

# Effects of L-arginine on refolding of lysine-tagged human insulin-like growth factor 1 expressed in *Escherichia coli*

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Received: 30 May 2011 / Accepted: 15 July 2011  
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**Abstract** Insulin-like growth factor 1 (IGF1), a therapeutic protein, is highly homologous to proinsulin in 3-dimensional structure. To highly express IGF1 in recombinant *Escherichia coli*, IGF1 was engineered to be fused with the 6-lysine tag and ubiquitin at its N-terminus (K6Ub-IGF1). Fed-batch fermentation of *E. coli* TG1/pAPT-K6Ub-IGF1 resulted in 60.8 g/L of dry cell mass, 18% of which was inclusion bodies composed of K6Ub-IGF1. Subsequent refolding processes were conducted using accumulated inclusion bodies. An environment of 50 mM bicine buffer (pH 8.5), 125 mM L-arginine, and 4 °C was chosen to optimize the refolding of K6Ub-IGF1, and 240 mg/L of denatured K6Ub-IGF1 was refolded with a 32% yield. The positive effect of L-arginine on K6Ub-IGF1 refolding might be ascribed to preventing unfolded K6Ub-IGF1 from undergoing self-aggregation and thus increasing its solubility. The simple dilution refolding, followed by cleavage of the fusion protein by site-specific UBP1 and chromatographic purification of IGF1, led

production of authentic IGF1 with 97% purity and an 8.5% purification yield, starting from 500 mg of inclusion bodies composed of K6Ub-IGF1, as verified by various analytical tools, such as RP-HPLC, CD spectroscopy, MALDI-TOF mass spectrometry, and Western blotting. Thus, it was confirmed that L-arginine with an aggregation-protecting ability could be applied to the development of refolding processes for other inclusion body-derived proteins.

**Keywords** Insulin-like growth factor 1 · Inclusion body · Refolding · L-arginine

## Introduction

Insulin-like growth factor 1 (IGF1) is a hormone isolated from human plasma and is called mecasermin or somatomedin C [1]. IGF1 is a truncated form of IGF1 precursor and consists of 70 amino acids from Gly<sub>33</sub> to Ala<sub>102</sub>. The tertiary structure of IGF1 is similar to that of proinsulin, because IGF1 and proinsulin have highly homologous sequences of amino acid [2]. It is released from various types of cells and mediates the growth-promoting effects of somatotropin. IGF1 also shows an insulin-like hypoglycemic activity which is controlled by an insulin-independent regulation. IGF1 is used as an adjunctive therapeutic reagent effective for treating diabetes patients with abnormal somatotropin or insulin receptors [3]. Recombinant DNA technology to over-express the IGF1 gene, and process optimization to produce large quantities of IGF1 in *Escherichia coli* [4–6] or *Saccharomyces cerevisiae* [7, 8] have been employed for physiological investigation and clinical trials.

IGF1 is commercially produced in recombinant cell lines and microorganisms. The recombinant IGF1s were generally produced as inactive and intracellular inclusion bodies in

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*E. coli*, but few successes have been announced concerning the soluble and active expression of IGF1 improved by the fusion of affinity tags [9, 10]. A commercially available analog of IGF1 (Long-R3-IGF1) is sold by GroPep Pty Ltd (Adelaide, Australia) as a supplement for mammalian cell culture and is also known to be accumulated as inclusion bodies when expressed in recombinant *E. coli* [11]. The extensive use of mammalian cell culture for commercial production of protein pharmaceuticals definitely requires a sufficient supply of highly pure IGF1.

High expression of the target genes in *E. coli* often leads to the accumulation of inactive and insoluble deposits of the corresponding proteins inside the cells, and such deposits are called inclusion bodies [12, 13]. They are easily distinguishable from other cell components due to their refractive characteristics. In spite of the requirement for additional refolding processes, expression of the target protein as an inclusion body has several attractive advantages. Inclusion bodies are easily collected and concentrated by simple centrifugation, because they consist exclusively of the target protein and show density differences from other intracellular proteins [14, 15]. In addition, their storage as aggregates protects them from protease-mediated degradation [13]. The refolding procedure required to obtain soluble and active proteins poses a bottleneck in every downstream scheme where protein aggregates should be resolved and folded into their authentic structures. Various strategies have been employed to achieve active proteins refolded from inclusion bodies [12, 13].

As mentioned, the expression of insoluble IGF1 in *E. coli* is known to be an important bottleneck for its mass production. For the cost-effective production of IGF1, in this study, IGF1 was fused with the 6-lysine (K6) tag and ubiquitin (Ub) at its N-terminus, and this engineered form was designated as K6Ub-IGF1. Ubiquitin (81 amino acids, Molecular weight 9248.4) was inserted between the K6 tag and IGF1 as the cutting site to be recognized by site-specific ubiquitin protease 1 (UBP1) [16]. Fed-batch fermentation of recombinant *E. coli* containing the recombinant IGF1 expression system was conducted to obtain high amounts of K6Ub-IGF1 in the form of inclusion bodies. Thus, to obtain the authentic IGF1 with high purity, we developed a scheme for dilution refolding using a self-aggregation inhibitor and purification using ion exchange chromatography.

## Materials and methods

### Construction of IGF1 expression system

*E. coli* DH5 $\alpha$  and TG1 were used as bacterial hosts for the manipulation and expression of the human IGF1 gene.

To fuse IGF1 with both the K6 tag and the Ub, or with only the Ub at its N-terminus, a 2-step polymerase chain reaction (PCR) was carried out with the Accupower HL PCR PreMix<sup>TM</sup> kit (Bioneer, Daejeon, Korea) and the GeneAmp PCR System 2400 (Applied Biosystems, Foster, CA, USA), programmed for 1 cycle of 95 °C for 10 min; 30 cycles of 94 °C for 30 s, 51 °C for 1 min, 72 °C for 1 min, and 1 cycle of 72 °C for 7 min. Plasmid pUC18-IGF containing the IGF gene was purchased from Cytokine Bank (Jeonju, Korea) and used as the template of the first PCR. Two DNA oligomers specific for the IGF1 gene, 5'-gtgctaaggcta agaggtggcggaccggaacgctgtgcgg-3' (Ub-I-F3) and 5'-tttgtt agcagccggatcctcattacgctgattggccgggtt-3' (I-SS-B-APT-R), were designed in order to fuse the IGF1 gene with the Ub coding region. The second PCR was carried out using two sets of PCR primers: 5'-gaaataattttgttaactttaagaaggagatataca tatgaagaaaaaaagaaaaacagatttcgtcaagactttgaccg-3' (APT-NdeI-K6Ub-F) and I-SS-B-APT-R primer for synthesis of K6Ub-IGF1; 5'-gaaataattttgttaactttaagaaggagatatacatatgca gatttcgtcaagactttgaccg-3' (APT-NdeI-Ub-F), and I-SS-B-APT-R primer for synthesis of Ub-IGF1. The first PCR product and plasmid pUC18-Ub harboring the ubiquitin gene were used as PCR templates. After digestion of the first and second PCR products, each DNA fragment was combined with plasmid pAPT which is an *E. coli* expression vector containing the *tac* promoter and the kanamycin resistance gene. The vectors constructed for expression of K6Ub-IGF1 and Ub-IGF1 were designated as pAPT-K6Ub-IGF1 and pAPT-Ub-IGF1, respectively. *E. coli* was transformed by the CaCl<sub>2</sub> treatment method and selected on LB solid medium containing kanamycin.

### Fed-batch fermentation

Fed-batch cultures were carried out in a 5 L jar fermentor (Kobitech, Incheon, Korea) containing 2 L of a Riesenberg medium, which contained per liter: glucose, 20 g; (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 4 g; KH<sub>2</sub>PO<sub>4</sub>, 13.5 g; citric acid, 1.7 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.4 g; trace metal solution, 10 ml. The trace metal solution contained per liter of 5 M HCl: FeSO<sub>4</sub>·7H<sub>2</sub>O, 10 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 2.25 g; CuSO<sub>4</sub>·5H<sub>2</sub>O, 1 g; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.5 g; Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O, 0.23 g; CaCl<sub>2</sub>, 2 g; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 0.1 g. A seed culture was prepared in a 500 ml-scale baffled flask containing 100 ml of Riesenberg medium [17]. The temperature was fixed at 37 °C. The pH was controlled at 6.8 using NH<sub>4</sub>OH (28%, v/v) which also served as a nitrogen source. Dissolved oxygen level was maintained at 40% of air saturation by controlling agitation speed and supplying pure oxygen when required. After batch-wise growth, feeding solution A containing 800 g/L glucose and 20 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O was added using the pH-stat feeding strategy [18]. When the pH rose to a value greater than its set point (6.8) due to the

depletion of glucose, a feeding solution B (600 g/L glucose, 20 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 50 g/L yeast extract) was automatically added to increase the glucose concentration in the culture medium. Expressions of K6Ub-IGF1 and Ub-IGF1 were induced by addition of 1 mM IPTG into the culture broth. Cell growth was monitored by measuring the optical density of culture broth at 660 nm using a spectrophotometer (UV-1601, Shimadzu Co., Kyoto, Japan). Protein content was measured by densitometric analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the Bio-Rad protein assay kit (Hercules, CA, USA) according to the manufacturer's instruction.

#### *Purification of denatured inclusion body*

After 32 h of fermentation, the cells were harvested by centrifugation at 6,000 rpm and 4 °C for 10 min. The wet cell pellets (100 g) were resuspended in 1 L of a resuspension solution consisting of 50 mM sodium phosphate buffer (pH 8.0) containing 100 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100. After cell disruption using a homogenizer (Microfluidics, Newton, MA, USA) at 10,000 psi and 4 °C for 2 passes, the insoluble pellets containing K6Ub-IGF1 or Ub-IGF1 inclusion bodies were isolated by centrifugation at 6,000×g and 4 °C for 30 min, and then washed twice with the resuspension solution supplemented with 2 M urea. The inclusion bodies were recovered by centrifugation at 12,000×g for 10 min, denatured at a concentration of 2 g/L in solubilization solution (30 mM sodium phosphate buffer (pH 7.5), 8 M urea, and 1 mM EDTA) for >4 h at room temperature, followed by the addition of 1 mM dithiothreitol for breaking of disulfide bonds. The solubilized inclusion bodies were purified with the HiTrap SP FF packed with SP Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) using buffer U consisting of 50 mM sodium phosphate buffer (pH 7.5) and 8 M urea. The amount of denatured fusion protein was determined by automatic detection at a wavelength of 280 nm in the ÄCTA prime (Amersham Biosciences, Uppsala, Sweden).

#### *Dilution refolding*

The dilution refolding process designed for this study was composed of 3 steps: formation of a molten globular structure by addition of urea solution (2~3 M) to the denatured protein, shuffling of disulfide bonds to minimize aggregate formation, and completion of refolding by lowering urea concentration to 0.1 M. The initial concentrations of denatured fusion protein were adjusted by diluting with buffer U to provide several final concentrations (30, 60, 120, 240, and 360 mg/L) at the end of the following dilution refolding process. The concentration of urea

contained in 40 ml of the denatured protein solution was reduced from 8 to 3 M at 4 °C during an 11 h period by the slow addition of buffer solutions with different pH values (20 mM Tris, pH 8.0; 50 mM bicine, pH 8.5; 20 mM Tris, pH 9.0; 20 mM Tris, pH 9.5) using a MasterFlex peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA). L-Cysteine (5 mM) and L-arginine (0~400 mM) were added to each buffer solution. Herein, the final concentration of urea was 2 M in 160 ml. Finally, each dilution refolding was completed by reducing the concentration of urea to 0.1 M by addition of the same pH buffer at 25 °C for 16 h. At the end of dilution, the final volume was 3.2 L, in which the formed aggregates were removed by filtration with a 0.45 µm PVDF filter. Soluble proteins composed of correctly folded and mis-refolded fusion proteins were obtained by centrifugation at 15,000×g for 30 min.

#### *Concentration of K6Ub-IGF1*

To concentrate the refolded protein and also to remove residual reagents, the solution containing the refolded K6Ub-IGF1 was subjected to a chromatographic process using a cation exchanger (SP-Sepharose Fast Flow) which was equilibrated with 30 mM of bicine buffer (pH 8.5) containing 0.1 M urea. Then, urea was removed from the column by slow feeding of the bicine buffer with a concentration gradient of urea (from 0.1 to 0 M). The concentrated K6Ub-IGF1 was eluted by a gradual increment (0 to 1 M) of NaCl concentration in 30 mM bicine buffer (pH 8.5) and then collected. Meanwhile, the solution containing the refolded Ub-IGF1 was subjected to cross-flow filtration (Minimate, Pall, Port Washington, NY, USA) with a 1,000 kDa of molecular cut-off, and 30 mM bicine buffer (pH 8.5) was used as a diafiltration buffer.

#### *Removal of tag from refolded fusion protein*

Ubiquitin protease 1 (AP Technology Co., Suwon, Korea) was used at room temperature for 12 h to cleave the peptide bond between Ub and IGF1 in refolded K6Ub-IGF1 or Ub-IGF1. Cleavage yield was calculated as follows: cleavage yield (%) = the molar concentration of soluble refolded IGF1/the molar concentration of soluble refolded fusion protein. IGF1 (7.7 kDa) was separated from uncut K6Ub-IGF1 (16.8 kDa) and Ub-IGF (16.1 kDa) by a size exclusion chromatography column equilibrated with 30 mM bicine buffer (pH 8.5), and then further concentrated by cross-flow filtration with a 1,000-kDa of cut-off. Concentration of the purified IGFs was measured by a reverse phase high pressure liquid chromatography (RP-HPLC, 1200 series, Agilent Tech., Santa Clara, CA, USA) equipped with a C18 column (Cat. No. 214TP, Vydac Co., Hesperia, CA, USA) heated at 50 °C. A mixture of water

(phase A) and acetonitrile (phase B) with 0.1% trifluoroacetic acid was used as a mobile phase. An acetonitrile–water gradient was programmed as follows: 0 to 27.5% for 10 min, 27.5 to 29% for 15 min, 29 to 90% for 5 min, and 10% for 10 min at a flow rate of 0.5 ml/min. Elution of proteins was monitored with an UV detector at a wavelength of 215 nm. The concentration of IGF1 in the samples was determined by comparing the peak areas of the sample and standard IGFs (Cat. No. I3769, Sigma, USA). Refolding yield was calculated as follows: refolding yield (%) = the molar amount of correctly refolded IGF1/the molar amount of denatured fusion protein.

### Analysis of IGF1

Western blot analysis of IGF1 was performed using the first antibody of a rabbit polyclonal IgG (Cat. No. H-70, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the second antibody of a goat anti-rabbit IgG conjugated with alkaline phosphatase (Cat. No. A3687, Sigma Co., USA). Other conditions followed the standard method. Circular dichroism (CD) analysis of IGF1 was carried out with a circular dichroism spectroscopy (J-715, Jasco, Tokyo, Japan) according to the manufacturer's manual. Samples containing 75  $\mu$ M protein were used after being dialyzed against 5 mM sodium phosphate buffer (pH 7.0). For accurate data acquisition, the spectrum was monitored 8 times at 25 °C. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometric analysis of IGF1 was done by the National Instrumentation Center for Environmental Management (Korea).

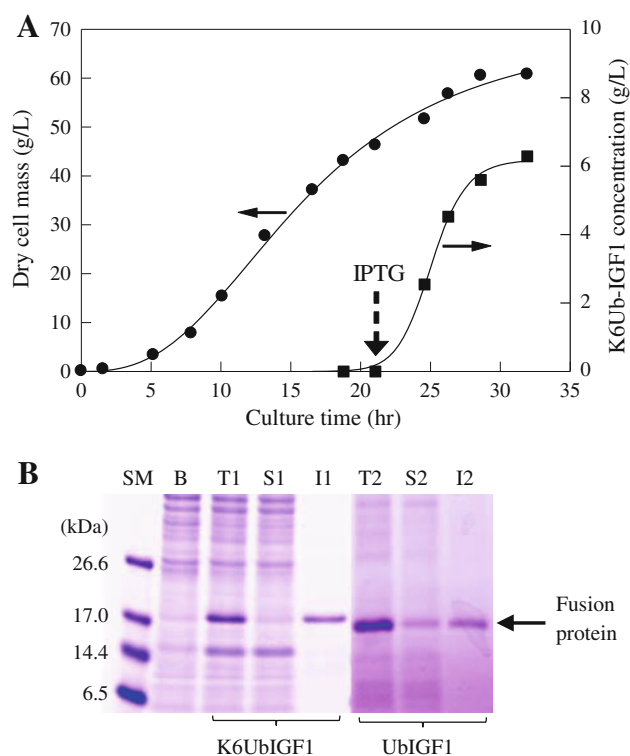
## Results and discussion

### Expression of K6Ub-IGF1 in recombinant *E. coli*

The structural analysis of IGF1 indicated that IGF1 contains a  $\alpha$ -helix structure in the inner area and hydrophobic regions on the surface [2]. Because the *N*-terminal of IGF1 is located on its surface and exposed outside, it seems that the functional tag which is fused at the *N*-terminal of IGF1 may have no adverse effect on the 3-dimensional structure or biological activity of IGF1. To enhance the possibility of expressing the target protein in a soluble form in *E. coli*, several types of amino acid tags have been fused with it. Among them, ubiquitin strongly contributed to the soluble expression of hybrid peptides cecropinA(1-8)–magainin2(1-12) and amyloid-peptide 42 in recombinant *E. coli* [9, 19]. Therefore, a positive effect of ubiquitin was expected to be demonstrated on IGF1 expression in this study. It should be extremely important that affinity tags

were attached to target protein by genetic engineering as a requisite for selective recovery of the protein. Cation exchange chromatography was expected to provide an efficient method for the purification of target protein fused with a strong polycation of tag [20]. The net charge on the lysine (K) molecules is a positive factor, and by all economic measures, the ion-exchange resin is cheaper than other resins. Therefore, as a general rule, the K6 tag was used to force the fusion protein to strongly bind to a cation exchange resin for an easy and cost-effective purification. Thus, in this study, two tags were combined with IGF1 at the *N*-terminus: the K6 tag for easy purification, and the Ub tag both for soluble expression and for the UBP1-mediated cleavage site.

To evaluate the effect of K6 and Ub tags on expression of IGF1, we constructed plasmids pAPT-K6Ub-IGF1 and pAPT-Ub-IGF1. Their transformants of *E. coli* TG1 strains were cultured to produce K6Ub-IGF1 and Ub-IGF1. The expression patterns of both fusion systems were compared using SDS–PAGE, after high cell density culture of recombinant *E. coli* TG1 strains was carried out by fed-batch culture in a 5 L bioreactor (Fig. 1). As a result, the pH–stat

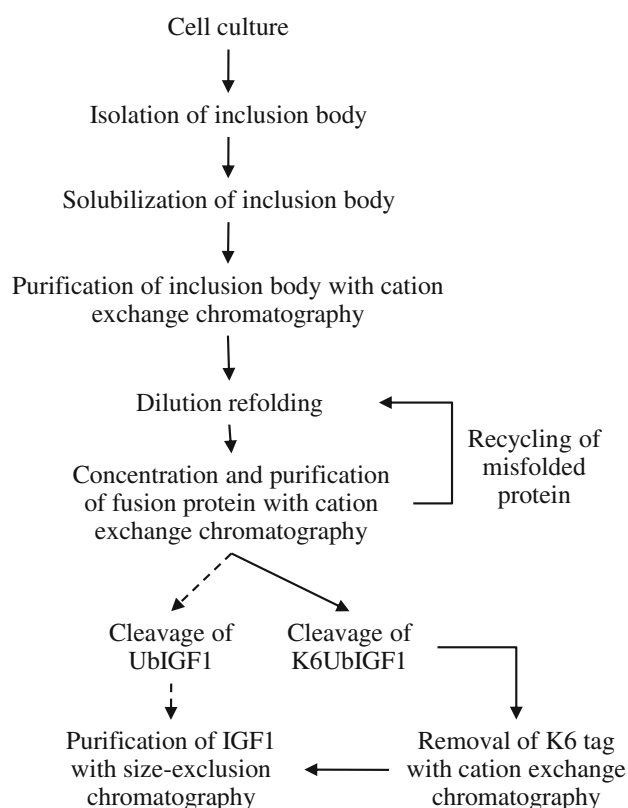


**Fig. 1** Fed-batch fermentation of recombinant *E. coli* TG1/pAPT-K6UbIGF1 (**a**) and SDS–PAGE analysis of K6UbIGF1 and UbIGF1 obtained after 32 h of fed-batch cultures (**b**). *1* the pAPT-K6Ub-IGF1 system, *2* the pAPT-Ub-IGF1 system, *lane B* total protein collected before induction, *lane T* total protein, *lane S* soluble protein, *lane I* insoluble protein, *SM* size marker

feeding of the glucose solution and IPTG induction at the late exponential phase led to the high accumulation of recombinant proteins. The K6Ub-IGF1 expression system with the K6 tag produced 60.8 g/L of dry cell mass with a K6Ub-IGF1 content of 18%, while the Ub-IGF1 expression system without the K6 tag showed a lower fermentation yield of 51.5 g/L dry cell mass with a Ub-IGF1 content of 15%. It was therefore suggested that the K6 tag improved cell growth and expression of the foreign gene. Of greater interest, as shown by SDS-PAGE analysis (Fig. 1b), the K6Ub-IGF1 was expressed in a different pattern from that of Ub-IGF1. Nearly all of the K6Ub-IGF1s were accumulated as insoluble inclusion bodies, perhaps due to inoperative in vivo folding in cells, and their concentration was estimated to be 6.3 g/L. When actually measured by a protein assay, the soluble K6Ub-IGF1 was estimated to be <5% of the total protein. By contrast, ~35% of the Ub-IGF1, which did not harbor K6 tag, was expressed in a soluble form, suggesting that the K6 tag might exert a negative effect on in vivo folding and thus interfere with soluble expression of K6Ub-IGF1. Considering that the surface of IGF1 is hydrophobic and the K6 tag contains a positive charge, it may be also assumed that the aggregation-prone property of K6Ub-IGF1 probably results from the intermolecular non-specific electrostatic interaction between the K6 tag and other parts of the fusion protein, as well as from hydrophobic interactions between IGF1 portions [21]. However, the advantage (high yield) of K6Ub-IGF1 greatly outweighed the disadvantage (insolubility) which was thought to be overcome by advances in in vitro refolding technology.

#### Effect of L-arginine on in vitro refolding of K6Ub-IGF1

Because the addition of a K6 tag led to the insoluble expression of Ub-IGF1, it is plausible to assume that the K6 tag might also affect the in vitro refolding process. In general, the soluble target protein in correct or mis-folded form and a small amount of insoluble aggregate in mis-folded form are formed simultaneously during in vitro refolding of inclusion bodies [22, 23]. L-arginine has been known as an additive used to minimize self-aggregation of denatured inclusion bodies by increasing their solubility during the refolding process. A report showing the positive effects of L-arginine on protein refolding indicated that L-arginine stabilized the exposed hydrophobic areas of a recombinant protein or suppressed aggregation of a partially folded intermediate during refolding [23, 24]. Though the exact mechanism of its positive role has not been clarified to date [25], it was expected that L-arginine might enhance the refolding yield by competitively inhibiting the intermolecular K6-protein interaction. Thus, the effects of L-arginine on in vitro refolding of the denatured

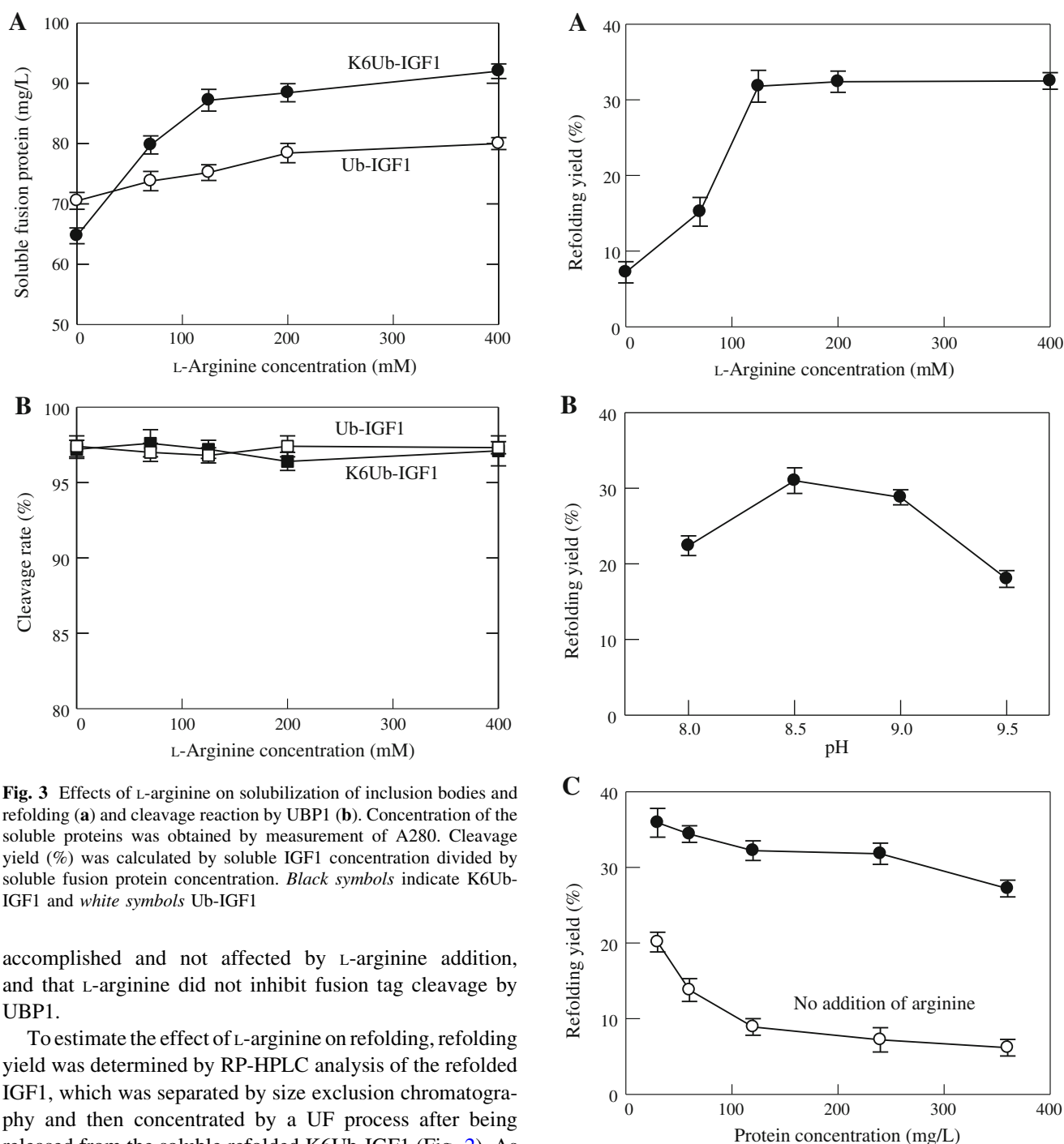


**Fig. 2** Scheme for production of IGF1 released from the refolded fusion protein. Dilution refolding was performed through purification of unfolded fusion protein. IGF1 was released from the refolded K6UbIGF1 or UbIGF1, and the mis-folded fusion protein was recycled into the dilution refolding process

fusion proteins, especially soluble ones, were investigated at different concentrations of L-arginine ranging from 0 to 400 mM, using procedures shown in Fig. 2. The final concentration of fusion protein was set to be 240 mg/L. As shown in Fig. 3a, the concentration of the soluble refolded proteins was proportional to the concentration of added L-arginine in the range of 0 to 125 mM. Addition of L-arginine increased the amount of soluble K6Ub-IGF1 by 1.4-fold, compared with a control without addition of L-arginine. In contrast, the effect of L-arginine on solubilization of Ub-IGF1 was less than that of K6Ub-IGF1 (Fig. 3a). The maximum concentration of soluble Ub-IGF1 ( $79 \pm 1$  mg/L) was obtained when L-arginine was supplemented at a concentration of >200 mM. Although K6Ub-IGF1 was produced in vivo as an insoluble inclusion body by recombinant *E. coli*, it was more easily refolded in vitro by the aid of L-arginine, than that of Ub-IGF1.

Irrespective of L-arginine addition, UBPI precisely spliced the peptide bond between IGF1 and Ub in both fusion proteins, and its cleavage yield was >96% (Fig. 3b). This indicates that refolding of the ubiquitin tag itself, which contains a cleavage site recognized by UBPI, was





accomplished and not affected by L-arginine addition, and that L-arginine did not inhibit fusion tag cleavage by UBP1.

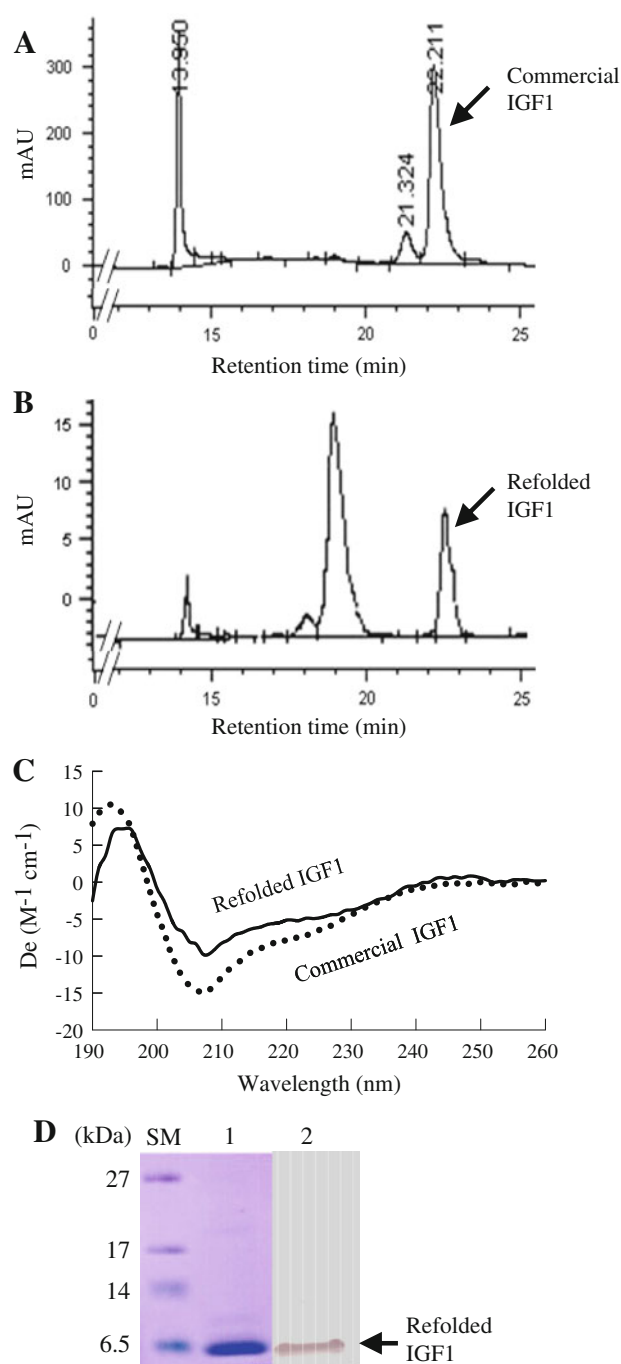
To estimate the effect of L-arginine on refolding, refolding yield was determined by RP-HPLC analysis of the refolded IGF1, which was separated by size exclusion chromatography and then concentrated by a UF process after being released from the soluble refolded K6Ub-IGF1 (Fig. 2). As shown in Fig. 4a, addition of L-arginine increased the refolding yield of K6Ub-IGF1 up to ~32% (a 4.4-fold increase in comparison with a case of no L-arginine addition). Since an L-arginine concentration of >125 mM gave a similar refolding yield (33%), 125 mM L-arginine was chosen as an optimum concentration for the further determination of refolding conditions.

# Optimal conditions for dilution refolding of K6Ub-IGF1

Conditions which affect the refolding process, other than the concentration of L-arginine, were also determined. In general, refolding yield is greatly dependent on the concentrations of added protein and redox reagents, and the acidity of the refolding buffer. The effects of these parameters were tested by procedures described in Fig. 2. As IGF1 is a cysteine-containing protein, the refolding buffer should be supplemented with thiol reagents to permit shuffling of the disulfides [12, 22]. In spite of its high efficacy, glutathione is expensive to use and the high intensity of cysteamine at 280 nm is an obstacle when assaying protein concentration. The  $\text{NH}_3^+$  residue of cysteamine is able to hinder the ionic interaction between the target proteins and cation exchangers [12]. When cysteamine and cystamine were used in refolding of K6Ub-IGF1, protein aggregates were observed at the early stage (data not shown). Thus, the L-cysteine was selected as a thiol reagent and was used in small amounts (5 mM) in this study to allow the shuffling of -SH residues between free and protein-bound cysteines [22].

In a test for the effects of acidity of the refolding solution, a moderately alkaline pH ( $\sim$ pH 8.5) gave the best results for refolding yield (Fig. 4b). At a pH ( $\sim$ pH 9.5) much higher than the theoretical isoelectric point (9.15) of K6Ub-IGF1, intermolecular interactions were intensified owing to increased negative charge on the surface of proteins, and refolding yield was considerably decreased. At pH  $<6.7$ , the refolding solution was clouded, due to precipitation of solid aggregates (data not shown).

A final protein concentration of 10–100 mg/L is generally applied in fast dilution refolding [13]. In this study, denatured proteins were subjected to the refolding process at the higher range of final concentration (30 to 360 mg/L), together with a pH of 8.5, and 125 mM L-arginine (Fig. 4c), resulting in refolding yields of 27–36%. When a high concentration (360 mg/L) of the denatured K6Ub-IGF1 was subjected to the refolding conditions with L-arginine, the refolding yield decreased only by 9%. However, without addition of L-arginine, a second-order aggregation reaction occurred during refolding, resulting in progressively lower yields (Fig. 4c). As mentioned earlier, both hydrophobic aggregation and intermolecular electrostatic interactions were responsible for such a side reactions during in vitro refolding as a function of protein concentration. Apparently, refolding without L-arginine was a reaction of the second or higher order, while refolding with L-arginine was a first-order reaction [22, 26]. In summary, the optimal conditions for the refolding of denatured K6Ub-IGF1 were determined to be 125 mM L-arginine concentration, 240 mg/L protein concentration, and a final pH of 8.5.



**Fig. 5** Instrumental and immunoblot analysis of recombinant IGF1 produced through refolding in this study. **a** and **b** Show HPLC diagrams. **c** Presents circular dichroism analysis. SDS-PAGE (lane 1) and Western blot analysis (lane 2) of refolded IGF1 are shown in **d**. **a** and dotted line in **c** denote a commercial IGF1, and **b** and solid line in **c** indicate refolded IGF1 in this study. The arrows point to IGF1s

## Confirmation of correct refolded IGF1

To verify the identified optimal refolding conditions, the refolded IGF1 in this study and a commercial standard IGF1 were compared by various analytical tools, including

RP-HPLC, circular dichroism (CD) spectroscopy, MALDI-TOF mass spectrometry, and Western blotting. The RP-HPLC analysis of IGF1s showed that the peaks of the standard and refolded IGF1s were detected at the same retention time (Fig. 5). Correctly refolded IGF1 had a retention time of 22.2 min. An unknown peak in front of the refolded IGF1 appeared to contain a mis-folded IGF1. The CD profile of renatured IGF1 was identical to that of the commercial IGF1. Measurement of *m/z* value by MALDI-TOF mass spectrometry indicated that the renatured and commercial IGF1s had molecular weights 7,612.97 and 7,616.50 kDa, respectively. Western blot analysis of the renatured IGF1 resulted in its correct binding with anti-IGF1 antibody. Consequently, instrumental analysis and immunoblot analysis indicated that the denatured and refolded IGF1 in this study had the same structural characteristics as the commercial IGF1.

## Conclusion

The recombinant IGF1s were generally expressed in an insoluble form, especially when fused with electrostatic affinity tags. When L-arginine was not supplied, the refolding yield was decreased by a second-order aggregation reaction; a major side reaction of *in vitro* refolding. Under the optimal refolding conditions using L-arginine, a large amount of K6Ub-IGF1 was successfully renatured, and the released IGF1 had a structure identical to that of commercial IGF1. Thus, it was speculated that L-arginine might be a good additive for protecting against aggregation during the refolding process.

**Acknowledgments** This work was financially supported by the Carbon Dioxide Reduction and Sequestration Research Centre (a 21st Century Frontier Program), and the Advanced Biomass R&D Center(ABC) of Korea Grant (2010-0029728) funded by the Ministry of Science and Technology of Korean government of the Republic of Korea.

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