Journal of Animal and Veterinary Advances 14 (1): 1-5, 2015
ISSN: 1680-5593
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Comparison of Three Methods for Refolding Recombinant Chicken Interleukin-18 Expressed in *E. coli*

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Abstract: It has been proved that artificial chaperone-assisted protein refolding can promote the refolding of chemically denatured proteins via the sequential addition of low molecular weight "artificial chaperones". The aim of the present study was to compare three methods for refolding recombinant Chicken IL-18 protein (rChIL-18) expressed in *E. coli*. The recombinant chicken IL-18 protein was efficiently expressed in inclusion bodies in the *E. coli* expression system. The inclusion bodies were thoroughly denatured with 6 M of Guanidine Hydrochloride (GH) and refolded by using the methods of artificial chaperone-assisted refolding, GH-deionized water diafiltration and GH-glutathione renaturation. Results of our comparative studies demonstrated that refolding yield of rChIL-18 was at least two times higher using the method of artificial chaperone-assisted refolding (42.45%) than those using GH-deionized water diafiltration (10.67%) and GH-glutathione renaturation (14.83%). The optimal SI values were observed at different concentrations of rChIL-18 proteins refolded by the methods of artificial chaperone-assisted refolding (150 μg L^{-1}), GH-deionized water diafiltration (600 μg L^{-1}) and GH-glutathione renaturation (400 μg L^{-1}). In conclusion, the method of artificial chaperone-assisted refolding is more efficient for the refolding of recombinant chicken IL-18 than those of GH-glutathione renaturation and GH-glutathione renaturation.

Key words: Artificial molecular chaperone, chicken IL-18, recombinant protein, inclusion bodies, protein refolding

INTRODUCTION

Interleukin-18 (IL-18) was originally identified as an Interferon-γ (IFN-γ)-inducing factor (IGIF) (Okamura *et al*., 1998; Ushio *et al*., 1996) and cloned from mouse liver (Okamura *et al*., 1995). IL-18 is a pleiotropic cytokine whose multiple biologic activities include induction of IFN-γ from T and Natural Killer (NK) cells, stimulation of Fas ligand-mediated cytotoxicity by NK and T cells, enhancement of the production of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), IL-2 and anti-CD3-induced T cell proliferation, inhibition of IgE synthesis by B cells and anti-tumor effects (Hayer-Hart *et al*., 1996). In addition, IL-18 can promote lymphproliferation response. To study biological functions of chicken IL-18, we have previously cloned the gene for chicken IL-18 in theexpression vector pGEX (pGEX-mChIL-18) and expressed chicken IL-18 in *E. coli* (Hu *et al*., 2005). However, high-level expression of chicken IL-18 in *E. coli* resulted in accumulation of expressed chicken IL-18 in the form of inclusion bodies. Generally, recombinant proteins expressed in *E. coli* are often in the form of inclusion bodies rather than their native form and therefore *in vitro* recovery of functional proteins through refolding is a main obstacle for protein production from *E. coli*. There are four steps involved in the process of recovery of bioactive proteins from inclusion bodies in *E. coli* expression system: isolation of inclusion bodies, solubilization of protein aggregates, refolding and purification of solubilized proteins. Among these four steps, solubilization and refolding are the most crucial ones for high recovery of functional proteins from inclusion bodies (Singh and Panda, 2005).

It has been proved that artificial chaperone-assisted protein refolding can promote the refolding of chemically denatured proteins via the sequential addition of low molecular weight "artificial chaperones" (Daugherty *et al*., 1998). Artificial chaperone-assisted refolding was inspired by the naturally occurring chaperones such as the prokaryotic GroEL/GroES System and involved a two step protocol. Artificial molecular chaperones, Cetyltrimethyl Ammonium Bromide (CTAB) and β-Cyclodextrin (β-CD), can combine and stabilize the instability conformation of other proteins. Artificial molecular chaperones can also promote the folding of nascent polypeptide chain, assembly or degradation of multimer and transmembrane transport of cell organ protein by controlled combination and liberation (Zhang and Chai, 1999). Recently, it was found that the unification of surfactant and β-CD has the effect of advancing refolding as well. Moreover, the

artificial chaperone-assisted refolding system consisting of surfactant and β-CD has similar function mechanism as molecular chaperones GroEL (Dong *et al*., 2002; Wang *et al*., 2004). In a previous study, we used the artificial molecular chaperones CTAB and β-CD to boost the renaturation yield of recombinant Chicken Interleukin-18 (rChIL-18) expressed in *E. coli* (Wang *et al*., 2008). The aim of the present study was to compare three methods for refolding recombinant Chicken IL-18 protein (rChIL-18) expressed in *E. coli*.

MATERIALS AND METHODS

Expression of recombinant chicken IL-18 (rChIL-18) in *E. coli*: The prokaryotic expression plasmid encoding the recombinant Chicken IL-18 (pGEX-mChIL-18) has been constructed in our laboratory. BL21 (DE3) competent cells were transformed with 2 μL (1 μg μL^{-1}) of pGEX-mChIL-18 plasmidand spread on LB plates containing 100 mg L^{-1} of Ampicillin (Amp) and allowed to stand at 37°C for 12-16 h. A single colony was chosen and diluted into 2 mL of 2×YT medium (pH 7.2) containing Tryptone (16 g L^{-1}), Yeast extract (10 g L^{-1}), NaCL (5 g L^{-1}) and Amp (100 mg L^{-1}) and shaked overnight at 37°C. The overnight culture was used to inoculate 25 mL of 2×YT culture, medium (1:100 dilution). The expression of recombinant chicken IL-18 was induced when the optical density at 600 nm reached 0.6 by adding Isopropyl-β-D-Thiogalactopyranoside (IPTG) to the final concentration of 0.2 mM and the bacterial culture was allowed to stand at 37°C for 4 h. To detect expression of recombinant chicken IL-18, Sodium Dodecylsulfate Polyacrylamide Gel Electropheresis (SDS-PAGE) assay was performed.

Extraction and resolution of recombinant chicken IL-18: Cells were harvested by centrifugation of 20 mL of induced culture at 5000 rpm for 10 min and cell pellets were resuspended in 2 mL of TE. Supersonic Wave Method was used to break down bacteria cells on ice under 450 W (30 sec each supersound and 30 sec each interval for 30 cycles). Furthermore, Gram staining was chosen to determine if cells were completely broken into pieces. After centrifugation at 8000 rpm for 10 min, the precipitates were harvested, resuspened in 2 mL of TE containing TritonX-100 (10 g L^{-1}) and allowed to stand at room temperature for 30 min. Inclusion bodies of chicken IL-18 were washed with 2 mL of TE containing TritonX-100 (10 g L^{-1}) on ice under 450 W (30 sec each supersound and 30 sec each interval for 10 cycles) using a ultrasonic cell disruptor. After centrifugation at 8000 rpm for 10 min, the supernatant was removed and the precipitates were harvested.

Denaturization of recombinant chicken IL-18 inclusion bodies: The harvested precipitates were denatured with 6M GH buffer (30 M DTT and 0.1M Tris-base, pH 8.0) and shaked at 120 rpm under 37°C for 1 h to completely denature and reduce the native structure of proteins.

Refolding of recombinant chicken IL-18: Artificial chaperone-assisted refolding approach, Guanidine Hydrochloride (GH)-deionized water diafiltration and GH-glutathione renaturation method were used for protein renaturation.

Artificial chaperone-assisted refolding approach: The blank renaturation solution was 0.1M Tris-base buffer (pH 8.0) containing 4.78 mM GSH, 0.478 mM GSSG and 1.195 mM EDTA. The experiment renaturation solution containing renaturation assistant was prepared using the blank renaturation solution. To bring the final concentrations of protein, CTAB and β-CD to the desired ones, following procedures were performed. Denatured protein solution and renaturation solution containing CTAB (20 mM) were mixed and allowed to stand for 15 min at constant temperature in a shaking incubator. The blank renaturation solution was added into the mixture and allowed to stand for 15 min at constant temperature in a shaking incubator. The renaturation solution containing β-CD (15 mM) was added into the mixed solution and allowed to stand for 1 h. After renaturation of recombinant chicken IL-18, magnetism agitation by deionized water was carried out. Moreover, the dialysate was changed once every 4 h for three times. All steps were carried out at 120 rpm under 37°C.

GH-deionized water diafiltration: The solution of GH-denatured protein was slowly transferred to a bag filter and renatured for 16 h at 4°C. Magnetism agitation by deionized water was carried out. The dialysate was changed once every 4 h for four times.

GH-glutathione renaturation approach: The denatured protein solution was then slowly transferred to renaturation solution which was being agitated by magnetism. When the final concentration of protein reached 0.1 g L^{-1}, GSH and GSSG were added into the renaturation solution to give final concentrations of GSH (1 mM) and GSSG (2 mM) and allowed to stand for 16 h at 4°C. Magnetism agitation by deionized water was carried out. The dialysate was changed once every 4 h for three times.

Analytical method: Bradford assay was carried out to determine protein concentrations. Recovery (%) was calculated as previously described (Hayer-Hart *et al*., 1996):

Recovery (%) = [(Concentration×Volume of re-natured protein solution)/Concentration×Volume of de-natured protein solution)]×100%

Biologic activity assay: Lymphocytic generation was measured using the MTT Method. Briefly, 5.0 mL of anticoagulated blood from healthy young SPF chickens were prepared to make blood-lymph cell suspension. The cells were resuspended in 0.5 mL of RPMI1640 containing 5 mL L^{-1} new-born calf serum, 100 U mL^{-1} Benzylpenicillin and 100 U mL^{-1} Streptomycin) and adjusted to the final concentration of 5×10^{8} cells/L. The resuspended cells (100 μL for each well) were added to a 96 well flat-plate and stimulated with refolded recombinant chicken IL-18, ConA and GST (expression of pGEX-6P-1 vector) at final concentrations of 150, 7.5 and 150 μg L^{-1}, respectively. Wells with cells, cells plus undenatured protein and RPMI1640 medium were used as controls (four wells each). The 96 well flat-plate was allowed to stand for 56 h at 37°C in a humidified atmosphere of 5% CO_2. After addition of 15 μL MTT (5 mg mL^{-1}) to each well, the cells were incubated for 4 h. For making cell lysates, 100 μL of lysis solution (50% DMF-20% SDS) were added to each well and the cells were incubated for 2 h at 37°C. The absorbance at 570 nm was measured using an ELISA plate reader. The level of lymphocyte conversion ratio was valued by Stimulation Index (SI). SI was calculated as following: SI = (A_{570} mean value of recombinant chicken IL-18 wells or GST wells-A_{570} mean value of b lank wells)/(A_{570} mean value of control wells-A_{570} mean value of blank wells).

Statistical analysis: The statistical differences between groups were determined by the Student's-test using SPSS and significant differences were evaluated at probability levels of <0.05.

RESULTS

Expression of recombinant chicken IL-18 in *E. coli*: The results of the SDS-PAGE analysis of recombinant chicken IL-18 are shown in Fig. 1. Recombinant chicken IL-18 was expressed in *E. coli* after induction by IPTG (0.2 mmol L^{-1}) for 4 h but not expressed without IPTG. The recombinant chicken IL-18 expressed with fusion protein has a molecular weight of about 44 kDa while the carrier protein GST of pGEX-6P-1 has a molecular weight of about 26 kDa which suggests that recombinant chicken IL-18 has a molecular weight of about18 kDa. The expression yield of the recombinant chicken IL-18 is 32% measured by Gel lamellar scanning assay.

Dialysis purification of recombinant chicken IL-18: Renaturated protein can be purified by dialysis of 24 mm bag filter. As showed in Fig. 1, recombinant chicken IL-18 fragment was obtained while hybridprotein was removed by SDS-PAGE.

Renaturation of recombinant chicken IL-18: The refolding yields of active recombinant chicken IL-18 were 10.67% using GH-deionized water diafiltration and 14.83% using GH-glutathione renaturation approach. The refolding yield of recombinant chicken IL-18 was 42.54% when the artificial chaperone-assisted refolding buffer was used which contained 0.98 M GH, 2.4 mM CTAB, 9.6 mM β-CD, 0.4 mM GSSG, 4.0 mM GSH, 1.0 mM EDTA and 0.1 M Tris-base.

Biologic activity assay of artificial chaperone-assisted refolding: IL-18 can promote lymphproliferation as described previously. Figure 2 shows lymphocyte multiplication effects of recombinant chicken IL-18, ConA and GST. Values of rpChIL-18 were highly significantly different compared to controls (4.38 vs. 1.00 SI, $p<0.01$) while values of ConA were highly significantly different compared to controls (4.20 vs. 1.00 SI, $p<0.01$). In contrast, GST was not significantly different from the control (1.00 vs. 1.00 SI, $p>0.05$). Figure 3 shows

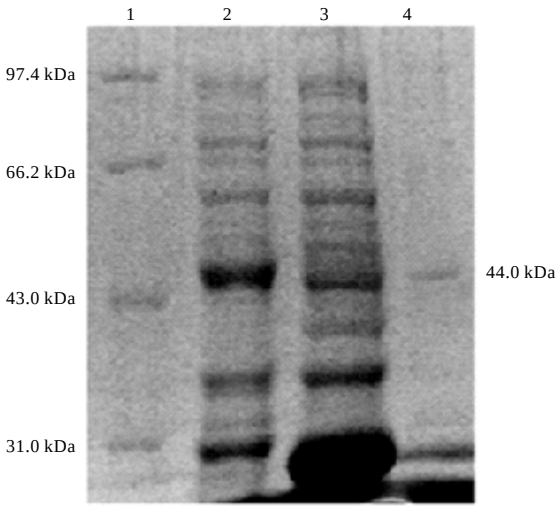

Fig. 1: Expression and purification of recombinant chicken IL-18. Lane 1: Low molecular protein marker, 2: ercombinant chicken IL-18 in *E. coli*, 3: carrier protein GST expressed in *E. coli*, 4: purified products of recombinant chicken IL-18

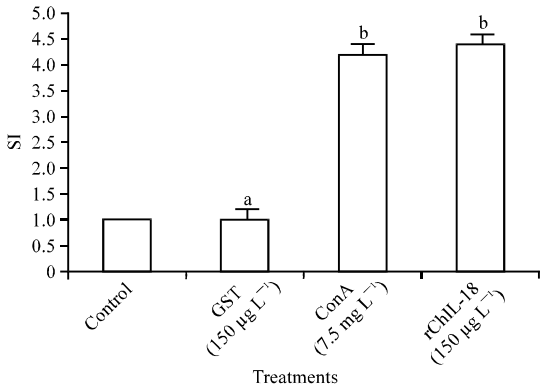

Fig. 2: Lymphocyte multiplication effects of GST, ConA and recombinant chicken IL-18 on blood-lymph cells of SPF chickens (6 chickens for each group). [a]p>0.05, [b]p<0.01

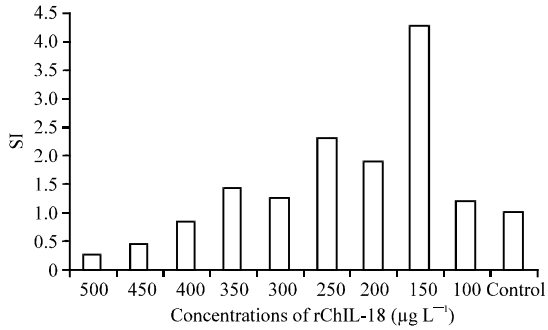

Fig. 3: Lymphocyte multiplication effects of recombinant chicken IL-18 on blood-lymph cells of SPF chickens

lymphcyte multiplication effects of recombinant chicken IL-18 in the range of 100-500 μg L^{-1}. The optimal effect of stimulation was observed at the concentration of 150 μg L^{-1}.

DISCUSSION

In this study, we compare three methods for refolding recombinant chicken IL-18 protein expressed in *E. coli*, the GH-deionized water diafiltration, the GH-glutathione renaturation approach and the artificial chaperone-assisted refolding. Result indicated that refolding yields of recombinant chicken IL-18 was promoted by artificial chaperone-assisted refolding and lymphproliferation response was promoted by protein obtained with artificial chaperone-assisted refolding method. The data suggest that recombinant chicken IL-18 possesses high biological activity.

Because *E. coli* was short of helper factors for facilitating protein folding and enzymes for protein modifications, expressed proteins were in the form of inclusion bodies when eukaryotic genee were over expressed in prokaryotic cells (Garman *et al*., 2000). Inclusion bodies were in the non-active forms when recombinant proteins were agglutinated in bacterium. Consequently, active proteins can be obtained after denaturation and renaturation of inclusion bodies. It was found that surfactant could assist protein refolding in the artificial chaperone-assisted refolding system with β-CD (Kurganov and Topchieva, 1998). It was reported that high concentrations of CTAB could assist lysozyme refolding using artificial chaperone-assisted refolding of lysozyme (Rozema and Gellman, 1996). The structure of surfactant-denatured lysozyme complex in the artificial chaperone-assisted refolding system has been characterized (Wang *et al*., 2004). In addition, synergistic effect between artificial molecular chaperones and GH in lysozyme refolding process has also been studied (Dong *et al*., 2002). Inclusion bodies are difficult to dissolve in water but they can be dissolved in denaturing agents such as carbamide and GH. After breaking into pieces by supersonic wave, addition of detergents such as Triton X-100 and/or low concentrations of chaotropic compounds either prior to mechanical cell breakage or for washing crude inclusion body preparations allow the removal of membrane proteins or other non-specifically adsorbed cell materials (De Bernardez *et al*., 1999; Georgiou and Valax, 1999; Sambrook and Russell, 2001).

The artificial chaperone-assisted refolding system consisting of detergent CTAB and β-CD involves a two-step protocol which is in somewhat reminiscent of the two-step binding-release mechanism of the GroEL/GroES System (Fenton and Horwich, 1997; Hartl, 1996). Firstly, CTAB was used to capture the nonnative protein. Secondly, β-CD was added to initiate folding by stripping CTAB from the protein-detergent complex. GH-deionized water diafiltration and GH-glutathione renaturation approach were also used to compare with the artificial chaperone-assisted refolding system. In this study, our strategy employs small molecules ("artificial chaperones") to promote protein folding of rChIL-18 from the chemically denatured state (Rozema and Gellman, 1995, 1996a, b). Results of our comparative studies demonstrated that refolding yield of rChIL-18 was at least two times higher using the method of artificial chaperone-assisted refolding (42.45%) than those using GH-deionized water diafiltration (10.67%) and GH-glutathione renaturation (14.83%). In addition, lymphproliferation responses of refolding products obtained by three different methods were compared. The optimal SI values were observed at different concentrations of rChIL-18 proteins refolded

by the methods of artificial chaperone-assisted refolding (150 μg L^{-1}), GH-deionized water diafiltration (600 μg L^{-1}) and GH-glutathione renaturation (400 μg L^{-1}).

CONCLUSION

The method of artificial chaperone-assisted refolding is more efficient for the refolding of recombinant chicken IL-18 than those of GH-glutathione renaturation and GH-glutathione renaturation.

ACKNOWLEDGEMENTS

We thank Dr. Pei-Hong Jiang, Dr. Hai Lin and Dr. En-Min Zhou for their kind assistance. This research was supported by a fund for research of HeZe University.

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