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An Improved RNA Isolation Method for Filamentous Fungus *Blakeslea trispora* Rich in Polysaccharides

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Abstract Isolation and purification of biologically active RNA from filamentous fungi is difficult because of the complex cell wall and the high level of polysaccharides which bind to or co-precipitate with RNA. Using benzyl chloride and guanidine thiocyanate, RNA was successfully isolated from *Blakeslea trispora* in which other RNA extraction methods and commercially available kits failed to deliver suitable results. The RNA isolated by this procedure appears to be relatively pure, as it has a ratio of absorbance at 260/280 nm of 1.8–1.9. The integrity of the RNA was further substantiated by RT-PCR and Northern hybridization, respectively. This procedure should be useful for isolating RNA from other filamentous fungi and, therefore, will serve as an important tool for the molecular analysis of these organisms.

Keywords RNA isolation · Benzyl chloride · Filamentous fungus · *Blakeslea trispora*

Introduction

Filamentous fungus *Blakeslea trispora* belongs to the order Mucorales within the class Zygomycetes. Because of its unique ability to produce high amounts of β-carotene in submerged cultures, it is the only species used for β-carotene production on an industrial scale. Carotenoid biosynthesis in fungi occurs via the mevalonate pathway [1]. The mevalonate pathway in *B. trispora* is highly active, and the flux through the pathway is higher than that in other species. Consequently, *B. trispora* is suitable for terpenoid production as a host strain. Although some advances have been made in *B. trispora* genetic engineering, there are still a few obstacles, such as plasmid transformation, selectable marker, and RNA extraction [2, 3].

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Isolation of high-quality RNA is critical for many molecular biological experiments, such as making cDNA libraries, isolating genes by reverse transcriptase-polymerase chain reaction (RT-PCR), investigating gene expression profiles, etc. The chemical compositions of different fungi are found to vary considerably [4], and even fungi belonging to the same genus or related genera can exhibit enormous variability in their chemical compositions. The chemotypic heterogeneity among species might hinder optimal RNA yield when using a single isolation protocol, and perhaps even closely related species may require significant different isolation protocols. A common problem encountered in such studies is that it is difficult to isolate pure and intact RNA from the fungal cultures because of the presence of high levels of polysaccharides. Furthermore, high molecular weight polysaccharides interfere with RT-PCR and Northern hybridization results [5, 6].

In this report, a procedure developed by Chomczynski and Sacchi [7] was modified and applied to the filamentous fungus *B. trispora*, which is referred to as benzyl chloride/guanidine thiocyanate (B/G) protocol and can be employed to effectively eliminate the effect of high levels of polysaccharides. Two other standard isolating methods were used to compare with B/G protocol, i.e., RNA extraction kits (E.Z.N.A.TM Fungal RNA Kit, Omega, USA) and Triton X-100/ethylene diamine tetraacetic acid (EDTA) protocol [8].

Materials and Methods

Organisms and Cultivation Conditions

B. trispora wild-type strain ATCC14271 (−) was maintained on potato dextrose agar (PDA) slants. The strain was grown on petri dishes at 28°C for 4 days and then at 20°C for 2 days to induce the formation of spores. Spores were harvested by rinsing the fully grown agar plates with a solution of 0.9% (*w*/*v*) NaCl. For the submerged culture, 10^7 spores of the strains were inoculated into 50 mL PDA liquid medium in 250-mL Erlenmeyer flasks. The flasks were shaken for 2 days at 180 rpm at 28°C without light. The moist mycelia were subsequently harvested by filtration and washed with double-distilled water.

Reagents

Solutions and reagents used were as follows: solution I—100 mM trihydroxymethylamino-methane–hydrochloride, 40 mM EDTA, pH 9.0; benzyl chloride (Sigma Aldrich, Steinheim, Germany), 10% sodium dodecyl sulfate (SDS); extraction solution (solution II)—4 M guanidine thiocyanate (Promega, Madison, USA), 25 mM sodium citrate,0.5% (*w*/*v*) sodium lauroyl sarcosine, 0.15 M β-mercaptoethanol (which has to be added into solution II just before use); phenol/chloroform/isoamyl alcohol (PCI) 25:24:1; chilled absolute ethanol and 70% ethanol; 2 M sodium acetate, pH 4.0; morpholinopropane sulphonic acid (MOPS) running buffer: 20 mM MOPS (Sigma Aldrich, Steinheim, Germany), 2 mM sodium acetate, 1 mM EDTA, pH 8.0; 0.1% DEPC-treated autoclaved double-distilled water.

RNA Extraction Protocols

In this paper, three protocols were studied. A modified one named B/G protocol was applied to isolate high-quality RNA from filamentous fungus *B. trispora*. In this protocol, benzyl chloride, potassium acetate, and absolute ethanol were used in the pretreatment and extraction process to get rid of polysaccharides, which is the main improvement of this protocol over the

procedure developed by Chomczynski and Sacchi [7]. Other two existing protocols (Triton X-100/EDTA protocol and RNA extraction kits) were used for comparison.

In B/G protocol, moist mycelia were pretreated with solution I before extraction. First, 5 mL solution I was added into the Erlenmeyer flask containing 1 g moist mycelia and gently vortexed. Then, 1 mL 10% SDS and 3 mL benzyl chloride were added into the flask, which was vortexed vigorously to make the mixture milky, and stirring was kept at 50°C for 1 h. Finally, the mixture was filtrated through the paper filters using vacuum pump to harvest the mycelia. The pretreated mycelia were subsequently frozen in liquid nitrogen for RNA extraction. In the extraction process, the frozen samples were first ground with mortar and pestle in the presence of liquid nitrogen. The resulting powder was transferred to a 1.5-mL Eppendorf tube containing 500 μl solution II and gently vortexed and inverted two or three times to homogenize the sample. After homogenizing the sample, 0.25 volume of absolute ethanol and 0.11 volume of 5 M KAc (pH 6.0) were added into the tube and inverted two or three times. Then, an equal volume of PCI was added into the tube and the sample was vortexed until thoroughly suspended (the whole homogenate turns to a turbid solution), and this was chilled on ice for 5 min. After centrifuging at 16,000×*g*, 4°C for 5 min, the upper clear aqueous layer was carefully removed and placed into a fresh Eppendorf tube. The obtained solution was retreated by PCI in the same manner. After treating by PCI, the obtained upper-phase solution was transferred into a fresh Eppendorf tube with an equal volume of chilled isopropanol and then incubated at −20°C for 10 min. Lastly, the precipitated RNA was collected from the supernatant by centrifugation at 16,000×*g*, 4°C for 5 min. The collected RNA pellet was washed by 70% ethanol and dried at room temperature and finally dissolved in DEPC-treated autoclaved double-distilled water.

The Triton X-100/EDTA protocol was carried out following the method reported by Dessel et al. [9], and extraction of total RNA by extraction kits was taken as described by the kits' manufacturer.

RNA Analysis

Purity was estimated by the absorbance ratio of A_{260}/A_{280}, to measure the contamination by the polyphenols, carbohydrates, or proteins. The yields were measured from absorbance A_{260} and the volume of RNA. Integrity was appraised with gel analysis for the intact 28S, 18S, and 5S ribosomal RNA.

RT-PCR Analysis

RT and subsequent PCR were carried out as described by the kits' manufacturer (Promega, USA) using a primer pair for the gene encoding *CarR* (P1: AAAGCCGTTTCACTCACA; P2: CACAGGACAAGCATACCA). RT-PCR conditions were: 45°C for 45 min, 94°C for 2 min, 40 cycles of 94°C for 30 s, 60°C for 1 min, 68°C for 2 min. The final cycle was followed by an extra extension step at 68°C for 7 min. The reaction product was electrophoretically separated on 1.5% agarose gel. After being stained with ethidium bromide, the gel was visualized and photographed under UV light.

Northern Hybridization

The *CarR* probe for radiolabel and hybridization was 397 bp. *CarR* gene fragment amplified with a pair of primers (CR1: 5′-TTGCTCTTCTTATGTATGGT-3′; CR2: 5′-ATAATTTCACTAGAAGGGAC-3′). The probe was radiolabeled using Random Primer

DNA Labeling Kit Ver. 2 from TaKaRa Company. Thirty grams of total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to Hybond-N+ nylon membranes (Amersham, England). The membranes were baked at 80°C for 1-h fixation. The fixed membranes were prehybridized at 42°C in prehybridization solution containing 50% formamide, 0.25 M NaCl, 0.25 M Na_2HPO_4, 10 mM EDTA (pH 8.0), 0.1% SDS, 100 g/mL salmon sperm DNA, and 5× Denhardt's reagent for 6 h. Hybridization was performed at 42°C in the same buffer solution for 24 h. The membranes were washed twice with a solution containing 0.3 M NaCl, 30 mM sodium citrate, and 0.1% SDS at 50°C for 30 min and then twice with a solution containing 25 mM NaH_2PO_4, 1 mM EDTA (pH 8.0), and 0.1% SDS at 50°C for 15 min. They were then exposed to FUJI X-ray film with an intensifying screen at −70°C for a week.

Results and Discussion

The success of the RNA isolation protocol may be judged by the quantity, quality, and integrity of RNA. The B/G protocol gave good RNA yield 110.7 μg per gram fresh weight of tissue. We obtained a A_{260}/A_{280} ratio of 1.89 when using the B/G protocol, which indicated that the presence of contamination was negligible [10].

The integrity of the RNA was checked by performing gel electrophoresis on a 1.5% agarose-formaldehyde gel. About 98% to 99% of total RNA is rRNA [11]; therefore, the integrity of an RNA sample can be determined by the integrity of ribosomal RNA. Both 28S and 18S rRNA bands were visible in samples isolated using method l. In addition, the 28S rRNA band was significantly more intense than the 18S rRNA band (Fig. 1a), indicating that RNA degradation did not occur. A faint background smear is also visible, probably corresponding to mRNA. A faint band, which may correspond to 5S RNA, is also visible at the bottom of the gel. However, 28S and18S rRNA bands were absent in Fig. 1b and c, suggesting that RNA isolated with methods 2 and 3 was degraded.

As indicated in Fig. 2, RNA prepared with method 1 described in this study served as a robust template for reserve transcription which resulted in the expected amplification of a 397-bp amplification. However, no amplifications were obtained when RNA isolated by either method 2 or 3, respectively, was used as the template.

When the RNA was subjected to Northern hybridization, transcription in the molecular weight range of 1.8 kb was detected (Fig. 3), suggesting that the isolated RNA is pure, without any contaminations, and amenable to use for other downstream applications. Northern hybridization often requires high-quality, intact RNA, free from any poly-

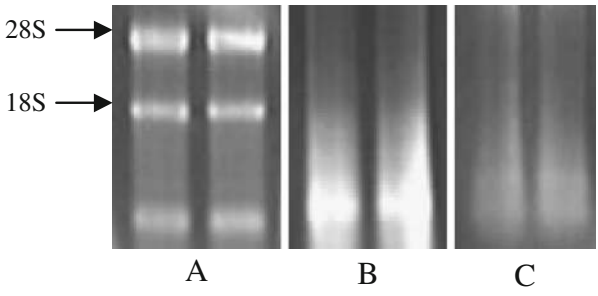

Fig. 1 Ethidium bromide-stained 1.5% agarose-formaldehyde gel electrophoresis of total RNA (5 μg) isolated from *B. trispora*. **a-c** Simultaneous isolation RNA using benzyl chloride and guanidine thiocyanate (method 1), Triton X-100/EDTA (method 2), and RNA extraction kit (method 3), respectively

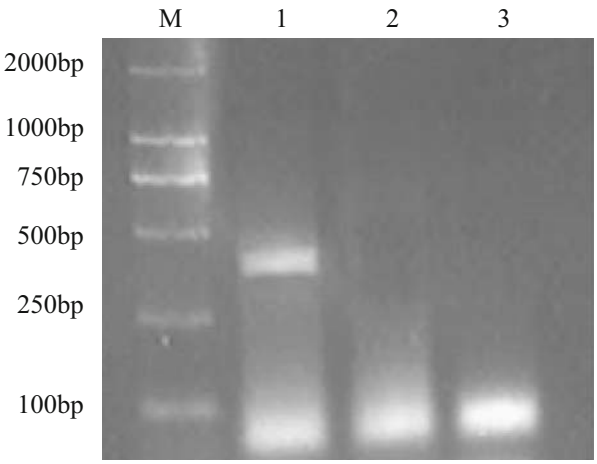

Fig. 2 RT-PCR products were detected on agarose gel. *M* DL-2000 Marker (2, 1, 0.75, 0.5, 0.25, 0.1 kb), *lane 1* by method 1, *lane 2* by method 2, *lane 3* by method 3

saccharides, proteins, and other inhibitors because nucleic acids form tight complexes with polysaccharides and form a gelatinous pellet containing embedded RNA [12].

The method of Majumdar et al. [8] and commercially available RNA kits for RNA isolation were applied to fungal tissues, but gave a poor result and failed to yield usable RNA for further investigations (Figs. 1b, c and 2). *B. trispora* contains a high level of polysaccharides whose content is higher than that of other fungal species, and the polysaccharides in the medium are so viscous that it is difficult to separate the mycelia from the liquid medium by a Buchner funnel [13]. Moreover, polysaccharides can interact with nucleic acids by forming insoluble complexes, affecting the yield and quality of RNA [14]. For the successful isolation of RNA from tissues rich in polysaccharides, it is important to prevent these contaminating substances from binding to nucleic acids. Therefore, benzyl chloride, potassium acetate, and absolute ethanol were used in the pretreatment and extraction process of the present method, respectively, to remove excess polysaccharides. Benzyl chloride is a chemical reagent, which, under the weak alkalic condition [15], can react with hydroxyl residues of polysaccharides, thus destroying the long chain of polysaccharides. For instance, it can split the chitin which is the substantial element of fungal cell walls. Based on this, the *B. trispora* mycelia were collected by filtrating through a Buchner funnel in the presence of benzyl chloride. Carbohydrates were selectively precipitated in the presence of 20% ethanol and 0.5 M K^+ [11]. Under these conditions, RNA remained in solution. A combination of the two steps in the present procedure was found effective for the almost complete removal of polysaccharides from total RNA preparations. If there are remaining polysaccharides, elimination of the final lower levels of

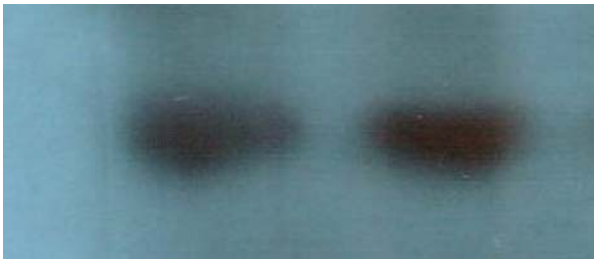

Fig. 3 Northern blot analysis: Northern blotting with *CarR* gene of *B. trispora*

contaminating polysaccharides was possible using absolute ethanol to precipitate total RNA from RNA water solution again.

Conclusion

The method described in this paper allowed the isolation of RNA from fungi rich in polysaccharides, for which other methods failed to deliver RNA suitable for reverse transcription and Northern analysis. The method reported here is, therefore, simple and efficient for the isolation of RNA from fungi that possess a wide range of properties that can interfere with RNA extractions and analysis. Furthermore, our method is not complicated and does not require long ultracentrifugation but is straightforward. The value of this method is that it should be suitable for isolating RNA from other filamentous fungi, and this protocol has been routinely used in our lab for isolation of RNA from different species rich in polysaccharides.

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References

1. Lee, P. C., & Schmidt-Dannert, C. (2002). *Applied Microbiology and Biotechnology*, *60*, 1–11 Medline. DOI 10.1007/s00253-002-1101-x.
2. Jayaram, R. (1986). *Proceedings of the National Academy of Sciences of the United States of America*, *83*, 7344–7347 Medline. DOI 10.1073/pnas.83.19.7344.
3. Quiles-Rosillo, M. D., Ruiz-Vazquez, R. M., Torres-Martinez, S., & Garre, V. (2003). *FEMS Microbiology Letters*, *222*, 229–236 Medline.
4. Mukhtar, M., Parveen, Z., & Logana, D. A. (1998). *Journal of Microbiological Methods*, *33*, 115–118 DOI 10.1016/S0167-7012(98)00047-5.
5. Yang, H., Wang, S. C., & Wang, Z. T. (2004). *Chin. Pharm. J.*, *39*, 254–256.
6. Li, B., Wang, B., Tang, K., Liang, Y. L., Wang, J., & Wei, J. M. (2006). *Colloids and Surfaces B: Biointerfaces*, *49*, 101–5 DOI 10.1016/j.colsurfb.2006.03.006..
7. Chomczynski, P., & Sacchi, N. (1987). *Analytical Biochemistry*, *162*, 156–159 Medline. DOI 10.1016/0003-2697(87)90021-2.
8. Majumdar, D., Avissar, Y. J., & Wyche, J. H. (1991). *BioTechniques*, *11*, 94–101 Medline.
9. Dessel, W. V., Mellaert, L. V., Geukens, N., Lammertyn, E., & Anne, J. (2004). *Journal of Microbiological Methods*, *58*, 135–137 Medline. DOI 10.1016/j.mimet.2004.03.015.
10. Keulen, G. V., Siebring, J., Rembacz, K. P., Hoogeveen, M., Tomczynska, , & Dijkhuizen, L. (2004). *Journal of Microbiological Methods*, *58*, 139–142 Medline. DOI 10.1016/j.mimet.2004.03.014.
11. Lopez-Gomez, R., & Gomez-Lim, M. A. (1992). *HortScience*, *27*, 440–442.
12. Sharma, A. D., Gill, P. K., & Singh, P. (2003). *Analytical Biochemistry*, *314*, 319–21 Medline. DOI 10.1016/S0003-2697(02)00689-9.
13. Shan, Z. P., Meng, Y., & Jiang, W. H. (2001). *Biotechnology (Chinese)*, *11*, 5–7.
14. Chirgwin, J. M., Pryzbyla, A. E., & Macdonald, R. J. (1979). *Biochemistry*, *18*, 5294–5299 Medline. DOI 10.1021/bi00591a005.
15. Raina, K., & Chandlee, J. M. (1996). *BioTechniques*, *21*, 1030–1032 Medline.