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ORIGINAL ARTICLE

Production of Universal Group O Red Blood Cells by Alpha-*N*-Acetylgalactosaminidase Enzyme Expressed in *Pichia pastoris*

Azam Molafilabi[1] · Majid Shahabi[1] · Houshang Rafatpanah[2] · Baratali Mashkani[3]

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Abstract Enzymatic removal of blood groups antigens A and B is an efficient method for production of universal red blood cells. In this research, an α-*N*-acetylgalactosaminidase (NAGA) enzyme was expressed in *Pichia pastoris* for digestion of the A blood antigen. DNA sequence of the gene NAGA, originally expressed in Elizabethkingia meningosepticum (NAGA-EM), was ordered for optimization and synthesis. It was then expressed in *P. pastoris* (KM71H and GS115 strains). Expression of the recombinant NAGA was evaluated by dot blot, SDS-PAGE, and Western blotting. The activity of the enzyme was measured using a synthetic substrate in addition to the conversion of group A red blood cells to the O cells. Expression of NAGA-EM with an apparent molecular mass of 55 kDa was verified by dot blot, SDS-PAGE and Western blot analysis. The maximum enzyme activity in the supernatant of KM71H was higher than that in the GS115 (250 vs. 200 U/ml). Treated group A RBCs did not react with the anti-A antiserum or with the sera from individuals with blood groups B and O. The results of this study indicated that NAGA-EM is an efficient enzyme for production of universal O blood cells.

Keywords Antigen A · α-*N*-acetylgalactosaminidase · Codon optimization · *Pichia pastoris* · NAGA-EM

✉ Baratali Mashkani
mashkaniba@mums.ac.ir

Azam Molafilabi
a.m.filabi@gmail.com

Majid Shahabi
m.shahabi@tmi.ac.ir

Houshang Rafatpanah
rafatpanahh@mums.ac.ir

[1] Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, IBTO Bldg, Hemmat Exp. Way, 1449613111 Tehran, Iran

[2] Immunology Research Center, Inflammation and Inflammatory Diseases Division, Mashhad University of Medical Sciences, 9177948564 Mashhad, Iran

[3] Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, 9177948564 Mashhad, Iran

Introduction

Individuals with blood group O, the most frequent blood group in the Northeast of Iran (34%) [1] and in other parts of the world including India (37%) [2], can only receive blood transfusion from the matched group. Additionally, blood group O is considered as a universal donor which can be transfused to other ABO groups in emergency situations. Therefore, maintaining the inventories of group O red blood cells (RBCs) is highly critical for blood banks.

Enzymatic removal of the *N*-acetylgalactosamine (α-GalNAc) and α-Gal moieties from A and B blood groups, respectively, has been suggested as an alternative for providing the supply of universal red blood cells [3]. Alpha-*N*-acetylgalactosaminidase (NAGA) is an exo-glucosidase, which hydrolyzes the α-*N*-acetyl D-glactoseamine bond from the glycoconjugates (EC.3.2.1.49) [3]. The NAGA enzyme has been isolated from different eukaryotic and prokaryotic sources. The optimum pH of 3–5.4 and non-specificity are the main limitation for using the enzymes of eukaryotic origin. [4–6]. On the other hand, the prokaryotic NAGA isolated from *Elizabethkingia meningosepticum* have high specificity and activity at physiological temperature and pH [3]. However the low-level expression is

the main obstacle for application of the native prokaryotic cells as a source for the enzyme production [7, 8].

The *Pichia pastoris* expression system is a fast, easy and more economical expression systems particularly for proteins rich in disulfide bonds and with post-translational modifications [9]. The presence of a methanol-inducible promoter and other molecular and genetic manipulations enabled *P. pastoris* to express heterologous proteins 10–100 times more efficient than *Saccharomyces cerevisiae* [10]. However, as previously reported [11, 12], heterologous gene expression in *P. pastoris* can be hampered by codon usage bias. It is partly due to the fact that synonymous codons are not used with equal frequency in the genome of different species. A common solution is replacing the rare codons in the target gene with the pattern of codon usage in the expression host. Many studies demonstrated that the codon optimization significantly increases the efficiency of heterologous protein expression in *P. pastoris* [13–17]. The aim of this study was to express a NAGA enzyme in *P. pastoris* for removing blood group A antigenic epitope from RBCs.

Materials and Methods

Enzyme Selection Criteria

The overall procedure was similar to the selection of the B-GalP enzyme [18]. A comprehensive literature review was conducted to select a NAGA enzyme based on the criteria listed below. The selected enzyme was expected to have optimal activity at the physiological blood pH (7.4) and to be an active enzyme in the form of a low molecular weight monomer.

Coding Sequence Optimization and Synthesis

The protein sequence of the selected enzyme was back-translated and optimized by Generay Biotech Co., LTD (Shanghai, China). The quality of sequence optimization was assessed by "rare codon analysis" tool (GenScript, USA). Rare codon analysis reports several optimization indicators such as Codon Adaptation Index (CAI), Codon Frequency Distribution (CFD), GC content and presence of negative *cis* elements in a coding sequence. According to the GenScript criteria, CAI below 0.8 and codons with less than 30% frequencies may interrupt the expression of the recombinant protein. Additionally, codon tables for the native and optimized sequences of NAGA were created by Geneious software (Biomatters, New Zealand) and compared with the codon table for the host, *P. pastoris* (Invitrogen, USA). Restriction sites for XhoI and XbaI were added to the 5' and 3′ ends of the optimized coding sequence. The construct was synthesized and cloned into pGH plasmid by Generay Biotech Co., LTD (China).

Expression of NAGA

The synthetic gene for NAGA was subcloned into pPICZαA vector (Invitrogen, USA) to be expressed as a fusion protein composed of NAGA, c-Myc epitope and His tags as previously described [11]. The α-factor signal allows the product to be secreted into the culture media. Then the construct electroporated into the competent KM71H and GS115 cells and the transformed cells were grown on the yeast extract peptone dextrose medium with sorbitol (YPDS). Single colonies grown on the YPDS plates were inoculated into 2 ml of BMGY medium and incubated 30 °C for 48 h at 250 rpm. When the OD_{600} reached 1.5, the BMGY medium was replaced with BMMY plus 2% methanol (day 0). The cultures were incubated at 25 °C, and samples of the supernatants were sequentially collected for the NAGA expression analysis on the eight consecutive days. The untransformed *P. pastoris* and the one transformed with pPICZαA empty vector were used as negative controls in the experiment.

NAGA Protein Expression Analysis

Supernatant samples of cultured GS115 and KM71H were analyzed by dot blot, SDS-PAGE and Western blot assays. The samples dot blotted onto the nitrocellulose membrane (Sigma-Aldrich, USA) and probed using the anti c-Myc mouse monoclonal IgG1 antibody (Santa Cruz Biotech., USA) and mouse anti-IgG conjugated with HRP (Sigma-Aldrich). The recombinant protein was visualized using a homemade ECL reagent [0.2 mM 3-aminophthalhydrazide (Fluka), 1.25 mM coumaric acid (Sigma-Aldrich) and 0.1% of H_2O_2 in 100 mM Tris buffer pH 8.5]. Finally, the chemiluminescence was recorded using an Alliance Mini instrument (UVItec Limited, Cambridge, UK). The supernatant samples were also resolved on SDS-PAGE and visualized using Coomassie Brilliant Blue R250 (Sigma-Aldrich). Finally, the proteins transferred onto nitrocellulose membrane, and then Western blotting was performed as described for dot blot analysis.

Purification of NAGA Protein

The supernatants were concentrated 25 times using Amicon Ultra-15 centrifugal filter units with 30 kDa cut-off (Merck Millipore, Billerica, MA). Affinity purification was performed using Ni–NTA affinity column (Parstous, Mashhad, Iran) according to the supplier's instruction. The samples collected from the concentrated and purified

enzyme were analyzed by SDS-PAGE and Western blotting.

NAGA Activity Assay

The activity of the NAGA enzyme was determined using the chromogenic substrate p-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (pNP-GlcNAc) (Sigma-Aldrich) in sodium phosphate buffer at 25° C. Hydrolysis of pNP-GlcNAc by NAGA results in releasing of *N*-acetylgalactosamine (GalNAc) and p-nitrophenol (yellow product with maximum absorbance at 405 nm and molar extinction coefficient of 17700). The absorbance was measured using a T60 UV–Vis spectrophotometer (PG Instruments, UK). The enzyme activity (Unit) was calculated as pNP (μM) produced in 1 min. Each experiment was repeated three times, and enzymatic activity was reported as Unit per ml of the culture supernatants.

Conversion of A Cells to O Cells

Group A RBCs were treated with the NAGA enzyme (200 U/ml of blood, 60 min, at 25 °C), and then tested for the presence of antigen A using human sera from blood group A, B, AB and O as the source of antibody. RBC agglutination was evaluated macroscopically and with low-magnitude microscopy.

Results and Discussion

Enzyme Selection

Based on the literature, four enzymes have been identified to be effective on blood group A antigen. Eukaryotic lysosomal α-NAGA enzymes were not selected in this study because of the optimal activity at acidic conditions, in addition to low substrate specificity and lack of activity on A1 antigens [19]. NAGA enzymes isolated from *Clostridium perfringens* were only suitable for enzymatic conversion of blood type A_2 to O [20]. The NAGA isoforms isolated from *Ruminococcus torques* of IX-70 strain had three unfavorable characteristics including high molecular weight of about 180–300 kDa, being active as a dimer and maximum activity at pH 5.8–6.8 [21]. According to the criteria described in the method section, these enzymes were assumed not to be suitable for the production of universal O blood cells.

The NAGA enzyme encoded by *Elizabethkingia meningosepticum* (UniProt accession No. AM039444) (NAGA-EM) has high specificity for cleaving terminal α-*N*-acetylgalactosamine in both A_1 and A_2 sugar moieties with optimal activity at pH 6.8 [19] and room temperature [3]. Additionally, it is active as a monomer, with a molecular weight of nearly 50 kDa. Altogether, this enzyme was chosen for expression in *P. pastoris*. This enzyme previously expressed in *E. coli* through direct cloning [14]; however, *P. pastoris* was selected here as the host to eliminate the possibility of lipopolysaccharides (LPS) contamination.

Sequence Optimization for Expression in *P. pastoris*

Sequence alignment of the native and optimized NAGA-EM revealed that the 20% of nucleotides were replaced during optimization without affecting the amino acid sequences. Rare codon analysis of the native sequence of the NAGA-EM revealed CAI value of 0.78 with 1% of codons with less than 30% frequency in the *P. pastoris* host and presence of 6 negative *cis* elements. Sequence optimization resulted in improving the CAI up to 0.91, replacement of all codons with less than 50% frequency and removal of all the negative *cis* elements. As a result, the frequency of the codons with 90–100% frequency was increased from 53% in the native to 73% in the optimized NAGA-EM sequence. GC content of the optimized NAGA gene was also 41.7%, which showed nearly 2% increase upon optimization.

NAGA-EM Expression Analysis

Figure 1 shows the results of dot blot analysis of the culture supernatants for the screening of the high-level expression day. Expression of the recombinant protein was detectable in both *Pichia* strains (KM71 and GS115) transformed with pPICZ-NAGA (columns 1 and 4, respectively). It seems that the NAGA-EM expression was started from day one after adding the methanol-contacting BMMY medium. However, the control samples (lanes 2, 3, 5 and 6) did not express the recombinant protein. The level of NAGA-EM product in KM-71 was greater than the GS-115. Additionally, the product level remained on a plateau state until day 7 in the KM-71 supernatant, but it declined dramatically after day 4 in the GS-115 supernatant.

Consistent with the dot blot analysis, NAGA-EM activity in the culture supernatant of the transformed KM71 reached maximum (250 U/ml) on day 5 and remained constant up to day 8 (Fig. 2). However, in the culture of transformed GS strain, the activity of the supernatant reached about 200 U/ml, but declined dramatically after day 3. These findings suggest that KM-71 may be the preferred host for production of NAGA-EM. Previously, KM 71 was also reported as a better host for expression of a cold-active β-galactosidase [18].

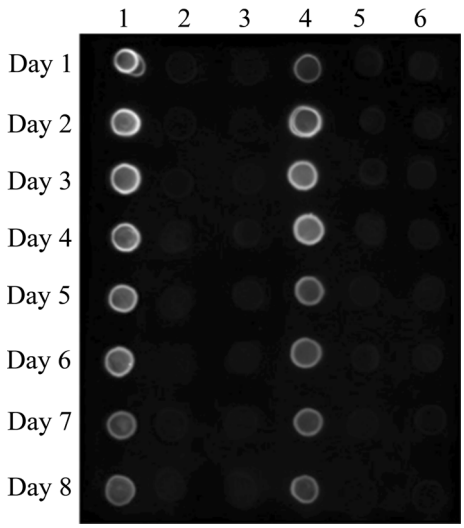

Fig. 1 Dot blot results of *P. pastoris* culture supernatants. The samples were collected over 8 consecutive days after starting induction. The product was probed with mouse anti-cMyc antibody followed by anti-mouse IgG-HRP. The antibody interaction was visualized using chemiluminescence reaction. Lanes 1 and 4: KM and GS transformed with pPICZ-NAGA; lanes 2 and 5: KM and GS transformed with the empty pPICZαA vector; lanes 3 and 6: untransformed KM and GS strains

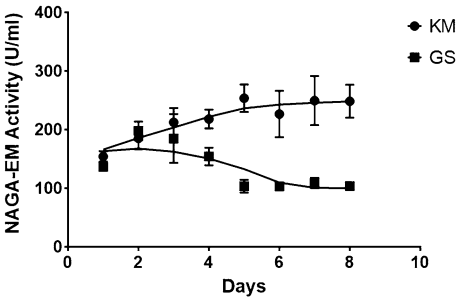

Fig. 2 Expression of the NAGA recombinant fusion protein in the culture supernatant of the *P. pastoris* strains KM71H and GS115. Lane M: protein marker, Lane 1: *P. pastoris* transformed with empty pPICZαA vector; 2: untransformed *P. pastoris*. Lanes 3–9 supernatants collected on days 1–7 from the culture of *P. pastoris* transformed with pPICZ-NAGA-EM. The protein bands corresponding to the apparent molecular mass of NAGA-EM are indicated by an arrow in the right

NAGA-EM Protein Analyses

The molecular weight of NAGA peptide with a c-Myc epitope and 6× His Tag was calculated to be about 55 kDa. Two protein bands with apparent molecular weight of nearly 55 kDa were detected in the culture supernatants of both KM (panel A), and GS (panel B) strains transformed with pPICZ-NAGA, but not in the control samples (Fig. 3).

Western blot analysis of three samples (culture supernatant, concentrated supernatant, and column-purified

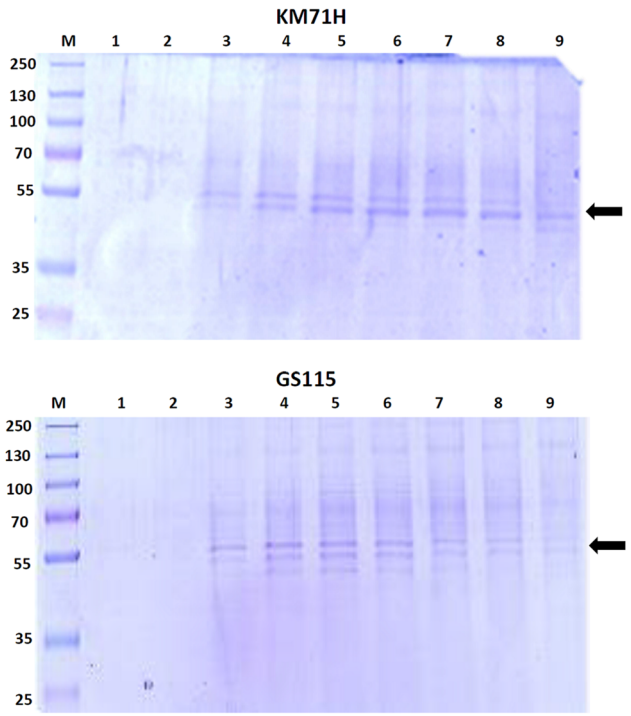

Fig. 3 Protein analysis of affinity purified NAGA-EM from the supernatants of GS115 (lane 1) and KM71H (lane 2). Western blot analysis of NAGA-EM in the crude supernatant of the fifth day from KM71H (lane 3), concentrated and purified NAGA-EM in lanes 4 and 5, respectively

product in lanes 3, 4 and 5 of Fig. 4) indicated that both bands were indeed NAGA-EM fusion proteins with c-Myc and His tag protein. According to Fig. 3, the intensity of the bands in SDS-PAGE analysis looked similar; however, comparing lanes 3 and 4 with lane 5 in Fig. 4, it seems that

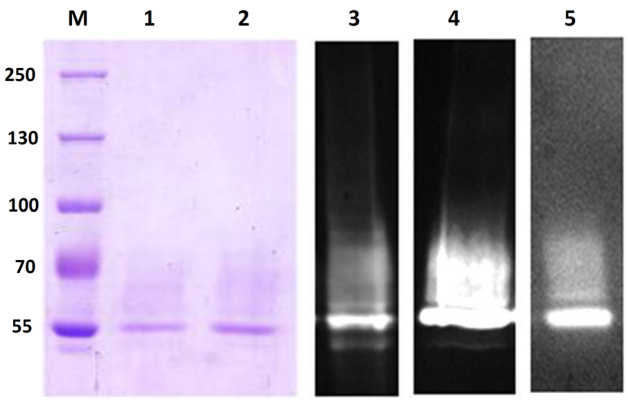

Fig. 4 Comparing the enzyme activity of the clones from GS115 and KM71H strain during 8 days of induction with methanol. Colon of KM71H strain has been shown as KM-PIC-NAGA and colon of GS115 strain has been displayed as GS-PIC-NAGA. Enzymatic activity assay was performed in sodium phosphate buffer at 25 °C using pNP-GlcNAc as a substrate. Each experiment was repeated three times. Enzymatic activity was measured in one microliter of supernatant in the yeast culture medium

the higher molecular weight protein was purified more efficiently with Ni–NTA affinity column. There was also smearing protein with MW above 55 kDa, which may result from heterogeneous glycosylation of the product.

Decreasing the intensity of low molecular weight product after purification suggest that the Ni–NTA affinity column may be not the most appropriate purification tool for this product. Therefore, alternative methods such as ultrafiltration should be applied to improve the purification process. Presence of less contaminating proteins in the culture supernatant of KM71H will facilitate the purification of the product, which can be considered as the third advantage of KM71 over GS115 as the host for NAGA-EM expression.

Conversion of RBC Group A to O Cells

In the macro and microscopic assessing, there was no sign of hemolysis or morphological changes in the treated RBCs. Figure 5 shows the result of blood group testing of group A cell after treatment with NAGA-EM enzyme. There was clear agglutination in the blood group typing of the A cells after adding standard anti-A antiserum or the serum from blood group B in both macroscopic and microscopic examination of the untreated cells (panels A1 and B1). Treatment with NAGA-EM prevented the interaction of the RBCs with the commercial anti-A antiserum (panel A2) and also with the sera of individual AB, A, B and O groups in panels B2-5, respectively.

This experiment confirmed that NAGA-EM was capble of cleaving group A antigen without damaging the treated RBCs. Lack of interaction between treated cells and anti-A antiserum as well as sera of individuals with blood group B and O indicated the complete removal of the blood group A antigen. Additionally, the treated cells did not agglutinated in the sera from B and AB blood groups, which ruled out the possibility of creating any new undesired antigenic epitopes on the NAGA-treated RBCs. Altogether, these findings confirmed that enzyme-treated blood cells should pass two important tests (blood type and crossmatching) for transfusion. Before advancing to the clinical trial, however, it is necessary to optimizing the quantity of the enzyme required to completely digest the cell surface antigens. This will require flow cytometry analysis of the treated cells for the residual A-antigens. Additionally, the enzyme may have interactions with the RBC membrane component, strong enough to resist washing steps. If so, it will be required to evaluate its possible consequences on the stability of the transfused RBCs and also the host responses.

Conclusion

Results of the dot blot analysis, consistently with the SDS-PAGE analysis and the enzyme activity assays, revealed that the expression of NAGA-EM recombinant protein started rapidly after induction in both *P. pastoris* strains and reached maximum on day 4. The KM-71 strain is the preferred host for production of the recombinant enzyme rather than GS-115. The enzyme is recommended for enzymatic conversion of RBCs group A to O cells. *P. pastoris* is generally recognized as a safe (GRAS) host; additionally, blood group typing and crossmatch testing indicated complete removal of the A blood group antigen from RBCs upon treatment with NAGA-EM. This evidence strongly indicates the safety of universal O blood cell produced by NAGA enzyme. However, further clinical trials are required before its widespread application to mitigate the constant shortage of the blood group O reservoir in blood banks.

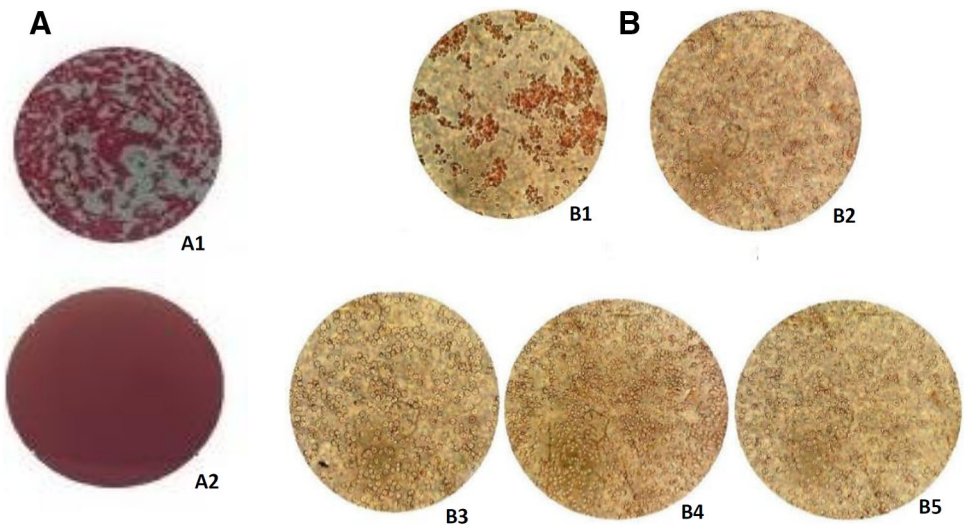

Fig. 5 Antigen A removal from RBCs. Panel A: Blood group typing using antiserum A, Unconverted A blood group (A1). A blood group converted by the recombinant NAGA-EM enzyme (A2); Panel B: Cross-match testing: Enzyme-converted group A RBCs reacted with different human serum groups. Control: Unconverted group A RBCs agglutinated with the B serum (B1), Converted RBCs with the AB serum (B2), with the A serum (B3), with the B serum (B4) and the O serum (B5)

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Compliance with Ethical Standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical Approval This project was approved by the local ethics committee (Code: IR.MUMS.FM.Rec.1394.228) at Mashad University of Medical Sciences, Mashhad, Iran.

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