Cloning, Expression and Purification of Glutathione Peroxidase of Antarctic Yeast *Rhodotorula mucilaginosa* AN5

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Abstract: Glutathione peroxidase (GPx) is a key enzyme in glutathione antioxidant enzyme system, which can remove H2O2 and other oxidants in organisms. In this study, the GPx gene was cloned, sequence analyzed, prokaryotic expressed and purified from Antarctic yeast Rhodotorula mucilaginosa AN5. The GPx gene sequence was amplified successfully by PCR and named RmGPx (GenBank No. KX164292). The open reading frame (ORF) of RmGPx was 498 bp and encoded 165 amino acids. The predicted molecular weight of the protein was 18268.6 Da and the theoretical isoelectric point was 8.37. The recombinant expression plasmid pET-28a-RmGPx was successfully constructed and transferred to E. coli. The protein expression was the optimum when induced by 0.2 mM IPTG at 37 ℃ for 4 hours. The protein was purified by an elution buffer of 100 mM imidazole in Ni NTA column. All study results supply the theoretical foundation for the functional analysis and application of GPx protein.

1 INTRODUCTION

Glutathione peroxidase (GPx) catalyzes the reduction of hydrogen peroxide by GSH and was first discovered in bovine erythrocytes (Mills 1957). Reactive oxygen species (ROS) are chemically active small molecules formed in the process of aerobic respiration and substrate oxidation. They participate in many biological processes, such as stimulating signal transduction, mediating cell apoptosis and defending against pathogen invasion (Bathige et al 2015). Trace ROS is indispensable in many biochemical processes, while excess ROS will cause cellular damages, such as DNA strand breakage, protein oxidation, polysaccharide depolymerization, membrane peroxidation and signal trans-duction damage (Zhang et al 2015). In order to remove excess ROS and maintain redox balance in cells, aerobic organisms have evolved various non-enzymatic and enzymatic antioxidant systems including GPx (Matés 2000).

GPx can scavenge free radicals, participate in signal transduction and immune response, and plays an important role in biological stress adaptation (Zhang et al 2018, Li et al 2021). In Arabidopsis and yeasts, the growth is inhibited by H_2O_2 produced by environmental stress, while catalase, ascorbate peroxidase and GPx can eliminate them and maintain metabolism balance (Smirnoff and Arnaud 2018). Zhang et al discovered that in corn (*Zea mays* L.) seedlings treated with polystyrene nanoplastics, the antioxidant enzyme activity of superoxide dismutase and GPx increased obviously, which indicated glutamate metabolic pathways appear to be closely related to plant mechanisms for tolerance/detoxification of nanoplastics (Zhang et al 2021).

As well known, Antarctic behaves in harsh environmental conditions. With the changes of air temperature and snow cover, sea ice is highly variable by strong gradients in temperature, salinity, space, and light (Thomas, Dieckmann, 2002). Sea-ice microorganisms suffer huge environmental stresses, which result in the cellular metabolism imbalance and active oxygen generation. So, polar organisms frequently produce more antioxidants or antioxidases to maintain the redox equilibrium

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(Núñez-Pons et al 2018). In this study, GPx gene was amplified by PCR from Antarctica yeast AN5. The recombinant expression plasmid was constructed, and the optimum induction expression and purification conditions of recombinant protein were analyzed. The study lays a foundation for the properties and functions analysis of GPx protein, and provides a reference for revealing environmental adaptation mechanisms of extreme organisms.

2 MATERIALS AND METHODS

2.1 Microorganisms and Growth

Antarctic yeast *R. mucilaginosa* AN5 was isolated from Antarctic sea ice collected by the 23th Chinese Antarctic Scientific Expedition. Yeast AN5 was grown in YEPD medium at 20 °C in an orbital shaker of 120 rpm. *E. coli* were kept in LB medium at 37 °C.

2.2 Cloning and Sequencing of GPx Gene

The yeast cells were collected by centrifugation and ground with a mortar and pestle in liquid nitrogen. Total RNA was extracted following the instruction of total RNA extractor and then removed the possible DNA contamination with gDNA eraser. The RNA was examined in 1.0% (w/v) agarose gel.

The first strand cDNA was prepared by the manufacturer's instruction of the PrimeScript RT reagent kit. The primers *RmGPx*-F1 (5'-ACGACCTCACAACGCTCAG-3') and *RmGPx*-R1 (5'-GTGGGAAAGGCGAGGATATT-3') were used for PCR amplification with cDNA. The PCR product was sequenced by Sangon Biotech.

2.3 Expression of the Recombinant Protein

According to the sequencing result of *GPx* gene, the forward primer *RmGPx*-F2 (5'-CGCGGATCCACCAGCGTCGCCTCTTTC -3') contained a *Bam*HI restriction site (underlined nucleotides) and reverse primer *RmGPx*-R2 (5'-CCCAAGCTTTGCGGACTCGGCGAGCG -3') contained a *Hin*dIII restriction site (underlined nucleotides) were designed to amplify the corresponding open reading frame (ORF). After PCR was performed, the product was purified, digested with *Bam*HI and *Hin*dIII and cloned into the same restriction enzyme sites of pET28a expression vector. The recombinant plasmid was transformed into *E. coli* BL21 cells. The transformants were selected on LB plates with 100 μg/ml kanamycin. Plasmid DNA in the positive clones was extracted with SanPrep column plasmid mini-preps kit and digested with *Bam*HI and *Hin*dIII. The cloned gene was verified by PCR reaction.

2.4 IPTG Induction Expression of GPx Gene

For the expression of *GPx* gene, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to LB medium containing 1 mM kanamycin. For the determination of the optimum induction time, every two hours, 5 ml culture cells were collected and mixed with 5x protein sample buffer, and boiled for 4 min. After a short centrifugation, the mixtures were conducted to SDS-PAGE electrophoresis to detect the expression of target protein. The electrophoresis was run at 120 V with 12.5% separating gel, and stained with Coomassie staining solution for 1 h followed by destaining in destaining solution.

For the determination of the optimum concentration of IPTG induction, when the transformants grew to mid-log phase, IPTG was added to the medium to the final concentration of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM, respectively. After incubation for 2 h, cells were collected by centrifugation and subjected to SDS-PAGE analysis.

2.5 Purification of the Recombinant Protein

The recombinant protein was expressed by 0.1 mM IPTG induction for 2 h. The collected cells were resuspended with cold 0.05 M phosphate buffer (pH 7.0) and broken by the ultrasonic technique for 10 min. After centrifugation, the precipitate was dissolved with 8 M urea. SDS-PAGE electrophoresis was used to detect protein expression in the supernatant and precipitate.

The recombinant protein was purified by Ni^{2+} column affinity chromatography. The sample was firstly washed with 5 bed volumes of washing buffer to remove contaminating proteins, and then the target proteins were eluted by elution buffer. The elution was detected by A_{280} value and SDS-PAGE assay.

3 RESULTS AND ANALYSIS

3.1 Cloning of RmGPx Gene

3.1.1 RNA Extraction

The total RNA of Antarctic yeast AN5 was extracted with the total RNA extractor kit, and electrophoretic detection is shown in Fig. 1. It can be seen that the extracted RNA has three obvious bands of 28s, 18S and 5S rRNA, indicating that the RNA is not degraded.

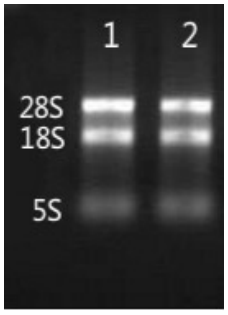


Figure 1: RNA analysis of Antarctic yeast AN5

3.1.2 PCR Amplification of RmGPx

The extracted RNA was reverse transcribed into cDNA by reverse transcription kit. Using cDNA as template, PCR amplification was performed with primers *RmGPx*-F1 and *RmGPx*-R1, and electrophoretic detection was shown in Fig. 2. According to the primer design, the target fragment was 671 bp, which was consistent with the expected size, and sequencing results showed the PCR product was correct sequence.

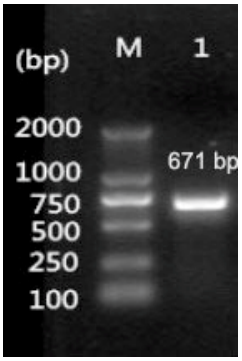


Figure 2: Gel electrophoresis analysis of target gene PCR product M, DNA Marker; Lane 1, PCR product.

3.2 Bioinformatics Analysis of RmGPx

PCR amplification and sequencing showed that the open reading frame of *RmGPx* gene was 498 bp, encoding 165 amino acids. The gene sequence was submitted to GenBank database with the sequence login number of kx164292. RmGPx protein predicted that the theoretical molecular weight was 18268.6 Da and the isoelectric point was 8.37, belonging to cytoplasmic protein. Blastp analysis of RmGPx protein showed that the similarity of RmGPx was the highest with GPx sequences of *Rhodotorula* sp. jg-1b (KWU44177), followed by *Rhodotorula toruloides* NP11 (EMS25797) and *Rhodotorula graminis*.

3.3 Construction of Plasmid Pet-28a-RmGPx

3.3.1 PCR Amplification of RmGPx

Using the cloned *RmGPx* gene fragment as the template, PCR amplification was carried out with primers *RmGPx*-F2 and *RmGPx*-R2. The electrophoresis results (Fig. 3) showed that the target fragment is 498 bp, and the size is consistent with the expected value.

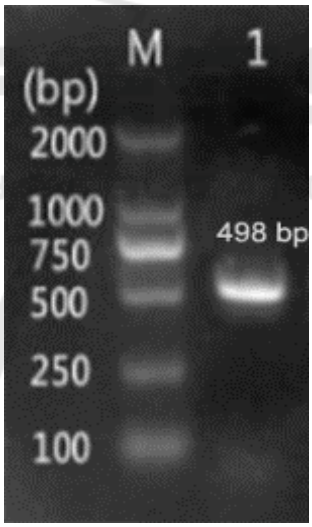
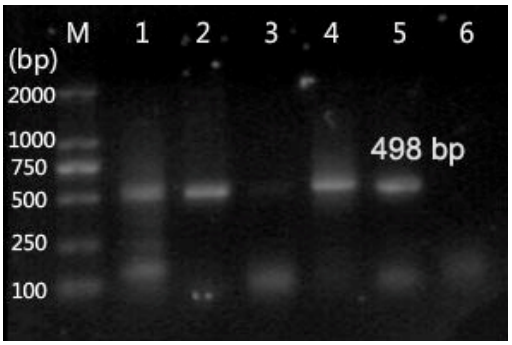


Figure 3: PCR amplification of RmGPx gene.

3.3.2 Identification of Positive Clone

RmGPx gene and vector pET-28a(+) were respectively digested by restriction enzymes *Bam*HI and *Hind*III. After purification and recovery, the digested fragments were connected in proportion, and then converted to DH5α competent cells. Positive clones were screened by colony PCR, and the results indicated that the target gene *RmGPx* was inserted in the plasmid successfully (Fig. 4).

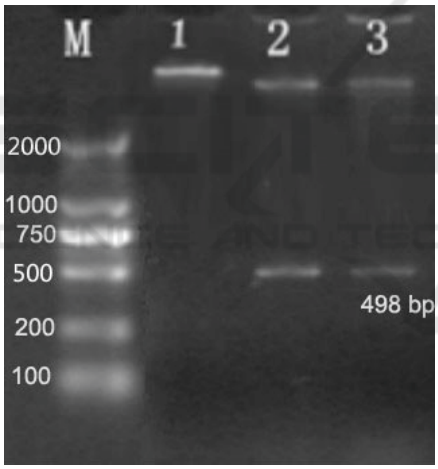


M, DNA Marker DL2000; 1, 2, 4 and 5, Positive clones

Figure 4: Screening of positive clones.

3.3.3 Identification of Recombinant Plasmid

The plasmid of positive clone was extracted and then identified by double enzyme digestion. The results in Fig. 5 indicated that the band size of 500 bp was consistent with the expected value, which proved that the recombinant expression plasmid was successfully constructed.



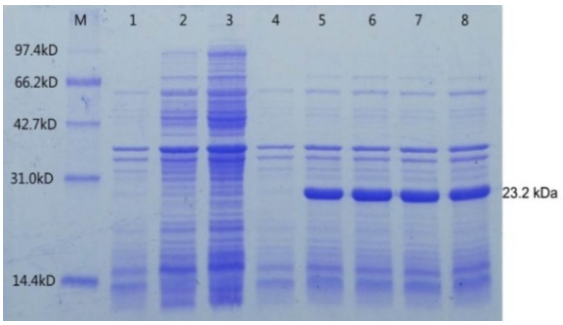
M, DNA Marker; 1, recombinant plasmid; 2 and 3, Enzymatic product.

Figure 5: Gel electrophoresis analysis of enzymatic products of recombinant plasmid.

3.4 Expression of Recombinant GPx

3.4.1 Optimizing of IPTG Induction Time

E. coli BL21(DE3) transformed with pET-28a-*RmGPx* was induced with 1 mM IPTG at 37 ℃. The protein electrophoresis results in Fig. 6 showed that an obvious new protein band was appeared, which was consistent with the expected value of 23.2 kDa. After band density detection, protein expression of Lane 6 was the highest, which indicated that 4 h was the optimum induction time.

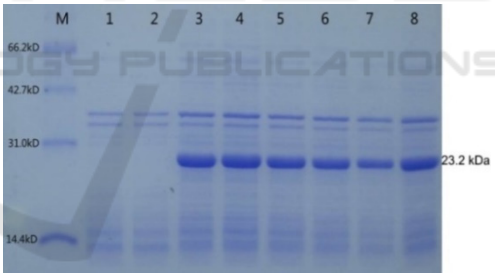


M, protein Marker; Lane 1-3, IPTG induction of wild bacteria at 0 h, 2 h and 4 h; Lane 4-8, IPTG induction of recombinant bacteria at 0 h, 2 h, 4 h, 6 h and 8 h

Figure 6: Expression of recombinant protein induced by IPTG at different time.

3.4.2 Optimizing of IPTG Induction Concentration

E. coli BL21(DE3) transformed with pET-28a-*RmGPx* was induced 4 h at 37 ℃ with different IPTG concentrations of 0.1-1.0 mM, respectively. The results shown in Fig. 7 indicated that Lane 6 was the highest density value, which demonstrated that 0.2 mM was the optimum induction concentration of IPTG.



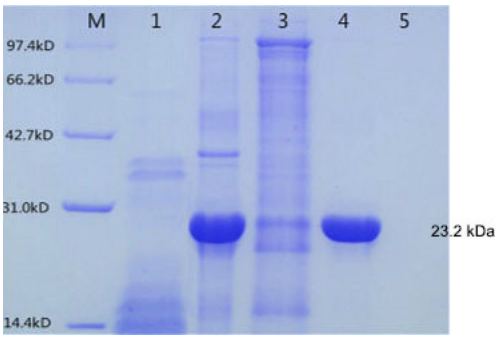
M, protein Marker; Lane 1, Wild bacteria without IPTG; Lane 2, Recombinant bacteria without IPTG; Lane 3-8, recombinant bacteria with IPTG induction of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM

Figure 7: Expression of recombinant protein induced by different IPTG concentration.

3.5 Purification of Recombinant GPx

The recombinant protein was added to the Ni column, and the protein impurity was washed off with binding buffer. Then the binding protein was eluted with elution buffer containing 50 mM, 100 mM and 200 mM imidazole respectively. The eluted samples were collected and concentrated, and then detected by electrophoresis, and the results were shown in Fig. 8. It could be seen that the elusion

buffer containing 50 mM imidazole could wash off the protein impurity, and 100 mM imidazole could elute GPx protein.



M, Protein marker; Lane 1, Recombinant bacteria without IPTG induction; Lane 2, Total protein of IPTG induced bacteria; Lane 3-5, Effluent fraction with 50, 100 and 200 mM imidazole buffer respectively

Figure 8: Purification of recombinant RmGPx.

4 CONCLUSIONS

In this study, the *RmGPx* gene was cloned, expressed and purified from *Rhodotorula mucilaginosa* AN5. The ORF of *RmGPx* was 498 bp encoding 165 amino acids. The predicted molecular weight was 18.3 kDa and the theoretical isoelectric point was 8.37. The optimum expression conditions were 0.2 mM IPTG at 37 °C for 4 hours. The protein was purified by elution buffer of 100 mM imidazole in Ni NTA column. All study results supply the theoretical foundation for the functional analysis and application of GPx protein.

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