Simple Purification of *Escherichia coli*-Derived Recombinant Human Interleukin-2 Expressed with N-terminus Fusion of Glucagon

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Abstract Simple procedures have been devised for purifying recombinant human interleukin-2 (hIL-2), which was expressed in *Escherichia coli* using sequences of glucagon molecules and enterokinase cleavage site as an N-terminus fusion partner. The insoluble aggregates of recombinant fusion protein produced in *E. coli* cytoplasm were easily dissolved by simple alkaline pH shift (8→12→8). Following enterokinase cleavage, the recombinant hIL-2 was finally purified by one-step reversed-phase HPLC with high purity. The ease and high efficiency of this simple purification process seem to mainly result from the role of used glucagon fusion partner, which could be applied to the production of other therapeutically important proteins.

Keywords: human interleukin-2, glucagon, N-terminus fusion, purification process

INTRODUCTION

Human interleukin-2 (hIL-2), formerly referred to as T-cell growth factor, is a lymphokine which is produced by lectin- or antigen-activated T cells. The reported biological activities of hIL-2 include stimulation and proliferation of natural killer cell, lymphokine-activated killer cell, and B cell [1], and induction of γ-interferone activity [2]. The *E. coli*-derived recombinant hIL-2 (rhIL-2) was successfully commercialized by Chiron (U.S.A.) as a therapeutic (Proleukin™) for renal cell carcinoma in 1992. The Food and Drug Administration (FDA) also permitted market-license of Proleukin™ as a recombinant therapeutic for malignant melanoma in 1998, and the recombinant hIL-2 is currently in clinical trial phase III for testing its therapeutic efficacy in treament of AIDS patients [3].

The human interleukin-2 comprises 133 amino acid residues (15 kDa) with a disulfide linkage (Cys58-Cys105) and is folded into a bundle-shaped protein consisting of four α helices [4]. A recent study has shown that when rhIL-2 was directly expressed in *E. coli*, the high hydrophobicity of rhIL-2 caused non-specific intermolecular aggregation and formed complex heterogeneous aggregate by non-specific intermolecular S-S linkages [5]. The insoluble inclusion bodies are easily separated from cell lysates [6,7] and however, the formation of highly heterogeneous aggregates, inter-linked by S-S bonds causes significant problem of insolubility unless they are completely denatured by using reducing agent(s). The denaturation of recombinant protein should be followed by dialysis and refolding processes and hence adds more complexity to downstream process. Recently the use of small α-helical peptide (*i.e.* glucagon) as an N-terminus fusion expression partner has been shown to be highly effective in inducing homogeneous aggregate formation of rhIL-2 with avoiding intermolecular S-S bond formation [5,8].

For the purification of *E. coli*-derived rhIL-2 which was expressed as a fusion protein involving N-terminus glucagon molecules, we report in present study that a simple downstream process has been developed, which comprises recovery of soluble fusion protein by simple alkaline pH shift, enterokinase digestion, and reversed-phase HPLC.

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MATERIALS AND METHODS

Recombinant Culture Broth

The induced culture broth containing the *E. coli* strain BL21(DE3)[pT7-G3IL2], which produces recombinant fusion hIL-2 (G3 · IL-2, 27 kDa) was kindly provided by Biochemical Process Engineering R.U. at KRIBB (Taejon, Korea). The N-terminus fusion tag G3 (12 kDa) comprises the sequences of consecutively-linked three glucagon molecules and enterokinase cleavage site (D4K) [5,8,9]. The cultivation media and bioreactor operation for high-level production of rhIL-2 in high-cell-density cultures are well described in a recent report by Saraswat *et al*. [9].

Recovery of Inclusion Bodies and Dissolution of G3 · IL-2 Fusion Protein

Following centrifuge of 10 mL culture broth (6,000 g, 15 min), the cell pellet was resuspended in 10 mL phosphate buffer (10 mM phosphate, 0.15 M NaCl, 10 mM EDTA, 1 mM DTT, pH 7.4). After centrifuge (6,000 g, 30 min), the precipitate was mixed with 10 mL urea (2 M) for 20 min, and the recovered protein aggregates by centrifuge was washed twice with 10 mL solution of 0.5%(v/v) Triton X-100 and 10 mM EDTA.

The recovered inclusion bodies were simply dissolved in 10 mL alkaline solution (0.05%(w/v) NaOH, pH 12). The alkaline pH of the resulting solution was shifted back to 8 by adding Tris buffer (0.5 M, pH 8), and subsequently the solution was subjected to enterokinase digestion to remove the fusion tag G3.

Enterokinase Cleavage and Purification of hIL-2

With the addition of urea (2 M) to the dissolved G3 · IL-2 solution above, the G3 · IL-2 at a concentration of 0.67 g/L was digested with enterokinase (EK) (Invitrogen, Groningen, The Netherlands) at a ratio of 0.1 U EK: 1 μg G3 · IL-2 for 16 h at 37°C.

To purify the recombinant hIL-2 from the digestion mixture containing cleaved G3, undigested G3 · IL-2, and enterokinase, the reversed-phase HPLC was used with C8 column (250 × 4.6 mm, Eka Nobel, Sweden). A binary buffer system of water and acetonitrile was used as mobile phase, and the samples were loaded onto the column and eluted with a linear gradient of 10-60% acetonitrile. The elution profile was monitored at 254 nm.

SDS-PAGE Analysis and Protein Quantification

After each step in the recovery and purification procedure, the proteins were electrophoretically separated via reducing (15 mM DTT) SDS-PAGE (12.5% polyacrylamide gel) and Coomassie-stained. The resulting protein bands were scanned using a densitometer (Bio-Rad, Hercules, CA, U.S.A.), and the percentage of G3 · IL-2 or hIL-2 in total proteins under analysis was carefully analyzed. The amount of total proteins under the same SDS-PAGE analysis was estimated using Lowry method, and then the recombinant protein (G3 · IL-2 or hIL-2) of interest was quantified on the basis of the result of percentage analysis above. The high purity of rhIL-2 in final purification product after reversed-phase HPLC was ascertained by silver staining of the same reducing SDS-PAGE gel.

RESULTS AND DISCUSSION

Recovery of G3 · IL-2 Fusion Protein

As recently reported by Park *et al*. [5,8,9], the N-terminus fusion tag G3 (12 kDa) comprises the sequences of consecutively-linked three glucagon molecules and enterokinse cleavage site (D4K). From the induced recombinant *E. coli* culture where the expression level of G3 · IL-2 is about 40% (lane 2 in Fig. 1), the inclusion bodies were isolated after cell disruption. Similar to the result of earlier study [5], the purity of G3 · IL-2 in the insoluble fraction was higher than 75% (lane 3 in Fig. 1), indicating that the inclusion bodies formed in the transformed *E. coli* cells are very homogeneous, thus simplifying the next stages of purification. As shown in Table 1 and Fig. 1, the insoluble aggregates were readily dissolved by simple pH shift to alkaline condition without using any denaturing agents. This makes a big difference compared with other cases of inclusion bodies where denaturation by reducing agents and subsequent renaturation are normally required. The recent study [5] has also shown that the G3 fusion partner played a significant role in the easy dissolution of inclusion bodies, that is, the G3 tag induced specific hydrophobic aggregation of G3 · IL-2 with avoiding non-specific intermolecular S-S linkages. After decreasing pH back to 8, the G3 · IL-2 fusion protein was recovered as a soluble recombinant protein with 100% yield (Table1).

The recombinant hIL-2 used in this study does not contain free cysteine residues because a free cysteine residue (Cys125) of native human interleukin-2 was switched by serine [8]. Since the soluble G3 · IL-2 fusion

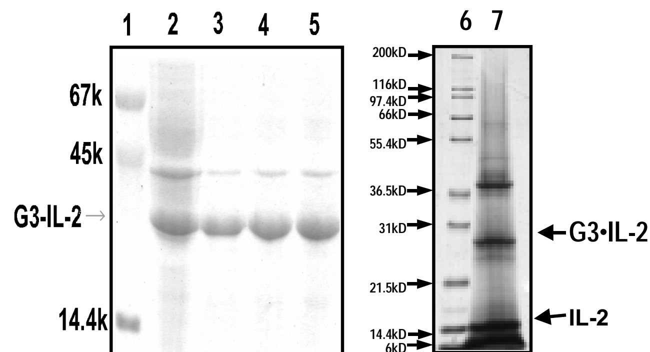

Fig. 1. Results of reducing SDS-PAGE analysis: lane 1, marker; lane 2, total cell lysates; lane 3, insoluble aggregates; lanes 4 and 5, solubilized aggregates by simple pH shift; lane 6, markers; lane 7, protein products of enterokinase cleavage reaction (% of G3 · IL-2 in the sample loaded to lanes 2, 3, 4, and 5: 40, 71, 79, and 77%, respectively).

Table 1. Result of recombinant hIL-2 purification

Purification Step	Component	Milligrams	Step yield (%)	Overall yield (%)
Insoluble aggregates	G3 · IL-2	231	100	100
Solubilization	G3 · IL-2	238	100	100
Enterokinase cleavage	hIL-2	71	30	30
Reversed-phase HPLC	hIL-2	67	94	28

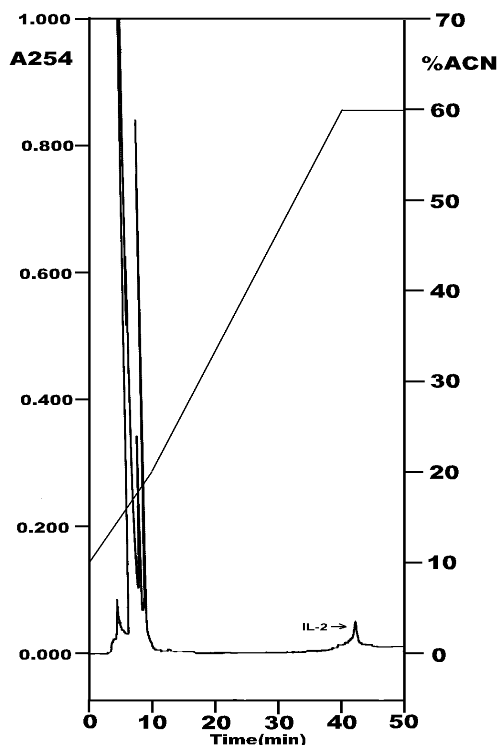

Fig. 2. Purification of recombinant hIL-2 by reversed-phase HPLC.

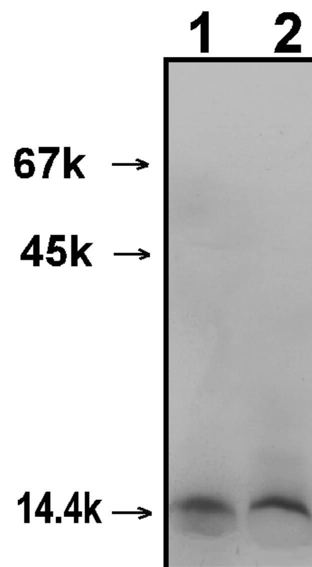

Fig. 3. Silver staining of final purified product separated by reducing SDS-PAGE (lanes 1 and 2 represent the silver staining results of the same purified sample).

protein at pH 8 was evidently monomerized by non-reducing SDS-PAGE [5], it is therefore presumed that the correct formation of intramolecular disulfide bridge (Cys58-Cys105) was highly probable while G3 · IL-2 fusion protein was dissolved by the simple pH shift (8→12→8).

Fusion Cleavage and Purification of rhIL-2

Since the high purity of G3 · IL-2 (> 80%) was also achieved in the dissolved aggregates (Fig. 1), the soluble G3 · IL-2 was cleaved by enterokinase without further purification of G3 · IL-2. The enterokinase (EK) cleaves the carboxy terminus of lysine at D4K site in a sequence specific manner. The result of EK cleavage reaction shows that the G3 · IL-2 was not completely processed though urea (2 M) was added to the reaction mixture (Fig. 1). (Two apparent bands appearing above G3 · IL-2 and below hIL-2 in lane 7 of Fig. 1 are Coomassie-stained EK and cleaved G3 tag, respectively.) Since the weight fraction of hIL-2 in G3 · IL-2 is 56% (*i.e.* 132 mg hIL-2 in 238 mg G3 · IL-2), 61 mg hIL-2 was not recovered due to incomplete cleavage process (Table 1). This might be because the D4K sequence was sterically hindered within the G3 · IL-2 molecule and hence EK access to the cleavage site was not efficient enough.

hIL-2 was finally purified by reversed-phase HPLC (RP-HPLC) to remove the cleaved G3 fusion partner and residual uncleaved G3 · IL-2. In Figs. 2 and 3, it is evident that hIL-2 was eluted in a single peak at 60% acetonitrile at 42 min of residence time and that the 42 min peak contained hIL-2 as a single protein. The eluted proteins in three large peaks at < 10 min of residence time are presumed to be the cleaved G3 fusion partner, residual uncleaved G3 · IL-2, and enterokinase (Fig. 2). It seems likely that the apparently higher hydrophobicity of hIL-2 provided the cleaved hIL-2 with higher binding affinity to the reversed stationary phase, thus making the separation easily accomplished. The recovery yield of hIL-2 in RP-HPLC step was 94%, and the overall recovery yield was about 28% in the whole downstream process (Table 1). Since RP-HPLC has been usefully employed to separate correctly-folded form from various mis-folded isomers [10,11], the single peak of purified hIL-2 might represent a single secondary structure of hIL-2. It is probably a native structure because a point mutation in rhIL-2 (Cys125Ser) [5,8] removed the chance for intramolecular disulfide mis-linkage as well as because it was previously confirmed that intermolecular S-S bridges between rhIL-2 molecules were effectively circumvented by G3 fusion partner [5]. The efficacy of simple downstream process demonstrated in this report seems to provide a firm basis for further process development, including process improvement (*e.g.* EK digestion process) and/or scale-up.

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