0273-2289/1559-0291 (Online)/06/133/0077/$30.00

Expression and Purification of a Mutant of Human Interleukin-2 in *Pichia pastoris*

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Received March 10, 2005; Revised September 29, 2005; Accepted October 20, 2005

Abstract

Interleukin (IL)-2 is a pharmacologically important cytokine secreted by T-lymphocytes. Recombinant IL-2 (rIL-2) has been modified and produced in many systems. Mass production of rIL-2 is the prerequisite for its wide application. Using a site-directed mutagenesis strategy, we first generated a gene coding for a new type of mutant of human IL-2 (MhIL-2), in which we replaced the cysteine-125 in human IL-2 with alanine, the leucine-18 with methionine, and the leucine-19 with serine. Then we investigated the possibility of its production of MhIL-2 in a *Pichia pastoris* system. High-level secreted expression of MhIL-2 was achieved by methanol induction. When purified with ultrafiltration, cation-exchange chromatography, and Sephadex G100 gel filtration, about 100 mg of MhIL-2 with high purity was obtained from 1 L of ferment supernatant. Biologic activity assay revealed that the purified recombinant protein displayed increased activity on proliferation of IL-2-dependent CTLL-2 cells. These results suggest that MhIL-2 is an improved IL-2 mutant that might hold great promise for clinical use, and that *P. pastoris* is an excellent system for the mass production of biologically active hIL-2.

Index Entries: Human interleukin-2; site-directed mutagenesis; *Pichia pastoris*; purification; increased activity.

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Introduction

Interleukin (IL)-2, initially known as T-cell growth factor, is a multifunctional immunoregulatory lymphokine *(1)*, and human IL-2 (hIL-2) can stimulate the growth, differentiation, and activation of T-, B-, and natural killer cells *(2,3)*. In recent years, the safe use of IL-2 for immunotherapy has been recognized, and clinical trials have denoted that IL-2-based therapy is promising for cancer and chronic infectious diseases, especially for human immunodeficiency virus infection and hepatitis C *(4–7)*. IL-2 is also used extensively as a tissue culture reagent owing to its requirement for the survival of T-lymphocytes in culture.

hIL-2 protein contains three cysteine residues, two of which form a disulfide bond that is required for biologic activity *(8)*. The free Cys125 is not involved in the recognition of IL-2 receptor, and it causes the formation of inclusion body *(9)*. It has been reported that mutants of recombinant hIL-2 (rhIL-2) in which the Cys125 was substituted for serine *(10)* or alanine *(11)* avoided the undesirable formation of protein aggregates. Other researchers reported that, compared to wild-type (WT) IL-2, a double mutant of IL-2 (L18M/L19S) displayed similar binding affinity to the IL-2 receptor, while exhibiting increased bioactivity.

During the past 15 yr, the methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins *(12)*. The increasing popularity of this particular expression system can be attributed to several factors, the most important of which are (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris*, and (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly.

Here we describe the expression of a triple mutant of rhIL-2 (L18M/L19S/C125A) in a *P. pastoris* system. A homogeneous and bioactive IL-2 variant was successfully expressed and then purified by the combination of ultrafiltration, cation-exchange chromatography, and Sephadex G100 gel filtration.

Materials and Methods

Molecular Reagents and Separating Medium

The yeast expression vector pPIC9K and Western blot kit were purchased from Invitrogen (Houston, TX). Plasmid pAO815-hIL-2 containing the native hIL-2 cDNA was kindly provided by Dr. Ying-He Hu. Primers were synthesized by Biocolor (Shanghai, China). Restriction enzymes, T4 DNA ligase, and high-fidelity thermostable polymerase Pyrobest were from Takara. The ultrafiltration membrane was from Millipore (Bedford, MA). The cation-exchanger cartridge was from Bio-Rad (Hercules, CA). Sephadex G-100 was from Pharmacia. Standard IL-2 was from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Construction of Expression Plasmid pPIC9K-MhIL-2

The sequence encoding the N-terminal 22 amino acids of mature hIL-2 was amplified from plasmid pAO815-hIL-2 using the following primers: upstream primer 1, 5′-GCACGTTACGTAAGCACCTACTTCAAGTTCTAC-3′; downstream primer 2, 5′-CTGTAAATCAGACATTAAATG-3′. The sequence encoding the C-terminal 118 amino acids of mature hIL-2 was amplified from plasmid pAO815-hIL-2 using the following primers: upstream primer 3, 5′-GCATTTAATGTCTGATTTACAG-3′; downstream primer 4,5′-GCGAATTCTCAAGTCAGTGTTGAGATGATGCTTTGCGC AAAGG-3′. The downstream primer 4 contains a site-directed mutagenesis sequence encoding Cys125→Ala125. Primers 2 and 3 were designed to be overlapped and paired, and they contain the site-specific mutagenic sequence encoding Leu18, Leu19→Met18, and Ser19. All the mutations are underlined. Thus, the 5′-end fragment of mutant IL-2 sequence, amplified with primers 1 and 2, could be integrated and fused to the 3′-end fragment of mutant IL-2 sequence, amplified with primers 3 and 4, to obtain intact MhIL-2 by polymerase chain reaction (PCR). PCR products of 5′- and 3′-end fragments of mutant IL-2 sequence were mixed and PCR cycles were performed as follows: denaturation at 94°C for 1 min, annealing and extension at 68°C for 5 min for 10 cycles. The resulting product, 5′- Met18-Ser19…. Ala125-3′ (MhIL-2), was used as PCR template, and primers 1 and 4 were added to the mixture to amplify the MhIL-2 gene. PCR cycles were as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles.

As a result of three rounds of PCR, a 400-bp fragment encoding rhIL-2 gene with three codon mutations—cysteine-125 to alanine, leucine-18 to methionine, and leucine-19 to serine—was obtained. It was digested with *Sna*BI and *Eco*RI and cloned into the same sites of pPIC9K to get pPIC9K-MhIL-2**.** The fragment was validated by restriction enzyme digestion with *Sna*BI and *Eco*RI and confirmed by DNA sequencing.

Yeast Transformation and Positive Clone Screening

To generate recombinants, GS115 cells were transformed with *Sal*I-linearized pPIC9K-MhIL-2 according to the Invitrogen manual. Cells were spread on RD plates and incubated at 30°C for 72 h until colonies formed. Fifty transformants were replica plated on minimal methanol (MM) and minimal dextrose (MD) medium for Mut^+ and Mut^s screening. The selected integrants were confirmed by PCR. An easy screening method was improved in which the culture medium was used as PCR template directly without extracting the total genomic DNAs of integrants. Two microliters of culture was added to a 20-μL PCR reaction system, and PCR were performed as recommended by Invitrogen's instructions.

To obtain multicopy integrants, 30 PCR-positive colonies were further screened for replica growth on increasing concentrations (0.25, 0.5, 0.75, 1.00, 1.5, 1.75, 2.00, 3.00, 4.00 mg/mL) of G418. Only colonies that could

grow on a concentration of 3.00–4.00 mg/mL of G418 were selected for expression analysis.

Expression and Purification of MhIL-2

Six colonies harboring the MhIL-2 cDNA and one control colony transformed with plasmid pPIC9K were inoculated into 25 mL of buffered glycerol-complex medium (BMGY), respectively, and were grown at 30°C until the culture reached an A_{600} of 2.0–6.0 (approx 24 h). Cells were harvested by centrifugation, resuspended in 200 mL of buffered methanol-complex medium (BMMY) to an A_{600} of 1.0, and induced to express at 30°C with the addition of methanol to a final concentration of 0.5% every 24 h to maintain induction. After 3 d, the culture was centrifuged at 3000*g* for 5 min to collect the supernatant. Twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining were employed to analyze the expression of different colonies. The colony with the highest expression level was chosen to produce rIL-2 on a large scale.

For the purification of rIL-2, the total protein in the supernatant was first concentrated by ultrafiltration using a 10-kDa molecular mass cutoff membrane. The concentrated protein was dialyzed with buffer A (50 m*M* HAc/NaAc, pH 3.5) for more than 24 h, then loaded onto a cation-exchange column (Bio-Rad Pac S, 1.5 × 30 cm) equilibrated with buffer A. MhIL-2 was eluted from the column with 50 m*M* HAc/NaAc, 0.55 *M* NaCl, pH 3.5; and the eluent was collected and concentrated. The condensed sample was further purified on Sephadex G100 gel filtration chromatography using 20 m*M* Tris-HCl, 20 m*M* NaCl, pH 7.4, as the elution buffer. Protein samples were analyzed by 12% SDS-PAGE. Purified IL-2 protein was subjected to electrophoresis under denaturing conditions. The Coomassie-stained SDS-PAGE gel was scanned with a UVP white/ultraviolet transilluminator. The yield of purified rhIL-2 was quantified using a MicroBCA kit and densitometric analysis of SDS-PAGE (Grab-it 2.5 and Gelwork software).

Western Blot Analyses

The identity of MhIL-2 was confirmed by Western blot. SDS-PAGE was performed by mixing the sample with sample buffer (0.5 *M* Tris-HCl, pH 6.8, 10% glycerol, 5% SDS, 5% β-mercaptoethanol, and 0.25% bromophenol blue), incubating at 100°C for 3 min, centrifuging for 1 min, and loading onto a 12% slab gel. After electrophoresis, the gel was transferred onto a nitrocellulose membrane with a Bio-Rad Mini-Trans Blot Cell in Bjerrum and Schafer-Nielsen transfer buffer (48 m*M* Tris, 39 m*M* glycine, and 20% methanol, pH 9.2) for 40 min at 20 V. The nitrocellulose membrane was probed with a 1:2000 dilution of polyclonal goat antihuman hIL-2 antibody and detected with a 1:1000 dilution of goat antirabbit IgG conjugated to alkaline phosphatase and 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium color development reagent.

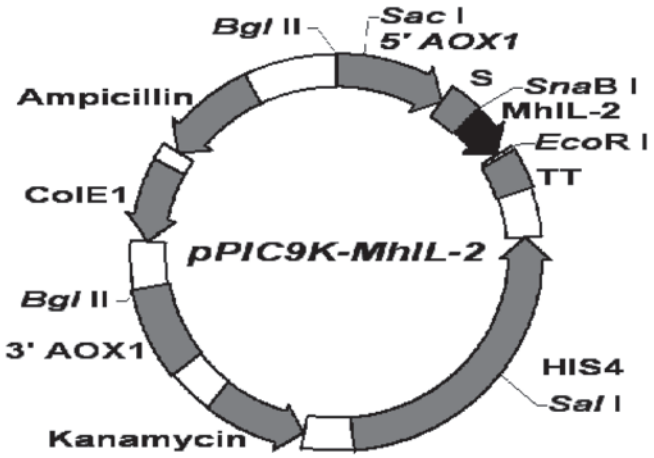

Fig. 1. Diagram of recombinant expression plasmid pPIC9K-MhIL-2.

Activity Analysis of MhIL-2

Activity assay of MhIL-2 was determined by IL-2-dependent CTLL-2 cell proliferation assay *(13)* and quantified by a modified MTT assay *(14)*. Standard IL-2 was (specific activity: 1.0×10^7 IU/mg) from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Results

Construction of MhIL-2 Expression Vector and Transformation Into P. pastoris

Using a site-directed mutagenesis strategy, we successfully obtained IL-2 (MhIL-2) gene with intentionally designed three-site mutations and inserted it into plasmid pPIC9K. Figure 1 presents a diagram of the expression vector. The recombinant plasmid with correct reading frame was confirmed by DNA sequencing, and the result of the DNA sequencing was identical to the gene for human WT IL-2 except that three mutation sites were consistent with our design. In the MhIL-2 gene, the codon for cysteine-125 was replaced with alanine, leucine-18 with methionine, and leucine-19 with serine (Fig. 2).

Recombinant plasmid pPIC9K-MhIL-2 was linearized with the restriction enzyme *Sal*I and transformed by a Pichia EasyCompTM transformation kit into GS115 Mut^+ strain of *P. pastoris*. Positive recombinant transformants were first selected from the clones growing on RDB plates by PCR method. When further screened for multicopy transformants by a gradient concentration of G418, six colonies were obtained.

Expression and Purification of hIL-2 in P. pastoris

On methanol induction, there was an obvious extra band around the molecular mass of 15 kDa, which was consistent with the expected

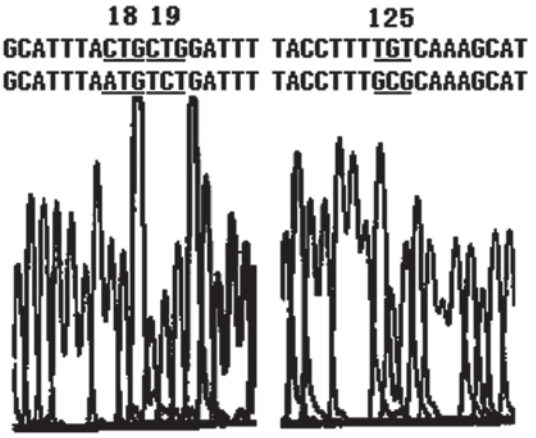

Fig. 2. DNA sequencing analysis of MhIL-2: upper lane, WThIL-2; lower lane, MhIL-2. Codons for the 18th, 19th, and 125th amino acid residues are underlined.

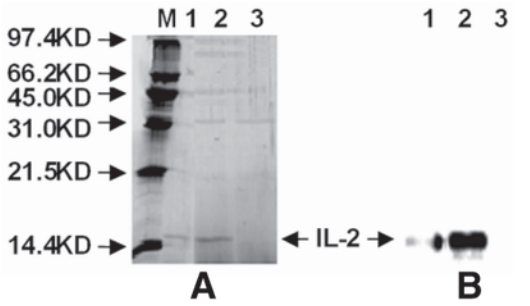

Fig. 3. SDS-PAGE **(A)** and Western blot **(B)** analysis of MhIL-2 expression. Lanes 1 and 2, supernatant sample from recombinants; lane 3, GS115 control; M, molecular mass markers.

molecular mass of MhIL-2 when compared with the colony transformed with vehicle plasmid pPIC9K (Fig. 3A). The identity of MhIL-2 was confirmed by Western blot, as shown in Fig. 3B. Colony no. 3 showed the highest expression level among the six colonies. rMhIL-2 expression accounted for about 31% of total secreted proteins.

The MhIL-2 protein was easily purified using ultrafiltration, cation-exchange chromatography, and gel filtration. An Econo-PacS cation exchanger was used to remove the most contaminant proteins via its strong ability to adsorb MhIL-2. Sephadex G-100 gel filtration chromatography was used to wipe out the minor contaminating proteins of high molecular weight. After the aforementioned three purification steps, MhIL-2 protein with high purity was obtained. The purified MhIL-2 migrated as about 15 kDa of protein in 12% SDS-PAGE, and 8 μg of pure rMhIL-2 concentrate was loaded onto the gel (Fig. 4). The yield was about 100 mg/L of culture. Table 1 summarizes the purification procedure. To facilitate the compari-

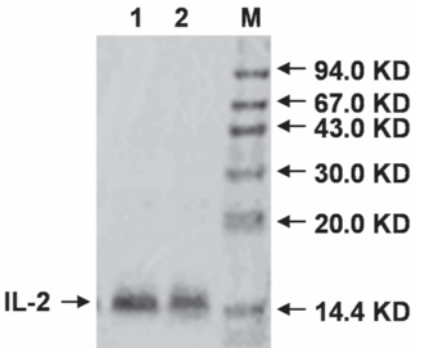

Fig. 4. SDS-PAGE analysis of purified MhIL-2. Lane 1, purified wild-type IL-2 (loaded ~10 μg); Lane 2, purified MhIL-2 (loaded ~8 μg); M, molecular mass markers.

Table 1
Summary of MhIL-2 Purification

Purification step	Total volume (mL)	Total protein (mg)	Purity (%)[a]	Recovery (%)
Ferment supernatant	200	134	31	100
Ultrafiltration	21	103.9	36	77.5
Cation exchange	54	38.2	80	36.8
Sephadex G-100	3	27.5	92	72

[a]According to densitometric analysis of SDS-PAGE.

son of biologic properties of mutant IL-2 and its WT, we also constructed WThIL-2 expressing *P. pastoris* strain and purified WThIL-2 by the aforementioned procedure.

Bioassay of Purified MhIL-2 Protein

Purified MhIL-2 was assayed for the activity on proliferation of CTLL-2 cells, while purified WT human IL-2 (WThIL-2) was applied as positive control. Both exerted potent stimulating effects on the proliferation of CTLL-2 cells in a dose-dependent manner. The concentration (156 pg/mL) of MhIL-2 to give half-maximal proliferation of CTLL-2 cells was about fivefold lower than that (580 pg/mL) of WThIL-2 (Fig. 5). WThIL-2 exerted a stimulating effect on the proliferation of CTLL-2 cells similar to that of standard IL-2 (data not shown).

Discussion

As a multiple functional cytokine, IL-2 shows great promise as a novel therapeutic for the treatment of cancer, AIDS, and many other diseases. The high efficient production of biologically active IL-2, with large quantity and low cost, is in urgent need for both further fundamental investigation and potential clinical application.

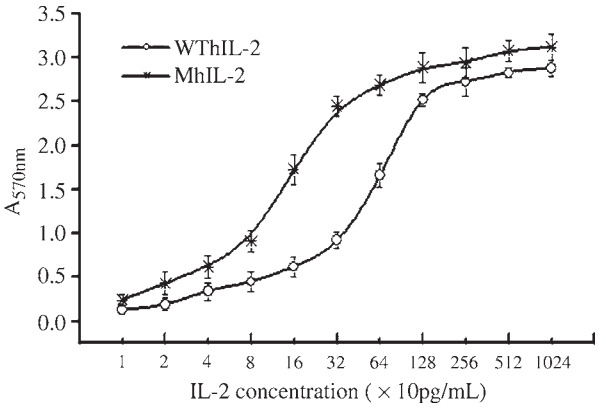

Fig. 5. Bioactivity assay of purified MhIL-2.

The production of WThIL-2 has been intensively investigated. Several processes have been developed for the production of rhIL-2 using *Escherichia coli (15–17)*. For example, by controlled feeding of postinduction media with an optimal composition of lactose and yeast extract, Saraswat et al. *(17)* produced rhIL-2 as an N-terminal G3 tag fusion protein in high-cell-density fed-batch cultures. The total expression level of this fusion protein was 9 g/L, but the final yield of purified IL-2 was not mentioned *(17)*. However, all the strategies concerning IL-2 production in *E. coli* need renaturation of inclusion body, which is a relatively cumbersome and costly procedure. WThIL-2 has also been tested for possible production in other systems, including insect cell, mammalian cell, and plant bioreactors, all of which are in an experimental phase *(18–20)*.

Convincing experimental results showed that an IL-2 variant containing adjacent point mutations (L18M/L19S) displayed increased activity and retained the innate binding affinity to its receptor *(21,22)*, which implies that this variant of IL-2 might have great potential as a substitute for WTIL-2 in basic research and clinical use. To our knowledge, there is no other systematic research regarding to the heterologous production of this variant.

Conclusion

We reported a system to efficiently produce a new type of mutant of hIL-2 with improved bioactivity. First, we generated the gene for this variant (L18M/L19S) reported before, meanwhile, the codon for Cys125 was replaced by Ala to avoid the undesirable mismatch of disulfide bond. Second, we investigated the possibility of the production of MhIL-2 in a *P. pastoris* system. As a eukaryote, *P. pastoris* has many advantages of a higher eukaryotic expression system, such as protein processing and protein folding. It is easier to manipulate and less expensive than mammalian or insect cells, with higher expression. Optimization of protein expres-

sion in a *P. pastoris* system includes the isolation of multicopy expression strains. A strain containing multiple integrated copies of an expression cassette can sometimes yield more heterologous protein than a single-copy strain. The level of G418 resistance can be roughly correlated to vector copy number *(23)*. In the present work, the multicopy transformants were successfully screened by a gradient concentration of G418, which ultimately resulted in high-level expression of MhIL-2.

Third, For the production of pharmacologic protein on a commercial scale, one of the fundamental problems exists in the purification procedure. We therefore investigated the isolation procedure of secreted MhIL-2 from the ferment supernatant. The major advantage of expressing heterologous proteins as secreted proteins is that *P. pastoris* secretes very low levels of native proteins, which facilitated the design of a simple and convenient purification process: starting with ultrafiltration, then using a cation-exchange column, and ending with gel filtration chromatography. The yield of purified IL-2 variant was 100 mg/L, with a purity >90% and a recovery of about 20%. Finally, we tested the bioactivity of purified MhIL-2 protein in vitro. When compared with standard WThIL-2, purified MhIL-2 showed much more potent biologic activity and was more stable in storage (data not shown). The strategy described in the present article may also be suggestive to the expression and purification of other functional proteins.

Acknowledgments

We are especially grateful to the China Postdoctoral Science Foundation (2005037721) and the Foundation for the Author of National Excellent Doctoral Dissertation of P. R. China (200355) for their financial support.

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