Expression and purification of the recombinant murine REG3α protein in *Pichia pastoris* and characterization of its antimicrobial and antitumour efficacy

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Regenerating islet-derived 3-alpha (REG3α) is a secreted intestinal antimicrobial protein, which shows antibacterial, anti-inflammatory and anti-apoptotic activities. It could significantly promote internal tissue regeneration and wound repair. In the present study, we expressed the codon-optimized murine REG3α in the yeast *Pichia pastoris* system. The secreted murine REG3α was captured using ProteinIsoTM Ni-NTA resin and further purified using a strong anion exchange resin Poros® 50 HQ. The final protein yield was 20 mg/L. The antibacterial activity and the anticancer efficacy of the recombinant REG3α were assessed using liquid growth inhibition assay, killing kinetics and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The results showed that the recombinant murine REG3α possessed antimicrobial activity against *Escherichia coli*, *Salmonella paratyphi A*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus pumilus* and *Micrococcus luteus*. Moreover, 100% killing against *E. coli* and *S. aureus* was observed after 30 min. The recombinant murine REG3α had a potent antitumour activity in a time-dependent and dose-dependent manner and had a negligible haemolysis activity against human erythrocytes. Taken together, *P. pastoris* is an efficient expression system for producing large quantities of antibacterial active REG3α for further research studies and clinical applications.

**Keywords:** REG3α; expression; purification; *Pichia pastoris*; antimicrobial; anticancer

Introduction

Regenerating islet-derived protein (REG), which belongs to the family of C-type lectins, is a protein, first isolated from pancreatic calculi of men, suffering from chronic calcifying pancreatitis in 1979.[1] Based on the primary structure of the proteins, the members of the family have been classified into four subgroups: REGI, REGII, REGIII and REGIV.[2] The REGIII subclass family comprises REG3α, REG3β, REG3γ and REG3δ. REGIII has been reported to possess anti-inflammatory and anti-apoptotic activity and the ability to promote wound repair.[3] REGIII can influence tissue regeneration after several tissue injuries, such as mucosal damage,[4] liver failure,[5] intestinal epithelial and Paneth cells impairment,[6] as well as pancreatic β-cells damage.[7] Furthermore, human REG3α (REG3A) and mouse REG3γ exhibit antibacterial activity by destroying the bacterial cell wall.[6,8] In addition, REG3A was found to be down-regulated in primary human gastric cancers, but to be up-regulated in primary HCCs.[9–12] REGIII may have a significant application value in clinical studies.

The high cost of chemical synthesis and extraction is one of the limiting factors that hamper the use of REG3α as a therapeutic agent. In recent years, the expression of recombinant proteins by using micro-organisms as host cells has become a more efficient alternative method. Recombinant human REG3A protein was expressed in *Escherichia coli* expression system,[13] which exhibited a fast and robust growth in bioreactors using a simple media. However, the recombinant protein existed in the form of inclusion bodies and the post-purification process was complicated. The eukaryotic expression system of *Pichia pastoris* has numerous advantages. Some of them include high-level and large-scale expression, cheap and easy purification, and post-translational modifications.[14,15] Based on the above, *P. pastoris* may be the best choice for the efficient expression of the murine REG3α.

In this study, in order to achieve an efficient expression of REG3α, pwPICZalpha expression vector was used to produce murine REG3α in a *P. pastoris* expression system. The study will lay the foundations for further development of a protein drug that might be used as a treatment for bacterial infections and cancer.
Materials and methods

Construction of plasmids encoding REG3α

The codon-optimized gene was designed based on the gene sequence of REG3α (Genbank accession NM_011259.1) according to the codon bias of *P. pastoris*. To facilitate the downstream purification, six histidines (6 × His tag) were added to the C-terminus. The entire REG3α gene, carrying *Xho*I and *EcoR*I restriction sites at each end, was synthesized by Invitrogen. The synthesized REG3α was cut out using *Xho*I and *EcoR*I restriction enzymes and cloned into a digested pwPICZalpha vector. Next, double digestion and DNA sequencing were used to confirm the final construction of REG3α in the pwPICZalpha vector. The resulting plasmid was designated as pwPICZalpha-REG3α.

REG3α expression and purification

The pwPICZalpha-REG3α was linearized by *Sac*I digestion and was transformed into *P. pastoris* strain X33 using the gene transfection instrument (NingBo Sciento Biotechnology Co., Ltd, Ningbo, China). The transformants were selected on yeast extract–peptone–dextrose (YPD) agar plates (10 g/L yeast extract, 20 g/L peptone, 15 g/L agar and 20 g/L dextrose) containing 100 μg/mL of zeocin. The positive transformants were cultivated on YPD agar and 20 g/L dextrose) containing 100 μg/mL of zeo- cin and incubated for 48 h at 25 °C, 225 rpm, as a seed culture. Next, 5% of the seed culture were transferred into 250 mL YPD and were cultured at 30 °C, 250 rpm. After 24 h the YPD was replaced with 250 mL yeast extract–peptone–dextrose (10 g/L yeast extract, 20 g/L peptone and 10 g/L glycerol). We continued to culture the yeasts at 30 °C at 250 rpm for another 24 h. Subsequently, the yeasts were centrifuged at 3000 rpm for 10 min and the pellet was resuspended in 125 mL buffered methanol-complex medium (BMMYC) (10 g/L yeast extract, 20 g/L peptone, 100 mmol/L potassium phosphate, pH 7.0, 13.4 g/L yeast nitrogen base without amino acids, 0.04 g/L biotin, 10 mL/L methanol and 10 g/L bacto casamino acids) and induced at 25 °C, 225 rpm for 48 h. We added methanol every 12 h to sustain a 0.5% level. The supernatant, containing the recombinant murine REG3α, was collected for protein purification after centrifugation at 3000 rpm at 4 °C for 10 min.

The culture supernatant was loaded on a ProteinIsoTM Ni-NTA resin column (TansGen, Beijing, China), pre-equilibrated with 10 column volumes (CV) of 50 mmol/L sodium phosphate, 0.3 mol/L NaCl, 10 mmol/L imidazole and 10 mmol/L Tris-HCl pH 8.0. The supernatant was eluted with 50 mmol/L sodium phosphate, 0.3 mol/L NaCl, 500 mmol/L imidazole and 10 mmol/L Tris-HCl pH 8.0 after an extensive washing with 50 mmol/L sodium phosphate, 0.3 mol/L NaCl, 10 mmol/L imidazole and 10 mmol/L Tris-HCl pH 8.0. The first purification fractions were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the protein of interest were pooled and dialysed using a 3.5 kDa cut-off Spectra/Por® membrane tubing (Spectrum Laboratories, Inc., CA, USA) against 20 mmol/L Tris-HCl pH 8.0, 1 mmol/L ethylene-diaminetetraacetic acid (EDTA) pH 8.0, 50 mL/L glycerol at 4 °C. The dialysis buffer was replaced once.

The dialysed protein was loaded on a Strong anion exchange resin Poros® 50 HQ column (Applied Biosystems, CA, USA), pre-equilibrated with 20 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 50 mL/L glycerol (10 CV), after this followed the washing of the column with 20 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 5 mL/L glycerol (8 CV). The bound protein was eluted into seven fractions with 100 mmol/L sodium borate and six fractions 200 mmol/L sodium borate in 20 mL/L Tris-HCl pH 8.0, 1 mL/L EDTA and 5 mL/L glycerol. The purification fractions were analysed using SDS-PAGE and western blot with mouse anti-His monoclonal antibody. The recombinant murine REG3α was concentrated by ultra centrifugal filter units and dialysed using a 3.5 kDa cut-off Spectra/Por® membrane tubing against phosphate buffered saline (PBS) pH 7.4 at 4 °C. The concentration of the recombinant murine REG3α was determined by bicinchoninic acid protein assay kit (Beyotime, Haimen, China).

Antibacterial activity assays

The antibacterial activity of the recombinant murine REG3α was assessed by liquid growth inhibition assay against a panel of micro-organisms, including *E. coli*, *Salmonella paratyphi A*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus pumilus* and *Micrococcus luteus*. The determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was done by a modification of a previously described method by using different micro-organisms.[18]

The bacterial or bacteriostatic effect was demonstrated using killing kinetics against *E. coli* or *S. aureus*, as previously described.[19] We incubated 20.30 μmol/L REG3α with the bacteria for 0, 10, 20, 30, 60, 90 and 120 min. At these time points, 5 μL from the mixture was serially diluted in PBS (pH 7.4) and was plated on nutrition broth agar. The plates were incubated at 37 °C for 24 h and the colonies were counted. The percentage of colony-forming units (CFU) was defined relative to the CFU obtained in the control (100% CFU at 0 min).

Cytotoxicity assay

The human hepatocellular carcinoma (HCC) cell line SMMC-7721 was treated with fresh medium, fresh medium containing the recombinant murine REG3α with different concentrations (0, 0.63, 1.27, 2.54, 5.08, 10.15 and 20.30 μmol/L) or PBS (pH 7.4), as a control, for 24,
48 and 72 h at 37°C in a 5% CO\textsubscript{2} humidified atmosphere. The results from each concentration were obtained in triplicate. After the treatment, the cytotoxic effect of REG3\textsubscript{a} to SMMC-7721 tumour cells was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The percent inhibitory rate (IR\%) of the treated cells was calculated by the formula:

\[
\text{IR\%} = 1 - \frac{(OD\ 490\ \text{nm} - OD\ 630\ \text{nm})_{\text{treated}}}{(OD\ 490\ \text{nm} - OD\ 630\ \text{nm})_{\text{control}}} \times 100\%
\]  

(1)

The half-maximal inhibitory concentration (IC\textsubscript{50}) value for SMMC-7721 cell line was evaluated using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA) and it was representing the concentration at which the viability of the tumour cells was reduced to 50%, compared with the untreated tumour cells.[20,21] The results are presented as means ± standard deviation.

**Haemolytic activity**

Human fresh blood was collected in a heparinized tube and centrifuged at 1500 rpm (800 g) for 10 min. The pellet was gently washed three times with cold PBS (pH 7.4) then the erythrocytes were resuspended in cold PBS (pH 7.4) and adjusted to 8%. About 100 \( \mu \)L of the erythrocyte suspension was added into a 96-well microtitre plate. Different concentrations (0.63, 1.27, 2.54, 5.08, 10.15, 20.30 and 40.60 \( \mu \)mol/L) of REG3\textsubscript{a} solution were added to each well and were incubated for 60 min at 37°C. Triton-X 100 (0.2%) and PBS were used as positive and negative controls, respectively. The release of haemoglobin of the supernatant was measured after centrifugation [3000 rpm (1000 g) for 10 min] by microplate reader (EL \( \times \) 808; Gene Co., Ltd., Hong Kong, China) at 490 nm.[20]

**Results and discussion**

**Expression and purification of the recombinant murine REG3\textsubscript{a}**

The yeast *P. pastoris* has been considered as a highly successful expression system for the production of a variety of heterologous proteins, because of its high production levels, easy manipulation, inexpensive growth media and post-translational modifications.[22–25] Previous studies have produced vaccine, candidate therapeutic protein and antimicrobial peptide using the yeast *P. pastoris*.[26–28] *P. pastoris* may be the best choice for the efficient expression of the murine REG3\textsubscript{a}. In our study, the murine REG3\textsubscript{a} carrying a 6×His tag in the C-terminus was expressed using a shake-flask system. The supernatant containing the secreted REG3\textsubscript{a} was collected and captured directly by ProteinIsoTM Ni-NTA resin through its His-tags (Figure 1). The second and third eluted fractions, containing REG3\textsubscript{a}, were collected and dialysed to remove the salts. Based on the anticipated isoelectric point (pI = 5.71), the second step purification was completed by a strong anion exchange resin Poros\textsuperscript{®} 50 HQ. Sodium borate was used to separate the murine REG3\textsubscript{a} from the yeast host’s protein and aggregates.[29] The pure REG3\textsubscript{a} was obtained from the first to the fourth fractions with 100 mmol/L sodium borate (Figure 2). The Western blot analysis using an anti-His monoclonal antibody confirmed the presence of the recombinant REG3\textsubscript{a} (Figure 3). The REG3\textsubscript{a} band was detected with the expected size (~14.8 kDa). These results demonstrated that the murine REG3\textsubscript{a} was successfully expressed and purified in *P. pastoris* system and we found that the *P. pastoris* expression system is on a higher level than *E. coli*’s expression system.[13] The final purification yield of murine REG3\textsubscript{a} was 20 mg/L from the original harvested supernatant in our study. Previously, Human Reg IV was expressed to a maximal expression level (20 mg/L) after 5% methanol induction.[30] Apart from this study, there are no additional reports of the production of a Reg family protein by *P. pastoris* system.

**Antimicrobial activity of the recombinant murine REG3\textsubscript{a}**

Human REG3A and mouse REG3\textsubscript{y} have been reported to inhibit the growth of gram-positive bacteria,[6] but whether other REGs possess antimicrobial activity was not clear. The antibacterial activity of the recombinant
murine REG3α was evaluated against a panel of bacteria by using MIC and MBC assays. The recombinant murine REG3α showed antibacterial activity against E. coli, S. paratyphi A, S. aureus, S. epidermidis, B. subtilis, B. pumilus and M. luteus (Table 1). The killing kinetics results showed that approximately 40%, 70% and 100% of E. coli were killed after incubation with 20.30 μmol/L concentration of the recombinant murine REG3α for 10, 20 and 30 min, respectively. Approximately 40%, 92% and 100% of S. aureus were killed after incubation for 10, 20 and 30 min, respectively (Figure 4). Our results showed that the recombinant murine REG3α possesses an antibacterial activity against gram-positive and gram-negative bacteria. Another study showed that RegIIIγ kills gram-positive bacteria by specifically binding with peptidoglycan.[6] Further research is needed to demonstrate the inhibitory mechanism of REG3α.

Antitumour efficacy of the recombinant murine REG3α

Human REG3A is overexpressed in liver carcinoma and is a paracrine hepatic growth factor promoting both proliferation

Table 1. Antimicrobial activity of the recombinant murine REG3α.

| Micro-organism          | CMCC No.  | MIC (μmol/L) | MBC (μmol/L) |
|-------------------------|-----------|--------------|--------------|
| Escherichia coli        | 44102     | 5.08–10.15   | 10.15–20.3   |
| Salmonella paratyphi A  | (B) 50001 | 5.08–10.15   | 10.15–20.3   |
| Pseudomonas aeruginosa  | (B) 10104 | NT           | NT           |
| Staphylococcus aureus   | 26003     | 2.54–5.08    | 2.54–5.08    |
| Staphylococcus epidermidis | 26069 | 2.54–5.08    | 2.54–5.08    |
| Bacillus subtilis       | 63501     | 2.54–5.08    | 2.54–5.08    |
| Bacillus pumilus        | (B) 63202 | 5.08–10.15   | 10.15–20.3   |
| Micrococcus luteus      | (B) 28001 | 5.08–10.15   | 10.15–20.3   |

Note: a–National centre for medical culture collections (CMCC).
MIC values are expressed as the interval of concentration b–c μmol/L, where b is the highest concentration tested, at which a microbial growth can be observed, and c is the lowest concentration yielding no detectable microbial growth. (The assay was performed in triplicate.)
MBC values are expressed as the interval of concentration d–e μmol/L, where d is the same mean as b and e is the lowest concentration tested that inhibited micro-organism growth or that killed more than 99.9% of the micro-organisms. (The assay was performed in triplicate.)
Not tested (NT).
and viability of liver cells in vivo.[10] However, the studies for the murine REG3α are very few and the antitumour efficacy of this protein are still not clear. In our study, we assessed the efficacy of the recombinant murine REG3α to SMMC-7721 tumour cells using MTT assay at different time intervals (24, 48 and 72 h) of treatment. As shown in Figure 5, the IR% of 20.30 μmol/L REG3α treatment was 70%, 76% and 79% at 24, 48 and 72 h, respectively. The results showed that the recombinant murine REG3α significantly inhibited the growth of SMMC-7721 tumour cells in both concentration-dependent and time-dependent manner. The IC50 values at 24, 48 and 72 h were 6.48, 3.75 and 1.01 μmol/L. Our results suggested that the recombinant murine REG3α may inhibit the growth of human HCC cells. The roles of the recombinant murine REG3α against human HCC were different from previous reports, related to the human REG3A. Moreover, the human REG3A is one of the few anti-acute liver failure (ALF) drug candidates and a free-radical scavenger that targets a broad spectrum of death effectors, favours liver regeneration and exhibits significant curative properties against ALF in mice.[31]

Haemolytic activity of recombinant murine REG3α

In our study, we have analysed the haemolytic activity of the recombinant murine REG3α by the level of human erythrocytes lysis. As shown in Figure 6, no lysis of the human erythrocytes was observed at 0.63, 1.27, 2.54 and 5.08 μmol/L REG3α solution. Almost no lysis was observed at 10.15 μmol/L REG3α and even at 20.30 μmol/L, and 40.60 μmol/L concentration of REG3α solution, under which the protein exhibited a potent antimicrobial and antitumour activity. Hence, its haemolytic activity could be negligible at the tested concentrations. This haemolytic activity analysis indicated that the recombinant murine REG3α is safe to the host. We speculated that the recombinant murine REG3α would be an ideal anticancer and therapeutic agent for inhibiting microbial infections, without posing any risks of antibiotic resistance and other side effects to the host organisms.

Conclusions

In summary, we have successfully expressed and purified the recombinant murine REG3α in P. pastoris system. The recombinant murine REG3α possessed antibacterial activity against certain gram-positive and gram-negative bacteria in vitro. Moreover, it showed a strong activity against the human HCC cell lines SMMC-7721 with negligible haemolytic activity. This suggested that this protein may be a potential novel therapeutic agent to use in the treatment of bacterial infections or cancer.

Disclosure statement

No potential conflict of interest was reported by the authors.
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