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Original Paper

Preparation of SARS-CoV-2 Polymerase Nsp12 and Optimization of Expression Conditions

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Abstract

The core component of transcription and replication of SARS-CoV-2 is Nsp12, a non-structural protein that function as an RNA-dependent RNA polymerase. Nsp12 is a key drug target for the development of anti-coronavirus drugs. The purpose of this study was to explore and optimize the expression conditions of Nsp12 of SARS-CoV-2 with the aim of establishing expression conditions to obtain high-purity Nsp12 protein. This study compared the "ice-water bath cooling" and "shaking table cooling" methods and explored the differences in cooling rates and protein expression and properties using the two cooling methods. The shaking table cooling method resulted in reduced amounts of ArnA protein in the Nsp12 protein samples compared with levels from the ice-water bath cooling method. The shaking table method enabled purification of high-quality Nsp12 protein and ensured stable output of the protein. These methods will enable subsequent exploration of the SARS-CoV-2 replication mechanism, the development of specific drugs and drug screening for anti-SARS-CoV-2 pneumonitis.

Keywords

SARS-CoV-2, RNA polymerase, protein expression, cooling rate

1. Introduction

The SARS-CoV-2 virus was responsible for the worldwide pandemic of the coronavirus disease of 2019, which has become a global crisis. Compared with the SARS virus in 2003, SARS-CoV-2 has a longer incubation period and stronger infectious ability, and the replication and transcription system of the virus plays an important role in this process.

The SARS-CoV-2 Nsp12 protein, a RNA-dependent RNA polymerase, is the core component of transcription and replication. High-purity Nsp12 protein is an essential biomaterial for the research of SARS-CoV-2, such as structural analyses of the SARS-CoV-2 transcription and replication complex

and the screening of specific drugs targeting Nsp12. SARS-CoV-2 Nsp12 is considered as the key drug target for the development of anti-coronavirus drugs. However, the expression and purification of Nsp12 protein is difficult. During the purification process, a hybrid protein tightly binds to Nsp12 and is difficult to remove by the common purification methods. Mass spectrometry revealed that this hybrid protein is ArnA. The presence of ArnA is an obstacle in obtaining a sufficient amount of high-purity Nsp12 protein. Ice-water bath cooling is commonly used in the laboratory to cool down the bacterial culture. During an Nsp12 purification experiment, because of a failure of the ice machine, the cooling system of the shaking table was used for cooling, and we obtained increased yields of Nsp12 with lower levels of the ArnA. Because all other condition parameters were unchanged, we suspected the cooling method was the influencing factor on reduced levels of the ArnA. This study will examine the effect of different cooling methods on the yield and properties of purified Nsp12.

2. Method

2.1 Routine Experimental Procedure

2.1.1 Construction of the Nsp12 Expression Vector

For molecular cloning means, we referred to the second edition of Molecular Cloning Experimental Guide [6], with slight changes. The full-length genome sequence of Nsp12 is available from the databases of GISAID and NCBI. The primers were synthesized by Jinweizhi Biotechnology Co., Ltd. In this construction we used pET-22b vector as the expression vector at the restriction endonuclease cleavage sites of BamHI and XholI.

Enzymatic digestion and recovery of vectors and fragments. PCR products were separated by agarose gel electrophoresis and purified using vazyme gel extraction kit. The products were digested by BamHI and XholI restriction endonucleases at 37 ℃. Ligation and transformation of fragments and vectors, screening and identification of positive clones. The digested vectors and fragments were mixed in a molar ratio of 1:5–1:10 and ligated overnight with T4 ligase at 16 ℃. The ligated product was added to E.coli DH5α cells on ice and the mixture was held on ice for 20 min. Cells underwent heat shock at 42°C for 45 s and were then placed on ice for 3 min. Next, 300 μL LB medium was added and cells were shaken at 37 ℃ for 60 min. The cells were plated on plates containing 100 mg/L ampicillin and incubated overnight at 37 ℃. On the next day, single clones were randomly selected from the plates and inoculated in 5 mL LB medium for culture for 10–15 h. Cells were harvested and plasmid was extracted with a plasmid extraction kit. Purified plasmids were subjected to enzyme digestion, and products were examined by agarose gel electrophoresis to identify positive clones. Plasmids corresponding to positive clones were sent to a sequencing company for sequencing.

2.1.2 Protein Expression

Protein expression and purification was conducted according to the second edition of the Protein Purification Guide [8], with slight changes. pET-22b-Nsp12 expression plasmid was transformed into E. coli BL21 (DE3) coated on a plate containing 100 mg/L ampicillin and cultured in a 37 ℃ incubator

overnight. On the following day, a single clone was selected and used to inoculate 5 mL of LB medium; the culture was incubated overnight on a 37℃ shaking table. Next, 5 mL of culture medium was inoculated into a conical flask containing 1L LB medium (ampicillin, 100 mg/L), and the medium was shaken at 37℃ for 3–4 h until the OD value of the medium was within 0.6–0.8. After cooled by an ice-water bath, the inducer IPTG was added to a final concentration of 0.4 mM. The medium was incubated overnight at 16℃ to induce expression of the target protein.

2.1.3 Purification

Immunoaffinity chromatography: The obtained supernatant containing Nsp12 was added to an elution column containing a nickel filler. The buffer solution containing 20 mM/L imidazole was added to the column to remove the ArnA. Then Nsp12 was competitively eluted using a buffer solution containing 200 mM/L imidazole. Ion exchange chromatography: Because of the charged nature of Nsp12, an anion exchange column was used for binding; the salt concentration of buffer solution was slowly increased, and the elution time of protein was determined by monitoring the characteristic absorption peak by the AKTA system and collecting the correct fraction. Gel exclusion chromatography: The protein solution purified by ion exchange chromatography was concentrated and centrifuged. The sample was loaded onto a gel exclusion column to separate proteins with different molecular weights.

2.2 Research on the Performance of Different Cooling Method

2.2.1 Cooling Rate Measurement Experiment for Different Cooling Methods

Before induction of E. coli, the culture should be cooled to 16℃. Commonly, culture is cooled rapidly by ice-water bath method. In this study, we used two different methods for culture cooling:

Two experimental groups were set in parallel: we prepare 24 bottles of cell culture medium, half of them were cooled by ice-water bath, and the other half were cooled by a shaking table air condition system. The two experimental groups started cooling from 37℃ to 16℃ at the same time, we mapped its temperature fluctuation by measuring its temperature. The group of shaking table cooling was carried out at 16 ℃, the cooling coefficient 0.5. with agitation by a shaking table at 180 r/min. For the group of ice-water bath, the culture medium was placed in ice-water (2L of crushed ice in 2 L of water) and was shook by hand to 16℃ ,then, moving the culture medium from the ice-water to the shaking table at 16℃, cooling coefficient 0.5, with agitation at 180 r/min .The temperature was measured once every 10 min to ensure that the temperature remained at 16℃.

2.2.2 Impurity Protein Elution Experiment for Different Cooling Methods

After cooling, cultures were centrifuged and the pellets were lysed; the lysates were centrifuged and the supernatants were collected for subsequent purification. The E. coli pellet was diluted by lysis buffer to 200 mL and purified using three columns containing 5 mL of Ni affinity medium (GE Healthcare). The supernatant was passed over the columns and then repeatedly passed through the same column for three times. After the residual supernatant in the column was washed out, the medium was re-suspended with 30 mL of lysis buffer, and samples were taken. After the sample was dried, another 30 mL of the lysis buffer was added to the column for three more times to obtain four fractions: 0 mL, 30 mL, 60 mL and

90 mL of protein samples (The lysis buffer composition was as follows: 150 mM NaCl, 25 mM Tris 8.0, 4 mM MgCl2, 10% glycerol, and 20 mM imidazole).

After 90mL of the washings, each column of affinity medium was further washed with 60mL of lysis buffer to a total washings volume of 150mL, and the medium was re-suspended with 30mL of lysis buffer and sampled again. Four microliters of each tube of samples were subjected to protein gel electrophoresis to detect the impurity removal effect, and the protein band brightness was calculated using ImageJ software, and the proportion of impurity proteins was calculated based on the measured values.

2.3 Data Analysis

Data were compared by T test, SPSS 23 software and GraphPad Prism softwares. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Comparison of Cooling Rates of Two Cooling Methods

The author compared the cooling rates of ice-water bath cooling group with shaking table cooling group. Data collection stopped when the temperature dropped to 16 ℃ during the cooling process. The data obtained were analyzed in GraphPad Prism 6.0 software .The results showed that it took 14 min to cool 1 L of cell culture from 37 ℃ to 16 ℃ by the ice-water bath cooling method, with an average rate of 1.5 ℃/min. However, it took 140 min to cool 1 L of cell culture from 37 ℃ to 16 ℃ by the shaking table cooling method, with an average rate of 0.15 ℃/min. These results illustrated the large difference in cooling rates between the two cooling methods.

3.2 Differences of ArnA Protein Binding Ability and Properties Resulted from Two Cooling Means

This study explored the differences in ArnA protein binding ability and properties between ice-water bath cooling group with shaking table cooling group. The results showed significant changes of the properties of ArnA with the ice-water bath cooling method compared with that of the shaking table cooling method.

Furthermore, the author found that the binding ability of ArnA and Nsp12 was weakened (Figure 1-2). Before impurities were washed, the proportion of ArnA in the ice-water bath cooling method was 55% compared with 50% in the shaking table cooling method. After impurities were washed with 90 mL buffer solution, the ArnA in the shaking table cooling group were reduced to 19%, which was a significant improvement compared with 52% in the ice-water bath cooling experimental group ($P<0.01$).

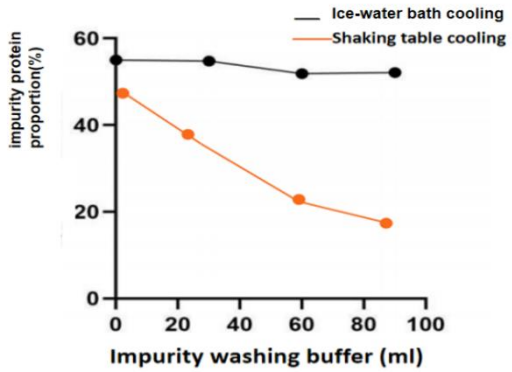

Figure 1. Curve of the Change of ArnA Ratio Caused by Gradient Washing in Two Cooling Methods

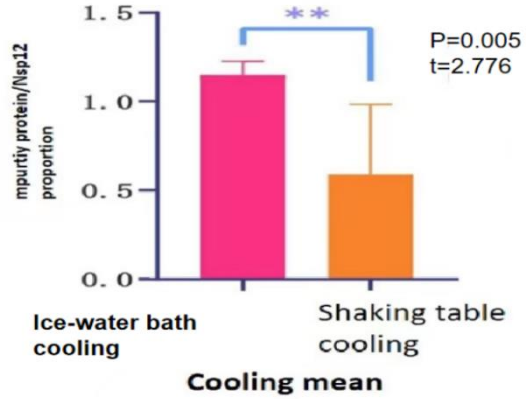

Figure 2. T-Test of the Change of ArnA Ratio Caused by Gradient Washing in Two Cooling Methods

4. Discussion

In this study, researcher evaluated the appearances of different methods for purifying SARS-CoV-2 Nsp12 protein. The author found that the presence of ArnA greatly reduce the yield and purity of Nsp12, so the follow-up work mainly focused on the optimization of Nsp 12 purification. The author noticed in preliminary research that changes in temperature reduction conditions influenced ArnA levels, and the author designed this study to explore the influence of temperature reduction on Nsp12. Our results showed that the cooling rate of the cooling system of the shaking table is slow. Under this mild cooling conditions, the expression of ArnA decreases, and it is easily eluted by buffer solution. This method greatly enhances the purity of Nsp12 obtained by immunoaffinity chromatography, which improves the separation effect of subsequent ion exchange chromatography and molecular sieve chromatography.

The AKTA protein purification system separates Nsp12 and ArnA through different peak positions. Through the peak diagram of target protein and hybrid protein, the difficulty of purification and the optimized effect can be illustrated. The overlap of two peaks means that two proteins were not well

separated before optimization. Figure 3 shows that the peak position remains unchanged after optimization, but the peak height is reduced, and the part mixed with the target protein is greatly reduced. The amount of Nsp12 protein of sufficient purity increased after optimization.

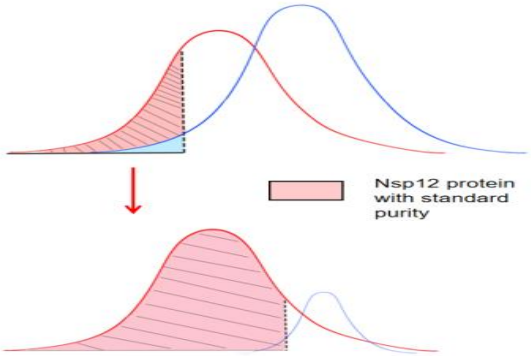

Figure 3. Schematic Diagram of Purification

The composition of lipopolysaccharide in cold-tolerant strains is quite different from that of the normal strains, which is mainly reflected in the high degree of unsaturation and higher density, this ensures the fluidity of cell membranes under low temperature. The composition of lipopolysaccharide changes greatly after Escherichia coli is stimulated at low temperature. Therefore, ice-water bath cooling may represent a severe low-temperature stimulus that brings the renewal of lipopolysaccharide components on E. coli surface to ensure the fluidity of cell membrane. The components renewal belongs to the stress reaction of bacteria, and the renewal of lipopolysaccharide components induces the up-regulation of the expression of the required modified protein ArnA. In addition, ArnA has a strong binding ability with Nsp12, which leads to the purification problems. In this study, changing the cooling method influences the yield and properties of the ArnA. Based on our research we speculate that a drastic drop in temperature will cause stress response in E. coli cells, leading its surface to change from saturated lipopolysaccharide to unsaturated lipopolysaccharide to ensure the fluidity of the cell membrane. The rapid renewal of lipopolysaccharide requires a lot of ArnA to be modified, inducing the overexpression of ArnA. The expression of ArnA may also be related to the degree and severity of cooling. ArnA exists in two 3D architectures, hexamer and tetramer, and the key carboxylase domain of the hexamer is closely oriented to the symmetric center, which is not conducive to its catalytic function. The author speculate that the tetramer may be the basic unit to enable the function of ArnA and makes it easy to combine with Nsp12. In response to a drastic drop in temperature , some of the hexamer is converted into tetramer for catalysis to protect cells, resulting in binding tight to Nsp12. Therefore, when the decline in temperature is slowed down, many hexamers loosely bound with Nsp12 can be easily eluted.

5. Conclusion

In conclusion, the author optimized the expression of Nsp12, reduced the production of ArnA protein, and purified high-quality Nsp12 protein using the shaking table refrigeration system to cool culture samples, instead of the rapid cooling of the ice bath, which ensured stable output of Nsp12 protein. These results lay a good foundation for structural analysis, drug screening and enzyme activity determination. To explore these possibilities, we are planning future studies to construct an ArnA-deleted strain for purification of Nsp12 protein; express ArnA and Nsp12 in vitro; and explore the difference of binding ability between ArnA and Nsp12 in different aggregation states.

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