Solid-phase refolding of poly-lysine tagged fusion protein of hEGF and angiogenin

Sang Joong Park1, Kang Ryu1, Young Gyu Chai2, Oh Byung Kweon3,
Seung Kook Park3 and Eun Kyu Lee1*

1Dept. of Chemical Engineering, 2Dept. of Biochemistry & Molecular Biology,
Hanyang University, Ansan, Korea 425-791,
3Central R&D Center, Daewoong Pharmaceutical Co., Ltd., Sungnam, Korea

Abstract

A fusion protein, consisting of human epidermal growth factor as a recognition domain and human angiogenin as a toxin domain, can be used as a targeted therapeutic against breast cancer cells among others. The fusion protein was expressed as inclusion body in recombinant *E. coli,* and when the conventional, solution-phase refolding process was used the refolding yield was very low due to severe aggregation, probably due to the opposite surface charge due to vastly different pI values of each domain. Solid-phase refolding process exploiting ionic interactions between the solid matrix and the protein was tried, but the ionic binding yield was very low regardless of the resins and pH conditions used. To provide higher affinity toward the solid matrix, six lysine residues were tagged to the N-terminus of the hEGF domain. When the cation exchange resins such as heparin- or CM-Sepharose were used as the matrix, the adsorption capacity increased 2.5-3 times and the subsequent refolding yield increased nearly 15 times compared to the conventional process.

INTRODUCTION

Targeted delivery of cytotoxins to specific cellular receptors using fusion protein technique has been used recently as a highly selective and effective approach in cancer treatments [1, 2]. In one case, human EGF (epidermal growth factor; pI 4.5, 6 kDa) was used as a recognition domain [3] and human angiogenin (pI 9.0, 14 kDa) was used as a toxin domain[4, 5]. EGF can act as a targeting marker for EGF receptors that are expressed on the surface of some tumor cells. After the fusion protein is internalized, angiogenin can act as a ribonucleolytic toxin killing the tumor cells. The C-terminus of the EGF was linked to the N-terminus of the angiogenin by a linker peptide consisting

of five amino acids (GGGGS.)

For mass production, this fusion protein (E5A) was expressed as inclusion body in *E. coli*, and several traditional refolding processes were applied. When a conventional refolding process consisting of rapid dilution for dilution followed by dialysis was used, refolding yield was very low due to severe aggregation. It was probably due to the opposite surface charge of the two domains at the refolding pH (i.e., 7.0) because the pI values of each domain are vastly different (4.5 for EGF and > 9.0 for angiogenin).

To circumvent the aggregation problem, we introduced a solid-phase refolding method. Basically solid-phase refolding consists of three sequential steps: adsorption of solubilized proteins to a surface of solid matrix, refolding on the surface of a solid matrix by washing off the denaturant, and elution from the solid surface. Solid-phase refolding process has some potential advantages. First, it can minimize the aggregation because protein-protein interactions can be systematically avoided. Second, we can maintain relatively higher protein concentration in refolding, which depends on the adsorption capacity of the protein to the given matrix. Besides the solid-phase refolding process can decrease the overall refolding process time, and bypass the additional post-refolding step of separating the monomers from the aggregate [6, 7, 8].

In this study, six lysine residues followed by a tetrapeptide of factor Xa cleavage sequence were tagged to the N-terminus of E5A (6L10E5A) to increase the binding capacity to a cationic exchanger and to provide an enzymatic cleavage site, respectively. The cleavage reaction was necessary to recover the monomeric form of the fusion protein after the refolding step. In this paper the detail procedure of applying the solid-phase refolding concept to the 6L10E5A refolding is presented. Also, the performance comparison between the conventional, solution-phase refolding and the new, solid-phase refolding is presented focusing on the refolding yield.

MATERIALS AND METHODS

Fusion protein expression

Human angiogenin DNA cloned into a pRSET A expression vector (Invitrogen), pRAng. Human EGF cDNA containing plasmid pTE105, pTED was used. Construction of plasmids pTEA 0,1 was designed to express the fusion proteins EGF-(gly)4ser-angiogenin in *E. coli*. The detailed plasmid construction was described elsewhere [2, 9] and this work was performed in Daewoong Pharmaceutical Co., Ltd. For recombinant *E. coli* fermentation GNPF medium was used, and to express the fusion proteins (E5A or 6L10E5A) 1 mM IPTG was added in the mid-exponential phase. The detailed fermentation and expression

procedure was described elsewhere [10].

Inclusion body isolation and solubilization

After the fermentation the cells were harvested by centrifugation (12000 rpm, 20 min). SDS-PAGE and western blotting were used to check the expression of a target protein, and the expression level was quantified by using a scanning densitometer. The isolated cells were added to a lysis buffer (100 mM sodium phosphate, 1% Tween 20, pH 7.0) and disrupted by sonication. The cell disrupt was centrifuged at 8000 rpm for 30 min to recover the inclusion body (IB) pellets, which were washed by washing buffer (50 mM sodium phosphate, 1% Tween 20, pH 7.0) three times. The washed IB was dissolved by solubilization buffer (8 M urea, 50 mM sodium phosphate, pH 7.0).

Solid-phase refolding

To find the suitable solid matrix and binding condition, equilibrium adsorption capacity of two solubilized proteins (E5A and 6L10E5A) was determined using different pH, ionic strength, and resins (CM-Sepharose, SP-Sepharose, Q-Sepharose, DEAE, Heparin-Sepharose, Phenyl-Sepharose, all from Amersham Pharmacia Biotech, Uppsala, Sweden). 0.5 ml resin was added to the solubilized IB solution (at 1 mg/ml). After mixing and sedimentation, the protein concentration in the supernatant was analyzed by Bradford assay to calculate the adsorption capacity. After the adsorption, urea was removed by urea-wash buffer (50 mM sodium phosphate, pH 7.0). The adsorbed fusion protein was solid-phase refolded during this step. The refolded proteins were eluted by elution buffer (50 mM sodium phosphate and 2 M NaCl, pH 7.0). RNase bioactivity of the fusion protein in the eluate was measured by tRNA assay.

RESULTS AND DISCUSSION

Fermentation

From the fermentation for E5A expression, final OD (at 600 nm) of 20, 6.7 g/L DCW, and IB dry weight of 2.1 g/L were obtained. The expression level was about 25% of the total proteins detected on the gel. The results of 6L10E5A fermentation were: final OD 25, DCW 4.7 g/L, and IB dry weight 3.4 g/L. The expression level was approximately 21% of the total proteins. The expression profile of 6L10E5A during fermentation was checked by Western blots to EGF antibody and angiogenin antibody. Some formation of E5A was observed before the induction, but the majority of the protein was formed after the induction.

Adsorption capacity of E5A

When the solubilized protein was refolded using a conventional method (rapid dilution followed by dialysis) [11], severe aggregation was observed with very low recovery yield. Also long refolding time, about 24 h, was necessary. To apply the solid-phase refolding, we needed to scout the suitable adsorption matrix and condition. Four types of ion exchangers (CM-Sepharose, Sephadex C-50, Q-Sepharose and DEAE-Sepharose) were tested at various pHs (6, 7, 8, 9, 10). Although binding yield varied in each case, adsorption capacity was below 30% (0.3 mg-E5A/ml-resin) in all the cases (Fig. 1.). Also, the adsorption capacity was not much affected by ionic strength (50, 100, 200, 300, and 400 mM were tested). Considering the fusion protein contained both positive and negative surface charges, we used mixed resins of CM-Sepharose and DEAE-Sepharose, but the yield was below 40%. Furthermore, hydrophobic interaction resin (Phenyl-Sepharose) was tested, but again the binding yield was very low. In sum, the solid-phase refolding technique was not deemed feasible because of the very poor affinity of E5A to any kind of the resins. It was mainly due to the fact that EGF and angiogenin have the contrary electric surface charge, which may hinder any ionic interactions by presenting both attractive and repulsive forces simultaneously.

Adsorption capacity of 6L10E5A

Six lysine residues were tagged to the N-terminus of E5A (6L10E5A) to provide higher affinity to cationic exchanger. The solubilized 6L10E5A IB was applied to several resins (CM-, SP-, Q-, DEAE-, and heparin-Sepharose) at various pHs. Fig. 2. indicated that the adsorption yield was quite improved: with CM-Sepharose at pH 6, for example, the binding yield was approx. 65%, which was 2.5 - 3 times improvement than the E5A case. Heparin-Sepharose showed the highest adsorption capacity (for example, approx. 88% binding yield at pH 6). The poly-lysine tag provided the higher affinity to cation exchanger as well as heparin. The intrinsic affinity of angiogenin toward heparin was believed to help improve the adsorption [12]. Based on this observation, heparin-Sepharose was used as the matrix of choice. The same experimental protocol was applied to Ni-NTA resin to confirm the adsorption of the 6L10E5A. Between pH 7 and 9, the adsorption yield was 70 +/- 5%.

Solid-phase refolding

Washed IB of 6L10E5A was dissolved in the solubilization buffer and 10 mg total protein was loaded to Heparin-Sepharose column (5 ml volume) at 2 ml/min, which was previously equilibrated with 8 M urea. After the solid-phase refolding the fusion protein was eluted at 1.5 M NaCl (Fig. 3.) The recovery yield was approx. 63.6%, which was about 15 times improvement than the conventional method. This was primarily due to the lesser degree of aggregation, intrinsic advantage of solid-phase refolding. Also the

refolding process time of the conventional method was about 30 h, but that of the solid-phase refolding was only 4 - 5 h. The heparin-Sepharose eluate showed approx. 65% RNase activity compared with pure angiogenin monomer. EGF bioactivity for the receptor binding is being checked by using a biosensor

CONCLUSION

Solid-phase refolding of the poly-lysine tagged fusion protein (6L10E5A) was able to achieve much-improved refolding yield when compared with the conventional, solution-phase refolding or the untagged protein (E5A). The poly-lysine tag provided stronger affinity toward cationic exchanger, resulting in improved binding. When the solid-phase refolding was performed in the heparin-Sepharose column, the refolding yield was increased about 15 times and the process time was reduced to approx. 8 fold. This work demonstrated that the solid-phase refolding method is able to provide much higher refolding yield by suppressing intermolecular interactions for aggregation.

REFERENCES

[1] Psarras, K., M. Ueda, T. Yamamura, S. Ozawa, M. Kitajima, S. Aiso, S. Komatsu and M. Seno, "Human pancreatic RNase1-human epidermal growth factor fusion; an entirely human 'immunotoxin analog' with cytotoxic properties against squamous cell carcinomas"(1998), *Protein Eng.*, 11, 1285-1292.

[2] Yoon, J. M., S. H. Han, O. B. Kown, S. H. Kim, M.H. Park and B. K. Kim, "Cloning and cytotoxicity of fusion proteins of EGF and angiogenin"(1999), *Life Science*, 16, 1435-1445.

[3] Hirata, Y. and A. N. Orth, "Epidermal growth factor (urogastrone) in human fluids: size heterogeneity"(1979), *J. Clin. Endocrinol. Metab.*, 48, 673-679.

[4] Shapiro, R., J. F. Riordan and B. L. Vallee "Characteristic ribonucleolytic activity of human angiogenin"(1986), *Biochemistry*, 25, 3527-3532.

[5] Wu, Y. N., S. K. Saxena, W. Ardelt, M. Gadina, S. Mikulski, C. D. Lorenzo, G. DAlessio, and J. Youle, "A study of the intracellular routing of cytotoxic ribonucleases "(1995), *J. Biol. Chem.*, 1270, 17476-17481.

[6] Creighton, T. E., "Process for the production of a protein"(1989), US Patent 4,977,248.

[7] Sinha, N. K. and A. Light, "Refolding of reduced, denatured trypsinogen and trypsin immobilized on agarose beads"(1975), *J. Biol. Chem.*, 250, 8624-8629.

[8] Stempfer, G., B. H. Neugebauer, and R. Rudolph, "Improved refolding of immobilized fusion protein"(1996), *Nature Biotech.*, 14, 329-334.

[9] Lee, K. M., Y. W. Koh, J. Y. Chung, S. K. Park and M. W. Park, "A novel gene coding human epidermal growth factor and preparing the same"(1997), US Patent 8,360,841.

[10] Lee, Y. S., "Fermentation and purification processes for fusion protein of human epidermal growth factor and angiogenin from recombinant *E. coli*."(1999), M.S. Thesis., Hanyang University, Dept. of Chemical Engineering, Korea.

[11] Kim, C. S. and E. K. Lee, "Effects of operating parameters in in vitro renaturation of a fusion protein of human growth hormone and glutathione S transferase from inclusion body"(2000), *Proc. Biochem.*, 36, 111-117.

[12] Soncin, F., D. J. Strydom and R. Shapiro, "Interaction of heparin with human angiogenin"(1997), *J. Biol. Chem.*, 272, 9818-9824.

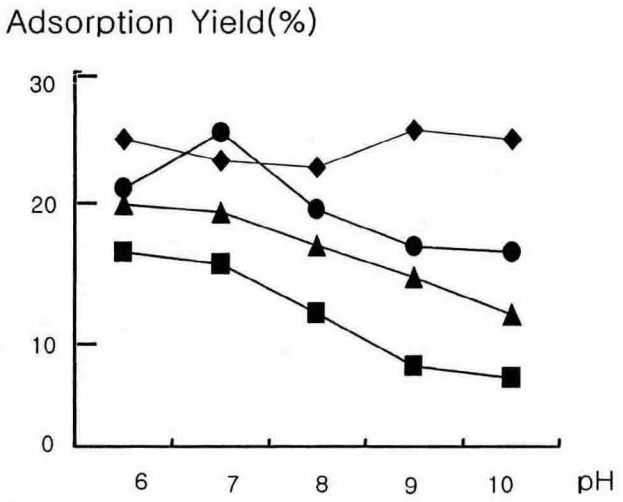

Fig 1. Adsorption yield of E5A in different resin and pH. Q-Sepharose(◆), CM-Sepharose(●), DEAE(▲), Sephadex C-50(□)

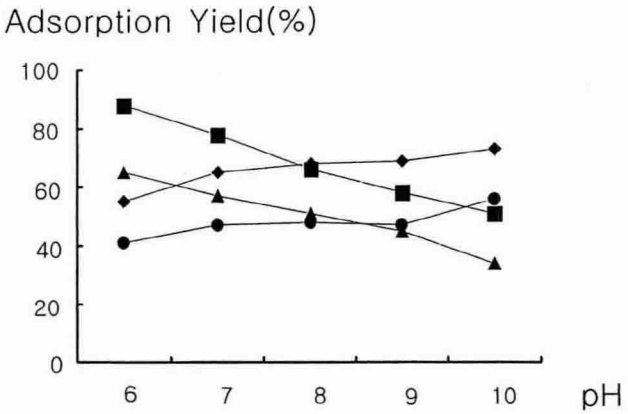

Fig 2. Adsorption yield of 6L10E5A in different resin and pH. Q-Sepharose(◆), CM-Sepharose(▲), DEAE(●), Heparin-Sepharose(◆)

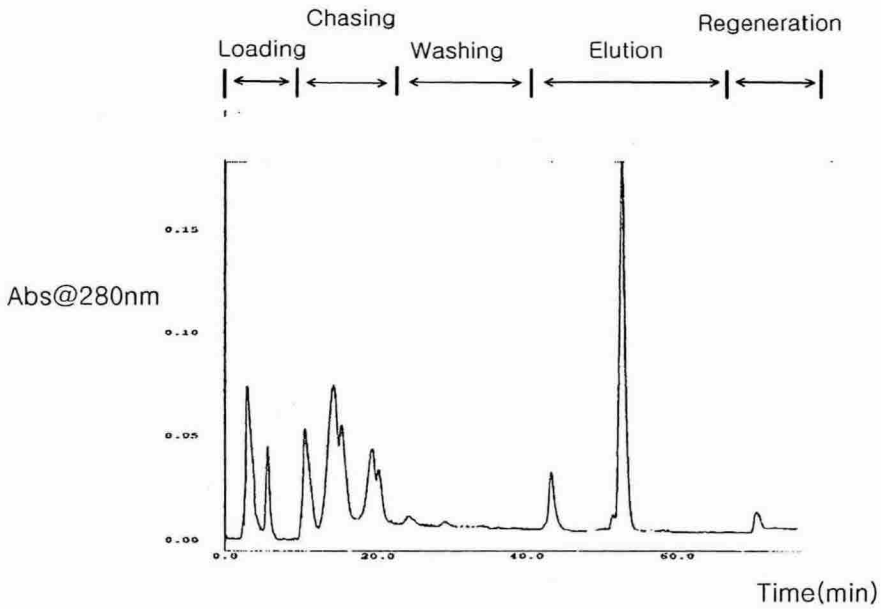

Fig 4. Heparin-Sepharose chromatogram from solid-phase refolding of 6L10E5A