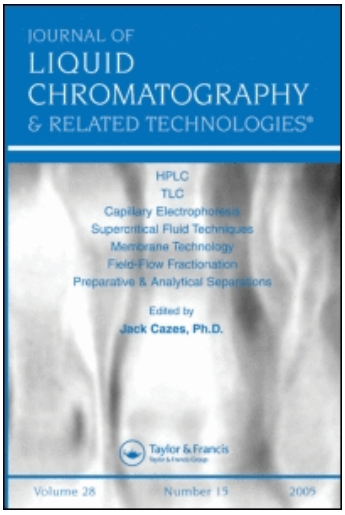

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 03 December 2003

To cite this Article Bai, Quan , Kong, Yu and Geng, Xin-du(2003) 'Studies on Renaturation with Simultaneous Purification of Recombinant Human Proinsulin from *E. coli* with High Performance Hydrophobic Interaction Chromatography', Journal of Liquid Chromatography & Related Technologies, 26: 5, 683 — 695
To link to this Article: DOI: 10.1081/JLC-120018414
URL: http://dx.doi.org/10.1081/JLC-120018414

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 5, pp. 683–695, 2003

Studies on Renaturation with Simultaneous Purification of Recombinant Human Proinsulin from *E. coli* with High Performance Hydrophobic Interaction Chromatography

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ABSTRACT

The renaturation with simultaneous purification of recombinant human proinsulin (rh-proinsulin) expressed in *E. coli* by high performance hydrophobic interaction chromatography (HPHIC) was investigated. The result indicates that the reduced/denatured rh-proinsulin, extracted with 8.0 mol L^{-1} urea solution in the presence of β-mercaptoethanol can be renatured and purified, simultaneously, in 45 min with HPHIC, resulting in the purity and mass recovery being more than 90% and 94%, respectively. The disulfide bonds of rh-proinsulin can correctly form on

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DOI: 10.1081/JLC-120018414 1082-6076 (Print); 1520-572X (Online)

the HPHIC column without the presence of reduced and oxidized glutathione (GSH, GSSG). The renaturation efficiency of rh-proinsulin with HPHIC was tested by enzyme cleavage in order to obtain insulin. The result was also confirmed with RPLC, SDS–PAGE, and MALDI-TOF, respectively. The renatured and purified rh-proinsulin can directly be enzyme-cleaved in the collected fraction containing the rh-proinsulin. Thus, the technology for the renatured and purified rh-proinsulin is very simple and fast.

Key Words: High performance hydrophobic interaction chromatography; Protein refolding; Purification; Recombinant human proinsulin.

INTRODUCTION

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 presence 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 an inclusion body, hardly dissolving into water, but easily dissolving in the solutions of 7.0 mol L^{-1} guanidine hydrochloride (GuaHCl) or 8.0 mol L^{-1} urea. Generally, rh-proinsulin can be partly renatured with dilution or dialysis methods.[2–4] The renaturation and separation of rh-proinsulin have been always carried out in steps. The purification of rh-proinsulin by five steps was reported in the American Patent in 1999;[3] its purity was only 80%. Ten years ago, one of the authors first reported that high performance hydrophobic interaction chromatography (HPHIC) could be used to as a tool for the investigation of the renaturation with simultaneous purification of denatured proteins.[5] With the new method, the denatured proteins can be completely, or partly, renatured by HPHIC with a chromatographic run in 40 min.[6–9] At the same time, the denaturant can be completely removed and the impure proteins can be separated from the aim-protein.

High performance hydrophobic interaction chromatography has been applied successfully in the process of the renaturation, with simultaneous purification of some recombinant therapeutic proteins expressed in *E. coli* in biotechnology, such as recombinant human interferon-γ (rhIFN-γ),[6,10] recombinant human granulocyte colony-stimulating factor (rhG-CSF),[11] recombinant human interferon-α (rhIFN-α),[12] and so on. The renatured efficiency is usually greater than 85%. Compared to the dilution or dialysis methods being only in the range of 10–15%, the bioactivity recovery of the former is always

2–3 folds of the latter. Recently, liquid chromatography (LC) was used as a tool for studying protein refolding and renaturation.[6,9]

However, there are three pairs of disulfide bonds in rh-proinsulin molecules. The rh-proinsulin expressed in *E. coli* has to be reduced and denatured with the denaturant solution including the reducing agent, such as β-mercaptoethanol or DTT. As it is well known, it is more difficult to renature the reduced–denatured protein than the proteins without disulfide bonds. We reported[7] that the reduced/denatured bovine insulin can be renaturated partly with forming correct disulfide bonds by HPHIC under oxidation conditions. In the paper, we try to do the renaturation with simultaneous purification of the reduced/denatured rh-proinsulin expressed in *E. coli* with HPHIC, but not under the presence of oxidized glutathione (GSSG). The question is, if its three pairs of disulfide bonds can be formed correctly, or incorrectly? If rh-proinsulin can be renatured with simultaneous purification in a single step, then, the enzyme-cleavage can directly be done in the collected fraction containing the renatured and purified rh-proinsulin. Thus, the whole process would be very simple and fast.

EXPERIMENTAL

Equipment and Material

A Shimadzu LC-6A was used including two pumps, gradient elution system, and UV detector. The HPHIC column was a 4×100 mm I.D. synthesized in our institute (Silica from Vydac Co., Herjbra, CA, particle diameter, 7 μm, average pore diameter, 30 nm). The end groups of the ligands of XDF-SGM1 and XDF-SFM2 are PEG 600 and phenyl, respectively. The reversed-phase column, ODS, was a 4×100 mm I.D. also synthesized in our institute (Silica from Vydac Co.).

Reagents

Urea (A.G), sodium hydrochloride (NaCl, A.G), potassium dihydrogen phosphate (KH_2PO_4, A.G), and Methanol (A.G) were obtained from Xi'an Chemical Reagent Company (China). Ammonium sulphate [$(NH_4)_2SO_4$, A.G] and β-mercaptoethanol (A.G) were obtained from Tianjin and Shanghai Chemical Reagent Company (China). Urea must be recrystallized. Trypsin (9900 U/mg) and carboxypeptidase (CPB, 180 U/mg) were from Sigma (St. Louis, MO).

Mobile phase for HPHIC: solution A: 3.0 mol L^{-1} ammonium sulphate + 0.05 mol L^{-1} potassium dihydrogen phosphate (pH, 7.0) and

solution B: 0.05 mol L^{-1} potassium dihydrogen phosphate (pH, 7.0). Mobile phase for RPLC: Solutions A: 90% water + 10% methanol + 0.03% hydrochloric acid and solution B: 10% water + 90% methanol + 0.03% hydrochloric acid.

Methods

Extraction of Recombinant Human Proinsulin Inclusion Body

One gram rh-proinsulin inclusion body was added to 10 mL solution of 8.0 mol L^{-1} urea, 3.0 mmol L^{-1} β-mercaptoethanol, 0.20 mol L^{-1} NaCl, 1.0 mmol L^{-1} EDTA in 0.10 mmol L^{-1} Tris–HCl, pH 8.0, and suspended by stirring overnight at 4°C. After centrifugation, β-mercaptoethanol was added to the supernatant to obtain a solution of 13.0 mmol L^{-1} and the mixture was incubated at 37°C for 3 h. After centrifugation, the solution was stored for use in separation or renaturation.

High Performance Hydrophobic Interaction Chromatography

The sample of rh-proinsulin extracted with 8.0 mol L^{-1} urea solution was directly injected into the HPHIC column equilibrated with the mobile phase, and then eluted with the gradient elution mode by the mobile phases A [3.0 mol L^{-1} ammonium sulphate + 0.05 mol L^{-1} potassium dihydrogen phosphate (pH, 7.0)] and B [0.05 mol L^{-1} potassium dihydrogen phosphate (pH, 7.0)]. Sample size was 0.5 mL of the rh-proinsulin solution (total proteins, 4 mg) containing about 0.4 mg rh-proinsulin. The flow-rate was 1.0 mL min^{-1} and the chart paper speed was 4 mm min^{-1}. All detections were done at wavelength 280 nm. AUFS is 0.08.

Enzyme Cleavage of Recombinant Human Proinsulin

According to Ref.[2] Tris–HCl buffer with pH 7.5, trypsin, and CPB with the enzyme/substrate ratios 1 : 100 and 1 : 400 were added to 0.080 mol L^{-1}, respectively. The Mixture was incubated at 37°C for 30 min and cooled down immediately. Isopropanol was added to be the final concentration of 40% to stop the cleavage. The enzyme-cleaved products were identified with RPLC and SDS–PAGE.

The Dectection of Recombinant Human Proinsulin Concentration

The rh-proinsulin concentration was detected according to the Lowry method.

RESULTS AND DISCUSSION

Effects of High Performance Hydrophobic Interaction Chromatography Stationary Phase on the Separation of Recombinant Human Proinsulin

Because proteins can be separated with HPHIC, according to the total result of the hydrophobic interaction force from the mobile phase used to push protein molecules to the HPHIC stationary phase, and the non-selective force between the strong non-polar part of the protein molecules and the HPHIC stationary phase, the properties of HPHIC stationary phase can have an effect on the separation of proteins. The extracted solution of the rh-proinsulin with 8.0 mol L^{-1} urea solution was separated with two kinds of HPHIC stationary phases, XDF-SGM1 (PEG 600) and XDF-SGM2 (phenyl), respectively. The obtained chromatograms were shown in Fig. 1. Figure 1(a) and 1(b) separately denote what was obtained from XDF-SGM1 and XDF-SGM2. With the comparison both from each other, in terms of the resolution of peak area, the XDF-SGM2 column shown in Fig. 1(b) more effective. The purity and the mass recovery of rh-proinsulin separated from both were separately shown in Table 1. The purity of the separated rh-proinsulin with the XDF-SGM1 column shown in Table 1 is 80% from SDS–PAGE and the mass recovery is only 73.1%. However, with the XDF-SGM2 column, the purity and mass

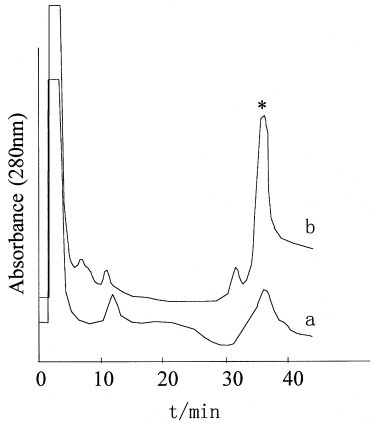

Figure 1. The chromatograms of rh-proinsulin separated by HPHIC with different packings. (a) XDF-SGM1, (b) XDF-SGM2, Flow rate 1.0 mL/min, detection wavelength 280 nm, and AUFS 0.08. Forty-five minutes non-linear gradient.
Key: *, rh-proinsulin.

Table 1. Comparisons of the purity and mass recovery of rh-proinsulin separated with two HPHIC stationary phases.

Stationary phase	Mass recovery	Purity
XDF-SGM1	73.1%	80%
XDF-SGM2	98.6%	84.5%

recovery were found to go up to 84.5% and 98.6%, respectively. These results indicate that XDF-SGM2 stationary phase should be used in this study.

Effects of Mobile Phase and pH on the Separation of Recombinant Human Proinsulin

Besides stationary phase, the composition of mobile phase should also effect the separation of proteins. Thus, the selection of suitable salts is important for obtaining better separation and greater bioactivity recovery of proteins with HPHIC. Six kinds of salts, such as ammonium sulphate, sodium hydrochloride, sodium acetate, ammonium acetate, ammonium hydrochloride, and sodium sulphate, etc., were selected. In terms of the mass recovery and purity of rh-proinsulin, the obtained results indicated two salts, ammonium sulphate and sodium hydrochloride, to be better, especially in the circumstance of the concentration of ammonium sulphate being $3.0\,\mathrm{mol\,L^{-1}}$. As it is well known, ammonium sulphate is the best salt and is widely used in HPHIC.[13] In addition, because of the function of salting-out of ammonium sulphate, it can stabilize the molecular conformation of proteins.

When a protein is separated with HPHIC, the protein can be absorbed on the HPHIC stationary phase at higher salt concentrations and eluted at lower salt concentrations. So, the buffer of the mobile phase may also have an effect on the separation of rh-proinsulin. The separation of rh-proinsulin was tested with the buffer solutions of potassium dihydrogen phosphate, Tri(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), and ammonium acetate. In terms of the mass recovery and purity of rh-proinsulin, the results showed that potassium dihydrogen phosphate and Tris–HCl were the best buffer solutions to be selected.

On investigating the effects of pH from 4.0 to 8.0 on the separation of rh-proinsulin, the results indicated that better separation of rh-proinsulin could be obtained in the pH range of 7.0 to 8.0. The mass recovery was greater than 90% and the purity is higher than 80%. Because proinsulin was enzyme-cleaved in the $0.080\,\mathrm{mol\,L^{-1}}$ Tris buffer solution and pH 7.5, the pH value of

the selected buffer solution of the HPHIC mobile phase should be comparable to that for enzyme cleavage. If it is successful, the separated rh-proinsulin with HPHIC can be directly cleaved by enzymes in the collected fraction.

Effects of Different Elution Modes for the Recombinant Human Proinsulin Separation

The selection of different elution modes may be used to improve not only the elution ability of proteins, but also the resolution. With 11 kinds of elution modes and the separation by the two kinds of HPHIC columns, the mass recovery and purity of rh-proinuslin were measured. Figure 2 only shows four of the chromatograms of rh-proinsulin obtained. Figure 2(a), 2(b), 2(c), and 2(d) denote the chromatograms of rh-proinsulin, marked by a star, obtained with impulse-elution; a linear gradient-elution for 25 min, two non-linear gradient-elutions for 45 min and 85 min, respectively. The comparisons of the mass recovery and purity of rh-proinsulin separated with the four kinds of elution modes are shown in Table 2. It is shown, that higher mass recovery and purity of rh-proinsulin can be obtained under the conditions of two non-linear gradient-elutions, with 45 min and 85 min. Although the purity and the mass recovery of rh-proinsulin can almost reach 100% with 85 min non-linear gradient-elution, it takes too long a time to accomplish it. So, non-linear gradient-elutions of 45 min should be selected.

In summary, the optimal condition of the separation of rh-proinsulin extracted with 8.0 mol L^{-1} urea solution by HPHIC, is a HPHIC column of XDF-SGM2; mobile phase, 3.0 mol L^{-1} ammonium sulphate, and elution mode, non-linear gradient of 45 min. Under the optimal chromatographic condition, rh-proinsulin extracted with 8.0 mol L^{-1} urea solution was separated by HPHIC, as shown in Fig. 2(c). The purity of rh-proinsulin was measured to be greater than 94% with SDS–PAGE (shown in Fig. 3) and its mass recovery, also, was more than 90%.

The Separation of Recombinant Human Proinsulin with RPLC

Because HPHIC can be used as a tool of renaturation with simultaneous purification of denatured proteins,[5–12] the rh-proinsulin extracted by 8.0 mol L^{-1} urea solution without any bioactivity, should be renatured in this manner. In order to test the renaturation efficiency of the rh-proinsulin with HPHIC, the fraction of the separated rh-proinsulin with HPHIC was collected and then separated with RPLC, results are shown in Fig. 4. If the purified rh-proinsulin can be simultaneously renatured with HPHIC, the retention time and the peak profile obtained by RPLC, should be the same

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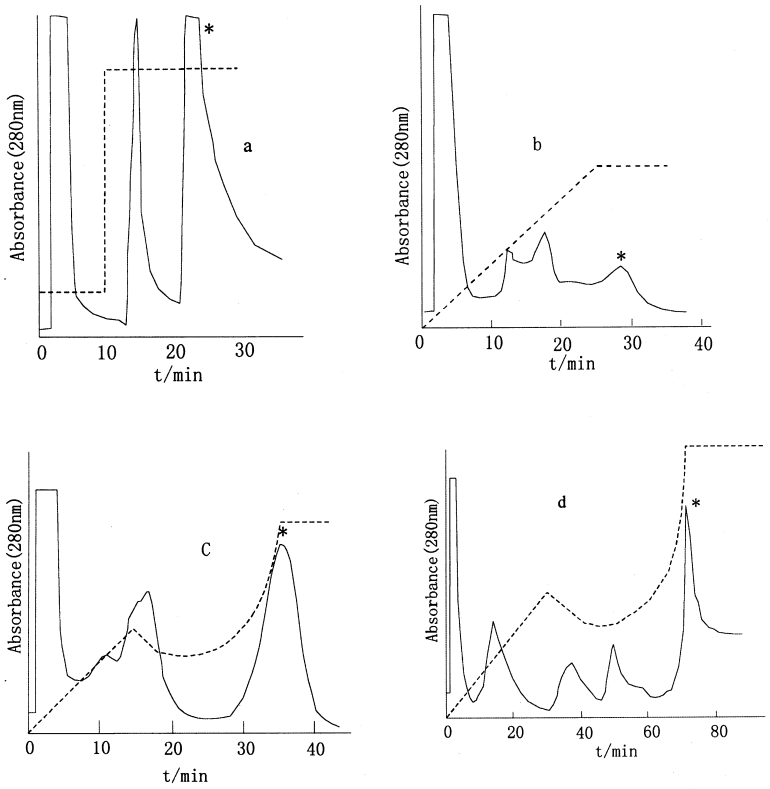

Figure 2. The effects of the different elution modes on the separation of rh-proinsulin with HPHIC. (a) impulse elution, (b) 25 min linear gradient, (c) 45 min non-linear gradient, (d) 85 min non-linear gradient. Flow rate 1.0 mL/min, detection wavelength 280 nm, and AUFS 0.08. *Key*: *, rh-proinsulin.

as that of standard rh-proinsulin. Figure 4(b) shows the chromatogram of the renatured and purified rh-proinsulin by RPLC, which had been collected, its fraction originally purified and renatured by HPHIC, and then re-purified with RPLC. As shown in this figure, only one peak had the same retention time and the peak profile as that of the standard rh-proinsulin. The result indicates that the molecular conformation of rh-proinsulin renatured with HPHIC was the same as that of the standard rh-proinsulin.

In general, the reduced/denatured protein can be renatured under oxidation conditions with the presence of GSSG/GSH. In the studies comparing the usual oxidation conditions, the GSSG/GSH was also added to the mobile

Table 2. Comparisons with the mass recovery and purity of rh-proinsulin separated with HPHIC under the different elution modes.

	Elution modes, min			
	Impulse elution	Linear gradient, 25 min	Non-linear gradient, 45 min	Non-linear gradient, 85 min
Mass recovery	120%	70.5%	95%	100%
Purity	48.6%	62%	94%	100%

phase employed. The results indicated that both are comparable. It was further proven, that the rh-proinsulin extracted with 8.0 mol L^{-1} urea solution can be really renatured with simultaneous purification by HPHIC.

The Enzyme Cleavage of Recombinant Human Proinsulin Renatured by High Performance Hydrophobic Interaction Chromatography

In order to test, further, that rh-proinsulin can be renatured simultaneously in the process of separation with HPHIC, the collected fraction of rh-proinsulin from HPHIC was directly cleaved by enzyme, according to the

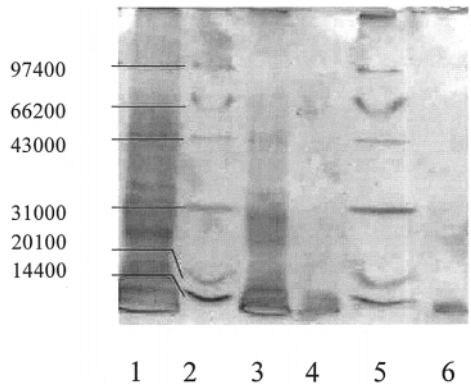

Figure 3. SDS–PAGE of the HPHIC fraction of rh-proinsulin. 1, 3. inclusion body of rh-proinsulin, 4, 6. The HPHIC fraction of rh-prosinsulin, 2, 5. Marker.

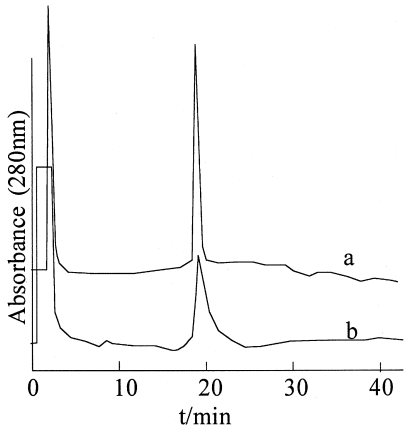

Figure 4. The chromatograms of standard rh-proinsulin and the fraction of rh-proinsulin separated with RPLC. (a) standard proinsulin, (b) the HPHIC fraction of rh-proinsulin. Flow rate 1.0 mL/min, detection wavelength 280 nm, and AUFS 0.08. Twenty-five minutes linear gradient elution.

method reported in Ref.[2] The enzyme-cleaved products were further separated with RPLC and tested by SDS–PAGE. The results are shown in Figs. 5 and 6, respectively. Figure 5(a) and 5(b) denote the standard rh-insulin and the enzyme-cleaved products of rh-proinsulin. Comparing Fig. 5(a) with 5(b), the insulin, denoted with a star, obtained by means of the enzyme cleavage of the rh-proinsulin, is what we desired. SDS–PAGE shown in Fig. 6, also confirms the conclusion obtained from Fig. 5. It is further proven, that the disulfide bonds of rh-proinsulin can form correctly.

In addition, in order to further prove that insulin can be obtained through the enzyme cleavage of rh-proinsulin, the collected fraction of the rh-insulin, denoted by star, from RPLC, shown in Fig. 5(b), and its molecular weight was measured with MALDI-TOF. The results show that the molecular weight is 11,456 Dalton equal 2-folds of standard human insulin (5701 Dalton). Because insulin can form the dimmer easily in the methanol solution, the molecular weight of insulin in the methanol mobile phase analyzed with MALDI-TOF is 2-folds of standard human insulin. However, although the difference of the molecular weight of the dimmer between the standard insulin and rh-insulin is 54 Dalton, and the deviation reaches to 4.5%, it is less than the molecular weight of glycin. In the presence of salt, the molecular weight measured with MALDI-TOF may be higher than the standard value.

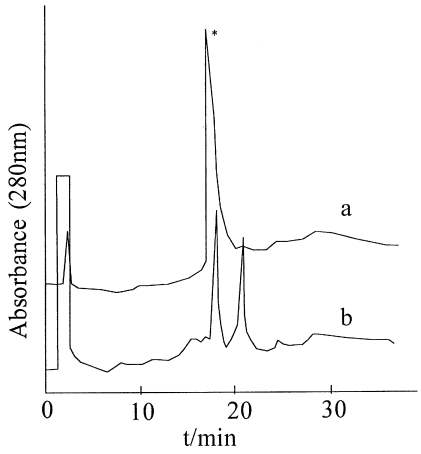

Figure 5. The chromatogram of enzyme-cleaved products of rh-proinsulin separated with RPLC. (a) standard insulin, (b) the enzyme-cleaved products of rh-proinsulin. Flow rate, 1.0 mL/min, detection wavelength 280 nm, and AUFS 0.08. Twenty-five minutes linear gradient elution.

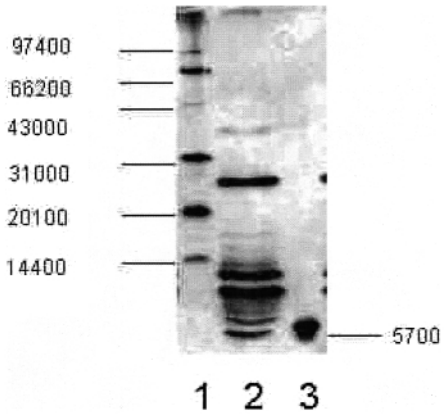

Figure 6. SDS–PAGE of enzyme-cleaved products of rh-proinsulin. 1. Marker, 2. enzyme-cleaved products of rh-proinsulin, 3. standard human insulin.

CONCLUSION

The extracted rh-proinsulin with $8.0\,\mathrm{mol\,L^{-1}}$ urea solution can be renatured and purified simultaneously by HPHIC. The disulfide bonds of rh-proinsulin can form correctly on the HPHIC column at the non-oxidation condition. This conclusion was proven with RPLC, SDS–PAGE, and MALDI-TOF. Compared to the usual dilution and dialysis methods, the renatured and purified rh-proinsulin, simultaneously, by HPHIC can be accomplished by only one chromatographic run within 1 hour, and the extracted rh-proinsulin by urea solution can be directly injected into a HPHIC column. It is unnecessary to exchange the enzyme-cleavage buffer solution, the rh-proinsulin can be enzyme-cleaved directly in the collected fraction containing rh-proinsulin. Thus, the process for the renatured and purified rh-proinsulin becomes very simple and fast. Under the optimal condition in this study, the purity of the obtained rh-proinsulin can approach more than 90% and the mass recovery to be more than 94%.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 29675017 and 39880003), the Foundation of the Young Teacher in the University of Ministry of Education P. R. C (Grant No. DF00308), the Natural Science Foundation in Shaanxi Province (Grant No. 2001H02), and the Foundation of the Committee of Education in Shaanxi Province (Grant No. 99JK101). The inclusion bodies of rh-proinsulin, expressed in *E. coli* and standard rh-proinsulin, were provided by Xuzhou Biochemical Pharmaceutical Plant, Jiangsu Province, China.

REFERENCES

1. Nilsson, J.; Jonasson, P.; Samuelsson, E.; Mols, T.; Stahl, S.; Uhlen, M. J. Biotechnol. **1996**, *48*, 241–250.
2. Yang, Z.H.; Du, X.; Tang, J.G. Appl. Biochem. Biotechnol. **1999**, *76*, 107–144.
3. Kim, C.K.; Kim, Y.I.; Pheu, J.N. Process for preparing human rh-proinsulin. United States Patent, No. 5952, 461, 1999.
4. Ladisch, M.R.; Kolmann, K.L. Biotechnol. Prog. **1992**, *8*, 469–478.
5. Geng, X.D.; Chang, X.Q. J. Chromatogr. **1992**, *599*, 185–194.
6. Guo, L.; Geng, X.D. Chinese. J. Biotech. **2000**, *16*, 661–666.

7. Bai, Q.; Wei, Y.M.; Geng, M.H.; Geng, X.D. Chem. J. Chinese. Univ. **1997**, *18*, 1291–1295.
8. Bai, Q.; Kong, Y.; Zhang, Y.J.; Geng, X.D. J. NW Univ. (Science Ed.) **2000**, *30*, 4851–4854.
9. Geng, X.D. *Guide to Theory of Modern Separation Science*; Higher Education Press: Beijing, 2001; 339–402.
10. Zhang, Y.D.; Zhang, L.H.; Geng, X.D. Chinese. J. Biotech. **1991**, *15*, 240–243.
11. Liu, T.; Geng, X.D. J. NW Univ. (science ed.) **1999**, *29*, 123–126.
12. Guo, L. Chin. J. Chromatogr. **2001**, *19*, 301–303.
13. Geng, X.D.; Guo, L.; Chang, J.H. J Chromatogr. **1990**, *507*, 1–23.

Received August 14, 2002
Accepted October 14, 2002
Manuscript 5933