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Expression and purification of non-*N*-glycosylated porcine interleukin 3 in yeast *Pichia pastoris*

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Abstract

Yeast *Pichia pastoris* has been widely utilized to express heterologous recombinant proteins. *Pichia pastoris* expressed recombinant porcine interleukin 3 (IL3) has been used for porcine stem cell mobilization in allo-hematopoietic cell transplantation models and pig-to-primate xeno-hematopoietic cell transplantation models in our lab for many years. Since the yeast glycosylation mechanism is not exactly the same as those of other mammalian cells, *Pichia pastoris* expressed high-mannose glycoprotein porcine IL3 has been shown to result in a decreased serum half-life. Previously this was avoided by separation of the non-glycosylated porcine IL-3 from the mixture of expressed glycosylated and non-glycosylated porcine IL-3. However, this process was very inefficient and lead to a poor yield following purification. To overcome this problem, we engineered a non-*N*-glycosylated version of porcine IL-3 by replacing the four potential *N*-glycosylation sites with four alanines. The codon-optimized non-*N*-glycosylated porcine IL3 gene was synthesized and expressed in *Pichia pastoris*. The expressed non-*N*-glycosylated porcine IL3 was captured using Ni-Sepharose 6 fast flow resin and further purified using strong anion exchange resin Poros 50 HQ. *In vivo* mobilization studies performed in our research facility demonstrated that the non-*N*-glycosylated porcine IL3 still keeps the original stem cell mobilization function.

Keywords

Porcine IL3; *Pichia pastoris* expression; purification; glycosylation; mobilization

Introduction

Hematopoietic stem cell (HSC) transplantation is a therapeutic strategy used for hematologic malignancies, immunodeficiency states, nonmalignant hereditary and acquired hematologic diseases, as well as inherited metabolic disorders [1, 2]. HSCs can be mobilized from the

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bone marrow into peripheral blood and collected by apheresis. This method of peripheral blood stem cell collection is frequently used clinically for obtaining HSCs for allogeneic and autologous HSC transplantation. Currently, injection of granulocyte colony-stimulating factor (G-CSF) is the clinical standard for mobilization of human HSC [1]. Porcine IL3 combined with porcine stem cell factor (SCF) has been shown to be more effective at stem cell mobilization than G-CSF in our preclinical miniature swine model. *Pichia pastoris* expressed porcine IL3 has been used for porcine bone marrow stem cell mobilization in an allo-hematopoietic cell transplantation model and pig-to-primate xeno- hematopoietic cell transplantation model in our lab for many years [3, 4, 5, 6, 7, 8]. However, since the *N*-glycosylation mechanism is different between yeast and mammalian cells [9], *Pichia pastoris* expressed high-mannose glycoprotein porcine IL3 could lead to rapid clearance *in vivo* [10]. This was avoided by use of the non-glycosylated porcine IL-3 which was separated from the mixture of expressed glycosylated and non-glycosylated porcine IL-3. This process was very inefficient and led to poor yields following purification.

In this study, we replaced all four potential porcine IL3 *N*-glycosylation sites with alanines. The non-*N*-glycosylated porcine IL3 was expressed and purified in *Pichia pastoris*. *In vivo* mobilization study demonstrated that removal of these glycosylation sites did not result in a decrease in the mobilization function

Materials and Methods

Plasmid construction

Using the published porcine IL3 gene sequence [11], the non-*N*-glycosylated porcine IL3 gene (Figure 1) was synthesized using the *Pichia pastoris* preferred codons [12]. Ten primers (Table 1) were designed to cover the full length of the non-*N*-glycosylated porcine IL3 gene (121 aa). There was an average 21 base overlap between neighboring primers. 10 pmol of the first and the last primer, and 2 pmol rest of the primers were used. PCR was performed at 95°C for 5 min, 25 cycles of 95°C for 30 sec, 55° for 30 sec, 72° for 1 min and an extension at 72° for 10 min. The PCR products were analyzed with 1% agarose gel electrophoresis, and the DNA fragment with the predicted size was cut out and extracted with QIAquick Gel Extraction Kit. The DNA was digested using *Xho*I and *Eco*RI, cloned into pWPICZalpha [13] and then sequenced. To facilitate the downstream purification, six histidines (6×His tag) were added to the C-terminus by PCR amplification using primers pIL3NGF and pIL3NGR (Table 1). The PCR product was digested with *Xho*I + *Eco*RI and cloned back into pWPICZalpha and sequenced to generate the non-*N*-glycosylated porcine IL3 construct carrying 6×His tag in its C-terminus.

Protein expression in *Pichia pastoris*

5–10 µg of the above constructed plasmid DNA was linearized by *Sac*I digestion for 3 h at 37°C, treated with Qiagen PCR purification kit, and transformed into *Pichia. pastoris* strain X33 using the Gene Pulser Xcell Electroporation system (Bio-Rad). Cells were spread on YPD agar plates (1% Bacto™ yeast extract, 2% Bacto™ peptone, 1.5% Bacto™ agar, 2% dextrose) containing 100 µg/mL of Zeocin and incubated at 30°C for 3–4 days. Six colonies were randomly picked and cultivated in test tubes containing 5 mL YPD (1% Bacto™ yeast extract, 2% Bacto™ peptone, 2% dextrose) at 30°C at 250 rpm for 24 h as growth phase I, then in YPG (1% Bacto™ yeast extract, 2% Bacto™ peptone, 1% glycerol) at 30°C at 250 rpm for another 24 h as growth phase II. The cultures were induced in 2 mL BMMYC (1% Bacto™ yeast extract, 2% Bacto™ peptone, 100 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base without amino acids (MP), 4×10^{-5} % biotin, 0.5% methanol and 1% Bacto™ casamino acids) for 48 h at 25°C at 225 rpm. 0.5% methanol was added at the

beginning and end of the day to sustain the methanol level. The culture supernatants were analyzed using 12% NuPAGE SDS gel under non-reducing conditions.

One clone was selected and cultivated in shake-flasks (scaled up from the above described small tube expression) for downstream purification. 100 units/mL of penicillin and 100 µg/mL of streptomycin were added to suppress bacterial contamination. The expressed protein in the supernatant was harvested by centrifugation at 3000 rpm at 4°C for 10 min prior to protein purification.

Protein Purification

Ni-Sepharose™ 6 fast flow resin (GE healthcare) was packed in a XK16/20 column (GE healthcare) for the first step purification. The column was equilibrated with 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, and 5 mM imidazole (10 CV). The sample was prepared by adding 0.5 M NaCl, 20 mM sodium phosphate pH 7.4, and 5 mM imidazole and filtering through crepe fluted filter paper (VWR) before being loaded onto the equilibrated column. The column was washed using 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, and 5 mM imidazole (8 CV). The bound proteins were eluted with 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 250 mM imidazole in eight fractions, which were analyzed using 12% NuPAGE® Bis-Tris gel stained with GelCode Blue stain reagent (Thermo Scientific). The fractions containing protein of interest were pooled and dialyzed against 20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5% glycerol at 4°C in a 3.5 kDa cut off Spectra/Por® membrane tubing (Spectrum labs). The dialysis buffer was replaced once.

Strong anion exchange resin Poros® 50 HQ (Applied Biosystems) in a XK16/20 column (GE Healthcare) was used for the second step purification. The column was equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol (10 CV). The dialyzed sample was loaded, then the column was washed with 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol (8 CV). The bound protein was eluted with 50 and 100 mM sodium borate in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol into eight fractions, respectively. The purification fractions were analyzed using 12% NuPAGE SDS gel. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific).

Western blotting

Protein samples were separated by electrophoresis using NuPAGE 12% Bis-Tris Gel and the gel was electro-transferred at 35 V onto nitrocellulose membrane filter paper using 1x NuPAGE® transfer buffer (Invitrogen). The membrane was thereafter blocked in 5% blotting grade blocker non-fat dry milk (Bio-Rad) in 1xPBS, 0.02% Tween 20 for 1 h with shaking and washed once with 1xPBS, pH7.4, 0.02% Tween 20 at room temperature with shaking. The non-*N*-glycosylated porcine IL3 was detected with mouse anti-His monoclonal antibody (1:500) (Invitrogen) and rat anti-mouse IgG-HRP (1:1000) (Invitrogen) in 5% non-fat dry milk in 1xPBS, 0.02% Tween 20. Detection of the proteins was done by TMB membrane peroxidase substrate (KPL Cat#:50-77-02), and color development was stopped with dH₂O.

In vivo mobilization study

Massachusetts General Hospital (MGH) Major Histocompatibility Complex (MHC)-defined miniature swine ranging in weight from 30–60 kg were used for this study. Mutated non-*N*-glycosylated porcine IL3 was compared to the original porcine IL3 developed by Biotransplant Inc [11] as control. This control porcine IL3 was named as porcine IL3-BTI to distinguish it from the non-*N*-glycosylated porcine IL3. Animals were treated for 5 to 7 days with a weight-based dose of recombinant porcine IL3 and porcine stem cell factor (0.1mg each of porcine IL3 and porcine SCF per kg recipient body weight for first 30 kg, 0.05 mg/

kg for each additional kg). The porcine IL3 was used in combination with porcine SCF to keep our established mobilization protocol consistent. All animals were pre-medicated using diphenhydramine (2–4 mg/kg, IM or IV) 30 min before each porcine IL3 injection. PBMC (peripheral blood mononuclear cells) collection was achieved by leukapheresis (COBE BCT Inc., Lakewood, Colorado, USA) starting after 5 days of cytokine injection.

The Cobblestone area forming cell (CAFC) [14] assay measures a spectrum of hematopoietic cells and allows committed precursor cells (day 7 to week 8) to be separated from more primitive HSC with long-term repopulating ability (day 28–35 CAFC). EUR-FLS-72/1C5A stromal cells originally derived from day -12 fetal livers of CBA×C57BL/10 F1 mice [15] were overlaid with various dilutions of PBMC to allow limiting dilution analysis of the precursor cells forming hematopoietic clones under the stromal layers (16 wells per dilution with eight 2-fold dilution steps). The cells were cultured at 37°C, 5% CO₂ for 8 weeks in CAFC medium [Iscove's modified Dulbecco's medium with GlutaMAX-1 (Invitrogen, Carlsbad, CA) supplemented with 25 ng/mL of porcine SCF, 2 ng/mL of porcine IL3, gentamicin (10 mg/mL), 2-mercaptoethanol (1×10^{-4} M), 12.5% fetal bovine serum, 12.5% horse serum, and hydrocortisone (1×10^{-6} M)] with weekly replacement of 80% of the medium. The percentage of wells with at least 1 phasedark hematopoietic clone of at least 5 cells (i.e. cobblestone area) beneath the stromal layer was determined weekly (or on week 5 to week 8). Frequencies of the week 8 CAFC subsets were calculated with L-cal statistical software (Stem Cell Technologies Inc.).

Results and discussions

Plasmid construction for the non-N-glycosylated porcine IL3

As shown in Figure 1 the four potential *N*-glycosylation sites (N24, N30, N43, N64) in the porcine IL3 gene were replaced with non-polar amino acid alanines. We expected that the four replacements would not influence the porcine IL3 mobilization function. The codon-optimized non-*N*-glycosylated porcine IL3 gene carrying 6x His tag (127 aa) in the C-terminus was synthesized (GenBank accession number: BankIt1492785 IL3 JQ028875). The 6xHis tag in the C-terminus was added to facilitate the downstream purification.

Pichia pastoris expression and purification of the non-N-glycosylated porcine IL3

The non-*N*-glycosylated porcine IL3 carrying 6xHis-tag in the C-terminus was expressed in shake flask cultures. Western blotting analysis confirmed the expression using mouse anti-His monoclonal antibody (data not shown). The secreted non-*N*-glycosylated porcine IL3 was captured directly by Ni-Sepharose 6 fast flow resin through its 6xHis-tag in the C-terminus mainly in the 2nd and 3rd fractions eluted (Figure 2A). The eluted fractions from the capturing step were pooled, concentrated down with Centricon Plus-70 (5 kDa cut off) and dialyzed to remove the salts for the second step purification. Based on the isoelectric point value (6.11) strong anion exchange resin Poros 50 HQ was chosen for the second step purification. In the second step purification sodium borate was applied to separate the non-*N*-glycosylated porcine IL3 from the glycosylated yeast host protein and aggregates [16]. As shown in Figure 2B we obtained the pure non-*N*-glycosylated porcine IL3 after the two-step purification. SDS-PAGE and Western blot analysis with anti-6xHis mAb for the final product is shown in Figure 3. After mutating the 4 potential *N*-glycosylation sites and adding the 6xHis tag in the C-terminus, the binding to the strong anion exchange resin Poros 50HQ improved dramatically under pH 8.0 which facilitated our second step purification. The absence of the *N*-glycosylation improved the expression and purification significantly. Prior to mutating the *N*-glycosylation sites, the expression level was ~10 mg/L before purification and 6.6 mg/L after two-step purification. After the mutation, the expression level improved to ~90 mg/L before purification and 75 mg/L after two-step purification.

Mobilization study in vivo for the non-*N*-glycosylated porcine IL3

To assess the ability of mutated non-*N*-glycosylated porcine IL3 to mobilize stem cells into the peripheral blood, we injected the non-*N*-glycosylated porcine IL3 or the control porcine IL3-BTI intramuscularly into MGH MHC-defined miniature swine. The frequency of CAFC activity following non-*N*-glycosylated porcine IL3 mobilization was comparable to the frequency of CAFC activity following porcine IL3-BTI control mobilization (Figure 4, Table 2). These results demonstrated that the non-*N*-glycosylated porcine IL3 can replace the porcine IL3-BTI as a mobilization reagent *in vivo*. The *in vivo* mobilization study also demonstrated that the 6xHis tag added in the C-terminus did not influence the mobilization function. Since the mobilization dose requirement is high (~0.7 mg/kg), cost-effectiveness, high production level, high purity, easy purification is needed. As presented in this study, the non-*N*-glycosylated porcine IL3 met all of the above requirements.

Highlights

1. The codon-optimized non-*N*-glycosylated porcine IL3 was expressed and purified in *Pichia pastoris*.
2. *In vivo* mobilization studies proved that the non-*N*-glycosylated porcine IL3 still keeps the original stem cell mobilization function.
3. This study successfully resolved the high-mannose *N*-glycosylation problem of *Pichia pastoris* expressed porcine IL3 for our hematopoietic stem cell transplantation model.

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atgccaactactacttttgcaaccaaagaactacttggctatgattcaagagattactaga
 M P T T T L Q P K N Y L A M I Q E I T R
 tctttggaggctttgactggttacttctgctaagtctttgactttgaacgagttggagact
 S L E A L T V T S A K S L T L N E L E T
 ttggttgctaacactttgttgagaccaaacttggaggctttcgttactttcgttgagaac
 L V A N T L L R P N L E A F V T F A E N
 cacttgaaggctattttctggtattaagaagaacttggagaagttcagaccaattttgcca
 H L K A I S G I K K N L E K F R P I L P
 acttctatgtctactgaggagccaatttctattgaggaggggtgacttgggtgacttcaga
 T S M S T E E P I S I E E G D L G D F R
 gctaagttgatggagtacttggttgttttgagagactctttgaagccaatgattactgag
 A K L M E Y L V V L R D S L K P M I T E
 Ccacaccaccaccaccactaa
 P H H H H H H -

Fig. 1.

Codon-optimized non-*N*-glycosylated porcine IL3 as well as its 6xHis tag DNA sequence and the derived amino acid sequence. The four potential *N*-glycosylation sites were replaced with alanines and shown in red. The six C-terminal histidines are additions to the native aa sequence.

Fig. 2. Purification for non-*N*-glycosylated porcine IL3: A) First step purification using Ni-Sepharose 6 fast flow resin. Lane 1: protein marker; Lane 2: sample; Lane 3: flowthrough; Lane 4–5: two 40 mL wash fractions; Lane 6–11: six 10 mL elution fractions using 250 mM imidazole. B) Second step purification using strong anion-exchange resin Poros 50HQ. Lane 1 and 14: protein markers; Lane 2: sample; Lane 3: flowthrough; Lane 4–5: two 40 mL wash fractions; Lane 6–13: eight 10 mL elution fractions using 50 mM sodium borate; Lane 15–22: eight 10 mL elution fractions using 100 mM sodium borate.

Fig. 3. SDS PAGE and Western blot analysis for non-*N*-glycosylated porcine IL3. From left to right, Lane 1: SDS-PAGE analysis of non-*N*-glycosylated porcine IL3 (0.6 μ g); Lane 2: Western blot analysis of non-*N*-glycosylated porcine IL3 (0.6 μ g) using anti-6xHis tag mAb; Lane 3: Protein marker

Fig. 4.

CAFC analysis to measure the frequency of stem cells mobilized into the peripheral blood *in vivo* following mobilization with mutated non-*N*-glycosylated porcine IL3 compared to porcine IL3-BTI. Each triangle represents the frequency of CAFC activity per 10⁶ cells plated of an individual animal mobilized with the non-*N*-glycosylated porcine IL3 combined with porcine SCF. Each square represents the frequency of CAFC activity of an individual animal mobilized with porcine IL3-BTI combined with porcine SCF. CAFC data from seven of the porcine IL3-BTI treated animals were previously published [14] (see Table 2). These data are included for comparison.

Table 1PCR primers to synthesize the codon-optimized non-*N*-glycosylated porcine IL3

PIL31
5'CCG CTC GAG AAG AGA GAG GCT GAA GCT ATG CCA ACT ACT ACT TTG CAA CCA AAG AAC3'
PIL32
5' CTC CAA AGA TCT AGT AAT CTC TTG AAT CAT AGC CAA GTA GTT CTT TGG TTG CAA AGT AGT 3'
PIL33A
5' GAG ATT ACT AGA TCT TTG GAG GCT TTG ACT GTT ACT TCT GCT AAG TCT TTG ACT TTG AAC3'
PIL34A
5' TGG TCT CAA CAA AGT GTT AGC AAC CAA AGT CTC CAA CTC GTT CAA AGT CAA AGA CTT AGC3'
PIL35A
5' GCT AAC ACT TTG TTG AGA CCA AAC TTG GAG GCT TTC GTT ACT TTC GCT GAG AAC CAC TTG 3'
PIL36A
5'GAA CTT CTC CAA GTT CTT CTT AAT ACC AGA AAT AGC CTT CAA GTG GTT CTC AGC GAA AGT3'
PIL37
5' AAG AAG AAC TTG GAG AAG TTC AGA CCA ATT TTG CCA ACT TCT ATG TCT ACT GAG GAG CCA3'
PIL38
5' AGC TCT GAA GTC ACC CAA GTC ACC CTC CTC AAT AGA AAT TGG CTC CTC AGT AGA CAT AGA3'
PIL39
5'GAC TTG GGT GAC TTC AGA GCT AAG TTG ATG GAG TAC TTG GTT GTT TTG AGA GAC TCT TTG3'
PIL40
5'CCG GAA TTC TTA TGG CTC AGT AAT CAT TGG CTT CAA AGA GTC TCT CAA AAC AAC3'
PIL3 NGF
5' CCG CTC GAG AAG AGA GAG GCT GAA GCT ATG CCA ACT ACT ACT TTG CAA C 3'
PIL3 NGR
5' CCG GAA TTC TTA GTG GTG GTG GTG GTG GTG TGG CTC AGT AAT CAT TGG CTT C 3'

Table 2

Summary of animals treated with porcine IL-3

Animal#	Weight (kg)	pIL3 -non-N-Gly	pIL3-BTI	pSCF	CAFC (/10 ⁶)	Reference
19425	48	+		+	29	this study
19866	48.1	+		+	76	this study
19431	49	+		+	30	this study
19424	30.9	+		+	22	this study
19082	55.4		+	+	27	this study
13665	38.4		+	+	6.65	Lima et al.
13817	39.4		+	+	32.03	Lima et al.
13062	83		+	+	9.28	Lima et al.
13666	94.7		+	+	23	Lima et al.
13901	104		+	+	10.04	Lima et al.
14127	26.6		+	+	14.67	Lima et al.
13471	95.8		+	+	75.71	Lima et al.