High Yield Refolding and Purification Process for Recombinant Human Interleukin-6 Expressed in *Escherichia coli*

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Abstract: Recombinant human interleukin-6 (hIL-6), a pleiotropic cytokine containing two intramolecular disulfide bonds, was expressed in *Escherichia coli* as an insoluble inclusion body, before being refolded and purified in high yield providing sufficient qualities for clinical use. Quantitative reconstitution of the native disulfide bonds of hIL-6 from the fully denatured *E. coli* extracts could be performed by glutathione-assisted oxidation in a completely denaturating condition (6*M* guanidinium chloride) at protein concentrations higher than 1 mg/mL, preventing aggregation of reduced hIL-6. Oxidation in 6*M* guanidinium chloride (GdnHCl) required remarkably low concentrations of glutathione (reduced form, 0.01 m*M*; oxidized form, 0.002 m*M*) to be added to the solubilized hIL-6 before the incubation at pH 8.5, and 22°C for 16 h. After completion of refolding by rapid transfer of oxidized hIL-6 into acetate buffer by gel filtration chromatography, residual contaminants including endotoxin and *E. coli* proteins were efficiently removed by successive steps of chromatography. The amount of dimeric hIL-6s, thought to be purification artifacts, was decreased by optimizing the salt concentrations of the loading materials in the ion-exchange chromatography, and gradually removing organic solvents from the collected fractions of the preparative reverse-phase HPLC. These refolding and purification processes, which give an overall yield as high as 17%, seem to be appropriate for the commercial scale production of hIL-6 for therapeutic use. *Biotechnol Bioeng* **62**: 301–310, 1999.

Keywords: interleukin-6; protein refolding; inclusion body; aggregation; purification

INTRODUCTION

Interleukin-6 (IL-6) is a cytokine, which is involved in diverse biological activities such as proliferation, differentiation, and maturation events in host target cells (Hirano et al., 1985; Simpson et al., 1997). Clinical use of hIL-6 in cancer therapy has been of great interest and functional agonists and antagonists for therapeutic use in IL-6-associated diseases are now in development (Ciapponi et al., 1997; Sporeno et al., 1996). Some groups have already reported hIL-6 production systems in recombinant *E. coli*, which can produce large amounts of insoluble inclusion bodies (Brakenhoff et al., 1987; Rock et al., 1992; Tonouchi et al., 1988; Yasukawa et al., 1990). However, problems concerning the low yield in the refolding process owing to the aggregate-prone property of hIL-6 (Simpson et al., 1997; Ward et al., 1995) remain to be solved. This property also makes it rather difficult to design a high-yield purification process applicable to the commercial scale production of hIL-6, which can efficiently remove residual contaminants from host cells and degradation or modification impurities of hIL-6 from the purified product.

Production of heterologous proteins as inclusion bodies in engineered *E. coli* is a common technique used to obtain valuable non-glycosylated proteins in large amounts. A recent study on refolding of denatured lysozyme (Hevehan et al., 1997) revealed that low concentrations of denaturants (guanidinium chloride or urea) can suppress the aggregation of refolding intermediates, even at high-protein concentrations and thus, increase the yields of refolded lysozyme recovered. This study emphasized the availability of non-denaturating concentrations of guanidinium chloride (GdnHCl) for designing industrial-scale refolding processes. However, denaturant-induced equilibrium unfolding studies on murine IL-6 (Ward et al., 1995; Zhang et al., 1997) have demonstrated that natively disulfide-bonded IL-6 forms partially unfolded conformations, which have a high tendency to self-associate at low denaturant concentrations. These studies also showed that fully disulfide-reduced IL-6 exhibits a higher tendency to self-associate than that of natively disulfide-bonded IL-6. Taken together, these results suggest that the refolding of hIL-6 by incubating disulfide-reduced denatured forms in partially denaturating conditions, as in the case of refolding lysozyme (Hevehan et al., 1997), will in principle, fall into the low efficacy category.

In this investigation, we have established a new refolding process for hIL-6, which consists of two steps. In the first

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step, high protein concentrations of natively disulfide-bonded hIL-6 were obtained from solubilized inclusion bodies under fully denaturating buffer conditions. In the second step, the native conformation of hIL-6 was obtained by rapidly removing the denaturant from the oxidized hIL-6 solution without any dilution. Aggregation during the refolding process was effectively suppressed. Refolded hIL-6 was purified successively by ion-exchange, reverse-phase, and gel filtration chromatographies with aggregation controlled by unique techniques to give a good recovery and provide enough high quality material to be evaluated in vivo free of responses to residual endotoxin and immunogenic impurities.

MATERIALS AND METHODS

Materials

Trifluoroacetic acid (spectroscopic grade), acetic acid, tris-hydroximethylaminomethane (Tris), reduced and oxidized glutathione (GSH and GSSG), ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, sodium hydroxide, and *Achromobacter* protease I (lysylendopeptidase; EC 3.4.21.50, 4.5 AU/mg) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Guanidinium chloride (GdnHCl) and HPLC-grade solvents were purchased from Nakarai tesque (Kyoto, Japan). All buffers were prepared with deionized water purified by the Milli-Q SP/UF system (Millipore, Tokyo, Japan). All other chemicals were reagent grade.

Recovery of Inclusion Bodies

Escherichia coli strain HB101 with an expression plasmid containing the trp promoter followed by the mature hIL-6 sequence was used as the expression host (Yasueda et al., 1990). The host cell was grown overnight in a 30 L fermentor and the cultured-broth (20 L) obtained was directly introduced into a high-pressure homogenizer (Type SHL-5; Alfa-Laval) for cell disruption. The cell homogenate was applied to a continuous centrifuge (type NO-U-5-HR; Kansai Centrifugal Separator Manufacturing, Osaka, Japan) at 7600*g*, and 1 L/min to recover the hIL-6 inclusion bodies. The collected inclusion bodies were suspended in 500 mL of washing buffer (20 m*M* Tris-HCl, 30 m*M* NaCl, pH 7.5), and then centrifuged at 8000*g* for 15 min. The pellet of inclusion bodies obtained was resuspended in 500 mL of 10 m*M* EDTA, pH 5.5, and stored at −80°C until use.

Solubilization of Inclusion Bodies and Air-Oxidation

The pellet of inclusion bodies was solubilized in 6*M* GdnHCl (13.8 mg/mL), adjusted to pH 5.5 with HCl and allowed to stand for 2 h at room temperature. Solubilized hIL-6 (1.5 mL) was rapidly diluted 10-fold to 1.38 mg/mL with 10 m*M* Tris-HCl, pH 8.5, containing varying concentrations of GdnHCl (0.6 ~ 6*M*), and then incubated for 16 h at 22°C with gentle stirring. After incubation, each solution was centrifuged and 12 μL of the supernatant part was analyzed by reverse-phase HPLC for the yield of soluble oxidized hIL-6.

Glutathione-Assisted Oxidation

The solubilized hIL-6 from above (13.8 mg/mL, pH 5.5) was rapidly diluted 10-fold to 1.38 mg/mL in 6*M* GdnHCl. Varying concentrations of reduced and oxidized glutathione were then added to 15 mL of diluted hIL-6, keeping the reduced/oxidized glutathione ratio at 5/1. Each supplemented solution was adjusted to pH 8.5 with 10 m*M* Tris-HCl and incubated for 16 h at 22°C with gentle stirring. Reverse-phase HPLC analysis for the yield of oxidized hIL-6 was performed in the same way as air-oxidation.

Large-Scale Refolding by Glutathione-Assisted Oxidation and Desalting Chromatography

The pellet of inclusion bodies containing 11 g of reduced hIL-6 was solubilized in 12 L of 6*M* GdnHCl, pH 5.5, and allowed to stand for 2 h at 22°C. Reduced and oxidized glutathione were added into the solubilized hIL-6 at the final concentrations of 0.01 m*M* and 0.002 m*M*, respectively. The supplemented solution was adjusted to pH 8.5 with 10 m*M* Tris-HCl and incubated for 16 h at 22°C. After the oxidation yield was confirmed by reverse-phase HPLC analysis, the solution was directly applied to a gel filtration column (Sephadex G-25 M, 44 cm internal diameter × 30 cm; Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 10 m*M* sodium acetate, pH 5.0, and the protein was eluted with the same buffer at a flow-rate of 750 mL/min. Eluted materials were detected by UV absorbance at 280 nm in all chromatography steps.

Purification of Oxidized hIL-6

Refolded hIL-6 (10 g) in 15 L of 10 m*M* sodium acetate, pH 5.0 was adjusted to 250 m*M* sodium acetate, pH 5.0 and applied to a cation-exchange column (CM-Sepharose FF, 26 cm internal diameter × 10 cm; Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 10 m*M* sodium acetate, pH 5.0. After the column was washed with 1 column volume of the same buffer, hIL-6 was eluted with a linear gradient of sodium acetate from 10 m*M* to 500 m*M* and a pH gradient from 5.0 to 5.5 (final elution buffer) over 10 column volumes at a flow-rate of 0.5 L/min. Fractions of 500 mL were collected and assayed by analytical cation-exchange HPLC by injecting 100 μL of each fraction.

Pooled hIL-6 fractions (1.2 g) in the cation-exchange chromatography were adjusted to pH 4.0 with formic acid and applied to a preparative reverse-phase HPLC column (Vydac 214TPB10, 5 cm internal diameter × 25 cm, The Separations Group, Hesperia, CA, USA) equilibrated with 0.1*M* sodium formate, pH 4.0. After the column was washed

with 1 column volume of the same buffer, hIL-6 was eluted with a linear gradient of 0–60% acetonitrile in the elution buffer (0.1*M* sodium formate, pH 4.0) over six column volumes at a flow-rate of 50 mL/min. Five fractions were collected, and assayed by the analytical reverse-phase HPLC by injecting 50 μg of hIL-6 from each fraction. Four runs of this chromatography were performed. Pooled hIL-6 fractions (800 mL) were applied to a gel filtration column (Sephadex G-25 M, 18 cm internal diameter × 18 cm, Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 20 m*M* acetic acid and 10% acetonitrile, and proteins were eluted with the same solvent at a flow rate of 120 mL/min. The hIL-6 (1760 mL) collected was kept at 22°C for 1 h and then applied to another Sephadex G-25 M column (30 cm internal diameter × 18 cm) equilibrated with 5 m*M* sodium acetate, pH 4.5, and run with the same buffer at a flow-rate of 350 mL/min.

Prior to the final purification step, hIL-6 was concentrated to greater than 8 mg/mL by cation-exchange chromatography. One gram of solvent-free hIL-6 (5 m*M* sodium acetate, pH 4.5) was adjusted to 175 m*M* sodium acetate, pH 5.0 and applied to a CM-Sepharose FF column (5 cm internal diameter × 2.5 cm) equilibrated with the 10 m*M* sodium acetate, pH 5.0. Adsorbed hIL-6 was eluted stepwise with 10 m*M* sodium citrate, pH 6.5 containing 50 m*M* NaCl at a flow rate of 30 mL/min. Three runs of this chromatography were performed. Finally, 50 mL of concentrated hIL-6 was applied to a preparative gel filtration HPLC column (Superdex-75 HR 60/600, 6 cm internal diameter × 60 cm, Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 10 m*M* sodium citrate, pH 6.0 and the proteins were eluted with the same buffer at a flow-rate of 24 mL/min. Monomeric hIL-6 fractions were pooled, filter sterilized (Millex GVHD-25020, Millipore, Tokyo, Japan) and stored at 5°C for characterization.

Analytical Methods

Analytical reverse-phase HPLC was performed using a Vydac C4 column (214TP54, 4.6 mm internal diameter × 250 mm, The Separations Group, Hesperia, CA, USA). The column was equilibrated with 32% acetonitrile and 0.1% trifluoroacetic acid/water, and the proteins were eluted with a linear gradient of up to 60% acentonitrile and 0.1% trifluoroacetic acid/water over 28 min at a flow rate of 1 mL/min at ambient temperature. The eluted materials were detected by UV absorbance at 280 nm (all analytical HPLC of hIL-6).

Analytical cation-exchange HPLC was performed using a SP-NPR column (35 mm internal diameter × 30 mm, Tosoh Corporation, Tokyo, Japan) equilibrated with 10 m*M* sodium acetate, pH 5.0, and the proteins were eluted with a linear gradient of up to 0.5*M* sodium acetate, pH 5.5 over 5 min at a flow rate of 1 mL/min.

Analytical gel filtration was performed using a Superdex 75 HR 10/30 (10 mm internal diameter × 300 mm, Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 10 m*M* sodium citrate, 8.7 m*M* sodium phosphate, pH 7.0 and the proteins were eluted with the same buffer at a flow rate of 0.8 mL/min.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Phastsystem and 20% homogeneous separating gels (Amersham Pharmacia Biotech, Tokyo, Japan) under non-reducing conditions according to the manufacturer's instruction. For GdnHCl-containing samples, GdnHCl was removed using a small prepacked desalting column (PD-10, Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 10 m*M* sodium acetate, pH 5.0 according to the manufacturer's instruction. Gels were visualized by silver staining (diamine method). Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and alpha-lactalbumin (14.4 kDa) were used as molecular weight markers.

Peptide mapping was performed by enzymatic digestion (S/E = 100/1 in molar ratio) and analytical reverse-phase HPLC. Purified hIL-6 (1 nanomole/21 μg in 10 m*M* sodium citrate, pH 6.0) was diluted into 0.1 mL of the digestion buffer (10 m*M* sodium phosphate, pH 7.0). Ten picomoles *Achromobacter* protease I enzyme (Wako Pure Chemical Industries, Osaka, Japan) dissolved in 0.01 mL of the digestion buffer was added to the hIL-6 solution, and the mixture was incubated for 12 h at ambient temperature. The whole solution was then injected directly into a Vydac C4 (214TP54, 4.6 mm internal diameter × 250 mm, The Separations Group, Hesperia, CA, USA) equilibrated with 0.1% trifluoroacetic acid/water, and the peptides were eluted with a linear gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid/water over 75 min at a flow rate of 1 mL/min. The eluted peptide fragments were detected by UV absorbance at 215 nm. Amino acid sequences of detected peptides were assigned by FABMS (JMS-HX110/HX110, JEOL, Tokyo, Japan) and automatic Edman degradation (model 470A, Applied Biosystems, Tokyo, Japan) of the collected peptide peaks.

The concentration of purified hIL-6 was determined by UV spectroscopy at 280 nm utilizing an extinction coefficient for hIL-6 of 0.47 mg^{-1} cm^2 at 280 nm, which was obtained experimentally using an hIL-6 protein standard measured by quantitative amino acid analysis. The concentrations of crude hIL-6 were determined by analytical reverse-phase HPLC (above) using purified hIL-6 as a protein standard.

Biological Assay

The hIL-6 titer was determined by an IgM-inducing assay using SKW6-CL4 cells, as previously described (Hirano et al., 1985).

Endotoxin and *Escherichia coli* Protein Contents

The amount of endotoxin in the hIL-6 samples was determined using the *Lymulus* amoebocyte lysate assay (Toxicolor system, Seikagaku Kogyo Inc., Tokyo, Japan) according to the manufacturer's instruction.

The *E. coli* protein (ECP) content of hIL-6 samples was determined by ELISA using polyclonal anti-ECP antibodies. The recombinant host cell was constructed by transforming *E. coli* with a vector identical to the production system but lacking the gene insert coding the product. They were then cultured in a fermentor (3 L), disrupted, and centrifuged in the same manner as the production. The pellet obtained was solubilized, purified by desalting and cation-exchange chromatographies using the same conditions for hIL-6 except for the scales to yield the semi-purified ECP mixtures. Rabbit and mouse polyclonal anti-ECP antibodies were obtained using these ECP mixtures and a highly sensitive sandwich ELISA system for ECP was constructed using anti-ECP antibodies, biotin-labeled alkaliphosphatase kit (AK 5001, Vectastain Vector Lab, Inc., Burlingame, CA, USA) and BCIP/NBT phosphatase substrate system (Kappel, Funakoshi, Tokyo, Japan). The detection limit of this ELISA for ECP was 1 ng/mL.

RESULTS

Production and Refolding of hIL-6

Recombinant hIL-6 was produced as insoluble inclusion bodies in the engineered *E. coli* strain HB101 containing an expression plasmid for mature hIL-6 sequence, as previously reported (Yasueda et al., 1990). After completion of the fermentation, the cultured broth was directly introduced into a high pressure homogenizer, and insoluble inclusion bodies recovered by continuous centrifugation. The pellet collected was resuspended in Tris-HCl buffer, centrifuged again, and the washed inclusion bodies finally suspended in 10 m*M* EDTA, pH 6.0 before being stored at −80°C until use.

Frozen inclusion bodies were thawed, and then solubilized in 6*M* GdnHCl, pH 5.5 for 2 h at ambient temperature without any disulfide reducing agents. Formation of the disulfide bonds at a concentration of 1.38 mg/mL denatured hIL-6 under several GdnHCl concentrations was examined in 15 mL volumes by air-oxidation. Each solution was centrifuged and obtained supernatant part was assayed by analytical reverse phase HPLC (Figs. 1,2). As shown in Figure 1B, most of extracted hIL-6 had reduced disulfide bonds before oxidation. It was found that the yields of oxidized hIL-6 after a 16 h incubation at 22°C were remarkably dependent on the GdnHCl concentrations used (Fig. 2). As the GdnHCl concentration increased from 0.6*M*, the yield of oxidized hIL-6 first decreased to give the minimum yield at 3*M* (12%), and then increased to give the maximum yield at 6*M* (87%). Solutions with GdnHCl below 4*M* became turbid after incubation for 16 h due to the presence of aggregation. The oxidation yield with 1*M* GdnHCl (62%) was distinctly lower than the maximum yield (87% in 6*M*), but the purity of the oxidized material as judged by reverse phase HPLC (Fig. 1F) was much better than that of the maximum yield (Fig. 2). Partially denaturating GdnHCl concentrations (2 ~ 4*M*), which are commonly used in oxidative refolding processes of many kinds of denatured proteins (Fischer et al., 1993), were not effective for refolding of reduced hIL-6.

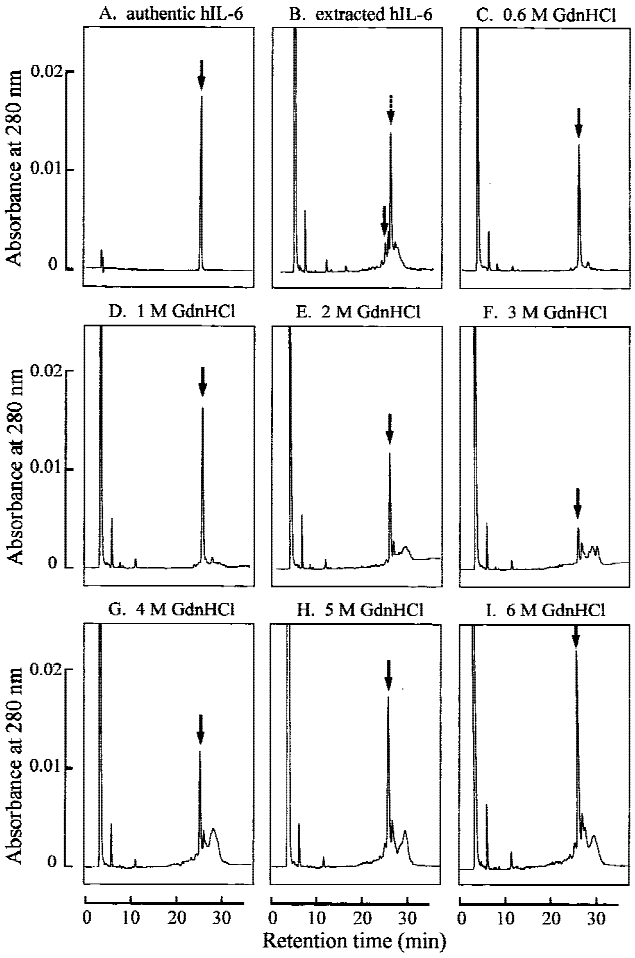

Figure 1. Air oxidation of denatured hIL-6. HIL-6 extracted in 6*M* GdnHCl (13.8 mg/mL) was diluted to 1.38 mg/mL in 10 m*M* Tris-HCl, pH 8.5 containing each concentration of GdnHCl. The solutions (15 mL) were incubated at room temperature for 16 h, and 12 μL of each was assayed by reverse-phase HPLC. Arrows and a dotted arrow show oxidized and reduced hIL-6, respectively.

Applications of thiol/disulfide redox buffer systems (glutathione system) in 6*M* GdnHCl were examined in the same 15 mL volumes (Fig. 3). Combinations of reduced and oxidized glutathione (GSH and GSSG) were added to the oxidation solutions, keeping the molar ratio of GSH/GSSG at 5/1, and the mixtures incubated for 16 h at 22°C, similar to the air-oxidation. The highest yield was obtained at 0.01 m*M* GSH/0.002 m*M* GSSG (105%, Fig. 3B), and this material showed a molecular weight equivalent to that of purified hIL-6 on SDS-PAGE (Fig. 4, lane 3). The reason why the yield was above 100% may be due to unresolved impurities in the oxidized hIL-6 peak. The combination of 1 m*M* GSH/0.2 m*M* GSSG, which is one of the popular conditions used in oxidative refolding of denatured proteins (Fischer et al., 1993), increased the yield a little (95%, Fig. 3C) as compared with that of air-oxidation (91%, Fig. 3A). However, the oxidized hIL-6 obtained under this condition

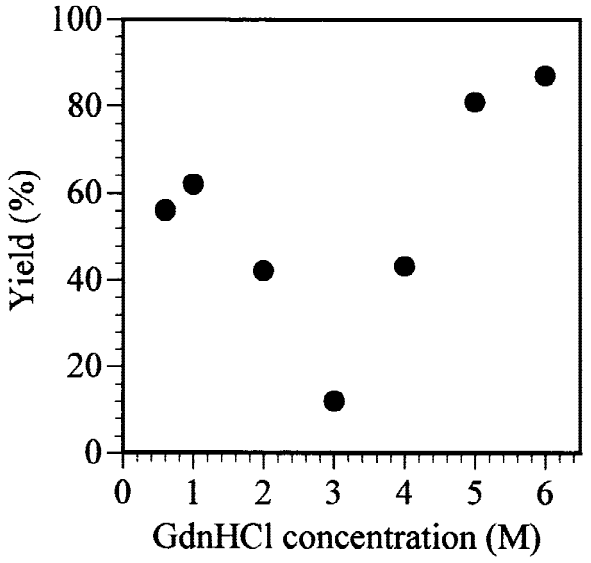

Figure 2. Yield of soluble oxidized hIL-6 in varying concentrations of GdnHCl (Fig. 1).

showed a contaminating band with a molecular weight a little higher than that of natively disulfide-bonded hIL-6 on SDS-PAGE (Fig. 4, lane 4). The amount of this contaminant band increased (Fig. 4, lane 5) and the oxidation yield decreased to 81% (Fig. 3D) with the combination of 5 m*M* GSH/1 m*M* GSSG. Peptide-mapping and Electrospray Ionization mass spectrometry analyses of this contaminant band revealed that it was probably due to an oxidation artifact which had one mixed disulfide bond with glutathione at Cys(44) or Cys(50) (data not shown). Consequently, the optimal oxidation condition was obtained using fully denaturating buffer containing 6*M* GdnHCl, 0.01 m*M* GSH, and 0.002 m*M* GSSG, at pH 8.5.

Large scale oxidation (12 L) was carried out almost quantitatively and the oxidized hIL-6 (0.9 mg/mL) was directly applied to a Sephadex G-25 desalting column equilibrated with 10 m*M* sodium acetate buffer, pH 5.0 (Fig. 5). The fraction (15 L) indicated by the arrow was collected and analyzed by reverse-phase HPLC (inserted chromatogram). Almost 95% of hIL-6 loaded was recovered (0.68 mg/mL) as one major peak with trace impurities as shown by analytical reverse-phase HPLC, and it had comparable bioactivity to purified hIL-6 in the IgM inducing assay (data not shown). Therefore, it was concluded that refolding of hIL-6 from inclusion bodies had been complete.

Purification and Characterization of Refolded hIL-6

Cation-Exchange Chromatography

Refolded hIL-6 (10 g in 10 m*M* sodium acetate buffer, pH 5.0) was adjusted to 250 m*M* sodium acetate, pH 5.0 and applied to the CM-Sepharose FF column equilibrated with 10 m*M* sodium acetate buffer, pH 5.0. Adsorbed hIL-6 was

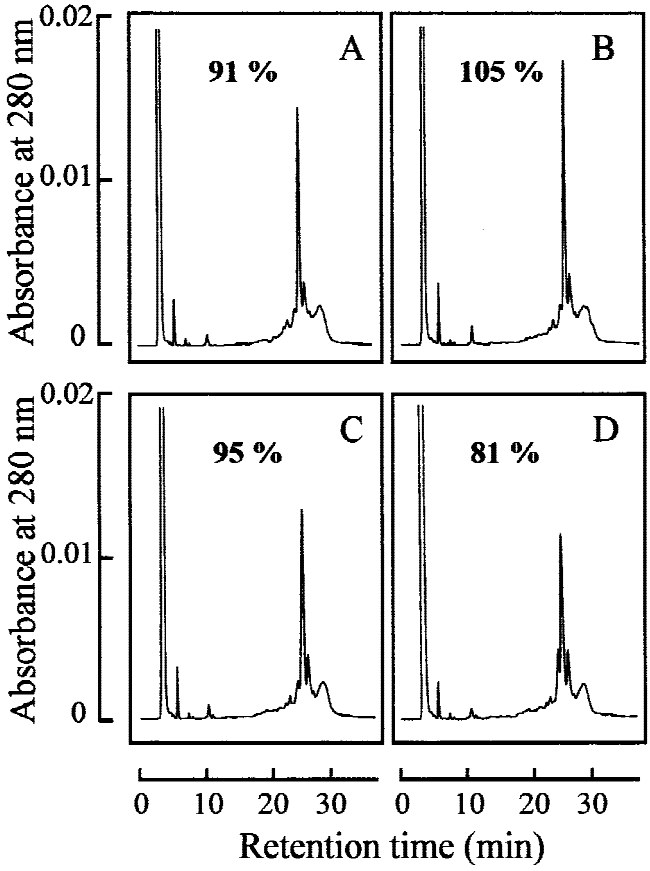

Figure 3. Glutathione-assisted oxidation of reduced hIL-6. Oxidation conditions were similar to those of air-oxidation (Fig. 1) except additions of reduced and oxidized glutathione (GSH and GSSG). Solutions were incubated for 16 h at 22°C. The yield of oxidized hIL-6 was assayed by analytical reverse-phase HPLC, shown inserted in each chromatogram. GSH/GSSG: A, air-oxidation; B, 0.01 m*M*/0.002 m*M*; C, 1.0 m*M*/0.2 m*M*; D, 5 m*M*/1 m*M*.

eluted with a linear gradient of up to 0.5 *M* sodium acetate, pH 5.5 (Fig. 6). Forty-eight percent of the loaded hIL-6 was recovered (4.8 g), and it showed a homogeneous peak in the analytical ion-exchange HPLC (inserted A in Fig. 6) with a more than 1000-fold decrease in the residual endotoxin content (Table I). When refolded hIL-6 (dissolved in 10 m*M* acetate, pH 5.0) was directly added to the CM-Sepharose FF column and eluted under the same conditions, dimeric hIL-6 appeared as a purification artifact and the yield of hIL-6 decreased to less than 35% (inserted B in Fig. 6).

Reverse-Phase HPLC

The hIL-6 collected (1200 mg) from ion-exchange chromatography was adjusted to pH 4.0 with formic acid, and loaded on a 50 mm internal diameter reverse-phase column equilibrated with 0.1*M* sodium formate, pH 4.0. Adsorbed hIL-6 was eluted with a linear gradient of 0–60% acetonitrile in 0.1*M* sodium formate, pH 4.0 (Fig. 7), and then assayed by analytical reverse-phase HPLC (inserted in Fig. 7). Fraction 3, which showed an almost homogeneous hIL-6

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