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Expression of human il-15 in Pichia pastoris final V postprint

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Abstract

Interleukin-15 (IL-15) is a pleiotropic cytokine and a member of the four -helix bundle family of cytokines which include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-15 exhibits a broad biological activity and induces the differentiation and proliferation of T, B and natural killer (NK) cells. In this study, a DNA fragment containing the mature human IL-15 sequence was cloned into pPICZaA vector, generating a fusion protein with the alpha factor signal sequence in the Nterminus and 6×His as well as c-Myc tags in the C-terminus. The resulting plasmid was integrated into the genome of Pichia pastoris strain X-33. Recombinant yeast transformants with high-level recombinant human IL-15 (rhIL-15) production were identified, which secrete as much as 75 mg/L rhIL-15 after 3 days of induction by methanol. The rhIL-15 was purified by Ni+-NTA affinity chromatography, followed by DEAE anion exchange, yielding over 95% highly purified rhIL-15. Mass spectrometry and MALDI-TOF-TOF analysis showed the purified rhIL-15 had larger molecular weights than expected, due to different degrees of N-linked glycosylation. The biological activity of the rhIL-15 proteins was measured by its ability to enhance cellular proliferation of CTLL-2 and NK cells. The results demonstrate that the experimental procedure we have reported here can produce a large amount of active recombinant human IL-15 from Pichia pastoris.

Full Text

Preamble

High-level expression and purification of active recombinant human interleukin-15 in *Pichia pastoris*

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Abstract

Interleukin-15 (IL-15) is a pleiotropic cytokine and a member of the four -helix bundle family of cytokines that includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. IL-15 exhibits broad biological activity and induces the differentiation and proliferation of T, B, and natural killer (NK) cells. In this study, a DNA fragment containing the mature human IL-15 sequence was cloned into the pPICZaA vector, generating a fusion protein with the alpha factor signal sequence at the N-terminus and 6×His as well as c-Myc tags at the C-terminus. The resulting plasmid was integrated into the genome of *Pichia pastoris* strain X-33. Recombinant yeast transformants with high-level recombinant human IL-15 (rhIL-15) production were identified, which secreted as much as 75 mg/L rhIL-15 after 3 days of induction by methanol. The rhIL-15 was purified by Ni+-NTA affinity chromatography, followed by DEAE anion exchange, yielding over 95% highly purified rhIL-15. Mass spectrometry and MALDI-TOF-TOF analysis showed that the purified rhIL-15 had larger molecular weights than expected due to different degrees of N-linked glycosylation. The biological activity of the rhIL-15 proteins was measured by its ability to enhance cellular proliferation of CTLL-2 and NK cells. The results demonstrate that the experimental procedure reported here can produce large quantities of active recombinant human IL-15 from *Pichia pastoris*.

Key words: Interleukin-15, *Pichia pastoris*, Expression and purification, N-linked glycosylation

1. Introduction

Interleukin-15 (IL-15) was discovered in 1994 by two different laboratories and characterized as a T cell growth factor [1, 2]. IL-15 has been shown to stimulate the proliferation of activated T cells, facilitate the induction of cytotoxic T-lymphocytes, and promote the generation, proliferation, and activation of NK

cells [3-8]. As a potent pro-inflammatory cytokine, IL-15 plays an important and complex role in autoimmune disease and inflammation [9, 10].

Previous studies have indicated that IL-15 is an effective vaccine adjuvant when administered as a plasmid DNA or recombinant protein in combination with DNA vaccines against infectious pathogens [11-13]. In addition, IL-15 plays a pivotal role in some hematological malignancies and exhibits anti-tumor effects [14-16]. Direct administration of IL-15 has shown anti-tumor effects in several preclinical mouse tumor models [17, 18].

In contrast to the well-defined biological activities of IL-15, methodologies to generate fully functional IL-15 protein in large quantities at low cost have barely been reported. Although commercial recombinant human IL-15 is available, its limited biological activity and high price have largely hampered its applications in both basic research and the clinic. Methods for the production and purification of rhIL-15 from *Escherichia coli* and Chinese hamster ovary (CHO) cells have previously been attempted and reported [19, 20]. However, there are no reports of its expression in *P. pastoris*, a system that has become popular in recent years due to its low cost, ease of genetic manipulation, capacity to grow to high cell density, and lack of pyrogenic endotoxins [21]. Many pharmaceutically important proteins have been successfully produced using this system for clinical applications [22, 23]. In this study, we expressed rhIL-15 using the yeast *P. pastoris* and report the establishment of a highly efficient expression system for biologically active human recombinant IL-15 in *P. pastoris* with potential for large-scale production in bioreactors.

2. Materials and Methods

2.1. Materials

P. pastoris strain X-33, expression vector pPICZaA, yeast nitrogen base (YNB), D-sorbitol, D-biotin, and BCA Protein Assay kit were purchased from Pierce (Guangzhou, China). Plasmid extraction kit, restriction enzymes, DNA polymerase, and T4 DNA ligase were purchased from Takara (Guangzhou, China). Primers 5' Factor and 3' Oxidase I were synthesized by Invitrogen (Shanghai, China). *Escherichia coli* transformants were selected on LB agar plates containing low salt (1% peptone, 0.5% NaCl, 0.5% yeast extract, and 1.5% agar) and 25 mg/L zeocin. *P. pastoris* transformants were initially selected on YPDZ plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) with 100 mg/L zeocin, then on YPDZ plates containing increasing concentrations of zeocin (from 500, 1000, 2000 to 3000 mg/L, respectively) for the isolation of high copy number recombinants. *P. pastoris* was grown in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen broth, 0.4 mg/L biotin, 1% glycerol) and induced in BMMY medium, which is the same as BMGY except that glycerol was replaced by methanol. Dialysis bags with molecular weight cut-off (MWCO) of 5 kDa were purchased from Millipore (Guangzhou, China). AKTA FPLC, Ni+-NTA columns (HisTrap™ HP,

5 ml), and DEAE-Sepharose FF ion exchange columns (HiTrap™, 1 ml) were purchased from GE Healthcare (Guangzhou, China). PNGase F was purchased from NEB (Guangzhou, China).

Commercial recombinant human IL-15 proteins were purchased from PEPROTECH (Guangzhou, China). Mouse CTLL-2 cells were purchased from ATCC (Beijing, China). Cord blood samples were collected at the Department of Gynecology and Obstetrics, South China Medical University (SCMU) (Guangzhou, China) for research purposes only, and this process was monitored by the Institutional Review Boards of SCMU. Usage of NOD/SCID/IL2Rg-/- (NSI) mice was approved by the Institutional Animal Care and Use Committee (IACUC) of the Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences.

2.2. Plasmid Construction

Human IL-15 cDNA (399 bp) was amplified by PCR from a plasmid encoding full-length human IL-15 cDNA (Genscript, Nanjing, China). An EcoRI site was introduced to allow in-frame cloning behind the a-factor secretion signal of pPICZaA, and a nucleotide sequence encoding the KEX2 cleavage site was placed upstream of IL-15. The forward and reverse primers used were 5' -GAATTCGGCATTCATGTCTTCATTTTG-3' and 5' -GCGGCCGCAGAAGTGTTGATGAACATTTG-3' , respectively. The PCR products were digested with EcoRI and NotI, and the digested fragment was inserted between the EcoRI and NotI sites of pPICZaA, where IL-15 is under the control of the alcohol oxidase 1 promoter on the vector. The resulting construct also contains a c-Myc tag as well as a 6×His-tag at the C-terminus.

2.3. Transformation of *P. pastoris* and Selection of Transformants

The expression vector was linearized by digestion with SacI and transformed into *P. pastoris* X-33 using the lithium chloride transformation method as recommended (Invitrogen, 2008). *P. pastoris* transformants were grown on YPDZ plates containing 100 g/ml of zeocin. Resulting colonies were transferred to YPD plates containing 500, 1000, 2000, and 3000 g/ml of zeocin, respectively. Colonies that grew on YPD plates containing 3000 g/ml of zeocin were picked and subjected to PCR amplification using the primers 5' factor and 3' Oxidase I. The correct transformants were selected for further studies.

2.4. Small-Scale Fermentation and Time Course Expression Study

Selected colonies from the YPD plate containing 3000 g/ml of zeocin were grown in 100 ml BMGY growth medium in 1000 ml flasks with constant shaking (250 rpm) at 30°C for 18 h until the OD600 measured between 6.0 and 8.0. The cultures were pelleted by centrifugation for 5 min at 2000 g. For the induction phase, the cell pellets were inoculated into 20 ml of BMMY induction media in 250 ml baffled flasks and grown for 120 h at 28°C with constant shaking at 250

rpm, as recommended by the manufacturer (Invitrogen, 2008). Each day, 0.2 ml of 100% methanol was added to the culture (final concentration 1.0%) to induce protein expression. One milliliter of cell culture was collected at 0, 24, 48, 72, 96, and 120 h after methanol addition. Protein concentrations in the collected supernatants were assayed with a Bradford protein assay kit. For analysis, 0.1 ml of each eluted solution was mixed with 0.5 ml of ice-cold acetone. After centrifugation, precipitates were resuspended in 40 l of 1×SDS loading buffer, heated at 95°C for 10 min, and the denatured samples were subjected to 15% SDS-PAGE analysis.

2.5. Large-Scale Fermentation and Purification

Scale-up expression was carried out in a 5 L baffled flask. *P. pastoris* harboring pPICZaA/hIL-15 with resistance to 3000 g/ml of zeocin was grown in 1 L BMGY medium with constant shaking (250 rpm) at 30°C until OD600 = 6.0-8.0. Cells were harvested and resuspended in a 1 L baffled flask containing 0.2 L BMMY medium, then cultured for 96 h. Methanol was added to the medium to a final concentration of 1.0% every 24 h. After the 96 h induction, all cell cultures were centrifuged for 15 min at 2000 g. Protein concentration in the supernatant was estimated by Bradford assay, and the supernatant was collected and dialyzed against 2 L buffer A (50 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole, pH 8.0) at 4°C overnight. After centrifugation at 20,000 g for 30 min at 4°C, the supernatants were loaded onto a 5 ml Ni+-NTA column pre-equilibrated with buffer A. The column was then washed with 60 ml buffer A to remove unbound proteins. Recombinant IL-15 was eluted with a linear gradient of 0-0.5 M imidazole in buffer B (50 mM Tris-HCl, 200 mM NaCl, 500 mM imidazole, pH 8.0). Fractions were collected and analyzed by SDS-PAGE (15% gel) followed by staining with Coomassie blue R250.

2.6. Deglycosylation Analysis of rhIL-15

The IL-15 recombinant proteins were eluted from the Ni+-NTA column and dialyzed in a dialysis bag against 2 L ice-cold dialysis buffer I (20 mM Tris-HCl, pH 8.0) overnight. After centrifugation at 20,000 g for 30 min at 4°C, the dialyzed IL-15 solution was filtered through a 0.22 m filter and loaded onto a DEAE-Sepharose FF ion-exchange column pre-equilibrated with 50 ml buffer I using an AKTA FPLC system. After loading, the column was washed with 100 ml buffer I and eluted with a linear gradient of 0-0.5 M NaCl in buffer I. Fractions containing protein were collected and assayed by 15% SDS-PAGE. Fifty microliters of purified rhIL-15 protein from the DEAE ion-exchange column was deglycosylated with PNGase F and then analyzed by 15% SDS-PAGE.

2.7. Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry

Two main protein bands from Coomassie-stained SDS-PAGE gel of deglycosylated rhIL-15 were excised and washed twice with 50 mM NH4OAc and 30%

acetonitrile (ACN) (40 l) for 30 min for destaining. The gels were dehydrated with 40 l of ACN for 3 min and completely dried under N2 for 20 min. The gels were digested with 50 g/ml trypsin in digestion buffer (50 mM NH4OAc and 0.5 mM CaCl2) for 1 h at room temperature, followed by addition of 40 l of digestion buffer (final pH 7.0) and incubation at 37°C for 16-20 h in a shaker bath. After digestion, samples were centrifuged at 10,000 rpm for 20 s, supernatants were collected, and the gels were extracted again with solvents at 30°C for 30 min in a shaker bath in the following order: 0.1% TFA (40 l), 30% ACN and 0.1% TFA (aq, 40 l), and 60% ACN and 0.1% TFA (aq, 40 l). After each extraction, samples were centrifuged at 10,000 rpm for 20 s and all supernatants were combined, including the initial supernatant (pH 4.0), dried under N2, and stored at -80°C until MS analysis using PerSeptive Biosystem' s Voyager-DE MALDI-TOF mass spectrometer (Framingham, MA/Applied Biosystems, Foster City, California, USA) equipped with a 337 nm nitrogen laser. Peptides from mass spectra of in-gel digest samples were matched against Swiss-Prot, NCBInr, and MSDB databases using the Mascot search engine (Matrix Sciences) for peptide mass fingerprinting.

2.8. Cell Proliferation Assays

2.8.1. CTLL-2 Cell Proliferation Assay CTLL-2 cells (ATCC) were washed three times in 10 ml fresh RPMI 1640 medium and centrifuged at 1000 g for 5 min. Cells were resuspended in RPMI 1640 medium containing 10% FBS, and 2×104 CTLL-2 cells were added to each well of a 96-well plate. Various concentrations of rhIL-15 (0, 0.01, 0.05, 0.25, 2.5, 25 ng/ml) were added to each well. The plates were then incubated under standard conditions (37°C and 5% CO2) for 48 h, and cell proliferation was determined using Cell Titer-Glo (Promega, Guangzhou, China) according to the manufacturer's instructions. Luminescence was read in a Wallac Victor absorbance/luminescence reader, and data were analyzed using GraphPad Prism software with four-parameter logistic curve fitting.

2.8.2. Natural Killer Cell Proliferation Assay *In Vitro* All primary samples were obtained with informed consent for research purposes, and procedures were approved by the Research Ethics Board of Guangzhou Institutes of Biomedicine and Health (GIBH). Mononuclear cells in human cord blood were separated by Ficoll-Hypaque density centrifugation (800 g, 20 min), and NK cells were collected from mononuclear cells by magnetic-activated cell sorting (MACS). NK cells were washed three times in 10 ml fresh RPMI 1640 medium and centrifuged at 1000 g for 5 min. Cells were resuspended in RPMI 1640 medium containing 10% FBS, and 1×106 cells were added to each well of a 24-well plate. rhIL-15 (50 ng/ml) was added to the plate wells, and the same volume of PBS was used as a negative control. The plates were then incubated under standard conditions (37°C and 5% CO2). Cells were collected at days 4 and 8, stained with APC-CD56 and FITC-CD3, and analyzed on a Beckman-Coulter FC500 flow cytometer for the proportion of live NK cells in the cultures.

2.8.3. Natural Killer Cell Proliferation Assay *In Vivo* NSI mice derived at GIBH have been described previously [24]. Animal experiments were performed in the Laboratory Animal Center of GIBH, and all animal procedures were approved by the Animal Welfare Committee of GIBH. All mice were maintained in specific-pathogen-free cages and provided with autoclaved food and water. Human NK cells were washed three times in 10 ml fresh PBS, then 3×106 human NK cells were injected into NOD/SCID/IL-2Rg-/- mice under the ophthalmic vein. The NK cell-treated SCID mice were divided into two groups; one group was treated daily with 200 g rhIL-15 for 4 days while the other group was treated with PBS. Mouse peripheral blood, spleen, and bone marrow were collected at day 5, lysed to remove red cells, and stained with hCD45-PE and hCD56-APC antibodies to detect human NK cells before analysis on a Beckman-Coulter FC500 flow cytometer.

3. Results

3.1. Construction of rhIL-15 Expression Vector and Transformation of *P. pastoris*

Using a custom-designed primer pair, the desired fragment of the human IL-15 gene was amplified by PCR (see Materials and Methods). The IL-15 gene was cloned into pPICZaA, where it is under the control of the Oxidase I promoter on the vector (Fig. 1a [Figure 1: see original paper]). The recombinant human interleukin-15 nucleotide sequence is as follows: 5' - GCTCCCATGACCCAGACAACGTCCTTGAAGACAAGCTGGGTCAACTGCTCTAACATGATCGATGAA 3' (contains a c-Myc tag and a 6×His-tag at the C-terminus). The resulting plasmid, pPICZaA/hIL-15, was further confirmed by sequencing before transformation into *P. pastoris* by the lithium chloride transformation method and plating on YPDS medium containing low zeocin (100 mg/L). After selection through increasing levels of zeocin, three colonies grown on YPDZ (3000 mg/L zeocin) were picked and subjected to PCR amplification using the primers 5' Factor and 3' Oxidase I. As shown in Fig. 1b, the amplified fragment was about 750 bp as expected, indicating that the pPICZaA/hIL-15 plasmid was successfully introduced into the *P. pastoris* genome.

3.2. Small-Scale Fermentation and Time Course Study

A single proven pPICZaA/hIL-15 colony of *P. pastoris* was grown in 100 ml of BMGY and induced with methanol in 20 ml BMMY for 5 days, with supernatant samples collected at 0, 24, 48, 72, 96, and 120 h. As shown in Fig. 2A [Figure 2: see original paper], an approximately 17 kDa secreted protein band was detected by Coomassie blue R250 staining at 48 h post-induction with methanol, gradually reaching a maximal level after 4 days of induction. The band decreased thereafter with substantial degradation, probably by yeast proteases secreted into the culture medium (Fig. 2). The size of the observed recombinant protein is similar to the estimated

molecular weight (MW) of rhIL-15 (the theoretical MW of 17.8 kDa, predetermined using the Expasy Compute PI/Mw tool, http://www.expasy.ch/cgi-bin/pi_tool) based on the predicted amino acid sequence: GIHVFILGCFSAGLPKTEANWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQM (c-Myc tag and 6×His-tag as indicated). Because mature native human IL-15 protein is a 15-17 kDa glycoprotein containing 133 amino acids with two potential N-linked glycosylation sites (NXS: N119NS and N127VT), it is likely that the three proteins (approximately 17, 22, and 55 kDa) represent rhIL-15 proteins with different degrees of N-glycosylation.

3.3. Large-Scale Fermentation and Purification with Ni+-NTA Column

After 3 days of induction with 1% methanol, all 0.2 L of culture medium was centrifuged and the supernatant collected. The recombinant IL-15 was eluted using a linear gradient of 0-0.5 M imidazole in buffer B (50 mM Tris-HCl, 200 mM NaCl, 500 mM imidazole, pH 8.0), and fractions were collected (Fig. 3a [Figure 3: see original paper]). The purified recombinant protein was recovered and analyzed by SDS-PAGE, revealing three major protein bands with molecular weights of 17, 22, and 55 kDa (Fig. 3b). Approximately 15 mg of protein was obtained from 200 ml of crude culture supernatant through purification with the Ni+-NTA column. As mature native human IL-15 has two disulfide bonds (positions C83-C133 and C90-C136), it may exist in a polyprotein state under native conditions. The results of SDS-PAGE conducted under native and denaturing conditions confirmed this state (Fig. 3c).

3.4. Deglycosylation Analysis of rhIL-15

After dialysis, the rhIL-15 was further purified using DEAE chromatography, which eliminated most contaminating proteins. It is well known that *P. pastoris* can attach N-linked glycans to secreted proteins. PNGase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. The purified proteins were deglycosylated by PNGase F, and SDS-PAGE results showed that the rhIL-15 proteins with molecular masses of approximately 22 and 55 kDa were indeed N-glycosylated proteins (Fig. 4 [Figure 4: see original paper]).

3.5. Mass Spectrometric Analysis

The two main protein bands were excised from the deglycosylated rhIL-15 gel and subjected to trypsin digestion. Mass spectrometric analysis by MALDI-TOF-TOF mass spectrometry, based on searching the vertebrate database, confirmed that both bands with different molecular masses were rhIL-15 protein (Fig. 5a [Figure 5: see original paper]-5b).

3.6. Biological Activity of rhIL-15

3.6.1. CTLL-2 Cell Proliferation Assay A CTLL-2 cell proliferation assay was conducted to determine the specific biological activity of the purified rhIL-15. As shown in Fig. 6 [Figure 6: see original paper], CTLL-2 cells were unable to proliferate in the absence of rhIL-15, while addition of as little as 0.01 ng/ml of rhIL-15 stimulated their growth. The cell proliferation assay therefore demonstrated the potent biological activity of rhIL-15 purified from *P. pastoris*.

3.6.2. NK Cell Proliferation Assay CTLL-2 is a transformed murine cell line, and its response may not reflect the activity of soluble IL-15 on lymphocytes from humans or other primate species. We therefore developed separate IL-15-dependent assays to measure NK cell expansion. In an *in vitro* assay, the proportion of live cells that were CD56+ CD3- NK cells at day 4 was 67.6% in wells exposed to rhIL-15, higher than the 43.7% observed in PBS-exposed wells. At day 8, this difference was more striking, with NK cells comprising 71.5% of live cells in rhIL-15-exposed wells versus only 16.8% in PBS controls (Fig. 7a [Figure 7: see original paper]). In an *in vivo* assay using a humanized mouse model, 3×106 human NK cells were injected into each mouse, and mice were treated intraperitoneally with 200 g IL-15 or 200 l PBS (n=3) once daily for 4 days. On day 7, mice were sacrificed to detect NK cells in peripheral blood, spleen, and bone marrow. The proportions of live cells that were NK cells in peripheral blood, spleen, and bone marrow were much higher in animals injected with rhIL-15 than in those injected with PBS (5.08%, 5.64%, and 0.028% respectively for rhIL-15 vs 0.00%, 0.003%, and 0.00% for PBS, as shown in Fig. 7b). These data confirm that purified rhIL-15 from *P. pastoris* can sustain human NK cells both *in vitro* and *in vivo*.

4. Discussion

The methylotrophic yeast *P. pastoris* is a single-celled microorganism that is easy to manipulate and culture. It is also a eukaryote capable of many of the same post-translational modifications as higher eukaryotic cells, such as proteolytic processing, disulfide bond formation, and glycosylation [25]. The *P. pastoris* system is also generally regarded as being faster, easier, and less expensive than other systems. Thus, *P. pastoris* is widely used for recombinant protein production [21]. Previously, IL-15 was produced using an *E. coli* expression system with low recovery of soluble protein. To our knowledge, this is the first report of highly efficient production and purification of active rhIL-15 (75 mg/L) using *P. pastoris*.

The yield of recombinant proteins is affected by a variety of factors at both genetic and cultural levels [25], so high-level secreted yields cannot be assumed using the standard protocol provided by the vendor. Previously, we found that optimization of the Kex2 P1' site residue could largely increase the *P. pastoris* secretory productivity of recombinant proteins [27]. In the current study, we

could not obtain high-level expression of rhIL-15 initially using standard protocols (data not shown). We therefore modified the rhIL-15 expression vector by replacing the Kex2 P1' alanine with proline. After optimizing the Kex2 P1' site residue, we ultimately achieved high-level expression of rhIL-15 in *P. pastoris*. Therefore, optimization of the Kex2 P1' site residue was a critical factor determining the level of rhIL-15 expression in *P. pastoris*.

Although *P. pastoris* has the capacity to perform many of the post-translational modifications of mammalian cells, its N-glycosylation pattern differs from mammalian systems [28]. The rhIL-15 obtained from *P. pastoris* was larger than the predicted molecular mass based on the amino acid sequence (17.8 kDa), suggesting that the various recombinant hIL-15 protein molecules purified were glycosylated to different degrees. PNGase F deglycosylation analysis confirmed that rhIL-15 proteins with molecular masses of approximately 22 and 55 kDa were N-glycosylated.

Interleukin-15 exerts powerful stimulatory effects on lymphocyte subsets that result in antiviral and anti-tumoral activities. Our study confirmed that glycosylated rhIL-15 from *P. pastoris* could stimulate mouse CTLL-2 cells and the proliferation of human NK cells both *in vitro* and *in vivo*. Recombinant human IL-15 produced from *P. pastoris* would be a useful tool for establishing humanized mouse models or could be further exploited as an antiviral and anti-tumoral medicine as well as a vaccine adjuvant.

In summary, by optimizing the Kex2 P1' site residue, we have established conditions that allow successful production of functional recombinant human IL-15 in *P. pastoris* for the first time, to the best of our knowledge. Large quantities of highly purified, active rhIL-15 would be an invaluable tool for investigating its structural and biochemical properties as well as facilitating its clinical applications.

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Figure Legends

Fig. 1 (a) Schematic illustration of the expression vector pPICZaA/hIL-15. (b) PCR results of rhIL-15 *P. pastoris* transformants. Lane M, DNA marker; lanes 1-3, transformants of pPICZaA/hIL-15; lane #, an untransformed colony as negative control.

Fig. 2 Time course of rhIL-15 expression in baffled flask. Culture supernatants were collected at the indicated times after methanol induction. The supernatants were precipitated with acetone, analyzed on 15% SDS-PAGE, and stained with Coomassie blue R250. Recombinant hIL-15 protein is indicated with an arrow.

Fig. 3 Purification of rhIL-15 expressed in *P. pastoris*. (a) Elution curves of rhIL-15 from Ni+-NTA chromatography column in buffer B. The eluents were collected at OD280 > 200 mAU, totaling 15 mL collected. (b) Recombinant hIL-15 was purified by Ni+-NTA column, separated on SDS-PAGE, and stained with Coomassie blue R250. Lane 1#, culture supernatants after 72 h

methanol induction; lane 2#, unbound protein in 72 h methanol induction supernatants; lane A, fraction. The purified rhIL-15 proteins are indicated with arrows. (c) rhIL-15 was purified by Ni+-NTA column, separated on SDS-PAGE under native and denaturing conditions, and stained with Coomassie blue R250.

Fig. 4 SDS-PAGE analysis of deglycosylated rhIL-15. The purified rhIL-15 proteins were deglycosylated by PNGase F. Lane M, protein markers; lane 1#, glycosylated rhIL-15; lane 2#, deglycosylated rhIL-15 by PNGase F; lane 3#, PNGase F (approximately 36 kDa). The bands indicated with arrows were excised and further analyzed by MALDI-TOF-TOF mass spectrometry.

Fig. 5 MALDI-TOF-TOF analysis of the purified deglycosylated rhIL-15. (a) Lower molecular weight protein band. (b) Higher molecular weight protein band.

Fig. 6 Bioactivity of purified rhIL-15. CTLL-2 cells were incubated with rhIL-15 samples (0, 0.01, 0.05, 0.25, 2.5, 25 ng/mL) for 48 h. Proliferation of CTLL-2 cells was determined using Cell Titer-Glo, and luminescence was read in a Wallac Victor absorbance/luminescence reader. Data represent the mean ± SD of triplicate tests; bars show mean and standard deviation of three independent experiments.

Fig. 7 (a) Purified rhIL-15 stimulates the proliferation of human NK cells *in vitro*. 1×106 human NK cells were incubated with rhIL-15 (50 ng/mL) for 8 days, with the same volume of PBS used as negative control. Cells were collected at days 4 and 8, stained with APC-CD56 and FITC-CD3, and analyzed on a Beckman-Coulter FC500 flow cytometer for the proportion of live NK cells in the cultures. (b) Purified rhIL-15 stimulates the proliferation of human NK cells in peripheral blood, spleen, and bone marrow in NSI mice. 3×106 human NK cells were injected into each mouse, and mice were treated intraperitoneally with 200 g IL-15 or 200 l PBS (n=3) once daily for 4 days. On day 7, mice were sacrificed to detect NK cells in peripheral blood, spleen, and bone marrow. (c) The percentage of CD45+CD56+ NK cells in peripheral blood, spleen, and bone marrow in rhIL-15- or PBS-treated NSI mice (n=3). Error bars represent SEM; *P<0.05.

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