# Expression, Solubilization and Refolding Of Antigen-Binding Fragments Of Antibodies Fused With Leucine Zipper In *Escherichia Coli*.

Panwad Ritthisan[1], Ojima-Kato Teruyo[1], Nagai Satomi[1], Kojima Takaaki[1], and Nakano Hideo[1]

***Abstract*—** Fragment antigen-binding (Fab) is used for several applications in research. However, overexpression of Fab in *E. coli* cells often leads to the accumulation of inclusion bodies, which limits the application for Fab production using *E. coli* expression system. Leucine zipper (LZ) forms a heterodimeric coiled-coil structure, the fusion of which peptides to Fab enhanced correct pairing of Hc and Lc, leading to more efficient formation of active Fab. The new Fab format is named as 'Zipbody'. In this study, we examined various refolding conditions to obtain a high amount of m6FabLZ, a leucine zipper-fused mouse Fab which binds to *E. coli* O157 and expressed in *E.coli* BL21 (DE3). The isolated inclusion body was soblubized in 3 M Urea using freeze-thawing method. The yield of purified m6FabLZ was 0.25 g from 1 L culture. The refolded showed a high affinity and specificity toward *E. coli* O157 in ELISA.

***Keywords*—** inclusion bodies, leucine zipper, solubilization, refolding, antibody

## I. Introduction

NOWADAYS, antibodies have become increasingly important for several applications. Fragment antigen-binding (Fab) are the most widely used and produced by recombinant processes[1]. However, overexpression of recombinant proteins in *E. coli* often resulted in the accumulation of protein aggregation as inclusion bodies. Inclusion bodies need extensive processing of isolation, solubilisation and refolding to recover a function of biological activity[2]. In general, inclusion bodies are solubilized by use of high concentration of denaturant. However, complete denaturation during solubilization causes a complete loss of secondary structure and leads to protein aggregation [3]. Mild solubilization processes have been reported to preserve native-secondary structures of solubilized proteins, which repress protein aggregation during refolding leading to a high yield of bioactive proteins.

From previous study, our group reported that the fusion of leucine zipper of C-terminal of Fab significantly increased the production of active Fab in *E. coli* cell-free protein synthesis system and *in vivo E. coli* expression system and named the new Fab format as Zipbody [4].

Panwad Ritthisan[1], Laboratory of Molecular Biotechnology, Department of Bioengineering Science, Graduate School of Bioagricultural Sciences, Nagoya University, Japan 464-8601

Nakano Hideo[1], Laboratory of Molecular Biotechnology, Department of Bioengineering Science, Graduate School of Bioagricultural Sciences, Nagoya University, Japan 464-8601

In this study, we examined various conditions of the protein refolding for zipbody to find highly efficient refolding procedures from inclusion bodies. We obtained a high amount of a zipbody, m6FabLZ, a leucine zipper-fused mouse Fab which binds to *E. coli* O157, from the inclusion bodies expressed in *E. coli* BL21 (DE3).

## II. Solubilization of m6FabLZ

The isolated of m6FabLZ was efficiently solubilized using freeze-thawing method with different urea concentration (2M, 3M and 4M) and 10 mM DTT.

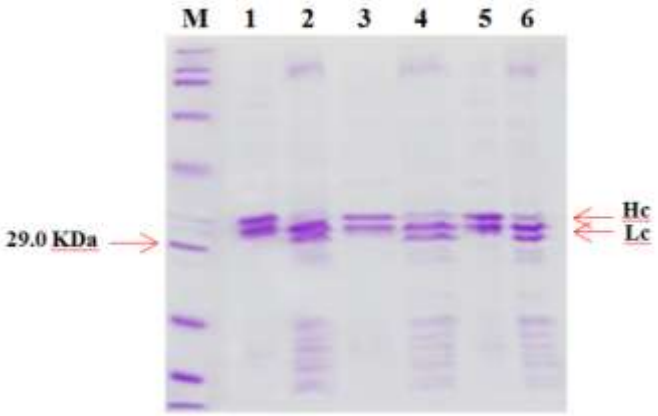


Fig. 1 SDS-PAGE analysis of m6FabLZ solubilization from inclusion bodies. M : marker, 1 and 2: supernatant and pellet from dissolved in 2 M urea, 3 and 4: supernatant and pellet from dissolved in 3 M urea , 5 and 6: supernatant and pellet from dissolved in 4 M urea .

## III. Refolding of m6FabLZ

The solubilized m6FabLZ inclusion bodies by dissolved in 3 M urea were chosen for the examination of refolding by dialysis. The solution was removed urea by dialysis using dialysis buffer (50 mM Tris-HCl (pH 8.0), 3 M urea and 100 mM NaCl) followed by dialysis in the same buffer with step wise reductions in urea concentration (2 M, 1 M, 0.5 M and 0 M). At the 1 M and 0.5 M stage stage, 50 fold molar excess of GSSG and 400 mM L-Arginine was added in the dialysis buffer and each dialysis step was incubated overnight at 4 C°. The soluble meterials after refolding were collected for analysis by SDS-PAGE and ELISA.

Figure 2 shows that the refolding yield was 65%. The yield of purified m6FabLZ was 0.25 g from 1 L culture. The refolded protein showed a high affinity and specificity toward *E. coli* O157 in ELISA (Figure 3).

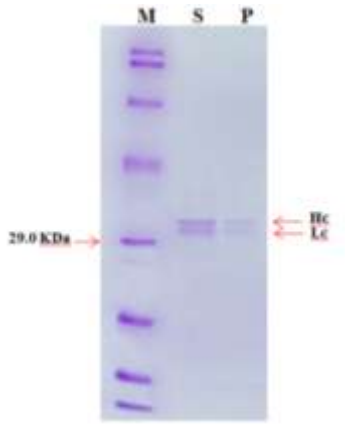


Fig. 2 SDS-PAGE analysis of m6FabLZ refolding. M : marker, S and P: supernatant and pellet after refolding.

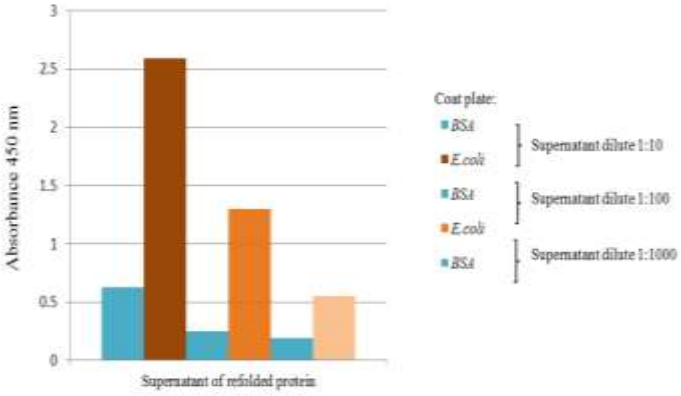


Fig. 3 ELISA results of refolded protein. The blue squares is negative control (1% BSA), the brown squares is supernatant of refolded protein diluted 1:10, the orange squares is supernatant of refolded protein diluted 1:100 and light orange squares is supernatant of refolded protein diluted 1:1000.

## IV. Conclusion

The high yield recovery of active antibodies from inclusion bodies is important for several applications of antibodies in clinical research, therapy and medical diagnostics.

In this study, we examined condition for solubilization and refolding of insoluble Fab protein. There are two important conditions to recover a high amount of bioactive proteins: first, solubilization of protein aggregates by freeze-thawing method with mild solubilization agent and reducing agent. Use of 3 M Urea preserved native-secondary structure of protein, which repress protein aggregation during refolding [5, 6] and 10 mM DTT prevented incorrect disulfide bonds by reduced nonnative inter- and intramolecular disulfide bonds [7]. Second, refolding of the solubilized protein by the step-wise dialysis and the incubation with GSSG and L-arginine at the appropriate stage (1 M and 0.5 M of urea). The GSSG promoted formation of disulfide bonds in proteins [8] and L-arginine suppressed aggregation and prevented incorrect of intramolecular disulfide bonds[9, 10]. In summary, refolding procedures in this study is easy, inexpensive, time-saving and can be utilized for a high yield recovery of Fab inclusion bodies produced in *E. coli.*

## References

[1] Frenzel A, Hust M, Schirrmann T. Expression of recombinant antibodies. Front Immunol 2013;4:1-20. http://dx.doi.org/10.3389/fimmu.2013.00217

[2] Clark EDB. Protein refolding for industrial processes. Current Opinion in Biotechnology 2001:202–7. http://dx.doi.org/10.1016/S0958-1669(00)00200-7

[3] Dill KA SD. Denatured states of proteins. Annu Rev Biochem 1991:795-825. http://dx.doi.org/10.1146/annurev.bi.60.070191.004051

[4] Ojima-Kato T, Fukui K, Yamamoto H, Hashimura D, Miyake S, Hirakawa Y, et al. 'Zipbody' leucine zipper-fused Fab in E. coli in vitro and in vivo expression systems. Protein Eng Des Sel 2016;29:149-57. http://dx.doi.org/10.1093/protein/gzw001

[5] Anupam Singh VU, Arun Kumar Upadhyay, Surinder Mohan Singh and Amulya Kumar Panda. Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. Microbial Cell Factories 2015:14:41. http://dx.doi.org/10.1186/s12934-015-0222-8

[6] Richard BR. Refolding solubilized inclusion body proteins. Methods Enzymol 2009:259-82.

[7] Rinas LFVaU. Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. Microbial Cell Factories 2004:3:11. http://dx.doi.org/10.1186/1475-2859-3-11

[8] Masaki Okumura MS, Hiroshi Yamaguchi and Yuji Hidaka. Acceleration of disulfide-coupled protein folding using glutathione derivatives. The FEBS journal 2011.

[9] Jing Chen YL, Yinjue Wang, Hong Ding, and Zhiguo Su. Different Effects of L-Arginine on Protein Refolding: Suppressing Aggregates of. Biotechnol Prog 2008.

[10] Kouhei Tsumoto MU, Izumi Kumagai, Daisuke Ejima, John S. Philo and Tsutomu Arakawa. Role of Arginine in Protein Refolding, Solubilization, and Purification. Biotechnol Prog 2004:1301-8. http://dx.doi.org/10.1021/bp0498793