

Matrix-Assisted Refolding and Purification of Proteins by a Novel Designed Anion Exchange Chromatography

Aziz Dashbolaghi

Department of Pilot Nano-biotechnology
Pasteur Institute of Iran, Tehran, Iran

Shohreh Khatami

Department of Biochemistry
Pasteur Institute of Iran, Tehran, Iran

Sorush Sardari

Drug Design and Bioinformatics Unit
Medical Biotechnology Department Biotechnology Research Center
Pasteur Institute of Iran, Tehran, Iran

Reza Ahangari Cohan

Nano biotechnology Department
Pasteur Institute of Iran, Iran

Masoud Ghorbani

Department of Virology
Pasteur Institute of Iran, Tehran, Iran

Dariush Norouzian*

Department of Pilot Nano-biotechnology
Pasteur Institute of Iran, Tehran, Iran

* Corresponding author

Copyright © 2015 Aziz Dashbolaghi et al. This article is distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Purpose: The refolding of recombinant human interferon α -2b is accompanied by low yield that could be due to high aggregation and multiplicity of steps in downstream processing. It is therefore essential to refold and purify the protein to simultaneously increase the production yield and reduce the required downstream steps.

Methods: Inclusion bodies were dissolved in Tris-HCl buffer containing 6 M guanidine and applied to a newly designed poly-arginine anion exchange chromatography system. Different reduced-oxidized glutathione amount were employed for refolding the target protein. The refolded proteins were applied to gel filtration chromatography and subjected to biological activity assessment.

Results: By increasing the sample volume, the refolding efficacy, purification factor and potency were significantly decreased. This reduction can be highest when only 2 column volumes of the glutathione redox pair presented in the column. The highest values of these factors were obtained when 4 column volumes of the glutathione redox pair offered to the column. To achieve better results, the proportion of sample volume and the amount of passed glutathione redox pair through column should be optimized. Consistent with the increase of sample volume to 1250 μ l at any levels of glutathione redox pair there was a significant drop in all factor values.

Conclusion: Poly-arginine anion exchange matrix is a new artificial chaperone that can improve the refolding and purification process simultaneously with high efficacy. Therefore, we present poly-arginine anion exchange chromatography as an alternative method for protein refolding with promising commercial and developmental potential.

Keywords: Matrix-assisted refolding, Recombinant human interferon α -2b, Poly-L-arginine, Anion exchange chromatography

Introduction

Recombinant protein technology was introduced as a promising approach to overcome the obstacles in the obtaining of a purified active therapeutic protein. But in some cases, especially prokaryote expression systems and high intricate proteins, produced protein did not have same functional activity as natural one. Biological studies elucidated the proper 3D-structure forming of a protein is a complicated process occurring in the cells by an advanced machinery folding system (Kandror O and . 1997). Many physicochemical studies were carried out to provide an in vitro condition for protein refolding process (Anfinsen CB 1961, CB 1973). Refolding of protein at large scale is a matter of commerce and remains as a state of the trade secret. However, there are still obstacles such as low yield and

multiplicity of steps in refolding of inclusion bodies in downstream processes that lead to a dramatic increase both time and cost of production. Therefore, it is vital to investigate new approaches to increase the yield of production. Attainment of higher yield, decrease the number of steps in process, time and cost of production powerfully promote researchers to find new lines of action for achievement of these goals (Machold C 2005). Hence, this study is focused on achieving a new and efficient approach for optimal refolding process.

The flexibility of chromatography applications allows the development of several strategies for refolding of proteins (Altamirano 1997, Machold C 2005). Matrix-assisted refolding (MAR) chromatography can be used to improve protein refolding yields by allowing higher protein concentrations and simultaneous purification of folded proteins. Such approaches are favorable for the downstream processing of recombinant proteins expressed in inclusion bodies. The other important advantage of MAR chromatography is that the desired protein can be concentrated to some extent (Machold C 2005). Furthermore MAR can be used coupled to additional chromatographic techniques, such as affinity chromatography (Shi 2003), hydrophobic interaction chromatography (Geng 2002), size exclusion chromatography (Shalongo 1987, Schlegl 2003); and ion exchange chromatography (IEX) (Suttner 1994, Machold C 2005). Each technique has its own advantages and disadvantages for a desired protein refolding process. In this article, we attempted to refold recombinant human interferon α -2b (rhIFN α -2b) by a newly designed poly-L-arginine (PArg) hydrochloride MAR that can also act as an anion exchanger. The new MAR chromatographic system, called poly-L-arginine anion exchange (PRAEX) chromatography, is designed by covalently binding of PArg to epoxy-activated Sepharose 6B beads. This MAR acts as an anionic exchanger chromatographic bed at below pH 12 and can be employed to purify and refold the desired proteins, simultaneously. The efficiency of this new system was investigated on refolding process of rhIFN α -2b as a model protein. In this investigation, we also answer the question, whether such a matrix can refold and purify rhIFN α -2b simultaneously with higher yield by acting as an artificial chaperone?

Materials and methods

1. Solubilization

The rhIFN α -2b inclusion body was supplied by the department of rhIFN α -2b production, Research and Production Complex, Pasteur Institute of Iran. It was thawed and then precipitated by centrifugation at 3000 g and at 4°C for 15 minutes. The supernatant was decanted and the pellet was weighed. Subsequently, 25 ml of denaturing buffer (Tris-HCl 50 mM, NaCl 50 mM and 8 M urea pH 7.0) containing 115 mg dithiothreitol (DTT, Merck, Germany) was added to each 0.3 g of pellet and slowly stirred for 2 hours to solubilize the protein. The insoluble fraction was then separated by centrifugation at 5000 g for 15 minutes at 4°C. The soluble protein was filtered through a 0.22-micron membrane filter. The protein

concentration was determined by Bradford protein assay using bovine serum albumin (Sigma Aldrich A4737) as standard (Bradford 1976). Finally, the protein concentration was adjusted to 5 mg/ml with denaturing buffer to minimize the effect of concentration on refolding yield. The reduced and denatured protein solution was loaded into the PRAEX chromatography column for a concomitant refolding and purification process. Volumes of sample containing 5 mg/ml of rhIFN α -2b were loaded into the PRAEX column at 250, 500, 750, 1000 and 1250 μ l in triplicate.

2. Matrix preparation and determination of attached PArg to the matrix

Epoxy-activated Sepharose 6B (Sigma-Aldrich, P4663) is a pre-activated chromatographic bed that can covalently bind to the amine groups. PArg was bind to epoxy activated Sepharose according to the manufacturer's instruction. The amount of PArg attachment to the matrix was calculated using difference in PArg concentrations, before and after binding. Bradford protein assay method was used for measuring of PArg concentration in all experiments by a serially diluted PArg and drawing standard curve.

3. Dynamic binding capacity measurement

Measuring the dynamic binding capacity (DBC) of matrix is an essential step in matrix design and optimization of protein refolding and purification by MAR chromatography. DBC is defined as the amount of desired protein that will bind to the matrix under special flow and loading conditions and was determined by frontal analysis (Tri Doa 2008). Therefore 1 mg/ml of reduced and denatured rhIFN α -2b was applied to approximately 0.5 ml of designed PArg matrix until breakthrough occurred. DBC was calculated as the mass of rhIFN α -2b corresponding to the breakthrough point.

4. Refolding efficacy and purification factor

The refolding efficacy and purification factor in PRAEX column was calculated using SDS-PAGE and Bradford protein assay method. Bound density of SDS-PAGE electropherogram was analyzed by densitometer (GS-800™ densitometer Bio-Rad, USA).

The percentage of refolding efficacy was calculated as equation 1:

$$\frac{\text{The amount of loaded rhIFN } \alpha\text{-2b}}{\text{The amount of eluted rhIFN } \alpha\text{-2b}} \times 100 = \text{Refolding Efficacy \%} \quad (1)$$

This factor indicates the amount of protein aggregated on the column which will be used to evaluate the efficacy of column for refolding process. The purification factor was calculated using equation 2:

$$\frac{\text{Soluble rhIFN } \alpha\text{-2b entered into the process \%}}{\text{Eluted rhIFN } \alpha\text{-2b \%}} = \text{Purification Factor} \quad (2)$$

5. Matrix assisted refolding

The purification steps were composed of two sequential column chromatography including novel designed of PRAEX chromatography and size exclusion chromatography (SEC). All solutions and buffers were HPLC grade and were filtered and de-gassed prior to use and applied to the column at the flow rate of 1 ml/min. In PRAEX, 15 ml prepared matrix was packed into the column (1.5 × 12 cm, Econo-Pac®) with column adaptor (Bio-Rad, USA). Column was equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.0. Before loading the sample onto the column, it was washed with the same buffer for 3 column volumes (CV). The flow rate was adjusted to 1 ml/min and the bed volume of columns was 15 ml. After loading the sample, the redox pair solution containing 1mM reduced and 0.1 mM oxidized glutathione or redox pair (in equilibration buffer) was added to the column in 2, 3 and 4 CV. Then the column was washed with buffer containing 0.25 M NaCl equilibration buffer. Subsequently protein was eluted in a step with elution buffer, consisting of 0.5 M NaCl in equilibration buffer. Finally the column was regenerated with 0.5 M NaOH. The eluted protein solution under the peak area was collected for the next purification step. In the second step, Sephadex G-75 gel (15 ml) was packed into the column (1.5 × 12 cm, Econo-Pac®) using a column adaptor (Bio-Rad, USA). After packing, the column was sanitized with 0.5 M NaOH and washed with 2 CV of PBS (pH 6.6 and 14). All packed columns were subjected to quality control according to the company's instruction by loading of 1% V/V acetone. Only columns with acceptable asymmetry factor range (0.8-1.5) were employed. The equilibrated columns were loaded with 0.5 mg of protein from the previous step and eluted with PBS (pH 6.6 and 14 ± 2 μS conductivity). The protein fractions were detected and collected at 280 nm wavelength based on standard rhIFN α-2b (Institute Pasteur, Iran) using a fraction collector unit equipped with ultraviolet detector (Bio-Rad, USA).

6. In vitro biological activity

Fifty μg/ml purified proteins were subjected to bioassay step. The biological equivalence assay of rhIFN α-2b is based on its inhibitory activity on the cytopathic effect of encephalomyocarditis virus on Hep2c cells. The potency was determined by protective effect of interferon against cell death and compared against a reference standard for interferon with defined activity. The standard rhIFN α-2b was obtained from National Institute of Biological Standards and Control, (NIBSC; Code 95/566). The bioassay was carried out according to British Pharmacopoeia 2012. The "relative potency" (potency) of the purified samples was determined using ParLin version 5.0 software and compared with standard.

Statistical analysis

All data were analyzed using Graphpad InStat software version 3.00 by Repeated Measures Analysis of Variance and Tukey-Kramer Multiple Comparisons post-hoc Test. Post-hoc test results are shown on plots.

Results and discussion

SDS-PAGE analysis results of after and before purification steps on PRAEX column and size exclusion are shown in figure 1. As it is depicted from the figure, the single band corresponding to about 19 KD was obtained. Thus SDS-PAGE analysis reveals the efficacy of the matrixes used to purify rhIFN α -2b.

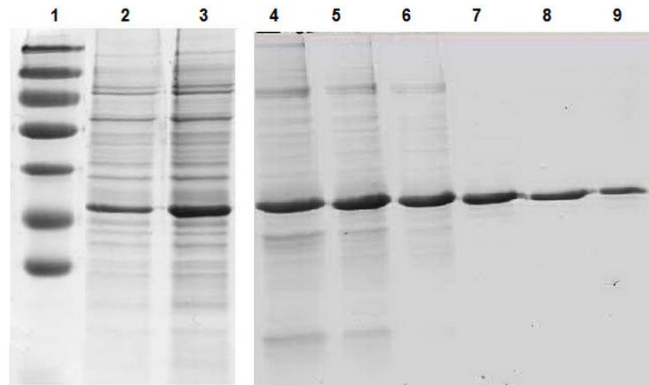


Fig 1: SDS-PAGE analysis of rhIFN α -2b applied to sequential purification steps [Lane 1: marker; lane 2 and 3 before purification with different concentrations; Lane 4 (250 μ l sample volume with 2 CV redox pair), 5 (250 μ l sample volume with 3 CV redox pair) and 6 (250 μ l sample volume with 4 CV redox pair): after PRAEX purification step; Lane 7, 8 and 9: Size exclusion purification step)

At the matrix preparation step, the initial concentration of PArg was 3240 μ g/ml and the amount of PArg binds to the matrix was about 356 μ g/ml. The DBC Value for this matrix was 44 mg/ml. This calculated DBC indicated a sufficient affinity of PRAEX matrix to the denatured and reduced rhIFN α -2b.

The effect of PRAEX matrix on refolding was studied using rhIFN α -2b as a model protein thereby refolding efficacy and purification factor were calculated for the designed matrix. Finally, after gel chromatography and purification of rhIFN α -2b monomers, potency was measured.

Statistical analysis of refolding efficacy, purification factor and potency (relative potency) showed all data had a normal distribution and therefore Repeated Measures Analysis of Variance was used to data analysis and significant determination. P values were calculated as 0.0001 (extremely significant), 0.0036

(very significant) and 0.0371 (significant) for purification factor, refolding efficacy and potency, respectively.

The results showed that by increasing the sample volume, refolding efficacy (Fig 2), purification factor (Fig 3) and potency (Fig 4) were significantly decreased (all p values are < 0.05). This reduction was highest when only 2 CV of redox pair was applied in the column. The highest values of these factors were obtained when 4 CV of redox pair offered to the column. To achieve better results, the proportion of sample loading and the amount of passed redox pair through column should be optimized. Consistent with the increase of sample volume to 1250 μL , at any level of redox pair, there was a significant drop in all factor values. According to the results, optimal point was 1000 μL sample volume with 4 CV of entered redox pair. High efficiency can be achieved in protein refolding by carefully selecting and adjusting the input equilibration buffer, flow rate, redox pair concentration and volume/concentration of loaded protein. So, the protein can simultaneously be purified and refolded more efficiently on PArg matrix. To improve the efficiency of purification, gradient method can be used instead of the simple stepwise elution method as employed in this study. Optimization of chromatographic conditions can increase in purification factor and refolding efficacy values.

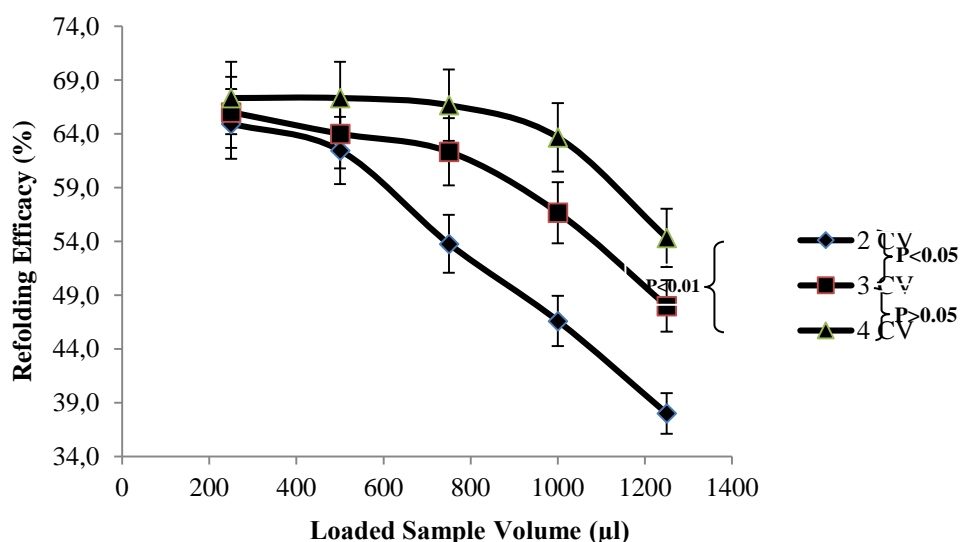


Fig 2: The percentage of refolding efficacy was plotted versus loaded sample volume with different column volumes of redox pair. Post-hoc test comparison results were presented.

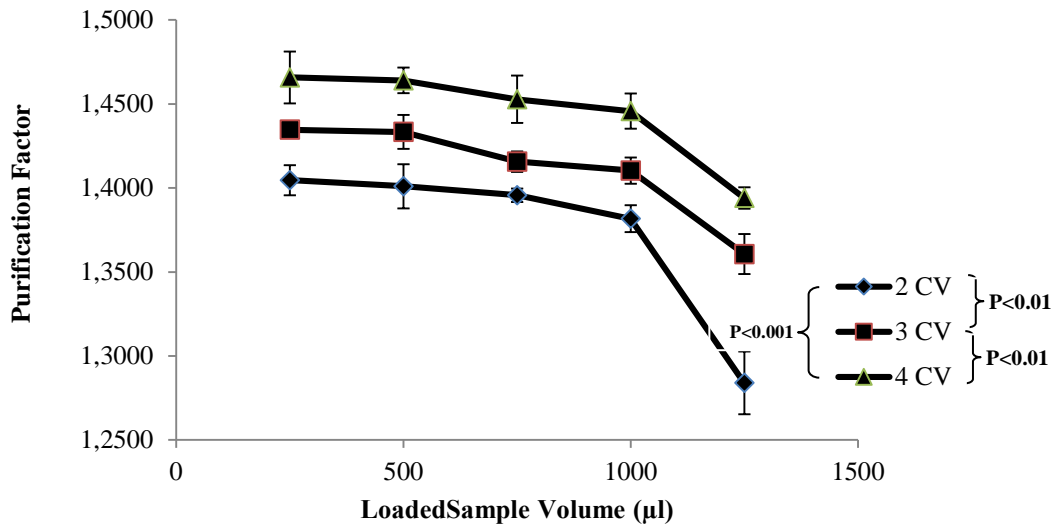


Fig 3: Purification factor was plotted versus loaded sample volumes with different column volumes of redox pair. Post-hoc test comparison results were presented.

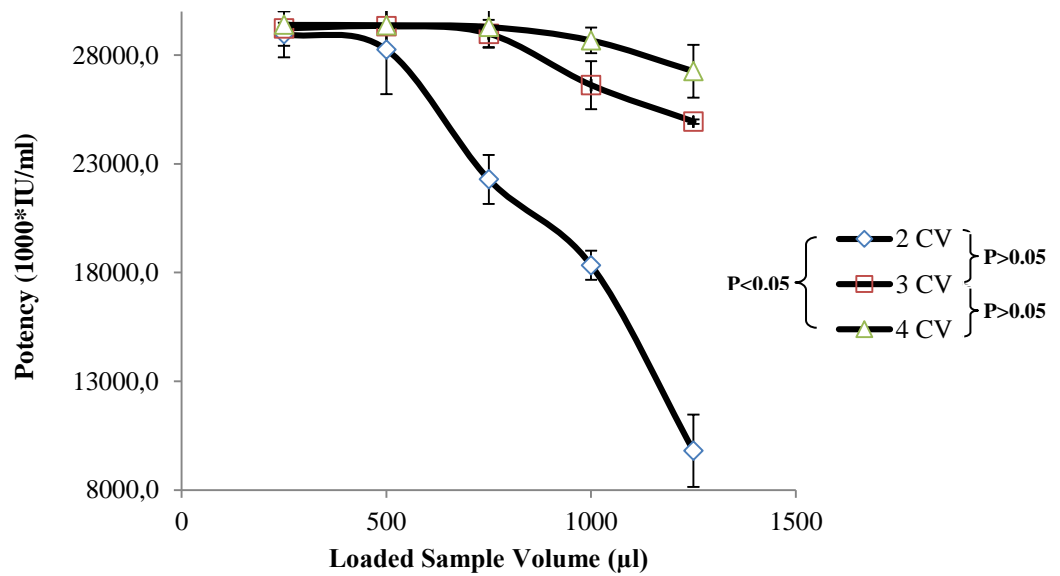


Fig 4: potency (IU/ml) versus loaded sample volume with different column volume of redox pair and post-hoc test comparison.

Non-covalent interactions usually enhance renaturation as well as mis-folded and aggregation of un-folded proteins. This could be due to competition between intra and intermolecular forces of proteins (interaction network) which can be driven by environmental conditions. Therefore, the protein molecules are able to form and reform to the various types of structures in order to reach the equilibrium. In

the presence of PArg, similar to a condition in which that a low amount of denaturing agent exists, protein molecules promote to form correct structure which could be due to the favorable refolding condition. In this condition, non-native molecules have high free energy level and can be return to the native structure. Therefore, equilibrium for structural reforming is toward correct folded structure by such systems. These mechanisms could involve in MAR efficiency of PRAEX and described high refolding efficacy of PRAEX (67.3%) as compared to other conventional anion exchange chromatography systems (15%) (Machold C 2005). By optimizing chromatographic conditions such as buffer, flow rate, sample volume and dimension of column chromatography, PRAEX increased purification factor and refolding efficacy more than 1.46 and 67.3 %, respectively. This novel system works on the basis of isoelectric point difference between rhIFN α -2b (5.67) and PArg (about 13.5) (Gasteiger E. 2005) enabling effective anion exchange. As most proteins have isoelectric points between 5 and 9, it is expected that this system will be applicable to other proteins(Landers Dec 23, 1996).

Moreover, the ability to perform the purification and refolding simultaneously becomes possible using this system. It is also suggested that other polymers containing guanidinic groups with higher stability can be used to increase the stability and efficiency of column.

Conclusion

PArg can be considered as a new artificial chaperone that can accelerate the refolding process and raise refolding efficacy. Therefore, PRAEX system can be introduced as an alternative method with considerable advantages. Moreover, instead of the epoxy-activated Sepharose matrix, other currently used matrixes can be applied to reduce the processing costs. By increasing of PArg density on the matrix and achievement of higher DBC value, large sample volume can be applied in PRAEX system. Therefore, it makes this system more affordable and suitable for using in industry. The used glutathione redox pair for the formation of disulfide bonds can be replaced with copper sulfate, vitamin C and other cheap materials.

No doubt, the advantages of PRAEX chromatography are undeniable but more studies are required to elucidate its real applicability. Moreover, PArg involved mechanisms must be identified by further investigations. In addition, it should be assessed that the results of current study can be obtained with the other proteins and can PRAEX be considered as a new generation of artificial chaperon?

Acknowledgements. This project was financially supported by Pasteur Institute of Iran and resulted from a PhD thesis. The authors wish to express their deep gratitude to all who provided support during the course of this research.

References

- [1] M. M. Altamirano, R. Golbik, R. Zahn, A. M. Buckle, A. R. Fersht, Refolding chromatography with immobilized mini-chaperones, *Proc. Natl. Acad. Sci. U.S.A.*, **94** (1997), 3576 - 3578. <http://dx.doi.org/10.1073/pnas.94.8.3576>
- [2] C. B. Anfinsen, E. Haber, M. Sela, F. H. White Jr., The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain, *Proc. Natl. Acad. Sci.*, **47** (1961), 1309 - 1314. <http://dx.doi.org/10.1073/pnas.47.9.1309>
- [3] M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem.*, **72** (1976), 248 - 254. <http://dx.doi.org/10.1006/abio.1976.9999>
- [4] C. B. Anfinsen, Principles that govern the folding of protein chains, *Science*, **181** (1973), 223 - 230. <http://dx.doi.org/10.1126/science.181.4096.223>
- [5] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel, A. Bairoch, Protein Identification and Analysis Tools on the ExPASy Server, *The Proteomics Protocols Handbook*, (2005), 571 - 607. <http://dx.doi.org/10.1385/1-59259-890-0:571>
- [6] X. Geng, Q. Bai, Mechanism of simultaneously refolding and purification of proteins by hydrophobic interaction chromatographic unit and applications, *Sci. China B*, **45** (2002), 655 - 669. <http://dx.doi.org/10.1007/bf03182538>
- [7] O. Kandror, M. Sherman, R. Moerschell, A. L. Goldberg, Trigger factor associates with GroEL in vivo and promotes its binding to certain polypeptides, *J Biol Chem*, **272** (1997), 1730 - 1734. <http://dx.doi.org/10.1074/jbc.272.3.1730>
- [8] J. P. Landers, *Handbook of Capillary Electrophoresis*, USA, CRC Press, 1996.
- [9] C. Machold, R. Schlegl, W. Buchinger, A. Jungbauer, Matrix assisted refolding of proteins by ion exchange chromatography, *Journal of Biotechnology* **117** (2005), 83 - 97. <http://dx.doi.org/10.1016/j.jbiotec.2005.01.004>
- [10] R. Schlegl, G. Iberer, C. Machold, R. Necina, A. Jungbauer, Continuous matrix-assisted refolding of proteins, *J. Chromatogr. A.*, **1009** (2003), 119 - 132. [http://dx.doi.org/10.1016/s0021-9673\(03\)00432-1](http://dx.doi.org/10.1016/s0021-9673(03)00432-1)
- [11] W. Shalongo, R. Ledger, M. V. Jagannadham, E. Stellwagen, Refolding of denatured thioredoxin observed by size-exclusion chromatography,

Biochemistry, **26** (1987), 3135 - 3141.
<http://dx.doi.org/10.1021/bi00385a029>

- [12] Y. Shi, C. Jiang, Q. Chen, H. Tang, One-step on-column affinity refolding purification and functional analysis of recombinant human VDAC1, *Biochem. Biophys. Res. Commun.*, **303** (2003), 475 - 482.
[http://dx.doi.org/10.1016/s0006-291x\(03\)00359-0](http://dx.doi.org/10.1016/s0006-291x(03)00359-0)
- [13] J. Suttnar, J. E. Dyr, E. Hamsikova, J. Novak, V. Vonka, Procedure for refolding and purification of recombinant proteins from Escherichia coli inclusion bodies using a strong anion exchanger, *J. Chromatogr. B: Biomed. Appl.*, **656** (1994), 123 - 126.
[http://dx.doi.org/10.1016/0378-4347\(94\)00078-6](http://dx.doi.org/10.1016/0378-4347(94)00078-6)
- [14] Tri Do, Frank Ho, Bettina Heidecker, Krista Witte, Lucille Chang, Laura Lerner, A rapid method for determining dynamic binding capacity of resins for the purification of proteins, *Protein Expression and Purification*, **60** (2008), 147 - 150. <http://dx.doi.org/10.1016/j.pep.2008.04.009>

Received: June 29, 2015; Published: August 5, 2015