Chinese Chemical Letters Vol. 14, No. 8, pp 824 – 827, 2003
http://www.imm.ac.cn/journal/ccl.html

Studies on the Renaturation with Simultaneous Purification of Recombinant Human Proinsulin with Unit of Simultaneous Renaturation and Purification of Protein in Semi-preparative Scale

Quan BAI*, Yu KONG, Xin Du GENG

Institute of Modern Separation Science, Kay Lab of Modern Separation Science in Shaanxi Province, Northwest University, Xi'an 710069

Abstract: The renaturation and purification of recombinant human proinsulin (rh-proinsulin) expressed in *E. coli* with the unit of simultaneous renaturation and purification of protein (USRPP) in semi-preparative scale was studied. The result shows that rh-proinsulin extracted with 8.0 mol/L urea can be renatured and purified simultaneously in 45 minutes with the USRPP (10×50 mm ID). The purity of rh-proinsulin was found to be more than 90% and the mass recovery to be more than 80%. The renaturation effect of rh-proinsulin with the USRPP was tested by enzyme cleavage for obtaining insulin. In addition, the result was further confirmed with RPLC, SDS-PAGE electrophoresis, and MALDI-TOF, respectively.

Keywords: Liquid chromatography, hydrophobic interaction chromatography, renaturation, preparation, recombinant human rh-proinsulin, biotechnology.

The recombinant human proinsulin (rh-proinsulin) is the precursor of insulin, which is connected with C- peptide between the carboxyl-terminal of chain A and NH_2-terminal of chain B of insulin. In the presences of trypsin and carboxypeptides B (CPB), the C-peptide can be cleaved from the special two peptides of rh-proinsulin and the recombinant human insulin (rh-insulin) can be, thus, obtained[1,2]. The rh-proinsulin expressed in *E. coli* exists as inclusion body, hardly dissolves in water, but easily dissolves in the solutions of 7.0 mol·L^{-1} guanidine hydrochloride (GuHCl) or 8.0 mol·L^{-1} urea. Generally, rh-proinsulin can be partially renatured with dilution or dialysis methods[2-4]. The renaturation and separation of rh-proinsulin have been always carried out by steps and taking about four days, but the mass recovery was about 30%. The purity of the five steps[3] purification method of rh-proinsulin is only 80%. If rh-proinsulin can be renatured with simultaneous purification in one step, the producing process of rh-proinsulin would be very simple and fast.

High performance hydrophobic interaction chromatography (HPHIC) could be used as a tool for the investigation of the renaturation with simultaneous purification of denatured proteins[5-9]. The unit of simultaneous renaturation and purification of protein (USRPP), with diameter being much larger than its length, is designed and employed for

*E-mail: baiquan@nwu.edu.cn

both laboratory and preparative scales. Some recombinant therapeutic proteins expressed in *E. coli* in biotechnology can be renatured and purified simultaneously with the USRPP in one hour with one step[6,10,11], and the bioactivity recovery is two to three times higher than by the usually methods. In this paper, the renaturation with simultaneous purification of rh-proinsulin expressed in *E. coli* was studied with the USRPP in semi-preparative scale.

Experimental

A Shimadzu LC-6A including two pumps, gradient elution system and UV detector was used. The size of the employed semi-preparative scale USRPP was a 10×50 mm I.D. The HPHIC packings were synthesized in our institute (Silica from Vydac Co., Herjbra, CA, USA, particle diameter 7 μm, average pore diameter 30 nm). The end groups of the ligands are phenyl. The reversed-phase column, ODS, was a 100×4 mm I.D. also synthesized in our institute (Silica from Vydac Co.)

The sample solution of rh-proinsulin extracted with 8.0 $mol \cdot L^{-1}$ urea was directly injected into the USRPP and then eluted with an non-linear gradient elution by the mobile phases A[3.0 $mol \cdot L^{-1}$ $(NH_4)_2SO_4$ + 0.08 $mol \cdot L^{-1}$ tris buffer (pH, 7.5)] and B[0.08 $mol \cdot L^{-1}$ tris buffer (pH, 7.5)]. Mobile phase for RPLC consisted of solutions A, 90% H_2O + 10% CH_3OH + 0.03% HCl and solution B, 10% H_2O + 90% CH_3OH + 0.03% HCl. Rh-proinsulin was enzyme-cleaved according to reference[2]. The rh-proinsulin concentration was detected according to the Bradford method[12].

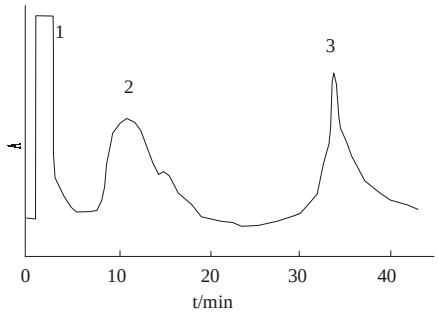
Results and Discussion

The process for simultaneous renaturation and purification of rh-proinsulin expressed in *E. coli* was studied with the semi-preparative scale USRPP. The chromatograms and SDS-PAGE are shown in **Figures 1** and **2**. The results indicate that a successful, simultaneous renaturation and purification of rh-proinsulin with mass recovery 80% and purity 90% was obtained in a 40 min run.

The fraction of the separated rh-proinsulin with USRPP was collected and then separated with RPLC (the result not shown). If the purified rh-proinsulin can be simultaneously renatured with USRPP, the retention time and the peak profile obtained by RPLC should be the same as that of standard rh-proinsulin. Really, it showed a positive result. It means that the molecular conformation of the renatured rh-proinsulin with the USRPP is the same as that of the standard rh-proinsulin, and further proves that the rh-proinsulin extracted with 8.0 $mol \cdot L^{-1}$ urea solution really can be renatured with simultaneous purification by USRPP.

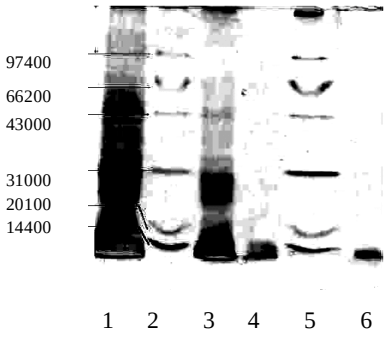
In order to prove this conclusion, the collected fraction of the renatured and purified rh-proinsulin by USRPP was directly cleaved with the enzyme, following the reference method[2].

Figure 1 The chromatogram of rh-proinsulin extracted with 8.0 mol·L^{-1} urea separated with semi-preparative scale USRRP



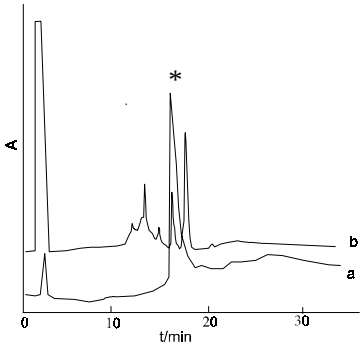
1. solvent, 2. impure protein, 3. rh-proinsulin.
Sample size, 7.0 mL of the rh-proinsulin solution having total proteins of 60 mg containing about 6 mg rh-proinsulin. The final concentration of rh-proinsulin 0.10 mg·mL^{-1}, the flow-rate 5.0 mL·min^{-1}, the chart paper speed 4 mm.min^{-1}. Detection at wavelength 280 nm. AUFS is 0.08.

Figure 2 SDS-PAGE of the HPHIC fraction of rh-proinsulin



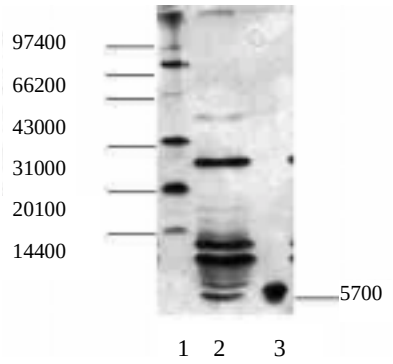
1,3. The inclusion body of rh-proinsulin extracted with 8.0 mol·L^{-1} urea, 2,5. Marker, 4,6. the HPHIC fraction of rh-prosinsulin

Figure 3 The chromatogram of enzyme- cleaved products of rh-proinsulin separated with RPLC



a. standard insulin, b. the enzyme-cleaved products of rh-proinsulin, flow rate, 1.0 mL·min^{-1}, detection wavelength 280 nm and AUFS 0.08, 25 min linear gradient elution.

Figure 4 SDS-PAGE of enzyme-cleaved products of rh-proinsulin



1. Marker, 2. enzyme-cleaved products of rh-proinsulin. 3. insulin

The enzyme-cleaved products were further separated with RPLC and tested by SDS-PAGE. The results are shown in **Figures 3** and **4**, respectively. The chromatograms a and b (**Figure 3)** denote the standard recombinant human insulin

(rh-insulin) and the enzyme-cleaved products of rh-proinsulin, respectively. The star denoted peak in **Figure 3** should represent the expected rh-insulin. SDS-PAGE shown in **Figure 4** confirmed further the result shown in **Figure 3**.

The molecular weight of the fraction shown in the chromatograms (**Figure 3**) with star denotation was measured with MALDI-TOF. The result showed that the molecular weight is 11,456 Dalton, being 2-folds of standard human insulin (5,701 Dalton). In other words, this fraction is not rh-insulin itself, but the dimmer of rh-insulin.

Conclusion

The extracted rh-proinsulin with 8.0 $mol \cdot L^{-1}$ urea solution can be renatured with simultaneous purification by the semi-preparative scale USRPP. The obtained rh-proindulin can be directly cleaved by enzyme. Comparing with the usual dilution and dialysis methods, the present method can be accomplished by only one chromatographic run within one hour. Under a suitable optimal condition, the purity of the obtained rh-proinsulin can reach to more than 90% and the mass recovery is more than 80%. This conclusion was proved by RPLC, SDS-PAGE, and MALDI-TOF.

Acknowledgment

The inclusion bodies of rh-proinsulin expressed in *E. coli* and standard rh-proinsulin were provided by Xuzhou Biochemical Pharmaceutical Plant, Jiangsu province.

References

1. J. Nilsson, P. Jonasson, E. Samuelsson, T. Mols, S. Stahl, M. Uhlen, *J. Biotechol.*, **1996**, *48*, 241.
2. Z. H. Yang, X. Du, J. G. Tang, *Appl. Biochem. Biotechnol.*, **1999**, *76***,** 107.
3. C. K. Kim, Y. I Kim, J. N. Pheu, *et al*., *United States Patent*, 1**999**, *No. 5952*, 461.
4. M. R. Ladisch, K. L. Kolmann, *Biotechnol. Prog.*, **1992**, *8*, 469.
5. X. D. Geng, X. Q. Chang, *J. Chromatogr.*, **1992**, *599*, 185.
6. L. Guo, X. D. Geng, *Chin. J. Biotech.*, **1992**, *16*, 661.
7. Q. Bai, Y. M. Wei, M. H. Geng, X. D. Geng, *Chem. J. Chin. Univ.*, **1997**, *18*, 1291.
8. Q. Bai, Y. Kong, Y. J. Zhang, X. D. Geng, *J. Northwest University* (science edd.), **2000**, *30*, 4851.
9. X. D. Geng, *Guide to Theory of Modern Separation Science*, published by Higher Education Press, Beijing, **2001**, p. 339.
10. Y. D. Zhang, L. H. Zhang, X. D. Geng, *Chin. J. Biotech.*, **1991**, *15*, 240.
11. T. Liu, X. D. Geng, *J. Northwest University* (science edd.), **1999**, *29*, 123.
12. M. A. Frederick, E. K. Robert, J. G. Seidman, *et al*, *Short Protocols in Molecular Biology*, published by Science Press, Beijing, **1998**, p. 332.

Received 27 August, 2002