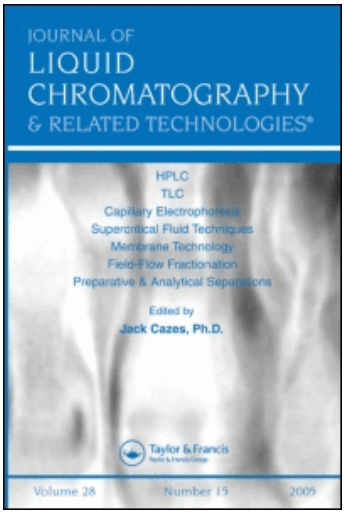

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Abstract: Mis-folded recombinant cytochrome c_3 (rcyt c_3) formed in the purification process was refolded with strong cation exchange chromatography, with tri-gradient of salt, urea, and pH in the mobile phase. The optimal concentration of urea was found to be 6.0 mol/L in mobile phase to refold the mis-folded rcyt c_3 under the experimental conditions. The recovery of the correctly refolded rcyt c_3 was calculated to be 57.0%.

Keywords: *Shewanella oneidensis*, Recombinant cytochrome c_3, Refolding, Strong cation exchange chromatography

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INTRODUCTION

Cytochromes c_3 (cyt c_3) are low potential tetraheme proteins, and have many potential uses such as bioremediation, bioelectronics, physicochemical analyses, and biotechnology.[1] Natural cyt c_3 are found almost exclusively in anaerobic bacteria, including sulfate reducing bacteria in the genus *Desulfovibrio*. At present, they can be produced by several expression systems, such as *E. coli*,[2,3] *Shewanella oneidensi*,[1,4] etc. In the *S. oneidensis* system, the highest yield of recombinant *Desulfovibrio vulgaris*, Miyazaki F cyt c_3 (rcyt c_3) was 8.3 mg/L-culture so far. Meanwhile, mis-folded rcyt c_3 was also found in the purification process, amounting to 10–30% of the total product. To further improve the yield of native rcyt c_3, we tried to refold the mis-folded rcyt c_3.

To refold denatured proteins, various methods have been proposed such as dilution, dialysis, reverse micelles,[5] chaperones,[6] polyethylene glycol,[7] tetrahydrofuryl alcohol,[8] surfactant,[9] antibodies,[10] and liquid chromatography.[11] Of these methods, liquid chromatography has been paid more attention due to its preparative ability in industry. Especially, ion exchange chromatography has been successively employed for the refolding of many proteins, such as papiloma virus HPV E7MS2,[12] fusion proteins of monomeric α-glucosidase,[13] recombinant secretory leukocyte inhibitor,[14] lysozyme,[15] and bovine serum albumin.[16] In the studies of protein refolding, how to renature mis-folded proteins avoiding aggregation is a usually encountered question.

This paper presents a procedure to refold the mis-folded rcyt c_3 to its native state with strong cation exchange chromatography (SCX) by tri-gradient of salt, urea, and pH in the mobile phase. Under the optimal conditions, the recovery of the correctly refolded rcyt c_3 was 57%, fulfilling the goal of improving the total yield of rcyt c_3.

EXPERIMENTAL

Host Strain and Vector

Shewanella oneidensis (*S. oneidensis*) strain MR-1 was used as a host cell for *Desulfovibrio vulgaris*, Miyazaki F (*Dv*MF) cyt c_3 expression.[4] The cells were transformed with pKFC3 vector,[17] which was derived from pKF19 k vector (TaKaRa Bio Co. Ltd., Japan, Gene bank accession No. D63847) containing *Dv*MF cyt c_3 gene as described previously.[17]

Media and Cultivation Condition

Transformants were microaerobically grown at 30°C, 90 rpm, and 48 h in 2 × YT media, adjusting the pH at 7.0 by 2 N NaOH. The media contained

200 μg/mL of kanamycine and 10 μg/mL of rifanpicine as described previously.[17]

Origination of Mis-folded rcyt c_3

Mis-folded rcyt c_3 originated from the separation and purification process of rcyt c_3, which included four steps.[18] In the first step, the supernatant containing rcyt c_3 was collected after centrifugating the broken cell supernatant treated with 50% and 70% ammonium sulfate, respectively. In the second step, rcyt c_3 (pI = 10.5) was separated from the supernatant with hydrophobic interaction chromatography. In the third step, the resulting rcyt c_3 fraction was dialyzed, then purified by SCX (SP Sepharose HP column, Hitrap™ 2 × 5 mL, Amersham Pharmacia Biotech) to give native rcyt c_3 and mis-folded rcyt c_3, respectively. In the fourth step, the mis-folded rcyt c_3 fraction after dialysis was frozen and dried for use as the next studied object for this paper.

Characterization of Produced rcyt c_3

The obtained rcyt c_3 and native *D. vulgaris* Miyazaki F cyt c_3 were lyophilized three times with 99.9% ^2H_2O and resuspended in deuterated 10 mM sodium phosphate buffer, p^{2}H 7.0. One-dimensional ^{1}H NMR spectra at 400 MHz recorded at 303 K on a Bruker DRX-400 NMR spectrometer confirmed that they were identical.

The mass recovery of the correctly refolded rcyt c_3 was calculated with regression analysis.

RESULTS

The refolding procedure of mis-folded rcyt c_3 included dissolving of the obtained mis-folded rcyt c_3 with urea, renaturation of mis-folded rcyt c_3 by SCX with tri-gradient of salt, urea and pH in the mobile phase, and finally, separation of the correctly refolded rcyt c_3 by the same SCX but with salt gradient in the mobile phase.

In detail, 2.0 mg mis-folded rcyt c_3 solid was dissolved in 1.0 mL of 6.0 M urea solution. The sample was then placed at room temperature for 24 hours. Two refolding modes were used. In the first one (namely, urea-salt gradient), 100 μL solution was injected into a SP Sepharose HP column (two 5 mL Hitrap™ columns were tandemly connected, Amersham Pharmacia Biotech) pre-equilibrated with solution A1 (6.0 M urea in 30 mM sodium phosphate buffer, pH 7.0). Then, it was eluted with 5 mL of solution A1, 10 mL of A1/B1 mixture with the gradient from 0 to 100%

(for a solution B1, 1.0 M NaCl, 30 mM sodium phosphate buffer, pH 7.0), and then 10 mL of 100% B1 at 0.8 mL/min flow rate to elute the correctly refolding rcyt c_3, which formed in the elution process. In the second one (namely, tri-gradient of urea-salt-pH), other chromatographic conditions including the solution composition and the gradient are the same as those in the first one, except that the pH values of solution A1 and B1 were 6.0 and 8.0, respectively. In these two cases, only one peak was found in the chromatogram (Figure 1). Its fraction contained the mixture of correctly refolded and mis-folded cyt c_3. In the second step, after dialysis against 30 mM sodium phosphate buffer, pH 7.0, the eluted fraction of this peak was loaded onto the same SP Sepharose HP column pre-equilibrated with solution A2 (30 mM sodium phosphate buffer, pH 7.0), then it was eluted with 80 mL of

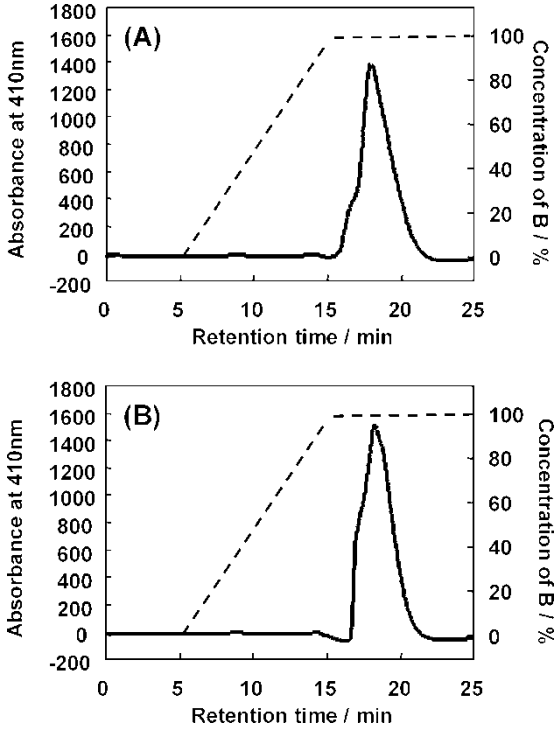

Figure 1. The refolding chromatogram of mis-folded rcyt c_3 by strong cation chromatography under two elution modes. Column: SP, 2 × 5 mL. Concentration of sample: 2.0 mg/mL, injection volume: 100 μL. Elution mode: buffer A1 with 5 mL, 10 mL of A1/B1 mixture with the gradient from 0 to 100% B1, then 100% B1 with 10 mL; Flow rate: 0.8 mL/min. (A) Buffer A1, 6.0 mol/L urea-30 mmol/L sodium phosphate, pH 7.0; Buffer B1, 1.0 mol/L NaCl-30 mmol/L sodium phosphate, pH 7.0. (B) Buffer A1, 6.0 mol/L urea-30 mmol/L sodium phosphate, pH 6.0; Buffer B1, 1.0 mol/L NaCl-30 mmol/L sodium phosphate, pH 8.0.

A2/B2 mixture with the gradient from 0 to 40% B2 (for solution B2, 1.0 M NaCl, 30 mM sodium phosphate buffer, pH 7.0), and then impulse elution with 20 mL of 100% solution B2 to separate the correctly refolded rcyt c_3.

After the separation by SCX with the salt gradient, the corresponding fraction obtained from the two elution modes gave mainly three peaks (Figure 2), of which the retention times of peaks 1 and 3 are the same as the native cyt c_3 and the initial mis-folded rcyt c_3, respectively, indicating preliminarily parts of mis-folded rcyt c_3 restore its native structure in the elution process with urea in the mobile phase, but the peak area was remarkably different. For a urea-salt gradient, the final peak area of peak 1, corresponding to the correctly refolded rcyt c_3, was very small (Figure 2A), while it was large (Figure 2B) for the tri-gradient of urea-salt-pH, that is, when a pH gradient was simultaneously added to the urea-salt gradient; the mass

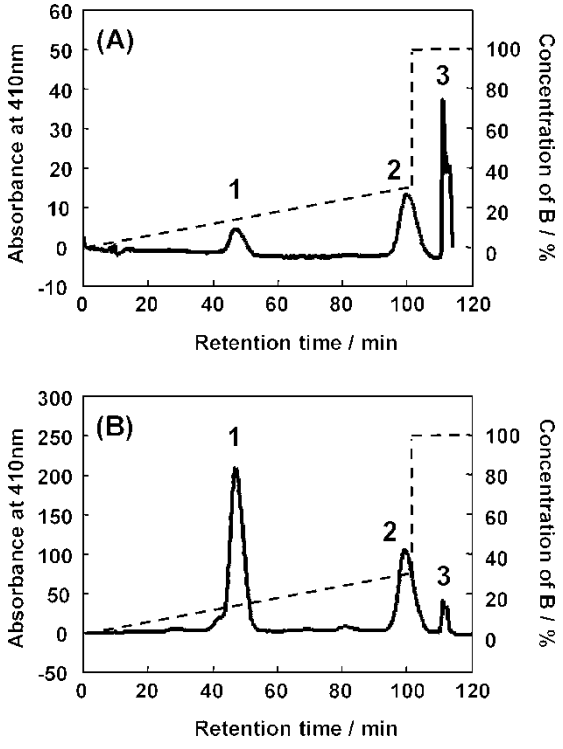

Figure 2. The separated chromatogram of the refolded rcyt c_3 mixture from Figure 1 by strong cation chromatography. (A), sample from Figure 1A; (B) sample from Figure 1B. Column: SP, 2 × 5 mL. Injection volume: 100 μL. Buffer A2, 30 mmol/L sodium phosphate buffer, pH 7.0; buffer B2, 1.0 mol/L NaCl-30 mmol/L sodium phosphate buffer, pH 7.0. Elution mode: 80 mL of A2/B2 mixture with the gradient from 0 to 40% B2, and then impulse elution with 20 mL of 100% solution B2; Flow rate: 0.8 mL/min.

recovery of the correctly refolded rcyt c_3 improved and, appropriately, that of the mis-folded rcyt c_3 decreased. The elution modes of the combined urea, salt, and pH gradient is the optimal one, and the mass recovery of the correctly refolded rcyt c_3 in Figure 2B was calculated to be 57% by regression analysis.

Characterization of rcyt c_3

The biological activity of rcyt c_3 has been confirmed in terms of its redox potentials and electron transfer kinetics with hydrogenase.[18] In addition, physical methods, i.e., NMR, and UV-vis, could be used to determine if the obtained cyt c_3 possesses the identical structure with the native cyt c_3, as reported by Ozawa, et al.[1] As stated above, the fraction of peak 1 in Figure 2, whose retention time is the same as native cyt c_3, was preliminarily confirmed to contain the correctly refolded rcyt c_3. To further confirm this, NMR were, therefore, used to determine the structure of the obtained rcyt c_3. Figure 3 is the NMR diagram of native cyt c_3 (A), the initial mis-folded rcyt c_3 (B), the fraction of peak 1 (C) and peak 2 (D) in Figure 2. The spectrum of peak 1 is

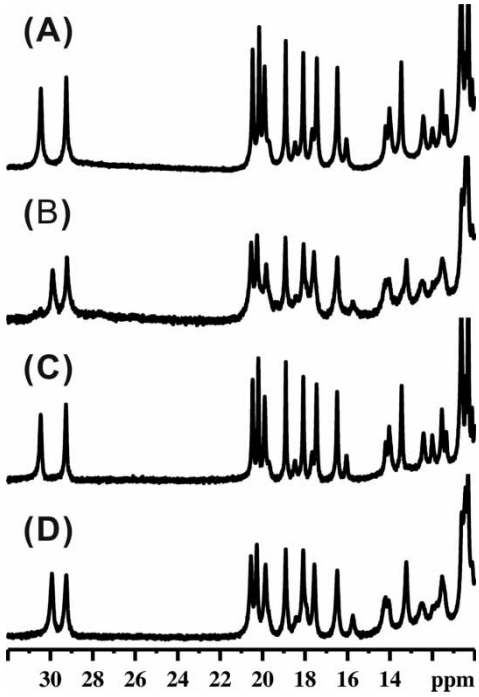

Figure 3. Four hundred MHz ^{1}H-NMR spectra of rcyt c_3. (A) authentic *Dv*MF cyt c_3; (B) mis-folded rcyt c_3; (C) peak 1 in Figure 2B; (D) peak 3 in Figure 2B.

identical with native cyt c_3, indicating that the obtained rcyt c_3 possess the same structure as native cyt c_3. A total 57% of mis-folded rcyt c_3 was correctly refolded via the procedure described in this paper.

DISCUSSION

The chromatographic condition, including column type, mobile phase composition, elution gradient mode, flow rate of mobile phase, and sample concentration, had influences on the refolding behavior of unfolded rcyt c_3 inside a SCX column. Of these factors, denaturant concentration in the mobile phase and elution gradient mode were found to be crucial to the renature of mis-folded rcyt c_3.

Effect of the Urea Concentration in the Mobile Phase on Refolding of Mis-folded rcyt c_3

To investigate the effect of denaturant concentration, the chromatographic experiment was carried out with 2.0, 4.0, 6.0, 7.0, and 8.0 M urea in solution A1, respectively, which constitutes a gradient of urea-salt along with salt in the mobile phase. Under these conditions, only one peak was found in the chromatogram. The resulting fractions were then separated by SCX with the salt gradient, respectively. No peak at the location of the target peak in the chromatogram was found at 2.0 and 4.0 M urea, while many peaks near the target peak appeared at 7.0 and 8.0 M (Figure not shown). At 6.0 M urea only one peak was on the location of native cyt c_3, and no other peaks near it were found in the chromatogram (Figure 2A). The fraction corresponding to this peak was proven to be the mixture of the correctly refolded and mis-folded cyt c_3. These results indicated that 6.0 mol/L urea was the optimal concentration of the denaturant agent for the refolding of mis-folded rcyt c_3.

Effect of the Elution Modes on the Refolding of Mis-folded rcyt c_3

The two elution modes in SCX, in which urea concentrations of solution A1 are both 6.0 mol/L, but one gradient is combination of urea and salt while the other is trigradient of urea-salt-pH, were used to illustrate the influence of the elution mode on the refolding of mis-folded rcyt c_3. In these two cases, only one peak was found in the chromatogram as stated above in the experimental section. Then, SCX only with the salt gradient was used to separate the fraction obtained from Figure 1. The results showed that the trigradient was of the optimal elution mode, with a refolding yield of 57%.

Although, the mechanism of protein refolding by ion exchange chromatography is unclear so far, the refolding procedure was inferred to include two stages. In the first stage, proteins are retained on the stationary phase due to the electronic interaction, and thus is separated from the denaturant and starts to refold to form a hydrophobic core. In the second stage, the hydrophobic core further folded to the native structure in the elution process.[19]

The function of the urea gradient, in which urea concentration decreased gradually to zero, is similar to the dilution method, but the difference is that unfolded protein was constantly interacting with the surface of the resins packed inside a column with the decrease of the denaturant concentration. Consequently, the formation of aggregation could be efficiently restrained, and the proteins start to refold to form the hydrophobic core and, further, to the native structure.[19] In liquid chromatography, the strong interaction between protein and stationary phase would cause the change of the molecular conformation.[20] Therefore, the pH gradient was used to improve the recovery by decreasing the surface charge of rcyt c_3 (pI 10.5) gradually with the change of pH from 6.0 to 8.0 in the elution solution, so as to gradually decrease the interaction between protein and stationary phase. As a result, such an elution mode with urea, salt, and pH gradient, simultaneously, in the mobile phase could favor the formation of the nature structure of rcyt c_3 during the elution process.

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REFERENCES

1. Ozawa, K.; Yasukawa, F.; Fujiwara, Y.; Akutsu, H. A simple, rapid, highly efficient gene expression system for multiheme cytochrome c_3. Biosci. Biotechnol. Biochem. **2001**, *65* (1), 185–189.
2. Herbaud, M.-L.; Aubert, C.; Durand, M.-C.; Guerlesquin, F.; Thöny-Meyer, L.; Doll, A. *Eschericia coli* can be able to produce heterologous tetraheme cytochrome c_3 when the ccm genes are co-expressed. Biochim. Biophys. Acta **2000**, *1481*, 18–24.
3. Costa da Conte, P.N.; Saraiva, C.L.M. Expression of a *Desulfovibrio* Tetraheme cytochrome c_3 in *Escherichia coli*. Biochem. Biophys. Res. Commun. **2000**, *268*, 688–691.
4. Ozawa, K.; Tsapin, A.I.; Nealson, K.H.; Cusanovich, M.A.; Akutsu, H. Expression of a Tetraheme Protein, *Desulfovibrio vulgaris* Miyazaki F cytochrome c_3, in *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. **2000**, *66* (9), 4168–4171.
5. Hagen, J.; Hatton, T.A.; Wang, D.I.C. Protein refolding in reversed micelles. Biotech. Bioeng. **1990**, *35* (10), 955–965.

6. Bulleid, N.J.; Freedman, R.B. Defective co-translational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes. Nature **1990**, *335*, 649–651.
7. Cleland, J.L.; Builder, S.E.; Swartz, J.R.; Winker, M.; Chang, J.Y.; Wang, D.I.C. Polyethylene glycol enhanced protein refolding. Bio/Technology **1992**, *10*, 1013–1019.
8. Shen, Y.H.; Wang, H.B.; Wei, Y.M.; Geng, X.D. Studies on the assisted actions of tetrahydrofurfuryl alcohol for the renaturation efficiency of α-chymotrysin by liquid chromatography. Chinese J. Anal. Chem. **2002**, *12* (30), 1435–1439.
9. Carlson, J.D.; Yarmush, M.L. Antibody assisted protein refolding. Bio/Technology **1992**, *10*, 86–91.
10. Buchner, J.; Rudolph, R. Renaturation, purification and characterization of recombinant fab-fragments produced in *Escherichia coli*. Bio/Technology **1991**, *9*, 157–162.
11. Geng, X.D.; Chang, X. High-performance hydrophobic interaction chromatography as a tool for protein refolding. J. Chromatogr. **1992**, *599*, 185–194.
12. Suttnar, J.; Dyr, J.E.; Hamsikova, E.; Novak, J.; Vonka, V. Procedure for refolding and purification of recombinant protein from *Escherichia coli* inclusion bodies using a strong anion exchanger. J. Chromatogr. B **1994**, *656* (1), 123–126.
13. Stempfer, G.; Neugebauer, B.H.; Rudolph, R. Improved refolding of an immobilized fusion protein. Nat. Biotechnol. **1996**, *14*, 329–334.
14. Hamaker, K.H.; Liu, J.; Seely, R.J.; Ladisch, C.M.; Ladisch, M.R. Chromatography for rapid buffer exchange and refolding of secretory leukocyte protease inhibitor. Biotechnol. Prog. **1996**, *12* (2), 184–189.
15. Li, M.; Su, Z.G. Separation and identification of different refolding components. J. Biotechnol. **2003**, *103*, 119–127.
16. Langenhof, M.; Leong, S.J.; Pattenden, L.K.; Middelberg, A.P.J. Controlled oxidative protein refolding using an ion-exchange column. J. Chromatogr. A **2005**, *1069*, 195–201.
17. Ozawa, K.; Takayama, Y.; Yasukawa, F.; Ohmura, T.; Cusanovich, M.A.; Tomimoto, Y.; Ogata, H.; Higuchi, Y.; Akutsu, H. Role of the aromatic ring of Tyr43 in tetraheme cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F. Biophys. J **2003**, *85*, 3367–3374.
18. Yahata, N.; Saitoh, T.; Takayama, Y.; Ozawa, K.; Ogata, H.; Higuchi, Y.; Akutsu, H. Redox interaction of cytochrome c_3 with [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F. Biochemistry **2006**, *45*, 1653–1662.
19. Geng, X.D.; Bai, Q. Mechanism of simultaneously refolding and purification of protein by hydrophobic interaction chromatographic unit and applications. Science in China (Series B) **2002**, *45* (6), 655–669.
20. Benedek, K. Thermodynamics of α-lactalbumin denaturation in hydrophobic-interaction chromatography and stationary phase comparison. J. Chromatogr. **1988**, *458*, 93–104.

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