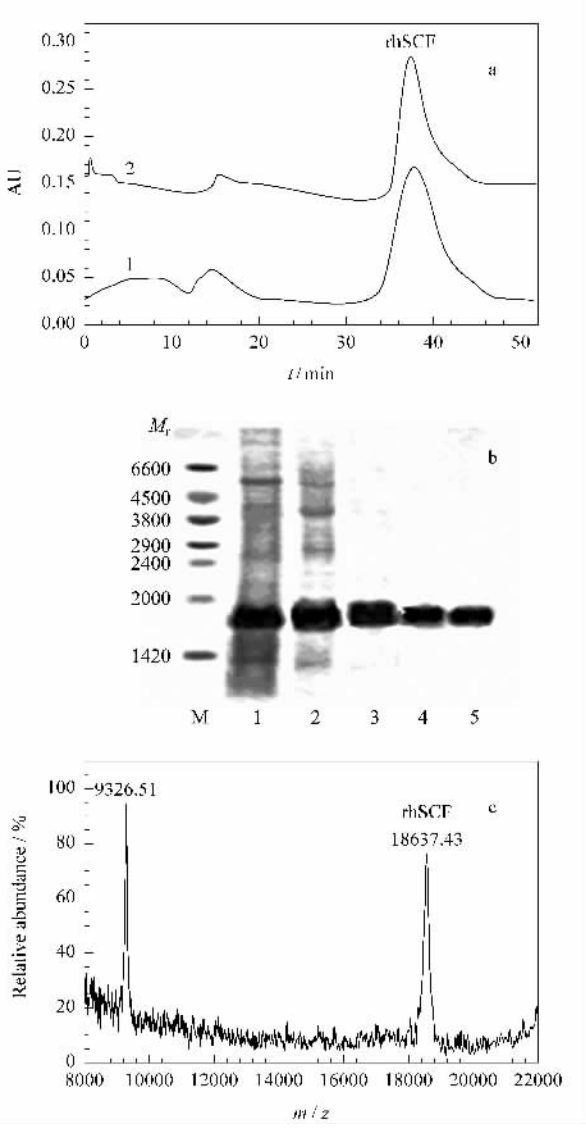

Fig. 3 Analysis of purified rhSCF

a. Chromatograms of purification rhSCF on different hydrophobic columns (PEG400). Curve 1: USRPP, 10 mm × 20 mm i. d.; flow rate, 2.0 mL/min. Curve 2: USRPP, 10 mm × 50 mm i. d.; flow rate, 5.0 mL/min. Mobile phase A: 3.0 mol/L $(NH_4)_2SO_4$, 50 mmol/L PBS, 4.0 mol/L urea, pH 7.0); mobile phase B: 50 mmol/L PBS, 4.0 mol/L urea, pH 7.0. Gradient: 0.01 – 10 min, 0 – 40% B; 10.01 – 25 min, 50% B – 30% B; 25.01 – 40 min, 30% B – 100% B; 50 min, stop.

b. SDS-PAGE analysis of rhSCF. Lane M: relative molecular mass marker; Lane 1: inclusion body solution; Lane 2: refolded by dilution; Lane 3: fraction refolded and purification of rhSCF by HPHIC; Lane 4: fraction refolded and purification of rhSCF by USRPP; Lane 5: the final bulk after preparation by USRPP (freeze-drying).

c. MALDI-TOF-MS analysis of lyophilized rhSCF after refolding with purification.

3 Conclusion

USRPP and traditional column could produce comparable mass recoveries for rhSCF, but the rhSCF obtained by USRPP possesses a higher specific bioactivity. One advantage of USRPP is that it has a diameter much larger than its length, the flow rate of the mobile phase is allowable to be rather high, which decreases the residue time of the sample in the sample loop, thereby, reducing the formation of precipitates [4]. In addition, if some precipitates form on the surface area of the filter frit, the column backpressure can still remain at a low level, because the precipitates only block a very small fraction of the total surface area. Thus, unlike traditional chromatographic columns, which can be blocked by precipitates, USRPP can maintain a normal chromatographic run. Our results show that (1) refolding and purification of recombinant proteins occurred more efficiently using USRPP rather than conventional column; (2) Since a combination of adsorption on STHIC and a urea gradient elution could markedly increase the purity, the mass recoveries and specific bioactivity of rhSCF, a reasonable conclusion is that the STHIC must have a moderate hydrophobicity and gentle environment for protein refolding; (3) USRPP has several attractive advantages, in particular low cost, short operational period, and easy scale-up. Thus it shows great potential for large-scale production of rhSCF; (4) It is expected that this procedure will be useful in the large-scale manufacture of rhSCF for therapeutic purposes. These data provide new evidence that PFLC is a reliable tool for the refolding with simultaneous purification of recombinant proteins.

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