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Research Article

Characterization and high-yield production of non-*N*-glycosylated recombinant human BCMA-Fc in *Pichia pastoris*

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B-cell maturation antigen (BCMA) fused at the C-terminus to the Fc portion of human IgG1 (BCMA-Fc) blocks B-cell activating factor (BAFF) and proliferation-inducing ligand (APRIL)-mediated B-cell activation, leading to immune disorders. The fusion protein has been cloned and produced by several engineering cell lines. To reduce cost and enhance production, we attempted to express recombinant human BCMA-Fc (rhBCMA-Fc) in *Pichia pastoris* under the control of the *AOX1* methanol-inducible promoter. To produce the target protein with uniform molecular weight and reduced immunogenicity, we mutated two predicted *N*-linked glycosylation sites. The secretory yield was improved by codon optimization of the target gene sequence. After fed-batch fermentation under optimized conditions, the highest yield (207 mg/L) of rhBCMA-Fc was obtained with high productivity (3.45 mg/L/h). The purified functional rhBCMA-Fc possessed high-binding affinity to APRIL and dose-dependent inhibition of APRIL-induced proliferative activity in vitro through three-step purification. Thus, this yeast-derived expression method could be a low-cost and effective alternative to the production of rhBCMA-Fc in mammalian cell lines.

Keywords: APRIL / B-cell maturation antigen / *N*-glycosylation / *Pichia pastoris* / Recombinant human protein

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1 Introduction

B-cell maturation antigen (BCMA; TNFRSF17) [1], a member of tumor necrosis family receptor (TNFR), and transmembrane activator, calcium-modulator, and cyclophilin ligand interactor (TACI; TNFRSF13) [2] both bind to a proliferation-inducing ligand (APRIL; TNFSF13) [3] and B-cell activating factor (BAFF; TNFSF13B) from the TNF superfamily [4] but with different affinities [5, 6].

The binding triggers various signaling pathways, which play pivotal roles in the regulation of the immune balance. Dissonance

of the BAFF/APRIL system has been linked to the development of various cancers and autoimmune disorders [6, 7]. Agents such as monoclonal antibodies blocking BAFF or APRIL have been proved effective against autoimmune diseases like systemic lupus erythematosus [8, 9].

rhBCMA-Fc, a recombinant human BCMA and IgG1Fc fusion protein, acts as a decoy receptor that competes with inherent receptors by binding to BAFF and APRIL, resulting in marker B-cell reduction, thereby blocking the pathological function induced by both ligands [10]. Since the demonstration of the treatment effect in the experimental autoimmune encephalomyelitis model of the BCMA-Ig fusion protein, a number of studies have shown the immunosuppressive therapeutic function of rhBCMA-Fc in autoimmune diseases and B-cell malignancies [11–14]. B-cell depletion achieved with BCMA-Fc provides a new approach for combination immunization with

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Abbreviations: APRIL, proliferation-inducing ligand; BAFF, B-cell activating factor; BCMA, B-cell maturation antigen; ECD, extracellular domain; rhBCMA-Fc, recombinant human BCMA and IgG1Fc fusion protein; TNFR, tumor necrosis family receptor; wt, wild-type; Non-*N*-Gly, non-*N*-glycosylated recombinant; Co-Non-*N*-Gly, codon-optimized-non-*N*-glycosylated recombinant

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insulin in treating type 1 diabetes [15]. Moreover, rhBCMA-Fc has been found to inhibit colorectal tumor growth induced by APRIL, supporting the role of BCMA-Fc for combination treatment of solid tumors [16].

rhBCMA-Fc has been designed and produced by numerous laboratories using mammalian hosts such as human embryonic kidney 293 cells and NS0 cells [12, 17]. However, recombinant protein produced from mammalian cells usually translates into high costs, low production efficiency, difficult purification, and risk of viral contamination. Therefore, the efficient production of rhBCMA-Fc at a low cost with easy purification to meet the demands of scientific research is urgently required.

Pichia pastoris is one of the most commonly used expression hosts owing to its strong and tightly regulated promoter of *AOX1* and well-established folding and modification machinery [18]. Moreover, it is an ideal expression system for producing secreted foreign proteins because it secretes few endogenous proteins into the culture medium, rendering its purification much simpler compared to other systems such as *Escherichia coli* [19]. Products like membrane proteins and bioactive peptides have been successfully obtained from *P. pastoris*, which could be a promising alternative to other expression systems and even chemical synthesis [20, 21]. Nevertheless, strategies to improve the production of rhBCMA-Fc in *P. pastoris* are warranted.

At the outset, different N-terminal forms of secreted protein usually occur since there are two different cleavage sites, *Kex2* and *Ste13*, in the sequence of the α -factor signal peptide. In a different study, a lysine and an arginine were added after the *XhoI* site in the 5' PCR primer to provide a *Kex2* cleavage site, thus allowing the signal peptide be cleaved after the Lys-Arg sequence to maintain the native N-terminal of the target protein [22]. For cloning convenience, we removed the extra *XhoI* site in the commercial vector pPIC9K. Besides, the *P. pastoris* system usually produces human origin protein with different glycosylation modifications, which leads to the production of heterogeneous target proteins. These modifications may limit its therapeutic applications because of the possibility of human immunogenicity [23]. Kobayashi et al. found that the expression of functional G-protein-coupled receptor was successful in *P. pastoris* by substituting amino acids at the N-glycosylation site to eliminate N-glycosylation [24]. Through the secretory pathway, the yeast expression system selects asparagine (N or Asn) residues within the peptide sequence N-X-S/T (X refers to any amino acid) to form a site for the covalent attachment of oligosaccharide (i.e. N-glycosylation). The replacement of N in the N-X-S/T sequence by Q (Gln) has therefore been performed to acquire uniform rhBCMA-Fc. Moreover, codon optimization has been used to circumvent the influence of foreign protein expression by codon usage bias [25, 26].

With the ultimate aim of scaling up the production of rhBCMA-Fc, we attempted to express rhBCMA-Fc in *P. pastoris* strain GS115 and characterize it.

2 Materials and methods

2.1 Strains, vectors, reagents, and growth conditions

The *E. coli* strain DH5 α (Biodev, China) was used for the propagation and manipulation of plasmids. *Pichia pastoris* strain GS115

and vector pPIC9K were obtained from Invitrogen (Carlsbad, CA, USA). All media for yeast cultivation were prepared according to the manufacturer's instructions from the Multi-Copy Pichia expression kit (Invitrogen, Carlsbad, CA, USA). Primer synthesis and DNA sequencing were performed by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China). Restriction enzymes and T4 DNA ligase were purchased from Fermentas (Shanghai, China). PCR purification and gel extraction kits were obtained from QIAGEN (Valencia, CA, USA). Anti-human BCMA/TNFRSF17 and anti-human APRIL/TNFSF13 antibodies were purchased from R&D (MN, USA) and Abcam (Shanghai, China), respectively. The Raji cell line obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) was grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% v/v FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

2.2 Homology modeling and molecular docking

Homology modeling and docking simulation were performed by Accelrys Discovery Studio (Discovery Studio, version 3.1). The structure of the BCMA-APRIL complex (PDB ID: IXU2) and IgG1Fc (PDB ID: 1H3U) were obtained from the protein structure database from the Protein Data Bank. The 3D model of the extracellular domain of BCMA was extracted from the BCMA-APRIL complex template. The BCMA-Fc fused protein model was constructed by homology modeling and then refined by an optimized geometry calculation of the mechanics using augmented CHARMM (Accelrys). After assessing the refined models based on geometry and energy, the optimal structure of BCMA-Fc was acquired and superimposed onto the extracellular domain (ECD) of BCMA. Then, we docked the modeled structure of BCMA-Fc with the crystal structure of APRIL extracted from the BCMA-APRIL complex using ZDOCK to acquire predictive complexes. After reranking with van der Waals, desolvation, and electrostatics energy terms, the best docking complex was finally selected.

2.3 DNA manipulation, plasmid construction, and sequencing

The sequence of the BCMA-Fc fragment was amplified by overlap PCR with a soft linker GSGGSG, using a sense primer containing a *XhoI* site, the *Kex2* protease recognition sequence (AAAAGA), and an antisense primer containing a *NotI* site. Thus, the *Ste13* protease cleavage site was removed to maintain the N-terminal homogeneity of BCMA-Fc. The extra *XhoI* site located at 4475–4480 of the commercial pPIC9K vector was mutated by overlap PCR. The resulting PCR product was ligated into pPIC9K between *NruI* and *SphI* sites to form a new modified vector with only 1 *XhoI* site. Then, the amplified PCR product of BCMA-Fc was digested with *XhoI* and *NotI* and ligated into the modified pPIC9K vector. The recombinant fragment was verified by restriction enzyme digestion and further confirmed by gene sequencing by the Shanghai Sangon sequencing service.

Table 1. Primers used in this study (restriction sites are italics, linker sequences are underlined and all mutations are in bold letters)

Primers	sequence (5'–3')
For pPIC9K modification	
P1-F	5'-GGCTCGCGAGCCCATTTATACCCAT-3'
P2-R	5'-CATGCATGCAAGGAGATGGCGCCCA-3'
P3-F	5'-GCGGGCGCGATCAAGAC-3'
P4-R	5'-TCTTGATCGCGCCCGC-3'
For BCMA-Fc fusion	
B-Fc-F	5'-CCGCTCGAGAAAAGAATGTTGCAGATGG-3'
B-Fc-R	5'-ATAAGAATGCGGCCGCTTATTTACCCGAGACAG-3'
Fc-F	5'-GGTTCGGCGGCTCTGGTTCTTGCCACCGTGCCAG-3'
B-R	5'-ACCAGAGCCGCCAGAACC CGCATTCGTTCTTC-3'
For non-N-glycosylation mutation	
B-Fc-F	5'-CCGCTCGAGAAAAGAATGTTGCAGATGG-3'
B-Fc-R	5'-ATAAGAATGCGGCCGCTTATTTACCCGAGACAG-3'
P9-F	5'-CAGCGTTATTTGTCAAGCAAGTGTGAC-3'
P10-R	5'-GTCACACTTGCTTGACAATAACGCTG-3'
P11-F	5'-AGGAGCAGTACCAAGCAGTACCGT-3'
P12-R	5'-ACGGTACGTGCTTTGTTACTGCTCCT-3'
For quantitative RT-PCR	
B-Fc-F	5'-CCGCTCGAGAAAAGAATGTTGCAGATGG-3'
B-Fc-R	5'-ATAAGAATGCGGCCGCTTATTTACCCGAGACAG-3'
Co-F	5'-ATGTTGCAATGGCTGGT-3'
Co-R	5'-TACTTACCTGGAGACAAAGA-3'
GAPDH-F5'	CATCTCTGCCCTCTGCTGA-3'
GAPDH-R5'	GGATGACCTTGCCACAGCCT-3'

To construct the non-*N*-glycosylated BCMA-Fc, 2 N (Asn) residues at the predicted *N*-linked glycosylation sites (N42, N133) were replaced with 2 Q (Gln) residues by site-directed mutation. The codons predicted to be less frequently used in *P. pastoris* were then replaced by the most frequently occurring triplets without changing the encoded amino acid sequence by Generay (Shanghai, China) based on the codon usage table of *P. pastoris* (<http://www.kazusa.or.jp/codon>). The confirmed sequence was digested by *XhoI* and *NotI* and cloned into modified pPIC9K to yield the reconstructed plasmid (pPIC9K-rhBCMA-Fc). All the primers used in this study are listed in Table 1.

2.4 Recombinant screening and shaking flask culture

The three different types of recombinant plasmids of pPIC9K-BCMA-Fc were designated as wild-type (wt), non-*N*-glycosylation (Non-*N*-Gly), and codon-optimized-non-*N*-glycosylation (Co-Non-*N*-Gly). They were linearized with *Sall* and transformed into GS115 by electroporation with a GenePulser system (Bio-Rad, Carlsbad, CA, USA) at 1.5 kV, 200 Ω , and 25 μ F. His⁺ transformants were selected on histidine-deficient minimal dextrose plates (2% glucose, 1.34% yeast nitrogen base with ammonium sulfate, 0.04% biotin) and screened by their level of resistance to different concentrations of geneticin (G418; 1, 2, and 4 mg/mL) on yeast extract-peptone-dextrose (YPD)-G418 plates (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) for 2–4 days. After analysis by PCR and sequencing, single colonies of the three different transformants exhibiting resistance to 2.0 mg/mL geneticin were inoculated into

5 mL BMGY (2% peptone, 1% yeast extract, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base (without amino acids), 0.4 μ g/mL biotin, and 1% glycerol) with constant shaking (250 rpm) at 30°C till the OD₆₀₀ reached approximately 4. The cells were pelleted by centrifugation at 3000 rpm for 15 min and transferred into 5 mL BMMY (2% peptone, 1% yeast extract, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base (without amino acids), 0.4 μ g/mL biotin, and 0.5% methanol). After incubation at 30°C with constant shaking at 250 rpm for 12 h, 1% v/v methanol was added every 24 h to induce protein expression. After 72-h induction, the cells were pelleted by centrifugation at 3000 rpm and 4°C for 5 min. All the supernatant samples of every 24 h were saved for SDS-PAGE analysis and further identification.

2.5 RT-PCR, protein determination, and Western blot analysis

The transcriptional levels of the wt BCMA-Fc and Co-Non-*N*-Gly BCMA-Fc strains were compared by RT-PCR analysis. Total RNA was isolated using TRIzol, and cDNA was synthesized using Superscript II reverse transcriptase following the manufacturer's instructions (Invitrogen, MN, USA). The relative RNA levels were estimated on the basis of agarose gel imaging with ImageJ 1.49. The amplification conditions were 95°C for 60 s; 35 cycles of 95°C for 20 s, 57°C for 20 s, and 72°C for 1 min; and 72°C for 5 min.

SDS-PAGE analysis was performed to compare the expression level of these different strains. Samples of every 24 h obtained from shaking flask culture were loaded on 12% gels followed by CBB R-250 staining. For immune blotting analysis, proteins resolved by SDS-PAGE were electrotransferred to a PVDF membrane using a wet electrophoretic transfer system (GE Healthcare, Bucks, UK) at 200 mA for 150 min. After the membrane was blocked in 5% skim milk prepared in TBS containing 0.05 % Tween-20 (TBST) at room temperature for 2 h, it was incubated with mouse anti-human BCMA monoclonal antibody (1:1000 dilution) overnight at 4°C. The membrane was washed thrice with TBST the next morning and incubated with HRP-conjugated goat anti-mouse IgG (1:5000 dilution) for 60 min at room temperature. The immunoreactive bands were visualized with PierceTM ECL Plus Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA).

2.6 Optimized expression of rhBCMA-Fc in fed-batch fermentation

Fermentation studies were carried out in 5-L bioreactors (Bioflo 3000 fermenter, New Brunswick Scientific, New Brunswick, NJ, USA) containing 3-L sterilized basal salts medium (4.25 mL/L orthophosphoric acid, 9.4 mM MgSO₄, 1 mM, CaSO₂, 16.4 mM K₂SO₄, 11.4 mM KOH, and 50 mL/L glycerol) plus 2 mL/L PTM₁ solution (24 mM CuSO₄, 0.53 mM NaI, 19.87 mM MnSO₄, 0.83 mM Na₂MoO₄, 0.32 mM boric acid, 2.1 mM CoCl₂, 0.15 mM ZnCl₂, 0.23 M FeSO₄, and 0.82 mM biotin). Ammonium was used as both a pH control reagent and nitrogen source. The dissolved oxygen level was maintained at over 35% of air saturation

Figure 1. (A) 3D structure of the BCMA-APRIL complex (PDB ID: 1XU2). (B) 3D structure of IgG1Fc (PDB ID: 1H3U). (C) Structure of the ECD of BCMA separated from the APRIL-BCMA complex. (D) Structure of BCMA-Fc without optimization. (E) Optimal structure of BCMA-Fc. (F) Optimal BCMA-Fc superimposed onto the ECD of BCMA and unmodified BCMA-Fc. Optimal BCMA-Fc is shown in blue; unmodified BCMA-Fc, gray; and the ECD of BCMA, red. (G) Optimal BCMA-Fc docked with APRIL. The docked complexes are superimposed onto the ECD of BCMA and unmodified BCMA-Fc. APRIL is shown in orange. The interaction surface of BCMA and APRIL is violet. The other models are as described above.

by a cascaded control of agitation speed and aeration rate. The Co-Non-*N*-Gly BCMA-Fc inoculum seeds were cultured in 200 mL YPD overnight and inoculated into the bioreactors. Three pH values (4.5, 5.5, and 6.5) and three temperatures (20, 25, and 30°C) were examined to optimize the protein yield. Continuous glycerol feeding was used as the sole carbon source. When the dissolved oxygen and pH suddenly increased markedly (indicated by complete consumption of all glycerol), pure methanol containing 2 mL/L PTM₁ solution was added into the fermenter at a gradient feeding rate from 0.8 to 4 mL/L to allow the culture to adapt to the methanol. After every 6 h, samples of the fermentation broth were collected to measure the OD₆₀₀ and analyzed by 12% SDS-PAGE. A BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the protein amount by measuring the absorbance at 562 nm on a microplate reader (Thermo Fisher Scientific, USA). Data were analyzed with Prism 6 (GraphPad Inc., San Diego, CA, USA).

2.7 Purification of rhBCMA-Fc

Cells in the fermentation culture were separated by centrifugation (4°C, 8000 × *g*, 15 min). The supernatant was concentrated by ultrafiltration through a 10-kDa cut-off Amicon Ultra-15 centrifugal filter (Merck Millipore, USA) and applied to Sephadex G-50 high-resolution columns (GE Healthcare, UK) equilibrated with 20 mM phosphate buffer (pH 7.0). The flow-through fractions containing target protein were collected and further purified by passing them through a Protein A column equilibrated with 20 mM phosphate buffer (pH 7.0) in advance at a rate of 0.8 mL/min. The column was washed with the same buffer at a rate of 1.2 mL/min to eliminate unbound proteins. rhBCMA-Fc then was eluted with 50 mM glycine (pH 3.0). After the elution fraction was neutralized with 1 M Tris-HCl buffer (pH 9.0), the eluant containing rhBCMA-Fc was collected for SDS-PAGE analysis and quantified by the bicinchoninic acid

assay. The N-terminal sequencing services were performed by Shanghai Genecore Biotechnologies (Shanghai, China). Finally, the purified rhBCMA-Fc samples were lyophilized and stored at −80°C until further use.

2.8 Identification of BCMA-Fc by MALDI-TOF MS

The purified fusion protein spots from fresh CBB-stained gels were excised and plated onto 96-well plates. After the spots were destained with 60 μL NH₄HCO₃ (50 mM) and 50% acetonitrile twice, they were dried with 60 μL acetonitrile twice. The dried slices were dissolved using ice-cold digestion solution (12.5 ng/μL trypsin plus 20 mM NH₄HCO₃) for 20 min and then incubated at 37°C for digestion overnight. Next, peptides were extracted with 60 μL extraction solution (5% formic acid in 50% acetonitrile) twice.

The peptide solution was dried under the protection of nitrogen, and 0.8 μL matrix solution (5 mg/mL α -cyano-4-hydroxycinnamic acid diluted in 0.1%TFA, 50% ACN) was added to dissolve the peptides. Then, the mixture was loaded onto a MALDI target plate and analyzed using an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). This work was conducted in collaboration with the Proteome Research Center at Fudan University.

2.9 Bioassay and characterization of rhBCMA-Fc

2.9.1 Protein A pull-down analysis

The binding affinity between purified rhBCMA-Fc fusion protein and APRIL was tested by the pull-down assay according to a previous report [27]. The active extracellular domain of human APRIL (hAPRIL) was expressed and identified in our laboratory. Purified rhBCMA-Fc (50 μg) diluted in 100 μL PBS was incubated with 50 μL prepared Protein A beads for 2 h at 4°C with

A

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1  ATGTTGCAGATGGCTGGGCGAGTCTCCAAAATGAATATTTTGACAGTTTGTTCATGCT
*****
1  ATGTTGCAAAATGGCTGGTCAATGTTCTCAAAACGAATACTTGCAGCTTTTGTTCACGCT
61  TGCATACCTTGTCAACTTCGATGTTCTTCTAATACTCCTCTCAACATGTCAGCGTTAT
*****
61  TGTATCCCATGTCAATTGAGATGTTCTTCTAACACTCCACCATTGACTTGTCAAAGATAC
121  TGTAAATGAAGTGTGACCAATTTCAGTGAAGAACGAATCGGGTCTGGCGGCTCTGGT
*****
121  TGTCAAGCTCTGTTACTAAGCTGTTAAGGGTACTAACGCTGGTCTGGTGGTCTGGT
181  ACATGCCACCGTGGCCAGCACCTGAACCTCGGGGGGACGTCAGTCTTCTCTTCCCC
*****
181  ACTTGTCCACCATGTCCAGCTCCAGAATTGTTGGGTGGTCCATCTGTTTCTTGTCCCA
241  CCAAACCAAGGACACCTCATGATCTCCCGGACCCCTGAGGTACATGCTGGTGGTG
*****
241  CCAAAGCCAAAGGACACTTTGATGATCTCTAGAATCCAGAAGTTACTTGTGTGTGTT
301  GACGTGAGCCACGAAGCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCTGGAGGTG
*****
301  GACGTTTCTCACGAAGCCAGAAGTTAAGTTCACTGGTACGTTGACGGTGTGAAGTT
361  CATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACACAGCAGTACCGTGTGGTCAGC
*****
361  CACAACGCTAAGACTAAGCCAAGAGAAGAACTACCAATCTACTTACAGATTGTTTCT
421  GTCCTCACCGTCTGCACAGGACTGGCTGAATGGCAAGGAGTACAAGTGAAGGTCTCC
*****
421  GTTTTGACTGTTTTCACCAAGACTGGTTGAACGTAAGGAATACAAGTGAAGTTTCT
481  AACAAAGCCCTCCAGCCCCATCGAGAAACCATCTCAAAGCCAAAGGGCAGCCCCGA
*****
481  AACAAAGGCTTTCAGCTCAATCGAAAGACTATCTTAAGGCTAAGGGTCAACCAAGA
541  GAACACAGGTGTACACCTGCCCCATCCCGGGATGAGTGACCAAGAACAGGTGACG
*****
541  GAACCAAGTTTACACTTTGCCACCATCTAGAGACGAATTGACTAAGAACAAGTTTCT
601  CTGACCTGCCTGGTCAAAGGCTTATCCAGCGACATCGCGTGGAGTGGGAGAGCAAT
*****
601  TTGACTTGTGTTAAGGGTTTCTACCCATCTGACATCGCTGTTGAATGGGAATCTAAC
661  GGGCAGCCGGAGAACTACTACAAGACCACGCT2CCGTGCTGGACTCCGACGGCCCTTC
*****
661  GGTCAACGAGAAACAACAAGACTACTCCACAGTTTGGACTCTGACGGTCCATTC
721  TTCTCTACAGCAAGCTCACCGTGACAAAGCAGGTGGCAGCAGGGGAACGCTTCTCA
*****
721  TTCTGTACTCTAAGTTGACTGTTGACAAGTCTAGATGGCAACAGGTAACGTTTCTCT
781  TGCTCCGTGATGATGAGGCTCTGCACAACCACTACACGAGAAGACCTCTCCCTGTCT
*****
781  TGTCTGTTATGCACGAAGCTTTCACAACCACTACACTCAAAGTCTTGTCTTTGTCT
841  CCGGGTAATA
*****
841  CCAGGTAAG

```

B

Figure 2. (A) Sequence alignment of wild-type (upper line) and codon-optimized non-*N*-glycosylated BCMA-Fc (lower line) based on the *P. pastoris* codon usage table. (B) mRNA levels determined by RT-PCR and normalized to GAPDH expression measured in parallel experiments. No significant difference was observed between wt and Co-Non-*N*-Gly BCMA-Fc ($p > 0.05$, *t*-test). Data are presented as the mean $\pm$ SEM of three experiments. a: BCMA-Fc, b: GAPDH, 1: wt BCMA-Fc, 2: Co-Non-*N*-Gly BCMA-Fc.

PBS used as a control and then mixed with 40 μ L hAPRIL diluted in 100 μ L PBS. After overnight incubation at 4°C on a rocker platform, the beads were washed with PBS 4 times and eluted by 0.1 M glycine-HCl (pH 3.0). All the samples were ready for SDS-PAGE analysis and Western blot assay using the anti-hAPRIL antibody.

2.9.2 Proliferation and apoptosis analysis

To analyze the biological activity of the BCMA-Fc fusion protein, the B lymphoma cell line Raji (1×10^4 /well) was cultured in 100 μ L RPMI 1640 without phenol red, supplied with 10% heat-inactivated FBS on a 96-well plate. Cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used to measure cell viability based on a previous study [28]. After we determined the optimal concentration (100 ng/mL) of APRIL to stimulate the proliferation of Raji cells, 100 ng/mL APRIL with different gradient concentrations of rhBCMA-Fc (0, 250, 500, 1000, and 2000 ng/mL) or corresponding concentrations of albumin (from BSA) as controls were added to each well for incubation in triplicate at 37°C for 24 h. After additional incubation for 5 h at 37°C, 10 μ L CCK-8 solution was added to each well, followed by gentle mixing on an orbital shaker. The absorbance of each sample was measured using the microplate reader at a wavelength of 490

nm. The effects were assessed as cell viability according to the following formula:

$$\text{Cell viability (\%)} = [(At - Ab) / (Ac - Ab)] \times 100\%,$$

where *At* is the absorbance of treatment wells (APRIL, BCMA-Fc, and CCK-8), *Ac* is the absorbance of control wells (APRIL, PBS, and CCK-8), and *Ab* is the absorbance of a blank well (CCK-8 only).

Statistical analysis was performed using the appropriate unpaired Student's *t*-test by Prism 6 and *p*-values < 0.05 were considered statistically significant. Data were presented as means $\pm$ SEM.

3 Results

3.1 Modeling analysis of the fusion protein

The model of the extracellular domain of human BCMA (BCMA-ECD) (Fig. 1C) was separated from the APRIL-BCMA complexes (Fig. 1A) (PDB ID: 1XU2) and linked to the N-terminal of IgG1Fc (Fig. 1B) (PDB ID: 1H3U) by a soft linker. The model of the fused protein BCMA-Fc is shown in Fig. 1D, and the refined optimum model is shown in Fig. 1E. Its

Figure 3. SDS-PAGE analysis of BCMA-Fc in shake-flask culture. (A) Supernatants of the transformant wt BCMA-Fc. Target bands are marked with braces. (B) Supernatants of the transformant non-*N*-Gly BCMA-Fc. (C) Supernatants of the transformant Co-non-*N*-Gly BCMA-Fc. Target bands are labeled with arrows. Lanes 1–4 were loaded with 20 μ L supernatants obtained every 24 h (0, 24, 48, and 72 h). (D) Western blot assay of the samples from (A), (B), and (C) at 24, 48, and 72 h.

superimposition onto BCMA-ECD and unmodified BCMA-Fc suggested that the difference between optimum BCMA-Fc and BCMA-ECD is less than 3.0 (Fig. 1F). The docking result between APRIL and the superimposed complexes (Fig. 1G) suggested that the influence of the IgG1Fc domain on BCMA's binding affinity to APRIL was not considered in this study.

3.2 Construction of the recombinant pPIC9K-BCMA-Fc expression vector

A flexible linker GGTCTGGCGGCTCTGGT was used to link BCMA-ECD and the Fc domain of IgG1. Next, the 849-bp gene fragment was inserted into the open reading frame of the modified pPIC9K vector.

Amino acid sequence analysis from the PROSITE website (<http://prosite.expasy.org>) showed that this transcription unit may form N-linked glycosylation and cause secretory protein heterogeneity. Therefore, the substitution of N by Q in N-X-S/T was performed by overlapping PCR.

The efficiency of heterologous BCMA-Fc production in *P. pastoris* can be greatly diminished by codon usage bias. Thus, some BCMA-Fc codons were altered into codons occurring with high frequency in the *P. pastoris* genome (Fig. 2A). The GC% content of the Co-Non-*N*-Gly-BCMA-Fc sequence rose to 56.54% from the original 42.17%, but there were no changes in the amino acid sequence except for 2 non-*N*-glycosylation sites mentioned above. Enhanced protein expression was demonstrated by SDS-PAGE performed below. However, no obvious difference was found between the mRNA levels of wt BCMA-Fc and Co-Non-*N*-Gly-BCMA-Fc strains by RT-PCR following normalization to GAPDH mRNA expression (Fig. 2B), suggesting that the difference in the expression level might be ascribed to translation efficiency related to codon usage bias.

3.3 Expression of recombinant pPIC9K-BCMA-Fc strain in shake-flask culture

Different degrees of glycosylation modification might occur in secretory target protein production in *P. pastoris*. This was verified by SDS-PAGE followed by CBB staining and Western blotting (Fig. 3A, D). The production of the wt BCMA-Fc strain presented as broad smear bands of ~45 kDa, a little larger than the expected 31.4 kDa. N was substituted by Q in N-X-S/T to obtain uniform rhBCMA-Fc. However, the expression level of the non-*N*-Gly strain decreased to such an extent that almost no bands were visible on the CBB-stained gel, necessitating, silver staining to visualize the bands. The band's molecular mass reduced to 35 kDa, and a > 35 kDa was also present (Fig. 3B). We speculate that the larger band might have been caused by O-glycosylation, or it might have just been a variant in the process of transcription splicing.

Next, codon optimization was carried out to improve the yield of the fusion protein. Two bands positioned around 35 kDa were clearly visible by SDS-PAGE followed by CBB staining (Fig. 3C, D), indicating that the expression of Co-Non-*N*-Gly-BCMA-Fc was successfully improved. The intensity of the expected band (<35 kDa) was stronger than that of the larger band with the passage of induction time in the western blot assay (Fig. 3D), further verifying the aforementioned speculation.

3.4 Optimized expression of rhBCMA-Fc in fed-batch fermentation

Since protein stability and yields can be influenced by cultivation pH, fed-batch fermentations were performed at different pH values (4.5, 5.5, and 6.5). When the cells were cultivated at pH 5.5, the glycerol was depleted at approximately OD₆₀₀ = 120, similar to that at 4.5. However, the maximum yield of

Figure 4. Expression optimization of rhBCMA-Fc in 5-L bioreactors and purification of rhBCMA-Fc, followed by optimized fermentation. (A) Time profiles of cell growth (OD₆₀₀) and protein production in culture supernatant at three different pH values during fed-batch cultivation. All fermentations were performed at 30°C and repeated thrice. (B) Time profiles of cell growth (OD₆₀₀) and protein production in culture supernatant at three different temperatures during fed-batch cultivation. All fermentations were performed at pH 5.5 and repeated thrice. (C) Fast protein liquid chromatogram of Protein A affinity. The elution peak is labeled with an arrow. (D) SDS-PAGE analysis of the purified BCMA-Fc. Lane M: standard protein marker (14.4–116.0 kDa), lane 1: elution fraction from the Protein A affinity column.

rhBCMA-Fc (207 mg/L) obtained at 68 h at pH 5.5 was higher than that at pH 4.5 (175 mg/L) (Fig. 4A). When the pH value was increased to 6.5, the cells grew very slowly compared to the lower pHs (4.5 and 5.5). It took almost 68 h to reach OD₆₀₀ = 100. The cell density decreased gradually after this point and the maximum yield (78 mg/L) was much lower than the fermentations at lower pHs.

We also evaluated the effect of temperature on rhBCMA-Fc production. Three temperatures (20, 25, and 30°C) were chosen to ascertain the optimal value. When the fermentations were performed at 30°C, the maximum yield reached 202 mg/L, which was 1.2-fold and 2.0-fold higher than that at 25 and 20°C, respectively. Clearly at 20°C, the cells took significantly longer to reach OD₆₀₀ = 120, with a much lower yield than that at 25 and 30°C (Fig. 4B).

Thus, finally, fed-batch cultivation was performed at 30°C and pH 5.5. The optimal yield (207 mg/L) was obtained with a high productivity (3.45 mg/L/h).

3.5 Purification of rhBCMA-Fc

rhBCMA-Fc was purified from fermentation culture supernatant in three steps: ultrafiltration, high-resolution gel-filtration chromatography, and Protein A affinity chromatography (Fig. 4C).

The purified rhBCMA-Fc was visualized by 12% SDS-PAGE gel followed by CBB staining. A clear band was observed close to our calculated molecular mass of 31.4 kDa (Fig. 4D). As for the larger band with an apparent molecular weight of ~35 kDa. We assumed that it might be a variant of the target protein or another form of rhBCMA-Fc with O-linked glycosylation. The results of SDS-PAGE and Western blotting during the shake-flask culture were consistent with our speculation. Finally, we obtained 35 mg purified rhBCMA-Fc per liter of culture supernatant, and the purity reached 90% according to SDS-PAGE analysis.

3.6 Identification of rhBCMA-Fc

N-terminal sequencing showed that the amino acid sequence at the N-terminus of rhBCMA-Fc begins with MLQMAGQCSQ, which indicated no degradation at the N-terminus.

Trypsin digestion of the purified rhBCMA-Fc was performed in-gel, and the cleaved peptides were analyzed by MALDI-TOF MS/MS. The resulting data showed a significant match with peptides searched by theoretical masses from the UniProt Knowledgebase (ca.expasy.org) (Fig. 5A); matched amino acid sequences in red are shown in Fig. 5B. Two of the target protein-derived peptides identified were YCQASVTNSV (Fig. 5C), which is identical to the extracellular domain of BCMA, and FN-WYVDGVEVHNAK (Fig. 5C), corresponding to the Fc segment.

Figure 5. Identification of BCMA-Fc by MALDI-TOF-MS/MS. (A) MALDI-TOF-MS spectrum of trypsin-produced peptides derived from purified rhBCMA-Fc. Mass values show the peptides corresponding to theoretical peptides of BCMA-Fc. (B) The amino acid sequence of rhBCMA-Fc, in which the matched peptide sequences are depicted in red. (C) The two tryptic peptides identified are YCQASVTNSVK (mass, 1256.5) [40–50] and FNWYVDGVEVHNAK (mass, 1677.6) [111–124], corresponding to the ECD of BCMA and the Fc fragment, respectively. Inset shows m/z values for decreasing amounts of tryptic peptides from rhBCMA-Fc.

These findings verified that the excised peptides represented the rhBCMA-Fc protein.

3.7 Bioactivity assay of rhBCMA-Fc in vitro

The binding activity of BCMA-Fc to APRIL was determined by a Protein A pull-down assay (Fig. 6A). High binding affinity be-

tween purified rhBCMA-Fc and APRIL was clearly demonstrated compared with the PBS control group.

Besides, a dose-dependent increase in the proliferation of Raji B lymphoma cells occurred in the presence of APRIL (Fig. 6B). When a gradient concentration of rhBCMA-Fc was supplied, a marked dose-dependent inhibition of the stimulating effect occurred. The inhibition was statistically significant ($p < 0.05$) compared to the BSA control group (Fig. 6C).

Figure 6. Bioactivity assay of rhBCMA-Fc in vitro. (A) SDS-PAGE analysis and Western blot assay of pull-down assay samples. M: standard protein marker (14.4–116.0 kDa), lane 1: hAPRIL alone, lane 2: rhBCMA-Fc alone, lane 3: hAPRIL and PBS incubated with Protein A agarose beads (PBS was used as a negative control), and lane 4: hAPRIL and rhBCMA-Fc incubated with Protein A agarose beads. (B) APRIL induced the proliferation of Raji B lymphoma cells. (C) Dose-dependent inhibition of APRIL's effect by rhBCMA-Fc. All the data are representative of three independent assays. * $p < 0.05$ compared with the control groups using Student's *t*-test.

The assays presented here demonstrate that rhBCMA-Fc possesses expected biological activity as an inhibitor of the effect of APRIL by competitively binding to its inherent receptors BCMA and TACI on the cell surface.

4 Discussion

In the present study, we successfully expressed and purified functional non-*N*-glycosylated rhBCMA-Fc with high yields in *P. pastoris*. However, despite the ability of yeasts to secrete high titers of properly folded and active foreign proteins, a common problem is heterogeneous N-termini of secreted proteins due to incomplete *Ste13* cleavage when using the α -factor secretion signal. Deletion of the *Ste13* protease site affects the secretion levels of mature heterologous protein with homogeneous N-termini since there are no EAEA repeats attached to the secreted product

in *P. pastoris* [22]. Therefore, we retained only the *Kex2* cleavage site.

The addition of high amounts of mannose enables oligomannose units to be linked to different sites, leading to a diverse structural heterogeneity of secreted foreign proteins (Fig. 3A) [29]. This renders purification difficult, and the protein might be immunogenic to the human body when used as a therapeutic product. As we hypothesized that *N*-glycosylation is not associated with the biological function of the recombinant protein, the two potential *N*-glycosylation residues were mutated by point mutation as suggested by Kobayashi et al. [24]. BCMA is a glycoprotein with a single *N*-glycosylation site at the N42 residue, and the *N*-glycan enhances the binding of the ligand to BCMA [30]. The non-*N*-glycosylated version was found to possess bioactivities; however, we could not estimate whether the activities were reduced or not compared to the control because the *N*-glycosylated version is difficult to purify. The effects of

mutation of *N*-glycosylation on the in vitro and in vivo functions of rhBCMA-Fc are poorly understood; therefore, more studies are required to elucidate this important aspect.

N-glycosylation can markedly affect the pharmacokinetic behavior of glycoproteins and their receptor interactions. Therefore, to attain full biological and therapeutic function, several combinatorial methods of engineering yeast (apart from mutating the *N*-glycosylation site) for producing human glycoproteins to achieve more human-like and homogenous *N*-glycosylation are becoming increasingly popular, such as the GlycoSwitch technology [31,32].

P. pastoris has codon usage bias, and the production of target protein can be increased one to fivefold by optimizing the heterologous protein-coding sequence based on the codon usage bias of host cells [33,34]. Our study also implies that codon optimization improved the production of rhBCMA-Fc to visible levels.

Various factors apart from pH and temperature might influence yield and productivity during fermentation, including inoculum density ($OD_{600} = 1\text{--}150$), methanol concentration, initial glycerol concentration, and induction time [35–37]. For example, different heterologous proteins need different induction times (16–60 h) for optimal production. Thus, the highest yield obtained in this study might not be the maximal achievable yield. Moreover, as the protein recovery was only 16.9%, more studies on the optimization of fermentation and purification conditions are required to obtain sufficient purified rhBCMA-Fc for further research.

To the best of our knowledge, this is the first report of high-yield expression of bioactive rhBCMA-Fc in *P. pastoris*, providing a promising alternative for scaling up the production of this fusion protein for both research and therapeutic purposes.

Practical application

The fusion protein of B-cell maturation antigen and Fc (BCMA-Fc) having sufficient immunosuppressive therapeutic function has been produced by mammalian expression hosts such as human embryonic kidney 293 cells. However, such hosts have drawbacks of high cost, low yield, and difficult control of production. Therefore, in the present study, we tried to achieve a high yield of soluble BCMA-Fc in *Pichia pastoris* via several optimization strategies. The purified fusion protein demonstrated high-binding affinity with its ligand and showed the expected bioactive characteristics. These results lay a solid foundation for scaling up the production of recombinant human BCMA-Fc in *P. pastoris* for both research and industrial purposes.

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