Immobilized metal ion affinity refolding of recombinant proteins

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Renaturation of recombinant proteins expressed in *E. coli* and accumulated as inclusion bodies by immobilized metal ion affinity chromatography (IMAC) technique was evaluated. Recombinant human interleukin-3 (IL-3), granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF), all possess metal-chelating sites in their sequence and were used for investigation of their renaturation upon denaturated macromolecule interaction with metalions charged Sepharose iminodiacetate (IDA) gels. The efficiency of correctly folded protein generation was studied depending on the concentration of guanidine-HCl in a loading buffer of inclusion bodies solution, type of metal ion, pH and protein loading. The IMAC procedure was shown to be promising and enabled to recover part of the target protein in non-denaturating conditions with the protein-dependent yield.

Key words: recombinant proteins, refolding, immobilized metal ion affinity chromatography

INTRODUCTION

Expression of recombinant proteins in *Escherichia coli* often results in formation of insoluble inclusion bodies [1]. They can usually be solubilized with chaotrophic agents which unfold the protein macromolecule. Hence, unfolded protein must then be refolded to the correct three-dimensional conformation of the native protein. Different steps and methods have been developed for isolation of recombinant eukaryotic polypeptides from inclusion bodies and for generation of their native conformation in solution [2–5]. Protein folding in solid state (immobilized or reversibly attached) became, as it has been shown [4, 5, 6–10], a more attractive approach, since it might be adapted for technological application. The reversible interaction of unfolded proteins with ion-exchangers [6, 7], or HPLC stationary phases that operate in hydrophobic interaction mode [8], in some cases gave high yields of protein renaturation. Recent studies on renaturation of constructed fusion proteins have shown that the procedure of iterative refolding of recombinant proteins, combining reversible interaction of the protein which contains hexa-histidine tail with the respective chelating adsorbent and the system of proteins renaturation, *e.g.*, pair reduced-oxidised glutathione, allowed to refold over 20 eukaryotic proteins or protein domains [9], while the fusion protein with hexa-arginine tail can be renaturated, as it has been shown [10], exploiting its reversible interaction with ion-exchanger at a high yield and high protein concentration. It is obvious that these renaturation approaches are rather general and might be expanded over the range of proteins.

Herein, we describe the use of IMAC procedure for the renaturation of recombinant proteins that possess naturally existing metal-binding sites in their sequence, if such sites are available for IMAC interactions in a denatured protein or its folding intermediates.

MATERIALS AND METHODS

Sepharose CL-6B and phenyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden), iminodiacetic acid (IDA) and epichlorohydrin were obtained from Fluka (Basle, Switzerland). All other chemicals were of analytical or reagent grade and obtained from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, USA) or Serva (Heidelberg, Germany). The harvested cells of *E. coli* (biomass) for the production of respective recombinant proteins were obtained from Biofa, AB (Vilnius, Lithuania).

E. coli lysate was prepared routinely as follows: 10 g (wet weight) of harvested cells was homogenized in 100 ml 0.1 M Tris-HCl, 5 mM EDTA buffer, pH 7.0–8.0, thereafter lysozyme (0.1%), Triton X-

100 (0.1%), phenylmethyl sulfonyl fluoride (1 mM) and β-mercaptoethanol (100 mM) were added, the cells were incubated for 1 h at room temperature and sonicated on ice 5 × 1 min. The suspension of destroyed cells was centrifuged at 13,500 g for 30 min. The collected pellets of inclusion bodies were washed twice with a solution of 0.15 M NaCl, 0.1% Tween 80, and twice with water. Washed inclusion bodies were solubilized in 10 mM Tris-HCl buffer, pH 7.0, containing 6.0–7.0 M guanidine-HCl (GdmCl). Before each IMAC experiment a freshly prepared stock inclusion bodies solution was used.

Sepharose CL-6B – IDA gel was prepared according to Porath and Olin [11] by reaction of an epichlorohydrin-activated matrix with disodium iminodiacetate. The content of iminodiacetate groups was controlled by gel capacity to adsorb Cu^{2+}ions. For this, 1.0 ml volume of Cu^{2+}-loaded and appropriately pre-washed gel was treated with 50 mM solution of EDTA and the content of metal ions was determined by atomic absorption spectrometry. Usually, IDA-gel with copper capacity of 38–42 μmol/ml was used for all the IMAC experiments.

Metal ion (Cu^{2+} Ni^{2+} Zn^{2+})-loaded gels were prepared as follows: metal-free IDA-gel pre-washed with 1.0 mM HCl and water was loaded with at least a ten-bed volume of a freshly prepared 20–50 mM solution of respective metal salts in water, the excess of loosely bound metal ions was removed by washing with water, and finally the adsorbent was equilibrated with the buffer of chromatography.

IMAC of recombinant proteins was performed on a 1.7 × 9.0 cm glass column (20 ml bed volume of the respective metal ion-charged Sepharose-IDA gel) at a flow-rate of 80–100 ml/h controlled by peristaltic pumping. A sample of a target protein inclusion bodies solution in 10 mM Tris-HCl buffer, 6.0–7.0 M GdmCl, pH 7.0 was diluted to adjust GdmCl to the appropriate concentration (see Results and Discussion) and applied to the column equilibrated with the starting 10 mM Tris-HCl buffer, pH 7.0, containing 1.0–6.0 M GdmCl. Not retained protein was washed out by passing a tened volume of a starting buffer at a flow rate of 80–100 ml/h, further, the same buffer without GdmCL, and finally, the elution of adsorbed protein was performed by a linear imidazole gradient. A LKB Uvicord S II was used for detection of absorbing fractions at 280 nm during chromatography. Additionally, the protein concentration in eluate fractions was determined spectrophotometrically at 595 nm by Bradford assay [12]. Protein identity and purity in the eluates were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [13].

Recombinant hG-CSF, the folding intermediates, the denatured, and the reduced denatured protein were analysed by reverse-phase HPLC by using a C-4 (Bio-Rad, Hi-Pore RP-304, 0.46 x 25 cm, 30 nm) reverse-phase column similarly as described [14]. Mobile phase: solvent A – 0.10% TFA, solvent B – 0.10% TFA, 90% CH_3CN. Chromatographic runs were performed by a linear gradient: 60–80% B over 40 min for G-CSF, 40–65% B over 50 min for GM-CSF, and 45–55% B over 20 min for IL-3 at a flow rate of 1.0 ml/min. Peaks were detected at 215 nm (Waters detector, Model 481). The amount of a correctly folded respective protein was determined according to its retention time (RT) and relative absorbance at 215 nm. If necessary, separate samples of individual purified recombinant proteins were used as references or internal standards for RP-HPLC of G-CSF, GM-CSF and IL-3, respectively.

RESULTS AND DISCUSSION

Recent studies performed by the group of Arnold and cowokers [15–17] on metal chelation by engineered proteins which have di-histidine motifs showed that such chelating sites are also effective in stabilizing folded proteins. It was shown [16] that high affinity binding of His-X_3-His cytochrome *c* with Cu(II)-IDA complex gained a protein stabilization with respect to unfolding induced by GdmCl [16]. Particularly based on this, and having in mind the role of specific ligands (metal ions, too) in folding process and the present knowledge on the folding pathways [1, 4, 5], we supposed to evaluate whether the renaturation of unfolded protein macromolecule which possesses in a native form well-defined metal binding motifs or is rich primarily in histidine residues might be achieved by IMAC.

In this regard, rhIL-3 which has been recently found [18] to contain a specific zinc-binding domain was chosen as a first representative for the detection of renaturation effect upon denatured protein interaction with Sepharose-IDA gel charged with zinc ions. The sample from a stock inclusion bodies solution in a buffer containing 7.0 M GdmCl was diluted 10 times with the equilibrating buffer containing 1.5 M GuHCl and loaded onto a Zn(II)-IDA-Sepharose column. After that the column was washed with the inverse gradient (a buffer, pH 7.0, containing 1.5 M GuHCl and a buffer without GuHCl). The retained target protein was eluted in non-denaturing conditions with 50 mM imidazole in the buffer of pH 7.0, or the elution of the retained protein was started by the pH gradient from pH 7.0 to pH 4.0, further returning to pH 7.0, and subsequent elution of the second protein fraction under 50 mM imidazole. In such cases the fraction of IL-3 was eluted from the Zn-IDA column by pH gradient and composed 25–39%, while 75–61% of the

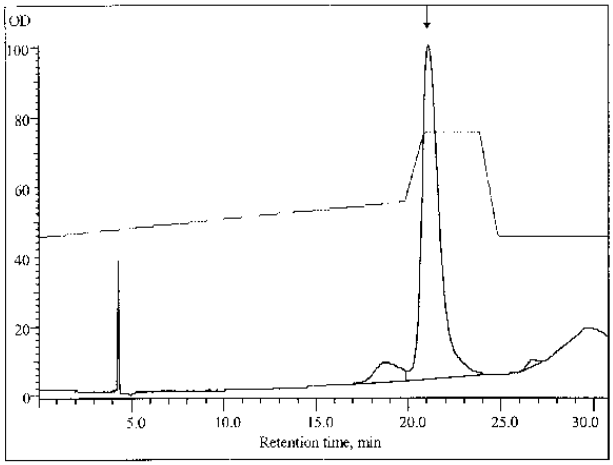

Fig. 1. RP-HPLC analysis of IL-3 removed from Zn(II)-IDA column under imidazole elution and rechromatographed on phenyl-Sepharose (the position of correctly folded protein is marked by the arrow)

total recovered protein was desorbed by imidazole. Since RP-analysis did show some difference in the retention behaviour of both fractions of IL-3 (referred as a correctly folded conformation), they were further chromatographed separately by a hydrophobic interaction mode on a phenyl-Sepharose column in 10 mM Tris-HCl buffer, pH 7.0, containing 2 mM EDTA and 1.4 M ammonium sulphate. In both cases IL-3 was similarly recovered from the column under 10 mM sodium phosphate buffer, pH 7.0, and yielded 72–73% (pH gradient fraction) and 61–72% (imidazole fraction). SDS-PAGE and RP-HPLC analysis (Fig. 1) of both fractions of IL-3 revealed their purity to be 90–93%. Both fractions of IL-3 were shown to possess a distinguishable biological activity of 4.6×10^6 U/mg and 10×10^6 U/mg for pH gradient and imidazole fraction, respectively. Thus, it appears possible that Zn-IDA gel is sensitive towards some type of local IL-3 conformational states. This may be in parallel with the determined [17] different retention behaviour of the properly folded and misfolded His-X_3-His IGFI variants on a Cu(II)-IDA column.

IMAC renaturation was expanded among the range of other human recombinant proteins which have a defined number of histidine residues in their polypeptide sequence, *e.g.*, rhG-CSF and rhGM-CSF. The polypeptide chain of G-CSF contained five histidine residues and unpaired cysteine-17 [19], which are of interest because via part of them the unfolded protein or incipient folding intermediates may be involved in the interaction with immobilized metal ions. The existence of the latter during the folding process of rhG-CSF was shown. Even more of these intermediate protein species (I_1 and I_2) as well as the conversion of intermediate I_2 to the native form can be detected by RP-HPLC [14]. With regard to this, the IMAC renaturation of G-CSF was studied in more detail by evaluating the type of metal ion, pH, protein loading and the concentration of GdmCl in a buffer of chromatography. Some data on G-CSF renaturation performed on Ni(II)-charged Sepharose-IDA column are summarized in Table and exemplified by Fig. 2.

Among the metal ions studied, most favourable are nickel ions. The Zn(II)-IDA complex is less convenient, if the column has to operate at a high concentration of GdmCl. On the other hand, the possible leakage of copper from its IDA-Sepharose column might influence Cu^{2+} ions-catalysed oxidative folding of a protein. As can be seen from Table, the relative amount of correctly folded form of G-CSF, if its renaturation was performed by IMAC on Ni(II)-charged-IDA-Sepharose column at pH 7.0, is strongly dependent on a protein loading and the concentration of GdmCl in the loading buffer. The effect of both these features influenced the strength of nickel ions chelation by less or more available histidine residues, since the concentration of imidazole which causes the elution of the protein varies as is seen from Table. It is worth to noting that the relative percentage of the native form of G-CSF in eluates that were recovered from Ni(II)-IDA column increased (Table and Fig. 2B-2C) in a time-dependent mode, in turn being dependent on the conditions of IMAC renaturation.

Table. **Relative amount of correctly folded conformation of G-CSF as a function of protein loading and concentration of GdmCl**

[GdmCl], M	Amount of loaded protein, mg	Amount of adsorbed protein, mg	Relative amount of correctly folded form, %	Protein recovery, %
3.0	155.2	128.5	9 (35)[x]	87 (50)[a]
3.0	84.6	75.3	26	78 (54)
3.0	45.5	43.0	41 (78)[xx]	75 (60)
3.0	27.5	27.5	44	83 (62)
2.4	93.6	88.0	54 (88)[xx]	15 (60)
5.0	94.2	89.5	33 (56)[xx]	75 (54)

[a] Concentration of imidazole which causes the appearance of protein concentration maximum.
[x] and [xx] Relative amount of the native form determined by RP-HPLC in the same sample after its stay at +4 °C for 120 (x) and 24 (xx) h, respectively.

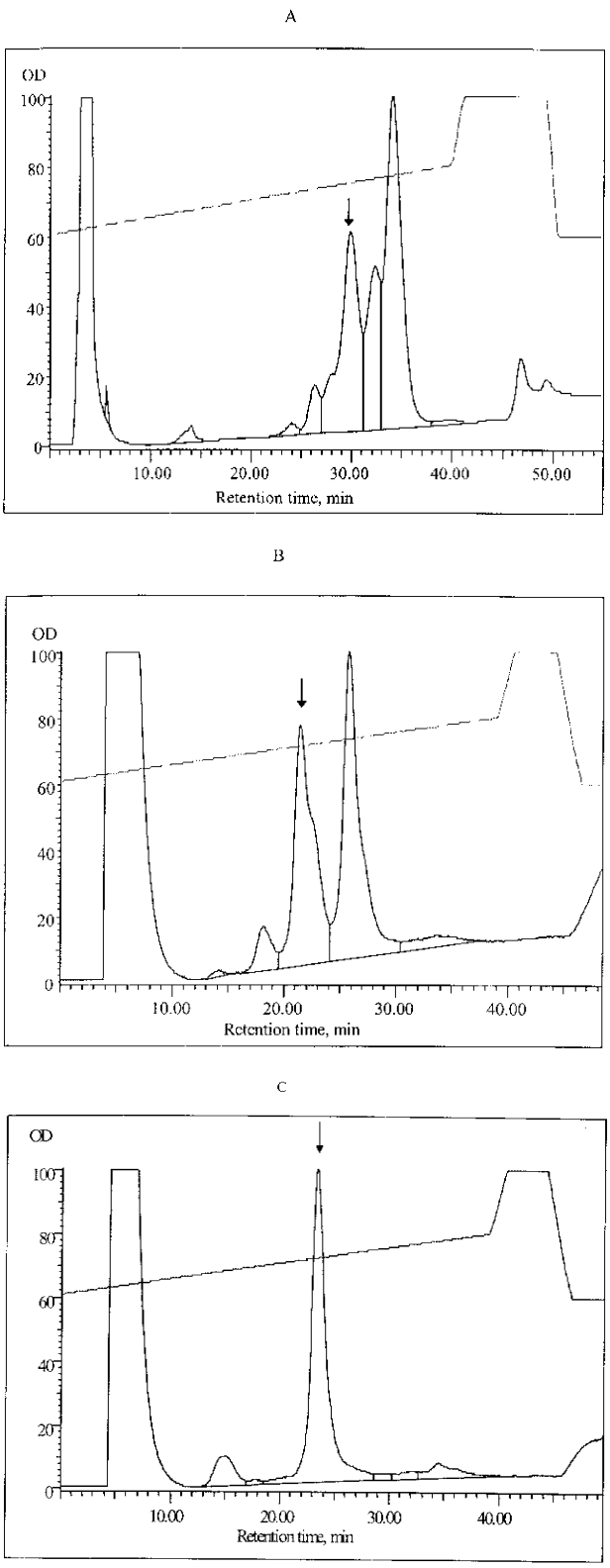

Fig. 2. RP-HPLC analysis of G-CSF. A – inclusion bodies solution in 10 mM Tris-HCl buffer, pH 7.0, containing 7.0 M GdmCl, B – sample of the protein recovered from Ni(II)-IDA column under imidazole elution, C – analysis of a sample as in B stored for 24 h at +4 °C (the arrows indicate the positions of nascent correctly folded conformation of G-CSF in A, and correctly folded protein in B and C, respectively)

Recombinant hGM-CSF possesses three histidine residues in a polypeptide sequence [20], therefore it also can be used as a model for IMAC renaturation. Similarly, as in the case of IL-3 and G-CSF, a sample of GM-CSF inclusion bodies solution was chromatographed on Ni(II)-IDA gel in a buffer, pH 7.0, containing 2.4 M GdmCl, and once again the fraction of the target protein was eluted under imidazole gradient in native conditions. RP-HPLC analysis (Fig. 3) of the eluate enabled to determine that GM-CSF was also in a properly folded conformation and its purity reached 40–84%.

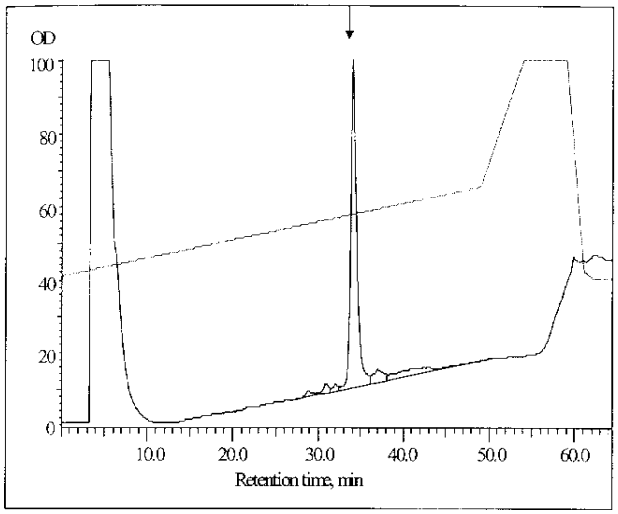

Fig. 3. RP-HPLC analysis of GM-CSF fraction recovered from Ni(II)-IDA-Sepharose under imidazole gradient (the position of correctly folded protein is marked by the arrow)

The data presented demonstrate the IMAC methodology to be applicable for the renaturation of recombinant proteins expressed in *E. coli* as an insoluble inclusion body, if the target protein has an appropriate metal-binding domain or is rich in histidine residues that are available for chelation in denatured protein or its folding intermediates. This renaturation procedure may be regarded as a more generalized approach expanding it into the range of recombinant proteins, *e.g.*, interleukins, colony-stimulating factors, interferons, etc.

References

1. Mitraki A, King J. Bio/Technology 1989; 7: 690–7.
2. Fischer B, Summer I, Goodenough P. Biotechnol Bioegn 1993; 41: 3–13.
3. Zardenetta G, Horowitz PM. Analyt Biochem 1994; 223: 1–6.
4. Jaenicke R. Phil Trans R Soc Lond B 1995; 348: 97–105.
5. Rudolph R, Lilie H. FASEB J 1996; 10: 49–56.
6. Hoess A, Arthur AK, Wanner G, Fanning E. Bio/Technology 1988; 6: 1214–7.
7. Creighton TE. In "Protein Structure, Folding, and Design", AR Liss, Inc 1986: 249–57.

8. Geng X, Chang X. J Chromatogr 1992; 599: 185–94.
9. Etzerodt M, Holtet TL, Thogersen HC. Protein Engineering 1995; 8: 89–90.
10. Stempfer G, Holl-Neugebauer B, Rudolph R. Nature Biotechnol 1996; 14: 329–34.
11. Porath J, Olin B. Biochemistry 1983; 22: 1621–30.
12. Bradford MM. Anal Biochem 1976; 72: 248–54.
13. Laemmli UK. Nature 1970; 227: 680–5.
14. Lu HS, Clogston CL, Narbi LO, Merewether LA, Pearl WR, Boone TC. J Biol Chem 1992; 267: 8770–7.
15. Arnold FH. Bio/Technology 1991; 9: 151–6.
16. Kellis JT, Todd RJ, Arnold FH. Bio/Technology 1991; 9: 994–5.
17. Arnold FH, Haymore BL. Science 1991; 252: 1796–7.
18. Smit V, van Veelen PA, Tjaden UR, van der Greef J, Haaijman JJ. Biochem Biophys Res Commun 1992; 187: 859–66.
19. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K. Science 1986; 232: 61–5.
20. Rozwarski DA, Diederichs K, Hecht R, Boone T, Karplus PA. Proteins: Structure, Functions, Genetics 1996; 26: 304–13.

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REKOMBINANTINIŲ BALTYMŲ RENATŪRACIJA AFININE CHROMATOGRAFIJA SU IMMOBILIZUOTAIS METALO JONAIS

S a n t r a u k a

Įvertintas rekombinantinių baltymų, akumuliuojamų *E. coli* ląstelėse intarpinių kūnelių pavidalo, renatūracijos vyksmas naudojant gimininggąją chromatografiją, kurioje specifinio ligando vaidmenį atlieka metalo jonai. Buvo tirtas žmogaus rekombinantinių interleukino-3, granulocitų kolonijas ir granulocitų makrofago kolonijas stimuliuojančių veiksnių, turinčių savo pirminėje sekoje metalo suršimo vietas, natyvios struktūros generavimas chromatografuojant denatūruoto baltymo makromolekules per Sefarozės-iminodiacetato į sorbentą, krautą metalo jonais. Baltymo korektiškos struktūros generavimo efektyvumas tirtas priklausomai nuo guanidino hidrochlorido koncentracijos intarpinių kūnelių ńhromatografijos tirpale, metalo jono prigimties ir chromatografijos pH. Parodyta, kad šiame baltymų renatūracijos procese įmanoma atgauti baltymą su jo visiškai atstatyta natyvia struktūra, ir išeiga, priklausoma nuo pastarojo prigimties.