Available online at www.sciencedirect.com
ScienceDirect
Chinese Chemical Letters 20 (2009) 1487–1490
CHINESE CHEMICAL LETTERS
www.elsevier.com/locate/cclet

Short communication

Refolding of reduced/denatured bovine pancreatic insulin with ion-exchange chromatography coupled with MALDI-TOF MS

Cui E. Lin, Quan Bai *

Institute of Modern Separation Science, Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, Northwest University, Xi'an 710069, China

Received 26 March 2009

Abstract

The refolding of the reduced/denatured insulin from bovine pancreas as the model protein was investigated with weak anion exchange chromatography (WAX) coupled with MALDI-TOF MS. The results indicated that the disulfide bonds almost cannot be formed correctly with the common mobile phase by WAX. However, with the urea gradient elution and in the presence of GSSG/Cyst as the ratio 1:6 in the mobile phase employed, the disulfide exchange of reduced/denatured insulin can be accelerated resulting in forming the correct three disulfide bonds. The protein refolding efficiency of reduced/denatured insulin can be increased from 3% to 34%. The effects of urea gradient and the oxidant and reductant groups, such as GSSG/GSH, Cyst, and GSSG/Cyst, on the forming the disulfide bonds of reduced/denatured insulin were investigated in detail. The results were further tested by the separation of the WAX fraction of reduced/denatured insulin with RPLC and MALDI-TOF MS.

Keywords: Protein refolding; Liquid chromatography; Reduced/denaturation; Insulin; MALDI-TOF MS

Many kinds of recombinant proteins have been produced in *E. coli* resulting in forming inclusion bodies. The challenge is how to convert the insoluble inclusion body proteins into soluble and bioactive proteins [1]. Liquid chromatography (LC) has been becoming one of the most interesting and exciting protein refolding method developed in recent years, which was named as protein folding liquid chromatography (PFLC) [2–7]. However, a lot of difficult problems are encountered during the folding of a denatured/reduced protein. It involves both thermodynamic problem (correct forming of many disulfide bonds existed in a protein molecule) and kinetic problem (rapid forming of disulfide bonds) [5]. Recently, one of the authors investigated the refolding of the reduced/denatured insulin using hydrophobic interaction chromatography (HIC) [8] and size exclusion chromatography (SEC) [9], but some problems still exist to be waiting for solving. For example, only according to comparing the retention time and peak profile between the refolded and native insulin with RPLC, it is not confirmed accurately and easily whether the disulfide bonds between chain A and B can be formed correctly or not, and how the stationary phase and mobile phase of LC used contribute to the formation of disulfide bonds in the refolding process, respectively. In general, the standard proteins with single chain, such as lysozyme and ribonuclease A, etc, were selected to investigate the refolding of the

* Corresponding author.
E-mail address: baiquan@nwu.edu.cn (Q. Bai).

1001-8417/$ – see front matter
doi:10.1016/j.cclet.2009.07.005

reduced/denatured proteins. However, it was difficult to determine whether the disulfide bonds can be formed correctly inner-amino chain or not. In this paper, we will select the standard insulin from bovine pancreas as the model protein to study the refolding of the reduced/denatured insulin with weak anion exchange chromatography (WAX) coupled with matrix assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF MS). Insulin is composed of two chains held together by two disulfide bonds. MALDI-TOF MS can be used to measure the molecular weight of refolded insulin. In addition, it can give much more information on the modifiers of chain A and B of insulin by some small molecules, such as urea, cyanuric acid and β-ME in denaturation solution and GSSG or GSH in the mobile phase used, which is very important to understand the mechanism of protein refolding on the liquid–solid surface.

1. Experimental

2 mg insulin (Sigma, USA) was dissolved in 1.0 mL reduced/denatured buffer containing 8.0 mol L^{-1} urea, 0.1 mmol L^{-1} Tris–HCl and 1 mmol L^{-1} EDTA at pH 8.5, and incubated for 3 h at 40 °C in the presence of 150 mmol L^{-1} β-ME, and then stored at 4 °C.

Ion exchange chromatographic condition: mobile phase I: solution A, 20 mmol L^{-1} Tris, pH 8.0; solution B, 20 mmol L^{-1} Tris + 1.0 mol L^{-1} NaCl, pH 8.0. Mobile phase II: solution A: 20 mmol L^{-1} Tris + 1.8 mmol L^{-1} Cyst + 0.3 mmol L^{-1} GSSG, pH 8.0; solution B: 20 mmol L^{-1} Tris + 1.0 mol L^{-1} NaCl + 1.8 mmol L^{-1} Cyst + 0.3 mmol L^{-1} GSSG, pH 8.0. Mobile phase III: solution A: 20 mmol L^{-1} Tris + 1.8 mmol L^{-1} Cyst + 0.3 mmol L^{-1} GSSG, pH 8.0; solution B: 20 mmol L^{-1} Tris + 1.0 mol L^{-1} NaCl + 8 mol L^{-1} urea + 1.8 mmol L^{-1} Cyst + 0.3 mmol L^{-1} GSSG, pH 8.0.

2. Results and discussion

The standard insulin was reduced/denatured with 8.0 mol L^{-1} urea and β-ME to become the chain A and B, respectively. 200 μL reduced/denatured insulin sample was injected directly on the DEAE WAX column, and then eluted using the mobile phase I with 30 min linear gradient. The chromatogram was shown in Fig. 1. Peak A is β-ME, peak B was collected and then separated with simultaneous desalting by RPLC (Fig. 2(1)). The fractions a–f in Fig. 2(1) were also collected, respectively, and lyophilized for MALDI-TOF MS analysis. The MS results indicated that fractions a–c and e–f in Fig. 2(1) were the chain A and B of insulin and their modifiers with urea, cyanuric acid and β-ME, respectively. Only fraction d has the same molecular weight (*m*/*z* 5733) and retention time as that of the standard insulin in RPLC, which indicated that only fraction d in Fig. 2(1) was refolded into its native state with the correct disulfide bonds by WAX. However, the mass recovery of refolded insulin is only less than 3%. As a result, it illuminated that the reduced/denatured insulin almost cannot be refolded into its native state under this condition.

It is necessary to provided redox surroundings by adding oxidant and reductant with certain concentrations in the refolding buffer solution, such as GSH/GSSG, the active sulfhydryls would form disulfide bonds quickly and correctly, and it will also promote the refolding of proteins greatly [10]. Therefore, the oxidant and reductant groups, such as

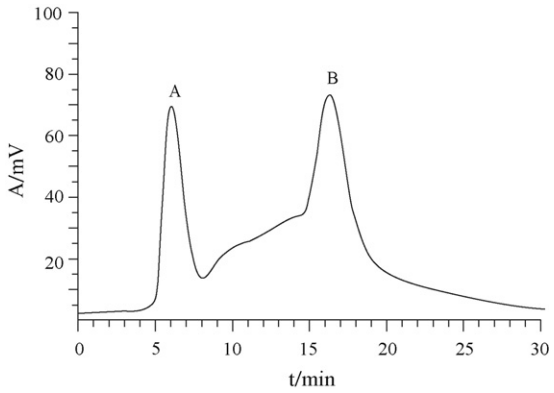

Fig. 1. The chromatogram of reduced/denatured insulin with 8.0 mol L^{-1} urea separated by WAX. Chromatographic conditions: column: DEAE sepherose, 75 mm × 10 mm I.D.; mobile phase I; 100% A–100% B, 30 min linear gradient; flow rate: 1.0 mL min^{-1}; detect wavelength: 280 nm.

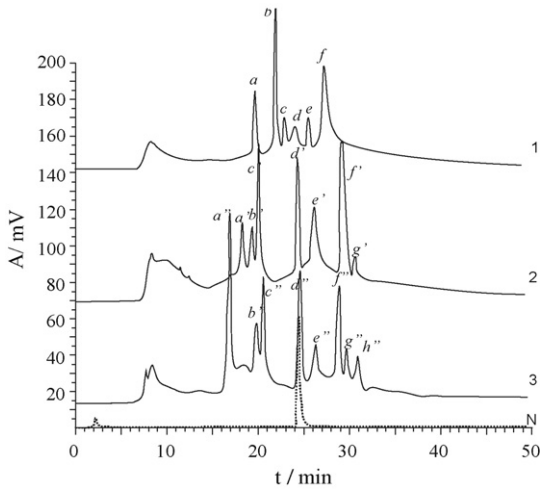

Fig. 2. The comparison of RPLC chromatograms of reduced/denatured insulin fractions separated with WAX using different mobile phases. Chromatographic conditions: column: YMC-ODS (10 μm, 12 nm), 150 mm × 4.6 mm I.D.; solution A, H_2O + 0.1% TFA; solution B, CH_3CN + 0.1% TFA; non-gradient elution: 0–5 min, 0–20% B, 5–50 min, 20–50% B, 50–60 min, 50–100% B; flow rate: 1.0 mL min^{-1}; detect wavelength: 280 nm; N. native insulin; (1) mobile phase I; (2) mobile phase II; (3) mobile phase III.

GSH/GSSG, cysteine (Cyst) and Cyst/GSSG, in a suitable ratio were added in the mobile phase, respectively, and their effects on the refolding of reduced/denatured insulin with WAX were investigated in detail. With the presence of Cyst/GSSG as the ratio 6:1 in the mobile phase II employed, the reduced/denatured insulin was refolded with WAX, and then the insulin fraction was separated with RPLC (shown in Fig. 2(2)). It shows that the peak d′ become more higher and larger than peak d shown in Fig. 2(1), and the mass recovery of refolded insulin is about 11%. The result indicates that the disulfide exchange of reduced/denatured insulin can be accelerated resulting in forming the correct three disulfide bonds. Therefore, the reduced/denatured insulin only can be refolded partly into its native state by WAX using oxidant mobile phase II.

For a successful oxidative refolding, one of main points is that how to depress the formation of both protein aggregates and intermolecular inappropriate disulfide bonds. If the intermediate states, which have native-like tertiary structure, are separately trapped from each other, the subsequently formation of the disulfide bonds may proceed successfully. Generally, with the presence of urea in mobile phase, it cannot only diminish the aggregate forming, but also increase the eluting strength of mobile phase [11]. Thus, 8.0 mol L^{-1} urea was added into mobile phase III (solution B), and the reduced/denatured insulin was refolded with WAX using oxidation mobile phase III. The refolded insulin fraction was separated by RPLC and eight peaks can be obtained (shown in Fig. 2(3)), and all these fractions were analyzed with MALDI-TOF MS (shown in Fig. 3). If the disulfide bonds between chain A and B can be formed correctly, the refolded insulin should have the same molecular weight as that of native one. From Fig. 3, it can be seen

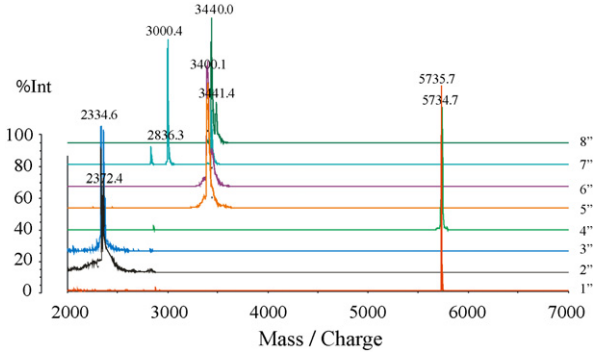

Fig. 3. The MALDI-TOF MS analysis of the fractions a″–h″ in Fig. 2(3). 1″–8″ donated the fractions a″–h″ in Fig. 2(3).

that peak d″ has the same retention time and the molecular weight as that of standard insulin, while peak a″ also has the same molecular weight but shorter retention time. Their mass recoveries are about 16% and 18%, respectively. Furthermore, the refolded insulin (peak d″) also has the same spectrums of UV–vis and fluorescence as that of native one (not shown here). The results indicated that using urea gradient elution and oxidation mobile phase III, the protein aggregation cannot only be depressed, but also much more chain A and chain B of insulin can be eluted to form the correct disulfide bonds resulting in the refolding efficiency of reduced/denatured insulin to be increased greatly. However, the refolded insulin of peak a″ with WAX has not the same retention time as the standard one in Fig. 2(3). They also have the different spectrums of UV–vis and fluorescence (not shown here). The results indicated that the refolded insulin maybe has the correct disulfide bonds, but it has the different conformation with that of native insulin. Therefore, the protein refolding efficiency of reduced/denatured insulin can be increased from 3% to 34%.

Based on the molecular mechanism of protein refolding in IEC, the refolding process of the reduced/denatured proteins in IEC processes instantaneously [11], because it involves carry out oxidation–reduction reaction which needs enough time. Thus the obtained peak shape, peak height, even retention of the refolded proteins in this instance may be a little different from that of the same protein in N state. Furthermore, it takes a certain time, such several, even over 10 h to accomplish the whole process of the oxidation–reduction reaction [11]. The contribution of the IEC column is to prevent from the aggregates of the unfolded protein molecules and make the –SH correctly closed together by the recognition of some specific amino acid residues to the stationary phase of the IEC and subsequently wait for accomplishing oxidation.

3. Conclusion

The refolding of the reduced/denatured insulin from bovine pancreas as the model protein was investigated using WAX coupled with MALDI-TOF MS. The results indicated that with the urea gradient elution and in the presence of GSSG/Cyst as the ratio 1:6 in the mobile phase employed, the reduced/denatured insulin can be partly refolded into its native state, and the disulfide exchange of it can be accelerated resulting in forming the correct three disulfide bonds. The protein refolding efficiency of insulin can be increased from 3% to 34%.

Acknowledgments

This work is supported by the National 863 Program (No. 2006AA02Z227), the Foundation of Key Subject Construct of Analytical Chemistry in Shaanxi Province and the Foundation of Key Laboratory of Modern Separation Science in Shaanxi Province (No. 05JS62).

References

[1] J.F. Kane, D.L. Hartley, Trends Biotechnol. 6 (1988) 95.
[2] X.D. Geng, X.Q. Chang, J. Chromatogr. 599 (1992) 185.
[3] X.D. Geng, Sci. Chin. (Ser. B) 45 (2002) 655.
[4] X.D. Geng, Q. Bai, C.Z. Wang, Protein Folding Liquid Chromatography, Science Press, Beijing, China, 2006, p. 1 (in Chinese).
[5] X.D. Geng, C.Z. Wang, J. Chromatogr. B 249 (2007) 69.
[6] M. Li, Z.G. Su, J.C. Janson, Protein Exp. Purif. 33 (2004) 1.
[7] A. Jungbauer, W. Kaar, R. Schlegl, Curr. Opin. Biotechnol. 15 (2004) 487.
[8] Q. Bai, Y. Kong, X.D. Geng, Chem. Res. Chin. Univ. 23 (2002) 1483.
[9] Q. Bai, Y. Kong, C.H. Dong, X.D. Geng, Sci. Chin. (Ser. B) 48 (suppl.) (2005) 55.
[10] E.D. Clark, Curr. Opin. Biotechnol. 12 (2001) 202.
[11] Y. Wang, B.L. Gong, X.D. Geng, Chin. Chem. Lett. 14 (2003) 828.