

Expression of recombinant human IL-4 in *Pichia pastoris* and relationship between its glycosylation and biological activity



Rui Li ^{a,b}, Chao Xie ^{c,e}, Yuan Zhang ^{a,b}, Bin Li ^{a,b}, William Donelan ^c, Shiwu Li ^c, Shuhong Han ^c, Xingli Wang ^b, Taixing Cui ^{b,d,*}, Dongqi Tang ^{a,b,*}

^a Center for Stem Cell & Regenerative Medicine, The Second Hospital of Shandong University, Jinan 250012, PR China

^b Shandong University Qilu Hospital Research Center for Cell Therapy, Key Laboratory of Cardiovascular Remodeling and Function Research, Qilu Hospital of Shandong University, Jinan 250012, PR China

^c Department of Pathology, Immunology, and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL 32610, USA

^d Department of Cell Biology and Anatomy, University of South Carolina of Medicine, Columbia, SC 29209, USA

^e College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, PR China

ARTICLE INFO

Article history:

Received 26 October 2013

and in revised form 21 December 2013

Available online 24 January 2014

Keywords:

N-glycosylation

Site-directed mutagenesis

Recombinant human IL4

Pichia pastoris

ABSTRACT

Secretory human interleukin 4 (hIL4) is an N-glycosylated pleiotropic cytokine. It is unknown if these N-linked glycans are required and essential for hIL4 protein stability, expression, secretion, and activity *in vivo*, and hIL4 expressed from *Pichia pastoris* yeast has not been tested to date. In this study, we successfully expressed human hIL4 in *P. pastoris*, the methylotrophic yeast, with a yield of 15.0 mg/L. Using the site-directed mutagenesis technique, we made two mutant hIL4 cDNA clones (N38A and N105L) and subsequently expressed them in *P. pastoris* to analyze the relevant function of each N-glycosylation site on hIL4. Our results demonstrate that the glycosylation only occurs at position Asn38, but not Asn105. The glycosylated form of hIL4 unexpectedly has lower biological activity and lower stability when compared to its non-glycosylated form. The implications of this are discussed.

© 2014 Elsevier Inc. All rights reserved.

Introduction

Glycosylation is ubiquitous and essential protein post/co-translational modifications in eukaryotic cells [1]. Glycan structures play biological roles in protein folding, protein stability, enzymatic function, inflammation, cancer, and immunity [2]. Glycosylation increases the heterogeneity and functional diversity of proteins [3].

There are three major types of glycosylation: (1) the carbohydrate is added to a carbon on a tryptophan side chain; (2) the formation of a glycosylphosphatidylinositol (GPI)¹ anchors (glypiation); and (3) phospho-linked, where the sugar is attached via the phosphate of a phospho-serine. Another classification system for protein-bound glycans is to describe them as N- or O-linked depending on the specific site of attachment. Great interest has been

directed to N-glycosylation due to its essential role in proper protein folding. N-linked glycosylation, in eukaryotes, is catalyzed by a membrane associated enzyme oligosaccharyl transferase (OT) [4], a protein complex localized in the lumen of the endoplasmic reticulum (ER). N-linked glycosylation occurs at the Asn-Xaa-Ser/Thr sequon and is a co-translational process [5]. After OT-catalyzed glycosylation, Glc3MAN9GlcNAc2 is transferred to the polypeptide chain, glycosidase I and II trim the two terminal glucose residues, and the protein folding cycle begins. This pathway in eukaryotes has been described in detail [6–8]. For many higher eukaryotes, such as in mammalian systems, protein N-glycosylation is a highly coordinated and complex process. A plethora of diverse monosaccharide units are added to glycoproteins, such as GalNAc, GlcNAc, galactose, fructose, and N-acetylneuraminic acid [6,9].

It has been relatively difficult to study glycoproteins because the glycan moieties usually have a heterogeneous chemical structure and conformation. *Pichia pastoris*, as a cellular host for the expression of recombinant protein, has become increasingly popular to use in recent times. Subsequent processing of newly glycosylated proteins occurs differently in *P. pastoris* compared with higher eukaryotic cells [10], however, *P. pastoris* is also a eukaryote, and thereby, provides the potential for producing soluble, correctly folded recombinant proteins [11] that have undergone the

* Corresponding authors. Address: Center for Stem Cell & Regenerative Medicine, The Second Hospital of Shandong University, Jinan 250012, PR China (D. Tang). Address: Department of Cell Biology and Anatomy, University of South Carolina of Medicine, Columbia, SC 29209, USA (T. Cui).

E-mail addresses: cuitaixing@sdu.edu.cn (T. Cui), tangdq@sdu.edu.cn (D. Tang).

¹ Abbreviations used: hIL4, human interleukin-4; Asn, asparagines; Man, mannose; GPI, glycosylphosphatidylinositol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; OT, oligosaccharyl transferase; LC-MS/MS, liquid chromatography-mass spectrometer.

post-translational modifications required for functionality [12]. Additionally, *P. pastoris* is easier to genetically manipulate and culture than mammalian cells and this system is more efficient for production of recombinant proteins [13].

Human IL-4 (hIL4) consists of 153 amino acids that is synthesized as a precursor containing a hydrophobic secretory signal sequence of 24 amino acids and contains six cysteine residues, with the potential to form three disulfide bonds [14,15]. The mature hIL4 contains 129 amino acids, including two potential N-glycosylation sites at Asn38 and Asn105 [16]. hIL4 is a 17 kDa monomeric glycoprotein secreted by T helper 2 (Th2) cells, NK T cells, mast cells, and basophils [17–20] as an immunoregulatory cytokine [21]. hIL4 is involved in various physiological and pathological conditions, especially atopic diseases such as allergic hypersensitivity and asthma [22–24], bone loss [25,26], and tumor immunology [27]. Blocking the activity of hIL4 might provide new therapeutic approaches to control allergies and inflammation [20].

Materials and methods

Construction of an expression plasmid

The human IL4 cDNA (129 amino acids) was amplified by PCR on a plasmid encoding full-length human IL4 cDNA using the primers 5'TCTCTCGAGAAAAGACACAAGTGGGATATCAC3' and 5'GAT-TCTAGATCAGCTCGAACACTTTGAA3'. PCR products were purified using a PCR purification kit (Invitrogen USA). The amplified PCR DNA was analyzed and confirmed by DNA sequencing. After cloning the PCR DNA into Xho1/Xba1 site of pPICZαA (Invitrogen, USA), the resulting plasmid pPICZα-IL4 was transformed into *Escherichia coli* (TOP10F) for plasmid preparation (Fig. S1).

Expression and purification of rIL4

The recombinant plasmid was linearized by digestion with SacI and transformed into *P. pastoris* X-33 cells. The transformation was performed using the lithium chloride method following the kit manual (Pichia Easycomp Transformation Kit, Invitrogen, USA). The transformed *P. pastoris* X-33 cells were selected on YPD (1% Yeast extract, 2% Peptone, 2% Dextrose, and 2% Agar) plates containing 100 µg/ml of Zeocin after incubation for 2–3 days at 30 °C. The Zeocin-resistant colonies were replated on an YPD plate supplemented with 500 µg/ml of Zeocin, and after incubation for 2–3 days at 30 °C, several colonies appeared on the YPD plate and the larger colonies were selected for protein expression. The pre-culture growth step was performed for 48 h with shaking (220 rpm, 30 °C) in a 50 ml centrifuge tube containing 5 ml YPD medium. The cells were then pelleted by centrifugation at 1000 rpm for 10 min at 4 °C and resuspended in 5 ml BMMH (100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4 \times 10^{-5}\%$ biotin, and 0.5% methanol) in a 50 ml centrifuge tube for induction of AOX1 expression. The culture was incubated at 30 °C with shaking (220 rpm). Every 24 h methanol (100%) was added to a final concentration of 0.5% to maintain induction and at different time intervals the supernatants were collected by centrifugation (1000 rpm, 25 min, at 4 °C). Then the most efficient rhIL4 secreting clone was selected by SDS–PAGE and Western blot analysis. The single colony with high yield was selected for large scale cell culture (Fig. S2).

The purification of rhIL-4 began with 100 ml of yeast culture. 100 ml supernatant was collected and dialyzed against 2 L buffer A (20 mM sodium acetate, pH 5.5) at 4 °C overnight. After centrifugation, the dialyzed supernatants were loaded onto a CM Sepharose column (1.5 × 18 cm) equilibrated with buffer A. After washing with buffer A, the protein was eluted with NaCl in a linear

gradient (0.0–1.0 M) in buffer A. Fractions were analyzed by SDS–PAGE and concentrated (Amicon Ultra 3 K device). Protein concentration was determined using the Bradford method with bovine serum albumin as a standard.

Glycosylation assay

The rhIL4 were digested with recombinant N-glycanase (PNGase F, New England BioLabs) according to the manufacturer's instructions. Deglycosylated rhIL4 and control were analyzed by SDS–PAGE and Western blotting.

Site-directed mutagenesis

Asparagine (Asn) at glycosylation site was mutated to alanine or leucine by using a site-directed mutagenesis kit according to the manufacturer's protocol (Stratgene USA). Oligonucleotide primers were used for mutagenesis as follows: N38A forward: TTTGCTGCCT CCAAGGCCACAACCTGAGAAGGAAACCTTC, reverse: TTCCTTCTCAG TTGTGGCCTTGGAGGCAGCAAAGATGTC, N105L forward: CCTGTGA AGGAAGCCCTGCAGAGTACGTTGGAAAACCTTC, reverse: TCCAACGTACTCTGCAGGGCTTCCTTCACAGGACAGGA. The restriction enzyme Dpn I was used to treat the samples according to the manufacturer's protocol. Plasmids were analyzed and confirmed by DNA sequencing.

SDS–PAGE and Western blotting

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 12% gel according to standard protocol. After electrophoresis the gels were either stained with Coomassie Brilliant Blue R-250 (Sigma) or the protein was transferred to a polyvinylidene fluoride (PVDF, Millipore) membrane. Rabbit polyclonal anti-human IL4 primary antibody was used (1:2500 abcam USA). Blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:30,000) and bands were detected using Amersham ECLTM Western Blotting Analysis System.

Determination of glycosylation sites

Identification of rhIL4 post-translational modifications was performed by mass spectrometry. The wild type rhIL4 protein band was excised from the gel following SDS–PAGE and Coomassie Blue staining. Tryptic digestion of samples was carried out as described previously [34,35]. The tryptic digests were injected into a NanoLC plus HPLC system, and analyzed by an LTQ-FT (linear ion trap, FT ICR; Thermo Fisher Scientific, San Jose, CA). All MS/MS spectra were searched and analyzed using Protein Prospector v4.25.4 [36] and Mascot Distiller v2.1.

Biological activity of rhIL4 in vitro

Spleen cell suspensions were obtained from C57BL/6 mice as previously described [37], with minor modification. CD19⁺ B cells were isolated through positive selection with microbeads (Miltenyi Biotec) coated with anti-CD19 antibody and Midi-Macs separation columns. Purity was checked by cytometry with mAb and was >97%. CD19⁺ cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, at a concentration of 3×10^5 cells/wells in 96-well plates at 37 °C in a 5% CO₂ atmosphere. Deglycosylated IL-4 (PNGase F treated) and glycosylated IL-4 were added at different concentration. The cells were harvested after 16 h incubation for cell flow cytometry analysis. Cells were incubated with the following mAbs: APC-conjugated anti-CD19 antibody (eBioscience), PE-conjugated

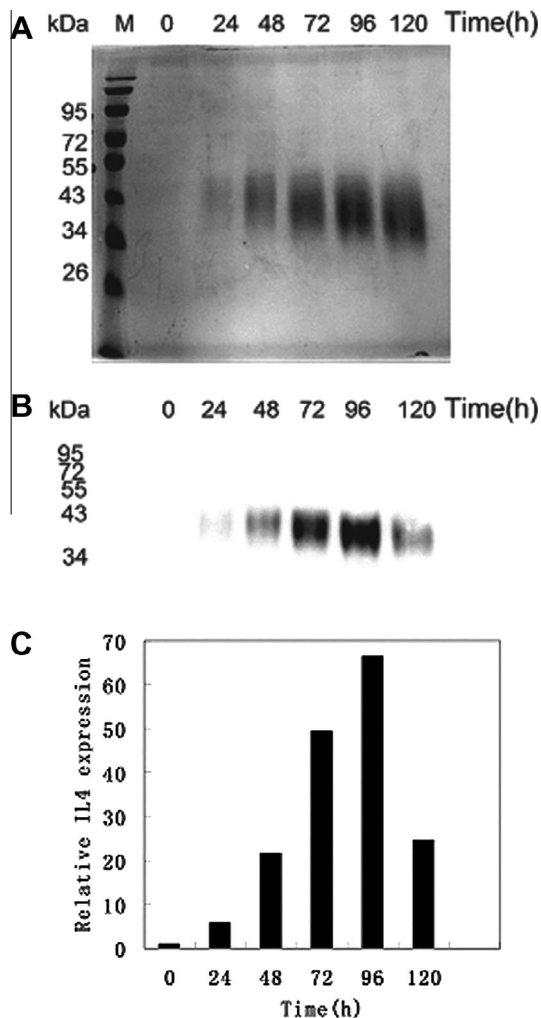


Fig. 1. Time-course dependent rhIL4 expression in *P. pastoris*. (A) Coomassie brilliant blue R-250 stained SDS-PAGE (10%) analysis of rhIL4 expression. Culture supernatants (40 μ l) were collected at 0, 24, 48, 72, 96 and 120 h, respectively. (B) Western blot assay time-course of rhIL4 expression. (C) The relative quantifications of data from Western blotting (B).

anti-CD23 antibody (eBioscience). Analysis was performed with an accuri[®] C6 Flow Cytometer and CellQuest software.

Protein stability

After 96 h methanol induction, the cell culture was divided into several parts and incubated at different temperatures (4 °C, RT and 37 °C) for 3, 7, and 10 days. Cells were pelleted by centrifugation at 3000g at 4 °C for 5 min and then we harvested supernatant and cells respectively. Intracellular protein was extracted according to the manufacturer's instructions of CellTic Y (C 4482 sigma). The stability of rhIL4 in the medium and intracellular cell was determined by Western blotting analysis.

Results

Expression of recombinant hIL4 from *P. pastoris*

The time-course expression of hIL4 in yeast is shown in Fig. 1. After induction with 0.5% methanol, supernatants were collected at indicated time points by centrifugation. The harvested supernatants were subjected to SDS-PAGE and Western blotting assays (Fig. 1A, B). Recombinant hIL4 (rhIL4) was successfully expressed in our optimized culture conditions in a time-dependent manner. The rhIL4 produced in *P. pastoris* appeared as a broad smear band following SDS-PAGE and Western blot analysis (Fig. 1A, B). The molecular mass of rhIL4 ranged from 34 to 50 kDa. The highest expression of hIL4 occurred at 96 h after induction (Fig. 1C).

N-linked glycosylation of the rhIL4

To examine whether the secreted rhIL4 protein was post-translationally glycosylated, rhIL4 isolated from supernatant was treated with or without the enzyme N-glycosidase F (PNGase F) for 2 h and subjected to SDS-PAGE and Western blotting analysis. After treatment, the molecular mass of rhIL4 was reduced to its expected molecular mass of 15 kDa (Fig. 2A, B), and the molecular mass of untreated rhIL4 was still 34–50 kDa (Fig. 2B). This indicates that the 34–50 kDa of rhIL4 from *P. pastoris* was the result of N-glycosylation.

Purification of rhIL-4

The isoelectric point of human IL-4 was 9.2. Extracellular rhIL-4 secreted by *P. pastoris* into the medium was purified to homogeneity by a single cation exchange chromatography method. The result revealed that about 1.5 mg of 97% pure protein was obtained from

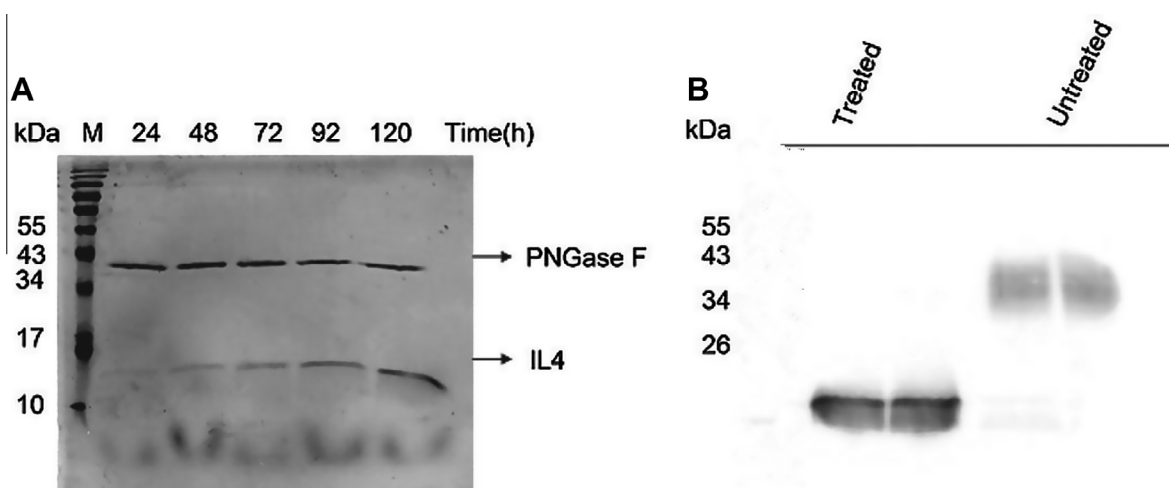


Fig. 2. rhIL4 is glycosylated when it expressed in *P. pastoris*. SDS-PAGE (A) or Western blot (B) of rhIL4 secreted fraction after incubation at 37 °C for 24 h with or without PNGase F treated as indicated.

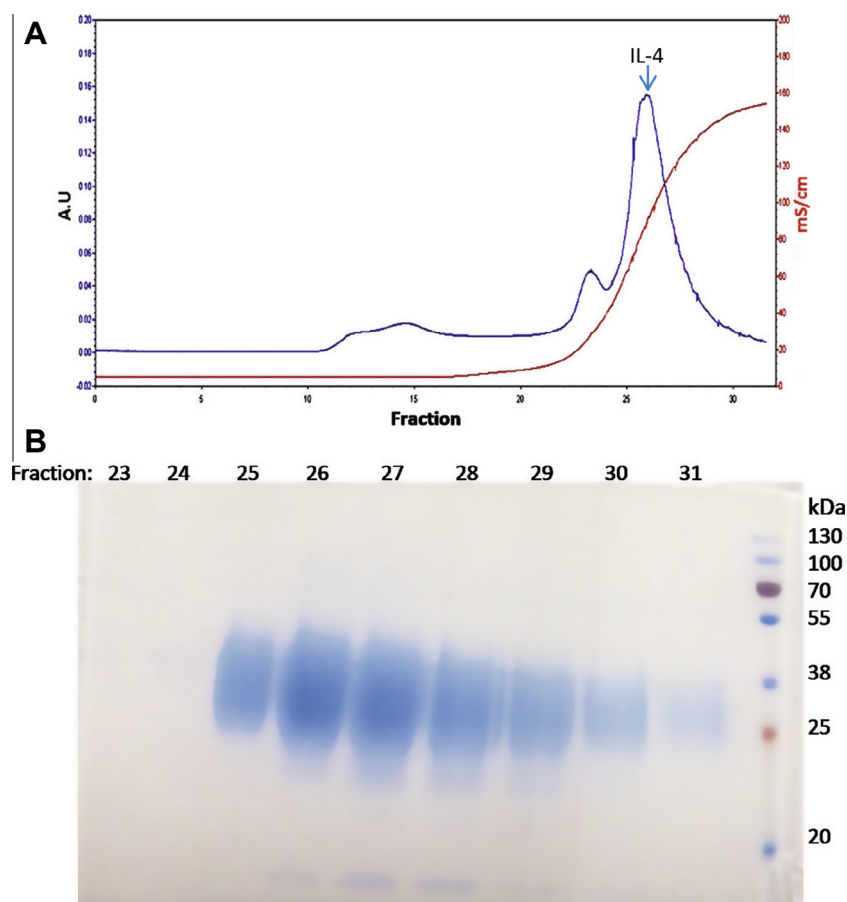


Fig. 3. Chromatographic profiles (A) and SDS-PAGE (B) analysis of the purified rhIL-4. Samples were run on CM-Sepharose columns (1.5×18 cm) under the following conditions: the loading and washing buffer (A) were 20 mM sodium acetate, pH 5.5; the flow rate was 1 ml/min; and the gradient elution (red line) was from 0.0 M NaCl to 1.0 M NaCl in buffer A. Fractions collected during the elution peak were separated by SDS-PAGE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Purification of rhIL4.

Purification step	Total volume (ml)	Total protein (mg)	Purity (%)	Yield ^a (%)
Supernatant after dialysis	100	30	ND	100
CM-sepharose CL-6B	7	1.6	97	55
Ultrafiltration	1.1	1.51	97	53

^a Estimated by SDS-PAGE and Western blotting.

a 100 ml culture after the single step purification (Fig. 3 and Table 1).

Identification of N-glycosylation site(s) of rhIL4

To determine if rhIL4 is glycosylated at two putative N-glycosylation sites at amino acid position 38 and/or 105 (Asn38, Asn105), we generated two hIL4 mutant clones N38A and N105L, respectively. Sequence analysis of the hIL4 mutant plasmids confirmed success of the mutagenesis (Fig. 4A, B). hIL4 with N38A mutation produced a protein completely lacking glycosylation with an expected molecular mass of 15 kDa (Fig. 4C). In contrast, hIL4 with Asn105 mutant (N105L) did not affect the glycosylation of the hIL4 protein. The apparent molecular mass for the Asn105 mutant was the same as for wild type hIL4 (Fig. 4D). Therefore, the Asn38 is the single N-glycosylation site on the hIL4 protein.

The study using variant N105L indicated that Asn105 has no contribution to glycosylation. LC-MS/MS was used to further examine whether Asn105 receives any post-translational modification. As expected, a non-modified asparagine (128 Da) between the y9 and y10 ions in the fragmentation spectrum was clearly identified. The LC-MS/MS spectrum indicated that there is not any change or modification at the Asn105 position in hIL4 (Fig. 5).

rhIL4 induces CD23 expression in B cells

Human IL4 can induce CD23 up-regulation in B cells. To determine whether glycosylation influences the biological activity of rhIL4, mice spleen B cells were incubated with either glycosylated rhIL4 or deglycosylated rhIL4. The expression level of CD23 in B cells was significantly increased after stimulation with both types of rhIL4 (Fig. 6). We compared the capacity of glycosylated rhIL4 and deglycosylated rhIL4 to upregulate cell surface CD23 expression. Our results demonstrated that the rhIL4 exhibited biological activity. Deglycosylated rhIL4, however, has higher biological activity (1–1.5 fold) when compared to glycosylated rhIL4 (0.5 fold) (Fig. 6B).

Protein stability

To determine if N-linked glycans were important for stability of the protein, we have compared the protein stability of glycosylated rhIL4 and non-glycosylated rhIL4 in cells or in culture medium at

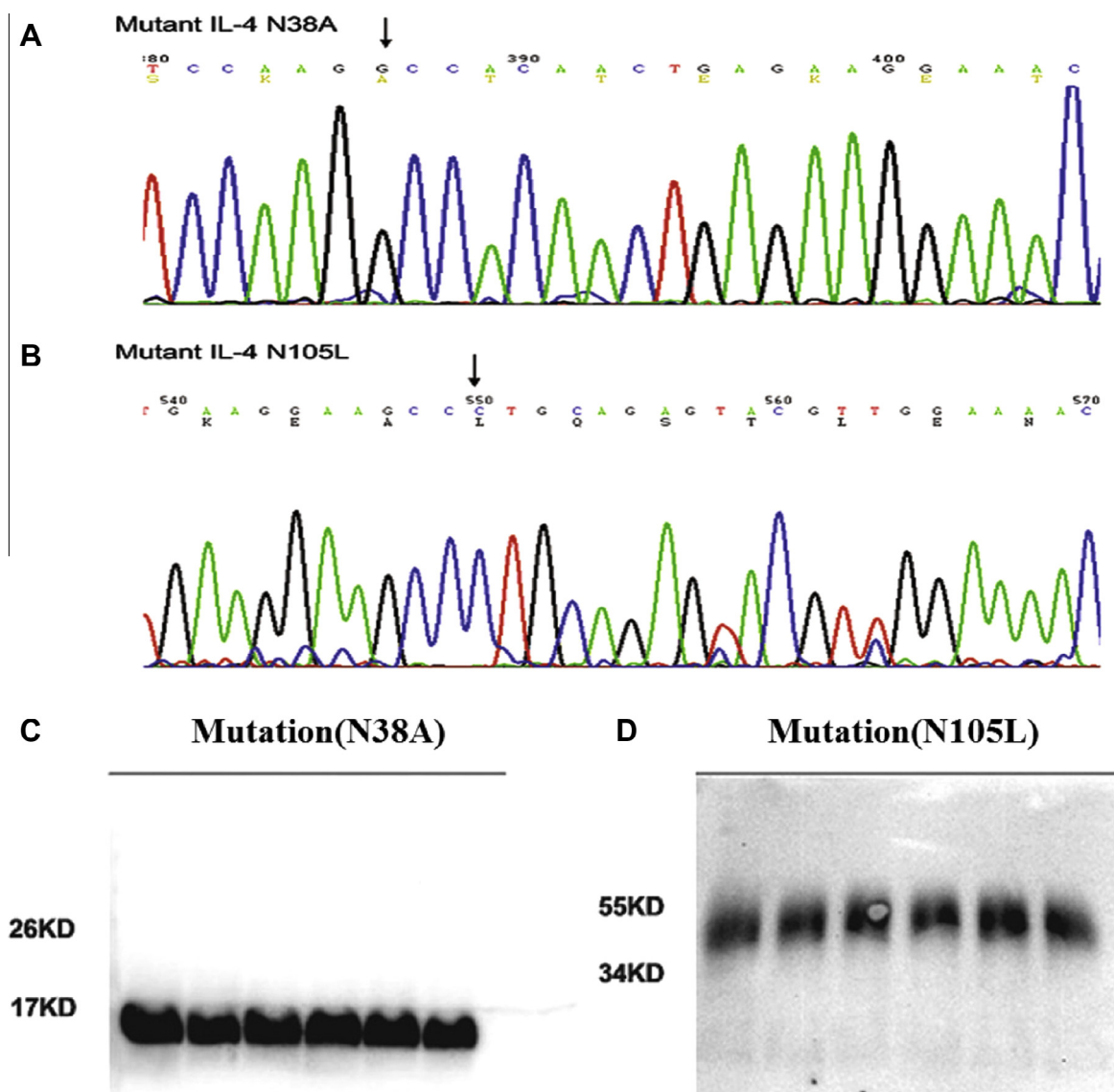


Fig. 4. Expression of variant N38A and N105L. Nucleotide sequences of variants N38A (A) and N105L (B) were analyzed and confirmed. Western blot analysis of hIL4 expression of variant N38A (C) and N105L (D). Supernatant (10 μ l) was collected from 8 individual clones at 96 h. The blots were probed with anti-hIL4 antibodies (1:2500).

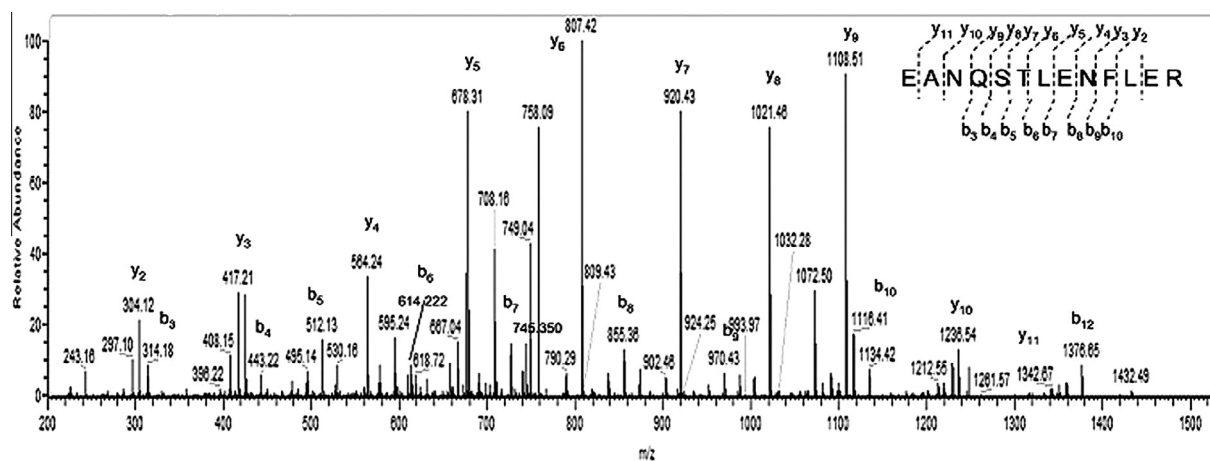


Fig. 5. Identification of non-modified asparagine (Asn105) in a peptide by MS. The peptide containing Asn105 was subjected to sequence analysis using LTQ-FT MS. The mass difference between either the y_9 and y_{10} ions is 128 Da, which corresponds to the asparagine residue at the amino acid 105 position. This confirms that asparagine 105 of hIL4 was not glycosylation site.

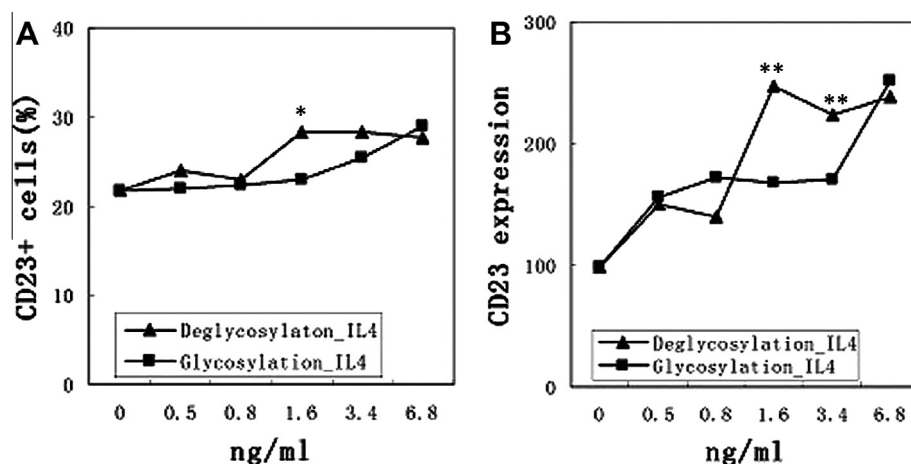


Fig. 6. CD23 expression induced by glycosylated rhIL4 and deglycosylated rhIL4. CD23 expression level on B cells isolated from C57BL/6 mice and stimulated for 16 h with different concentrations of glycosylated rhIL4 or deglycosylated rhIL4. Quantifications of CD23⁺ cells in total B cell population (A), and CD23 expression level in CD23⁺ B cell (B). The number is mean of fluorescence intensity followed by Student's *t* test **p* < 0.05, ***p* < 0.001.

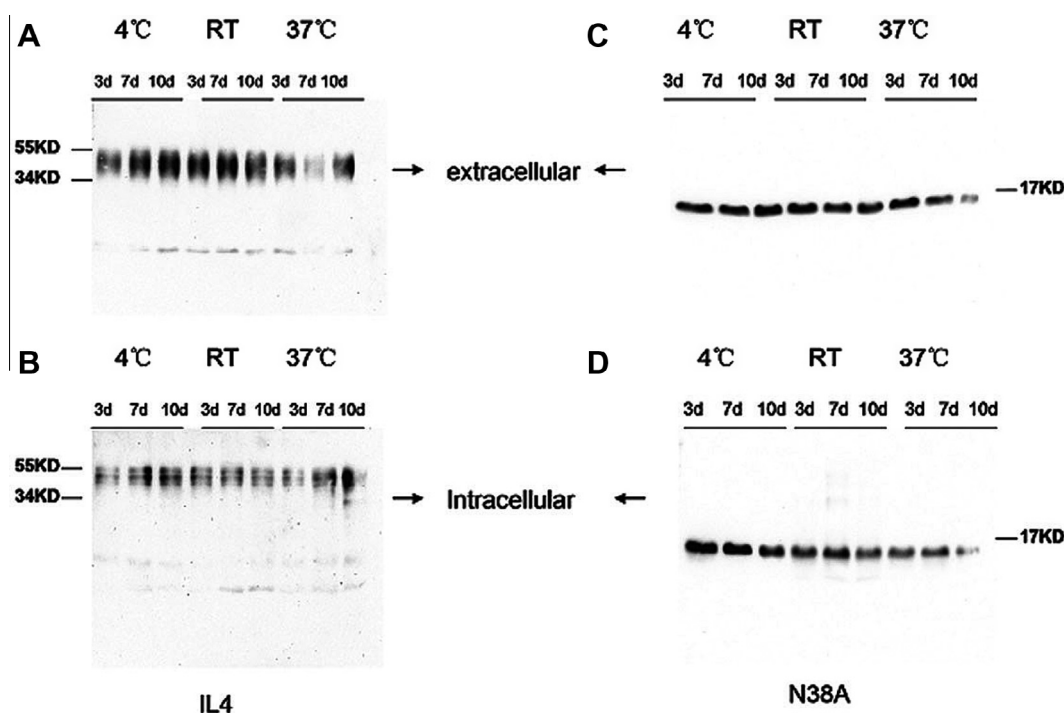


Fig. 7. Stability of glycosylated rhIL4 and non-glycosylated rhIL4 (N38A). Western blotting analysis of the supernatant (20 µl) or cell lysate (40 µl) from wild-type rhIL4 expression yeast (A, B) and 20 µl of supernatant or 40 µl of cell lysate from mutant rhIL4 (N38A) expression yeast (C, D).

different temperatures and days. As shown in Fig. 7, the non-glycosylated rhIL4 is very stable, both in the cell and in the culture medium. There is only a small percentage of protein that was degraded at 37 °C for 10 days. The glycosylated rhIL4 is quite stable in the medium, but is unstable in cells as demonstrated by the high level of degradation when compared to the non-glycosylated form.

Discussion

Biologically active rhIL4 protein was successfully expressed in *P. pastoris* yeast with a yield of 15.0 mg/L. The *P. pastoris* yeast expression system is a useful experimental tool for heterogeneous protein production. In previous studies, soluble hIL4 was produced constitutively in *E. coli* [28,29]. However, human IL4 is a

glycoprotein that participates in physiological and pathological processes in the body, and to our knowledge, the fully glycosylated hIL4 had not been produced so far. It is well known that the *P. pastoris* yeast expression system has the ability to secrete glycosylated and biologically active protein into culture medium. Our laboratory used this expression system to produce soluble hIL4 and obtained a high yield, providing the ability to study the relationship between the glycosylation and biological function of hIL4 protein.

Detailed examination of the effect of N-glycosylation and specifically which glycosylation site plays the major role in human IL4 biologically activity was studied. Treatment of the rhIL4 glycoprotein with PNGase F, caused a decrease in the molecular mass to approximately 15 kDa. Based on this result, we generated two rhIL4 with N-glycosylation point mutations to study the functional

role of each potential N-glycosylation site. Mutants (N38A and N105L) were cultured and induced for rhIL4 secretion. Our study confirmed that Asn38 is the single glycosylation site of hIL4, while Asn105 is not involved.

It was demonstrated that IL4 up-regulated CD23 expression which plays an important role in the differentiation and activation of B lymphocytes [30,31]. To determine whether glycosylation influences biological activity of hIL4, we analyzed the level of CD23 in B cells treated with the rhIL4. Our results demonstrated that glycosylated rhIL4 has lower biological activity than non-glycosylated rhIL4.

The lower biological activity of glycosylated rhIL4 may be caused by degradation following cellular uptake. Previous studies have shown that N-glycans of some growth factors (i.e. human LIF) bear mannose 6-phosphate (Man-6-P) residues. Man-6-P residues are required for efficient uptake in cultured cells, thereby reducing extracellular levels and shortening the protein's half-life. Failure to modify the N-glycans leads to protein stabilization and accumulation in the extracellular space which may have implications for disease pathogenesis [32]. *P. pastoris* produces core N-glycans which contains larger phospho-mannans than from *Saccharomyces cerevisiae*, and both the elongated and the core oligosaccharides are known to contain phosphate [33]. These studies may help explain why the glycosylated rhIL4 expressed from *P. pastoris* has lower biological function when compared to its deglycosylated form. The glycosylated IL4 may have a shorter half-life due to rapid internalization and degradation by cells in culture.

Funding

This study was supported by Shandong University, PR China.

Acknowledgments

We would like to thank Dr. Jinghua Yang for LC-MS/MS analysis. We also are indebted to Dr. Xiangdong Wang for much helpful discussion and information regarding the *Pichia pastoris* protein expression system.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pep.2014.01.005>.

References

- [1] P.E. Goss, M.A. Baker, J.P. Carver, J.W. Dennis, Inhibitors of carbohydrate processing: a new class of anticancer agents, *Clin. Cancer Res.* 1 (1995) 935–944.
- [2] A. Varki, Biological roles of oligosaccharides: all of the theories are correct, *Glycobiology* 3 (1993) 97–130.
- [3] J.W. Dennis, M. Granovsky, C.E. Warren, Protein glycosylation in development and disease, *Bioessays* 21 (1999) 412–421.
- [4] E. Weerapana, B. Imperiali, Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems, *Glycobiology* 16 (2006) 91R–101R.
- [5] B. Yan, W. Zhang, J. Ding, P. Gao, Sequence pattern for the occurrence of N-glycosylation in proteins, *J. Protein Chem.* 18 (1999) 511–521.
- [6] A. Helenius, M. Aebi, Intracellular functions of N-linked glycans, *Science* 291 (2001) 2364–2369.
- [7] A. Helenius, M. Aebi, Roles of N-linked glycans in the endoplasmic reticulum, *Annu. Rev. Biochem.* 73 (2004) 1019–1049.
- [8] E.S. Trombetta, A.J. Parodi, Quality control and protein folding in the secretory pathway, *Annu. Rev. Cell Dev. Biol.* 19 (2003) 649–676.
- [9] S. Wildt, T.U. Gerngross, The humanization of N-glycosylation pathways in yeast, *Nat. Rev. Microbiol.* 3 (2005) 119–128.
- [10] R. Daly, M.T. Hearn, Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production, *J. Mol. Recognit.* 18 (2005) 119–138.
- [11] E. Boer, G. Steinborn, G. Kunze, G. Gellissen, Yeast expression platforms, *Appl. Microbiol. Biotechnol.* 77 (2007) 513–523.
- [12] R.K. Bretthauer, F.J. Castellino, Glycosylation of *Pichia pastoris*-derived proteins, *Biotechnol. Appl. Biochem.* 30 (Pt 3) (1999) 193–200.
- [13] Z. Li, A. Moy, K. Sohal, C. Dam, P. Kuo, J. Whittaker, M. Whittaker, N. Duzgunes, K. Konopka, A.H. Franz, J. Lin-Cereghino, G.P. Lin-Cereghino, Expression and characterization of recombinant human secretory leukocyte protease inhibitor (SLPI) protein from *Pichia pastoris*, *Protein Expr. Purif.* 67 (2009) 175–181.
- [14] C. Carr, S. Aykent, N.M. Kimack, A.D. Levine, Disulfide assignments in recombinant mouse and human interleukin 4, *Biochemistry* 30 (1991) 1515–1523.
- [15] B.M. Curtis, S.R. Presnell, S. Srinivasan, H. Sassenfeld, R. Klinke, E. Jeffery, D. Cosman, C.J. March, F.E. Cohen, Experimental and theoretical studies of the three-dimensional structure of human interleukin-4, *Proteins* 11 (1991) 111–119.
- [16] M.R. Walter, W.J. Cook, B.G. Zhao, R.P. Cameron Jr., S.E. Ealick, R.L. Walter Jr., P. Reichert, T.L. Nagabhushan, P.P. Trotta, C.E. Bugg, Crystal structure of recombinant human interleukin-4, *J. Biol. Chem.* 267 (1992) 20371–20376.
- [17] D.L. Weiss, M.A. Brown, Regulation of IL-4 production in mast cells: a paradigm for cell-type-specific gene expression, *Immunol. Rev.* 179 (2001) 35–47.
- [18] B. Min, W.E. Paul, Basophils and type 2 immunity, *Curr. Opin. Hematol.* 15 (2008) 59–63.
- [19] M. Kronenberg, L. Gapin, The unconventional lifestyle of NKT cells, *Nat. Rev. Immunol.* 2 (2002) 557–568.
- [20] T.D. Mueller, J.L. Zhang, W. Sebald, A. Duschl, Structure, binding, and antagonists in the IL-4/IL-13 receptor system, *Biochim. Biophys. Acta* 1592 (2002) 237–250.
- [21] S. Maeda, Y. Yanagihara, Inflammatory cytokines (IL-4, IL-5 and IL-13), *Nihon Rinsho* 59 (2001) 1894–1899.
- [22] D.Y. Leung, Molecular basis of allergic diseases, *Mol. Genet. Metab.* 63 (1998) 157–167.
- [23] E. Maggi, The TH1/TH2 paradigm in allergy, *Immunotechnology* 3 (1998) 233–244.
- [24] M.E. Stern, K.F. Siemasko, J.Y. Niederkorn, The Th1/Th2 paradigm in ocular allergy, *Curr. Opin. Allergy Clin. Immunol.* 5 (2005) 446–450.
- [25] W.B. van den Berg, L.A. Joosten, E. Lubberts, F.A. van de Loo, P.L. van Lent, Cytokine directed targeting in chronic arthritis, *Eur. Cytokine Netw.* 11 (2000) 481–482.
- [26] S.G. Moreno, G. Laux, M. Briemeier, G.W. Bornkamm, M. Conrad, Testis-specific expression of the nuclear form of phospholipid hydroperoxide glutathione peroxidase (PHGPx), *Biol. Chem.* 384 (2003) 635–643.
- [27] Z. Li, L. Chen, Z. Qin, Paradoxical roles of IL-4 in tumor immunity, *Cell. Mol. Immunol.* 6 (2009) 415–422.
- [28] T. Stoyan, U. Michaelis, H. Schooltink, M. Van Dam, R. Rudolph, P.C. Heinrich, S. Rose-John, Recombinant soluble human interleukin-6 receptor. Expression in *Escherichia coli*, renaturation and purification, *Eur. J. Biochem.* 216 (1993) 239–245.
- [29] A. van Kimmenade, M.W. Bond, J.H. Schumacher, C. Laquai, R.A. Kastelein, Expression, renaturation and purification of recombinant human interleukin 4 from *Escherichia coli*, *Eur. J. Biochem.* 173 (1988) 109–114.
- [30] B. Bettler, H. Hofstetter, M. Rao, W.M. Yokoyama, F. Kilchherr, D.H. Conrad, Molecular structure and expression of the murine lymphocyte low-affinity receptor for IgE (Fc epsilon RII), *Proc. Natl. Acad. Sci. USA* 86 (1989) 7566–7570.
- [31] G. Delespesse, H. Hofstetter, M. Sarfati, Low-affinity receptor for IgE (FcERII, CD23) and its soluble fragments, *Int. Arch. Allergy Appl. Immunol.* 90 (Suppl 1) (1989) 41–44.
- [32] J. Barnes, J.M. Lim, A. Godard, F. Blanchard, L. Wells, R. Steet, Extensive mannose phosphorylation on leukemia inhibitory factor (LIF) controls its extracellular levels by multiple mechanisms, *J. Biol. Chem.* 286 (2011) 24855–24864.
- [33] F.D. Ziegler, J. Cavanagh, C. Lubowski, R.B. Trimble, Novel schizosaccharomyces pombe N-linked GalMan9GlcNAc isomers: role of the Golgi GMA12 galactosyltransferase in core glycan galactosylation, *Glycobiology* 9 (1999) 497–505.
- [34] A. Joubel, R.J. Chalkley, K.F. Medzihradszky, H. Hondermarck, A.L. Burlingame, Identification of new p53 acetylation sites in COS-1 cells, *Mol. Cell. Proteomics* 8 (2009) 1167–1173.
- [35] F. Chu, D.A. Nusinow, R.J. Chalkley, K. Plath, B. Panning, A.L. Burlingame, Mapping post-translational modifications of the histone variant MacroH2A1 using tandem mass spectrometry, *Mol. Cell. Proteomics* 5 (2006) 194–203.
- [36] R.J. Chalkley, P.R. Baker, L. Huang, K.C. Hansen, N.P. Allen, M. Rexach, A.L. Burlingame, Comprehensive analysis of a multidimensional liquid chromatography mass spectrometry dataset acquired on a quadrupole selecting, quadrupole collision cell, time-of-flight mass spectrometer: II. New developments in protein prospector allow for reliable and comprehensive automatic analysis of large datasets, *Mol. Cell. Proteomics* 4 (2005) 1194–1204.
- [37] P. Mina-Osorio, M. Rosas-Ballina, S.I. Valdes-Ferrer, Y. Al-Abed, K.J. Tracey, B. Diamond, Neural signaling in the spleen controls B-cell responses to blood-borne antigen, *Mol. Med.* 18 (2012) 618–627.