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Refolding Recombinant Human Granulocyte Colony Stimulating Factor Expressed By *E. coli*

A Case Study Using the Unit of Simultaneous Renaturation and Purification for Proteins

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Refolding of recombinant proteins expressed in *Escherichia coli* is known as a bottleneck in their production and product manufacturing. Refolding yields are often low using traditional methods such as dialysis, dilution, or diafiltration. Liquid chromatography (LC) has recently been applied to protein refolding to increase yields (**1**, **2**). In the 1990s, one of us first presented the refolding of rhIFN-γ by high-performance hydrophobic-interaction chromatography (HPHIC) (**3**), with its bioactivity two- to threefold better than that of a dilution method, and obtained a patent based on this method (**4**). Since then, HPHIC has been applied to the refolding of many proteins.

Product Focus: Protein products from microbial expression systems

Process Focus: Downstream processing

Who Should Read: Process development and manufacturing

Keywords: HPLC, protein folding, purification, *E. coli* expression, hydrophobic-interaction chromatography

Level: Intermediate

Based on that method, a USRPP (unit of simultaneous renaturation and purification of proteins) (Photo 1) was designed for both laboratory and preparative applications. Its thickness (depth) is only 10 mm, but internal diameters can range from 10 to 500 mm (**5**, **6**). Obtained recoveries of bioactivity and mass of proteins by the USRPP can be compared with those for most chromatographic columns. With gradually increased internal diameters and a column height kept at 10 mm, this kind column does not look like a typical chromatographic column but more like a "cake," so it has also been called a *chromatographic cake*.

A recombinant human therapeutic protein extracted by a highly concentrated denaturant solution can form precipitates in the mobile phase of a chromatographic process. They can block HPHIC columns. But in the USRPP, precipitates block only a very small fraction of frits because of a much greater cross-section area on top. This prevents significant increases in back pressure.

The USRPP has been applied to refolding with simultaneous purification of several proteins, such as proinsulin (**7**), recombinant human stem cell factor (**8**), and recombinant human interferon-γ (rhIFN-γ) (**9**). When using a unit with a 10 × 200 mm i.d., rhIFN-γ was obtained at a purity of >95% in two hours. Its specific activity was measured at 8.9 × 10^7 IU/mg, which is 24 times that for protein recovered by a traditional method, but the run time was only 0.045 of that for the same traditional method (**9**). And 700 mL of rhIFN-γ solution (extracted by 7 mol/L guanidine hydrochloride) containing 2.0 g of total proteins could be loaded onto a larger USRPP with a 10 × 300 mm i.d. (**9**).

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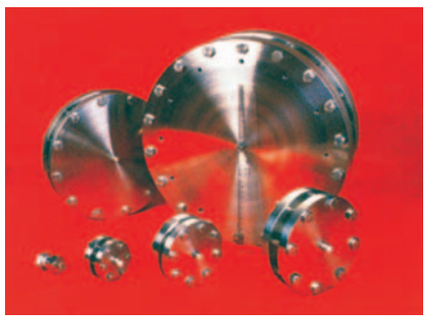


Photo 1: Kelin USRPP set (WWW.KELIN2Y.COM)

Granulocyte colony stimulating factor (G-CSF) stimulates proliferation of specific bone marrow precursor cells and their differentiation into granulocytes. It has been shown to improve survival rates of test subjects in several published studies (**10, 11**). Because the supply of natural human G-CSF is very limited — and it has been confirmed that the recombinant form exhibits most if not all of the biological properties of the native molecule (**12**) — only product must be made in large quantity through genetic engineering can meet clinical needs. But insoluble and inactive aggregates (inclusion bodies) form when rhG-CSF is expressed in *E. coli*. Here, we report refolding rhG-CSF expressed in *E. coli* with simultaneous purification by a USRPP packing with HPHIC packing materials (USRPP-HPHIC). This method could shorten processes for rhG-CSF production and decrease production costs, thus laying a good foundation for large-scale production.

Experimental Details

Instruments: We used the LC-10Avp high-pressure liquid chromatography system from Shimadzu (www.shimadzu.com) with Kelin fast protein purification columns and USRPP in dimensions of 10 × 20 mm i.d. and 10 × 50 mm i.d. For the 10 × 200 mm i.d. USRPP, we used an ÄKTA explorer 100A chromatographic system from Amersham Pharmacia Biotech (now GE Healthcare, www.amershambiosciences.com). We bought Kelin fast protein purification columns and USRPPs from Shaanxi Xida Kelin Gene-Pharmacy Co. Ltd. in Xi'an, China (www.kelin2y.com/indexe.html).

Chemicals: We obtained bovine serum albumin (BSA) and reagents for gel electrophoresis from Sigma (www.sial.com). Low–molecular-mass protein markers came from Amersham Pharmacia Biotech (GE Healthcare). All other chemicals were analytical grade.

Preparation Procedures: After expressing rhG-CSF in M9 medium, *E. coli* cells were recovered by centrifugation and disrupted by sonication. We recovered inclusion bodies with centrifugation and solubilized them in 8.0 mol/L urea, 1.0 mmol/L EDTA, and 50 mmol/L Tris (pH 8.0) using detailed procedures as referenced (**13**).

Refolding and Purification: We carried out our chromatographic run at room temperature. The USRPP-HPHIC (10 × 200 mm i.d.) was equilibrated with solution A consisting of 1.2 mol/L $(NH_4)_2SO_4$ and 0.05 mol/L Tris at pH 8.0. We directly injected a 200-mL sample of solubilized and denatured rhG-CSF in an 8.0 mol/L urea solution containing 7.9 mg/mL of total protein into the USRPP-HPHIC. After washing the column with solution A to achieve baseline UV absorbance, we eluted rhG-CSF with a linear gradient from 100% solution A to 100% solution B (0.05 mol/L Tris at pH 8.0) in 40 minutes at a flow rate of 10.0 mL/min, with a delay of 100 min. UV detection was set at 280 nm.

Analysis: We analyzed our refolded rhG-CSF by electrophoresis, concentration determination, and bioactivity assay according to published methods (**13**).

Results and Discussion

When LC is used for protein refolding, interactions between target proteins and the stationary phase prevent protein aggregation. As a strong purification technique, LC can complete refolding with simultaneous purification in one step. And the USRPP is a chromatography column of special shape. So it has four functions (Figure 1): to completely remove denaturant, to renature several proteins simultaneously, to separate renatured proteins from impure proteins, and to easily recycle waste denaturant.

Figure 1: Four functions of the USRPP

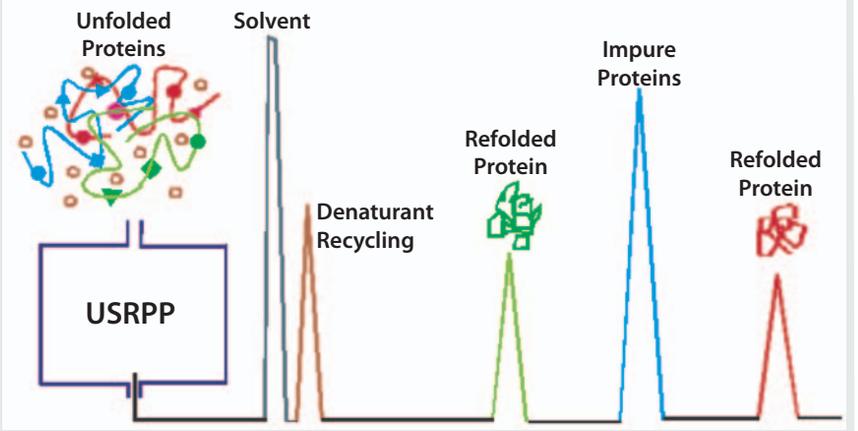
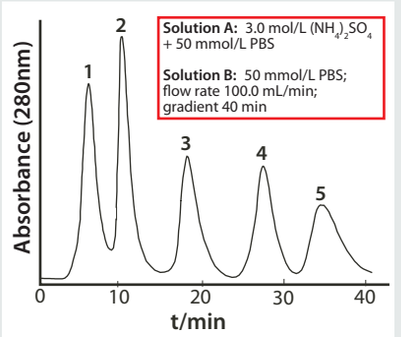


Figure 2: Chromatogram of five standard proteins separated by USRPP-HPHIC (10 × 200 mm i.d.); chromatographic conditions described below



[0] ~100% B
[1] Cytochrome C
[2] Myoglobin
[3] Lysozyme
[4] α-Amylase
[5] Insulin

The USRPP offers very good resolution for protein separation (Figure 2) and very high-activity recovery for protein refolding. For example, the active recovery of ribonuclease A is 94.4%, and the active recovery of lysozyme is 95.9% (**5**). The resolution and activity assure successful refolding with simultaneous purification for most denatured proteins.

A New Type of USRPP: The USRPP offers very good performance and decreases the cost for manufacturing process development. A series of simple and cheap columns (Photo 2) was manufactured for use in investigating chromatographic conditions at laboratory scale. Because

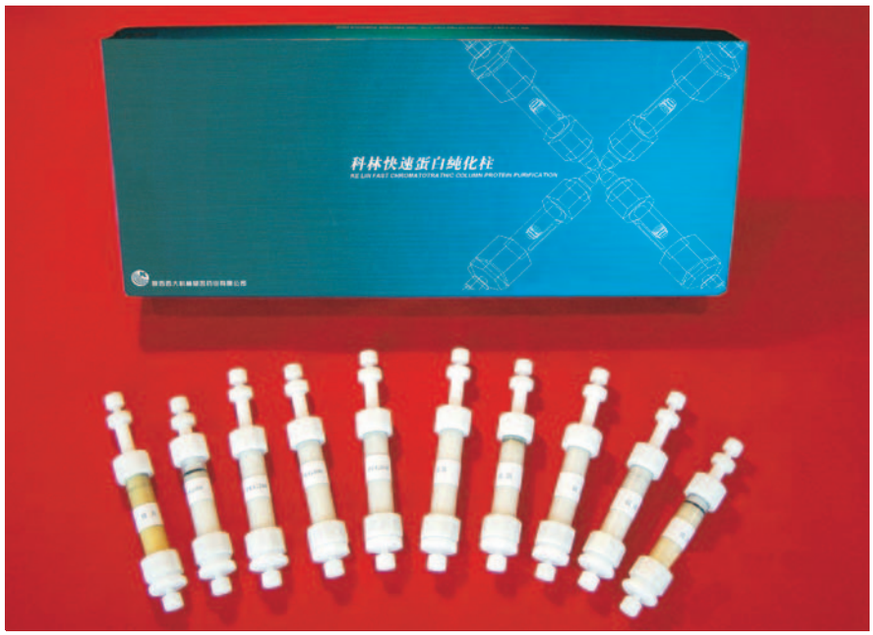


Photo 2: Kelin fast protein purification columns (WWW.KELIN2Y.COM)

Figure 4: Comparing production technologies of rhG-CSF produced by *E. coli*; (TOP) typical production technology (**2**) in three steps; (BOTTOM) new production technology with one step

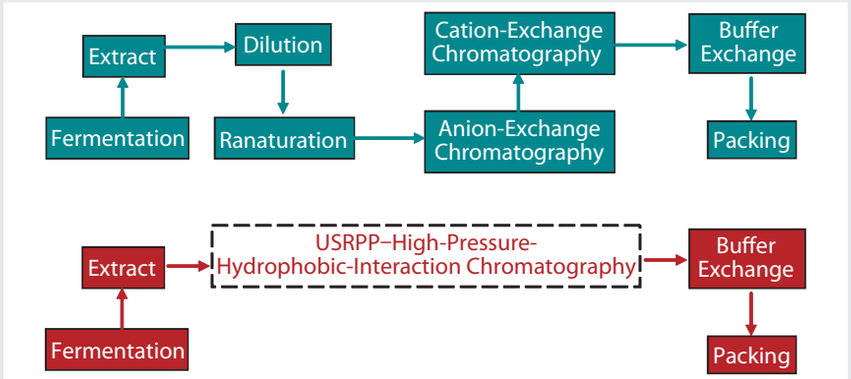
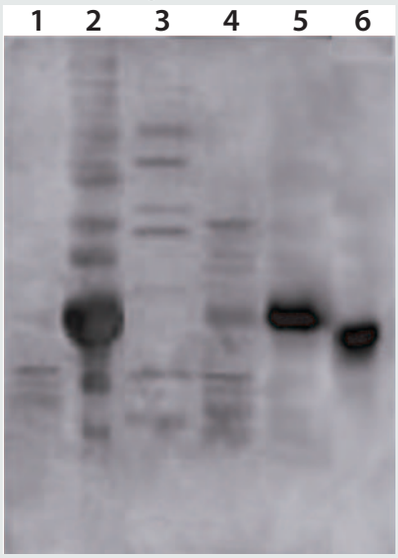


Figure 3: SDS-PAGE analysis of rhG-CSF during refolding with simultaneous purification at large scale



[1] Effluent collected during pumping the first 100-mL sample

[2] rhG-CSF solution extracted by 8.0 mol/L urea from inclusion bodies

[3] Molecular weight marker (from the bottom, 14.4, 20.1, 31.0, 43.0, 66.2, and 97.4 kD)

[4] Effluent collected during pumping a later 100-mL sample

[5] Reduced state of rhG-CSF after refolding with simultaneous purification using HPHIC (10 × 200 mm i.d.)

[6] Nonreduced state of rhG-CSF after refolding with simultaneous purification using USRPP-HPHIC (10 × 200 mm i.d.)

supports with larger particles were used, the resolution of these columns is slightly lower than for USRPPs.

To determine which kind of column could be applied to the refolding of rhG-CSF, we tested six kinds of fast protein purification columns packed with HPHIC materials using different end groups: phenyl, furfural, PEG 200, PEG 400, PEG 600, and PEG 800. We directly injected rhG-CSF (from *E. coli* inclusion bodies solubilized by 8.0 mol/L urea) into a column that had been equilibrated with a certain concentration of ammonium sulfate solution. With elution by descending concentration of ammonium sulfate, we obtained refolded rhG-CSF.

Results indicated that the column with an end group of PEG-400 was the best choice to produce rhG-CSF with a higher specific activity at a higher mass recovery. This may be because it is moderately hydrophobic, which probably accommodates rhG-CSF molecules well.

Scaling Up: Based on results obtained using the fast protein purification column, we gradually scaled up our process of refolding with simultaneous purification for rhG-CSF using USRPPs with dimensions of 10 × 20 mm i.d., 10 × 50 mm i.d., and 10 × 200 mm i.d. When the industrial-scale USRPP (10 × 200 mm i.d.) was used, we began with 200 mL of solubilized and denatured rhG-CSF in 8.0 mol/L of urea solution containing 7.9 mg/mL of total protein. So more than 1500 mg of total protein could be loaded in one chromatographic run. The rhG-CSF obtained had a specific activity of 2.3 × 10^8 IU/mg, a purity of more than 95.4% (Figure 3), and a mass recovery of 36.9%.

Figure 4 compares old and new production technologies for rhG-CSF at industrial scale. The USRPP can replace four steps in the traditional process (dilution, renaturation, anion-exchange chromatography, and cation-exchange chromatography) in rhG-CSF production (**14**), significantly reducing run time and cost. Our method 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 rhG-CSF. We plan to use a larger USRPP-HPHIC of 10 × 500 mm i.d. (Photo 3) to further enlarge our process.

A Method with Potential

The USRPP shows that HPHIC can be applied to protein refolding with simultaneous purification. We used Kelin fast protein purification columns successfully in our investigation of

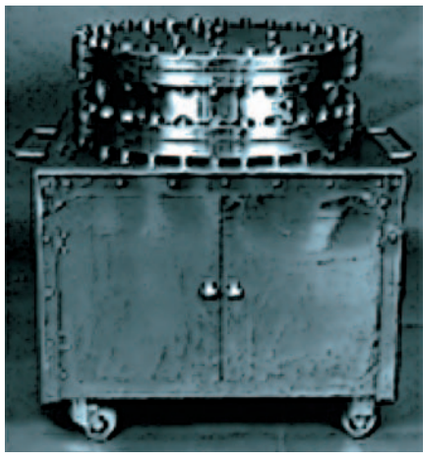


Photo 3: 10 × 500 mm I.D. USRPP

refolding with simultaneous purification of rhG-CSF. This process is easily enlarged by using USRPP at different scales. At 10 × 200 mm i.d., we refolded and purified rhG-CSF at industrial scale with good results. We believe this protocol is superior to traditional processes for large-scale production of rhG-CSF.

Acknowledgments

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