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Solubilization and Refolding with Simultaneous Purification of Recombinant Human Stem Cell Factor

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Abstract Recombinant human stem cell factor (rhSCF) was solubilized and renatured from inclusion bodies expressed in *Escherichia coli.* The effect of both pH and urea on the solubilization of rhSCF inclusion bodies was investigated; the results indicate that the solubilization of rhSCF inclusion bodies was significantly influenced by the pH of the solution employed, and low concentration of urea can drastically improve the solubilization of rhSCF when solubilized by high pH solution. The solubilized rhSCF can be easily refolded with simultaneous purification by ion exchange chromatography (IEC), with a specific activity of 7.8×10^5 IU·mg^{-1}, a purity of 96.3%, and a mass recovery of 43.0%. The presented experimental results show that rhSCF solubilized by high pH solution containing low concentration of urea is easier to be renatured than that solubilized by high concentration of urea, and the IEC refolding method was more efficient than dilution refolding and dialysis refolding for rhSCF. It may have a great potential for large-scale production of rhSCF.

Keywords Recombinant human stem cell factor · Solubilization of inclusion bodies · Protein refolding · Purification · Ion exchange chromatography · Protein folding liquid chromatography

Introduction

Stem cell factor (SCF, also called steel factor or c-kit ligand) is a multipotent hematopoietic growth factor for early progenitor cells of different lineages [1, 2]. Stem cell factor can act on hematopoiesis by stimulating the survival and proliferation of stem cells and progenitor cells. It is also crucial for mast cell production and function and plays an important role in the development of melanocytes, germ cells, and intestinal pacemaker cells [1]. SCF exists naturally as membrane-anchored and soluble isoforms as a result of alternative RNA splicing and proteolytic processing [3]. Each SCF monomer contains two intra-chain

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disulfide bridges, Cys^{4}-Cys^{89} and Cys^{43}-Cys^{138}, as well as three potential N-linked sites of glycosylation, Asn^{65}, Asn^{72}, and Asn^{120}. The presence or absence of glycosylation does not affect its specific activity [4]. Potential therapeutic applications of SCF in clinic trials include the treatment of anemia, boosting the mobilization of hematopoietic stem/progenitor cells to the peripheral blood for harvest and transplantation, and increasing the effectiveness of gene therapy [1, 5].

Recombinant human SCF (rhSCF) has been expressed in *Escherichia coli* by many laboratories including ours [6]. But rhSCF protein often forms insoluble and inactive inclusion bodies in *E. coli*. A general strategy for recovery of active rhSCF from inclusion bodies involves cell lysis, extraction and cleaning of inclusion bodies, solubilization of inclusion bodies, and refolding into its native conformation [7, 8]. rhSCF inclusion bodies were usually solubilized by high concentration of denaturants, such as 8.0 $mol \cdot l^{-1}$ urea or 7.0 $mol \cdot l^{-1}$ guanidine hydrochloride (GuHCl), reducing agents, such as dithiothreitol or β-mercaptoethanol (β-ME), are added to reduce all disulfide bonds. Then, the denatured protein is transferred into a nondenaturating environment to shift the folding equilibrium toward its native conformation. This is normally achieved by removing the denaturants through dilution or dialysis in the presence of reduced glutathione (GSH) and oxidized glutathione (GSSG). However, refolding yields are typically low. Low refolding yields are attributed to mass loss of protein by aggregation because of nonspecific hydrophobic interactions. Dilution of the solubilized/denatured protein significantly increases sample volume, bring difficulty to subsequent chromatographic purification process, and increase costs. Therefore, to develop a new protocol to recover active rhSCF from inclusion bodies is very necessary for the production of rhSCF from *E. coli*.

It was reported that high concentrations of urea or guanidine hydrochloride (GuHCl), being strong denaturants, result in the loss of existing native-like secondary structures of the target protein in the inclusion bodies [9] and lead to easy aggregation during protein refolding. In recent years, high pH solution has been used to solubilize proteins in inclusion bodies expressed in *E. coli* [10–12], and the results showed that this solubilization method is beneficial to protein refolding. Recently, liquid chromatography (LC) has been used to refold proteins with higher yields [13–17]. The main advantage of the LC refolding method is that it not only prevents the unfolded protein molecules from aggregating with each other but also simultaneously purifies or partially purifies the protein during the chromatographic process; thus, it is called *protein folding liquid chromatography* (PFLC) [13, 18]. Ion exchange chromatography (IEC) is a widely used chromatographic method for protein purification; it was reported that about 70% of protocol for protein purification involved IEC, and now, IEC has been becoming one of the most frequently used LC refolding methods and has been applied to many proteins with high yields [19–28].

In the presented work, high pH buffers were used to solubilize rhSCF expressed in *E. coli* as inclusion bodies; the high pH buffer component and the solubilization conditions were optimized, then the solubilized rhSCF was refolded by dilution, dialysis, and IEC, respectively, and the refolding results were compared with the urea solubilized rhSCF.

Experimental

Instruments

LC-10A high-performance liquid chromatograph (Shimadzu, Japan), consisting of two LC-10ATVP pumps, one SPD-10AVP UV-Vis detector, one SCL-10AVP controller, and one

Rheodyne 7725 injection valve. All chromatographic data was collected and evaluated using the class-VP data system. Strong anion exchange chromatographic packings were prepared in our laboratory and packed into a column (10×1.2 cm I.D.). The electrophoresis apparatus were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). An Avanti^{TM} J-25 centrifuge (Beckman coulter$_{TM}$, USA) was used for centrifugation. A 5 l fermentor (Braun, Germany) was used to express protein.

Chemicals

Acrylamide and bis-acrylamide, GSH, and GSSG are of analytical grade, obtained from Sigma (USA). Tris, glycine, and sodium dodecyl sulfate (SDS) were obtained from Amersco (USA). Bovine serum albumin (BSA) was from Sigma Chemicals (USA). Molecular mass marker was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade.

Expression of rhSCF

A fed-batch fermentation was carried out in a 5-l bioreactor with a working volume of 4 l, with 10 g·l^{-1} glycerol, 5 g·l^{-1} tryptone, 5 g·l^{-1} yeast extract, and M9 salts. Fermentation was performed at 32 °C, and the pH of medium was maintained at 7.2 by 5 mol·l^{-1} NaOH with the dissolved O_2 concentration held at 30%. When the culture reached an OD_{600} of 4, the temperature was shifted to 42 °C to induce rhSCF synthesis. The culture was harvested at an OD_{600} of 7.8 (= cell dry wt 5.6 g·l^{-1}). The bacteria were harvested and resuspended in a 0.05 mol·l^{-1} NaH_2PO_4/NaOH, pH 7.4 by centrifugation for 10 min at 25,000×*g*, 4 °C.

Recovery of rhSCF Inclusion Bodies

The cells were thawed at room temperature and cleaned up with 0.020 mol·l^{-1} Tris–HCl (pH 8.0), and then, the suspension was centrifuged at 7,000 rpm and 4 °C for 10 min after washing. The supernatant was discarded. After freezing at −20 °C for 12 h, 100 g of the frozen cells were thawed at room temperature and resuspended in 1,000 ml of 0.050 mol·l^{-1} Tris–HCl buffer (pH 8.0) containing 1.0 m mol·l^{-1} ethylenediaminetetraacetic acid (EDTA). The cells were lysed by sonication on ice-water bath. The lysates were centrifuged at 14,000 rpm for 20 min to collect the insoluble protein aggregates. The pellet (protein aggregates and cell debris) was washed with 500 ml of the following solutions: 0.020 mol·l^{-1} Tris–HCl (pH 8.0) containing 0.010 mol·l^{-1} EDTA and 2.0 mmol·l^{-1} β-ME, 0.020 mol·l^{-1} Tris–HCl (pH 8.0) containing 2.0 mol·l^{-1} urea and 2.0 m mol·l^{-1} EDTA, and 0.020 mol·l^{-1} Tris–HCl (pH 8.0) containing 70% 2-propanol, respectively. Finally, the inclusion bodies were washed with 0.02 mol·l^{-1} Tris–HCl (pH 8.0). After each washing step, the suspension was centrifuged at 14,000 rpm and 4°C for 15 min, the supernatant was discarded. Then, the pellet fraction containing rhSCF inclusion bodies were obtained and stored at −20 °C.

Solubilization of rhSCF from Inclusion Bodies

Several batches of 1.0 g of purified rhSCF inclusion bodies were solubilized in 20 ml of solution I (0.05 mol·l^{-1} Tris containing 0.05 mol·l^{-1} Na_2HPO_4 with different pH adjusted by hydrochloride acid or sodium hydroxide), solution II (0.05 mol·l^{-1} Tris, pH 12.5 containing 0.05 mol·l^{-1} Na_2HPO_4 and 2.0 mol·l^{-1} urea), solution III (8.0 mol·l^{-1} urea

containing 0.1 mol·l^{-1}Tris, pH 8.0; 0.02 mol·l^{-1} EDTA; and 0.1 mol·l^{-1} β-mercaptolethanol). For solubilization by solutions I and II, the rhSCF suspension was adjusted by using 0.1 mmol·l^{-1} HCl to pH 10.0 and was continuously stirred for 2 h; after that, the pH was adjusted to 8.0 by using 0.1 mmol·l^{-1} HCl, then the suspension was centrifuged at 14,000 rpm for 20 min to remove insoluble debris, and the supernatant was kept at 4 °C for renaturation and purification. For solubilization by solution III, the rhSCF inclusion bodies was solubilized with continuous stirring for 4 h, then the suspension was centrifuged at 14,000 rpm for 20 min, and the supernatant containing rhSCF was collected for further use.

Procedures for the Refolding with Simultaneous Purification of rhSCF by IEC

Chromatographic runs were carried out at room temperature using a strong anion exchange column (10×1.2 cm I.D.) and connected to a LC-10A high-performance liquid chromatograph. The column was equilibrated with solution A consisting of 1 mmol·l^{-1} EDTA, 20 mmol·l^{-1} Tris (pH 8.0), 1.0 mmol·l^{-1} GSH, and 0.1 mmol·l^{-1} GSSG. Four hundred microliters of sample solution containing the solubilized and denatured rhSCF was directly injected into the column. After washing the column with 10 ml of the solution A, the refolding with simultaneous purification of rhSCF was accomplished after a linear gradient elution from 100% A to 100% B (solution B consisted of solution A plus 1.0 mol·l^{-1} NaCl) in 30 min with a delay of 10 min at a flow rate of 2.0 ml·min^{-1}. The profile was recorded with a UV detection at 280 nm.

Refolding of rhSCF by Dilution

Four hundred microliters of sample solution containing the denatured rhSCF was diluted 100-fold with 20 mmol·l^{-1} Tris (pH 8.0), 1 mmol·l^{-1} EDTA, 1.0 mmol·l^{-1} GSH, 0.1 mmol·l^{-1} GSSG, then the solution was left for 24 h at 4 °C. After refolding, the rhSCF was purified by IEC.

Refolding of rhSCF by Dialysis

The, denatured rhSCF solution was dialyzed against 20 mmol·l^{-1} Tris (pH 8.0), 1 mmol·l^{-1} EDTA, 1.0 mmol·l^{-1} GSH, 0.1 mmol·l^{-1} GSSG at 4 °C with continuous stirring for 48 h; the buffer was renewed each 4 h during the dialysis. After refolding, the rhSCF was purified by IEC.

Analytical Procedures

Electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a Tris–SDS–glycine buffer system in the presence of a reducing agent was used to detect the purity of the purified rhSCF contained in the fractions after IEC. Electrophoresis was performed for 1 h at 250 V using 15% polyacrylamide gels.

Determination of Protein Concentration and Mass Recovery

The protein concentration was estimated by Bradford quantitative protein determination assay using BSA as standard. The mass recovery (R_m) of rhSCF was defined as

$$R_m = m_{G,F}/m_{G,IB} = (C_F \cdot V_F \cdot P_F)/(C_{IB} \cdot V_{IB} \cdot P_{IB}) \quad (1)$$

where, $m_{G,F}$, the mass of rhSCF in the finally obtained rhSCF solution (mg); C_F, total protein concentration in the finally obtained rhSCF solution (mg·ml^{-1}); V_F, volume of the finally obtained rhSCF solution (ml); P_F, purity of rhSCF in the finally obtained rhSCF solution; $m_{G,IB}$, the mass of rhSCF in the injected solution of inclusion bodies (mg); C_{IB}, total protein concentration in the injected solution of inclusion bodies (mg·ml^{-1}); V_{IB}, volume of the injected solution of inclusion bodies (ml); and P_{IB}, purity of rhSCF in the injected solution of inclusion bodies.

Bioactivity Assay of rhSCF

The bioassay for the bioactivity of the renatured rhSCF was determined using a UT-7-dependent cell line as described previously [29].

Results and Discussion

Effect of pH on the Solubilization of rhSCF Inclusion Bodies

It was previously shown that proteins in inclusion bodies expressed in *E. coli* can be solubilized by high pH solution [10–12], the obtained denatured proteins is easy to be renatured with relatively high yields. In the presented work, the same amount of rhSCF inclusion bodies were solubilized by 0.05 mol·l^{-1} Tris buffer containing 0.05 mol·l^{-1} Na_2HPO_4 with pH from 8.0 to 13.5, respectively, and the solubilization was monitored by determining protein concentration using UV absorbance at 280 nm and by determining

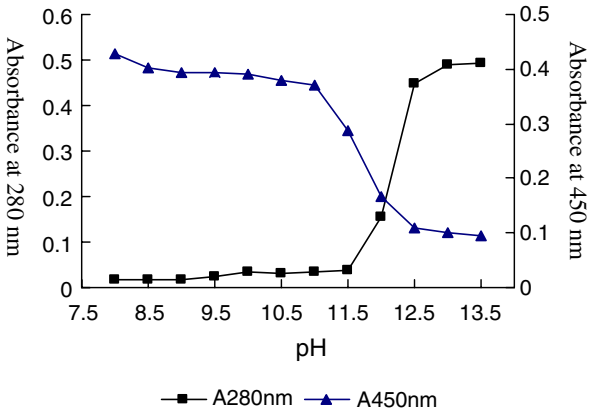

Fig. 1 Solubilization of rhSCF at different pH. One gram of rhSCF inclusion bodies was solubilized in 20 ml of 0.05 mol·l^{-1} Tris buffer containing 0.05 mol·l^{-1} Na_2HPO_4 at pH from 8.0 to 13.5

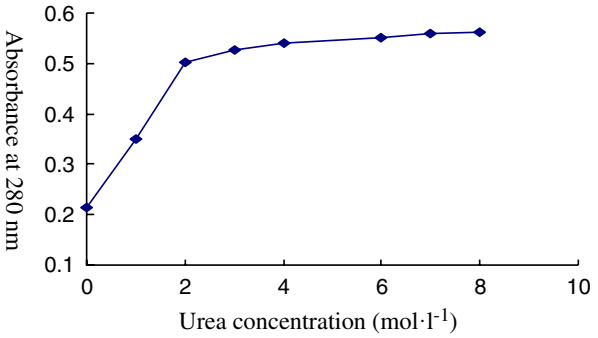

Fig. 2 Effect of urea concentration on the solubility of rhSCF inclusion bodies. One gram of rhSCF inclusion bodies was solubilized in 20 ml of 0.05 mol·l^{-1} Tris containing 0.05 mol·l^{-1} Na_2HPO_4 at pH 13.0 and 0~8.0 mol·l^{-1} urea

turbidity using visible absorbance at 450 nm. The results were shown in Fig. 1. It can be seen from this figure that solubilization of rhSCF inclusion bodies was very poor and hardly influenced by the pH in the pH range from 8.0 to 11.5. Remarkable enhancement of solubilization was observed with further increasing of pH, with a maximum at pH 13.0.

Effect of Urea on the Solubilization of rhSCF Inclusion Bodies

Urea is a widely used solubilizing agent for inclusion bodies, and it was also usually used to solubilize rhSCF inclusion bodies. From the above experimental results, rhSCF inclusion bodies can be solubilized with a high pH buffer. However, what is the result when high pH and urea were combined to solubilize rhSCF inclusion bodies? Figure 2 shows the solubilization effect of high pH buffer containing different concentration of urea. The results show that the solubilization of rhSCF inclusion bodies was greatly increased by introducing low concentration of urea in 0.05 mol·l^{-1} Tris buffer containing 0.05 mol·l^{-1} Na_2HPO_4 at pH 13.0; an approximate plateau was approached when the urea concentration was enhanced to 2.0 mol·l^{-1}.

Refolding with Simultaneous Purification of the High pH Solubilized rhSCF by IEC

Liquid chromatography has been recently applied to protein refolding; its main advantages are that proteins can be purified simultaneously during protein refolding; refolding yields are relatively high. IEC is a most commonly used LC method for protein refolding. Here,

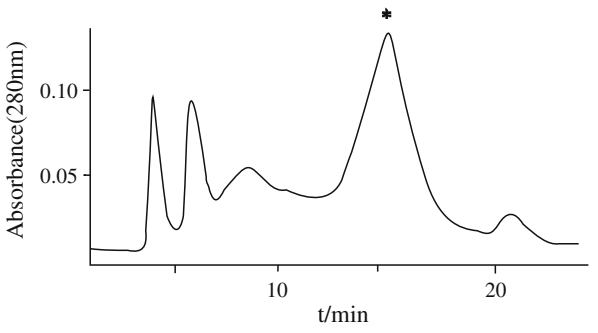

Fig. 3 Chromatogram of rhSCF refolded by IEC. Chromatographic conditions: gradient/linear gradient from 0% B to 100% B in 30 min, with a delay of 10 min; flow rate is 2.0 ml·min^{-1}; mobile phase A: 0.05 mol·l^{-1} Tris (pH = 8.0), 1 mmol·l^{-1} EDTA, 1.0 mmol·l^{-1} GSH, 0.1 mmol·l^{-1} GSSG; mobile phase B: the mobile phase A containing 1 mol·l^{-1} NaCl; the *asterisk* denotes rhSCF

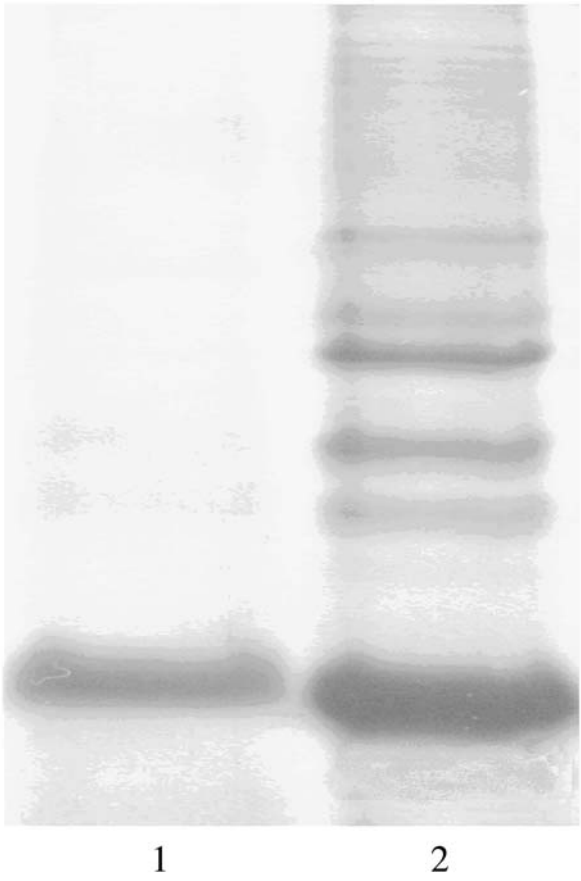

Fig. 4 SDS–PAGE analysis of rhSCF. *1* rhSCF refolded by IEC with simultaneous purification; *2* extract of rhSCF inclusion bodies by high pH buffer containing 2.0 mol·l^{-1} urea

IEC was used to refold rhSCF solubilized by high pH buffer containing 2.0 mol·l^{-1} urea; the chromatogram is shown in Fig. 3. The whole IEC refolding process could be accomplished in 1 h, including the equilibration and elution program. The obtained rhSCF has a specific bioactivity (SB) of 7.8×10^5 IU·mg^{-1}, a mass recovery (MR) of 43.0%, and a purity of 96.3% (Fig. 4). For comparison, the rhSCF solubilized by high pH buffer containing 2.0 mol·l^{-1} urea was also refolded by dilution and dialysis, and the above used three refolding methods were also applied to refold the rhSCF solubilized by 8.0 mol·l^{-1} urea. The results are shown in Table 1. It can be seen from this table that all of the mass

Table 1 Comparison of results for rhSCF solubilized and refolded by using a different method.

rhSCF sample	$SB_{dilution}$ (IU·mg^{-1})	$MR_{dilution}$ (%)	$SB_{dialysis}$ (IU·mg^{-1})	$MR_{dialysis}$ (%)	SB_{IEC} (IU·mg^{-1})	MR_{IEC} (%)
$rhSCF_{urea}$[a]	$(3.3\pm0.94)\times10^5$	18.8±1.53	$(4.5\pm1.1)\times10^5$	16.8±1.07	$(7.6\pm1.8)\times10^5$	36.4±3.10
$rhSCF_{pH}$[b]	$(4.7\pm0.86)\times10^5$	25.4±2.16	$(5.4\pm1.4)\times10^5$	26.2±1.79	$(7.8\pm1.5)\times10^5$	43.0±2.93

[a] $rhSCF_{urea}$ presents the rhSCF solubilized by 8.0 mol·l^{-1} urea.

[b] $rhSCF_{pH}$ presents the rhSCF solubilized by 0.05 mol·l^{-1} Tris (pH 13.0) containing 0.05 mol·l^{-1} Na_2HPO_4 and 2.0 mol·l^{-1} urea.

recovery for rhSCF solubilized by high pH buffer containing 2.0 mol·l^{-1} urea are higher than that solubilized by 8.0 mol·l^{-1} urea, no matter which refolding method was employed, and their specific bioactivities were comparable. This may attribute to that rhSCF solubilized from the inclusion bodies without disturbing its existing native-like secondary structure in the high pH buffer [9, 30], which helped in lowering the extent of protein aggregation during rhSCF refolding. It can also be seen from Table 1 that both of the SB and MR of the rhSCF refolded by IEC are higher than those refolded by dilution or dialysis, no matter which solubilizing method was used.

In the previous literature [4], rhSCF expressed in *E. coli* as inclusion bodies was solubilized by 8 mol·l^{-1} urea solution, refolded and oxidized by dilution refolding with a buffer containing low concentration of urea and glutathione for 60 h; the renatured rhSCF solution was concentrated by ultrafiltration and buffer exchanged by using diafilter, then the crude protein solution was primarily purified by acid precipitation, and filtration was used to remove the precipitated contaminant. After that, several chromatographic steps were used to further purify the rhSCF. Firstly, strong cation exchange chromatography was applied, then reversed-phase chromatography, strong anion exchange chromatography, and size exclusion chromatography were followed in sequence. The final yield of rhSCF was only 18%. In the present work, rhSCF inclusion bodies were solubilized by high-pH buffer with a low concentration of urea, and the solubilized rhSCF was refolded by strong anion exchange chromatography. As a result, rhSCF was also purified during the refolding process without further treatment, and a mass recovery of 43.0% was obtained; it was much higher than that in the early work [4].

Conclusions

The effect of pH and urea on the solubilization of rhSCF inclusion bodies was investigated; the results indicate that the solubilization of rhSCF inclusion bodies was significantly influenced by the pH of the solution employed, and low concentration of urea can drastically improve the solubilization of rhSCF using high pH solution. The solubilized rhSCF can be easily refolded with simultaneous purification by IEC with relatively high efficiency. The rhSCF solubilized by high pH solution containing low concentration of urea is easier to be refolded than that solubilized by high concentration of urea, and the IEC refolding method was more efficient than dilution refolding and dialysis refolding for rhSCF. The reported solubilization and refolding method may also be useful for other proteins produced in *E. coli* as inclusion bodies.

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