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Isolation of high quality RNA from *Polyporus umbellatus* (Pers.) Fries

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Abstract

Background: The dried sclerotium of medicinal fungus *Polyporus umbellatus* (Pers.) Fries has many pharmacological functions such as diuretic and anticancer activity, in which high-content polysaccharides may play an important role. However, RNA isolation is difficult in filamentous fungi and lacking in *P. umbellatus*. **Results:** Five methods for RNA extraction from five strains collected from four provinces were assessed for their ability to recover a high-quality RNA applicable for sequence-related amplification polymorphism (SRAP) PCR and GDP-D-mannose pyrophosphorylase (GMP) gene expression profiles. Both A260/A280 and A260/A230 ratios of the best Trizol Plus + RNAiso-mate for Plant Tissue method are around 2 with a yield of 1122.00 ± 0.21 ng μl^{-1}. The Trizol method also showed good quality with the yield 469.60 ng μl^{-1}. The SRAP PCR amplified clear and polymorphic bands in all five cDNA samples transcribed from RNA by using primer Me4-Em4. GMP gene fragment (1251 bp) was successfully amplified by RT-PCR, suggesting the integrity of isolated RNA. **Conclusion:** All these results showed that the total RNA isolated by this protocol is of sufficient quality for subsequent molecular applications.

Keywords: chuling, medicinal fungus, *Polyporus umbellatus*, RNA isolation

INTRODUCTION

Chuling (*Polyporus umbellatus* (Pers.) Fries), a medicinal fungus that belongs to Polyporaceae, Basidiomycetes, is widely distributed in China, including Shaanxi, Yunnan, etc. (Zhang et al. 2010). Its dried sclerotium has been used as diuretic in Chinese medicine for more than 2500 years (Yuan et al. 2004; National Committee of Pharmacopeia, 2010). Recently, the sclerotium has been reported to exhibit other pharmacological functions such as *in vivo* anticancer activity (Zhao et al. 2010). These potent pharmacological properties have attracted worldwide interest in developing this medicinal fungus, whereas over-digging wild materials has made its wild resources drastically decrease in China (Zhang et al. 2010).

However, molecular investigations on this fungus are very limited, and its RNA isolation is lacking. This may be attributed to the higher polysaccharide content contained in this species that can severely interfere with DNA, RNA and protein purification (Lönneborg and Jensen, 2000; Sánchez-Rodríguez et al. 2008; Zhu et al. 2012). Our past work showed that the intracellular polysaccharide contents ranged from 23.56 to 64.50 mg g^{-1} fresh mycelium weight among seven mycelial strains which sclerotium collected from seven provinces of China (Zhang et al. 2010). Like other filamentous fungi, *P. umbellatus* has rigid cell walls (Guo and Xu, 1992) which make RNA isolation more difficult.

Few methods for RNA extraction from fungi have been reported in the past years. These described methods often are difference because of different second metabolites contained in different fungal species (Sánchez-Rodríguez et al. 2008; Yang et al. 2010). On the other hand, to investigate the molecular mechanism of polysaccharide accumulation in Chuling, the intact and high quality RNA need to be obtained at first.

In this paper, we firstly described simple methods to efficiently remove polysaccharide and other secondary metabolites from five mycelia strains of *P. umbellatus* using Trizol Plus + RNAiso-mate for Plant Tissue to obtain high quality of RNA which showed good results for reverse transcription PCR and sequencing.

MATERIALS AND METHODS

Sclerotium materials

Five sclerotium strains of *P. umbellatus* were collected from Shaanxi, Henan, Heilongjiang and Sichuan provinces (Zhang et al. 2010). They were identified and preserved at local laboratory of regional institute after collection. The protocol of activation culture for each sclerotium was described by Zhang et al. (2010) and every mycelium was stored at -20ºC for use.

RNA extraction protocols

Total RNA was isolated from mycelium after activation culture by using five isolation methods, namely, CTAB-LiCl, Trizol Plus + RNAiso-mate for plant tissue (TaKaRa, Dalian), Trizol method (Qiagen, Beijing), Trizol Plus and Trizol Reagent, respectively. The latter three isolation methods were followed by their corresponding commercial kits' instructions. The CTAB-LiCl method was combined with Wang et al. (2005) and our work on lotus (*Nelumbo nucifera* Gaertn. ssp. *nucifera*) (unpublished data) that was described as follows. In a pre-chilled mortar and pestle, 0.1 g mycelium was finely ground using liquid nitrogen, and quickly transferred to 1 ml extraction buffer pre-warmed by 65ºC for 5 min. RNA extraction buffer contained 2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2.0 M NaCl, 0.5 g/L spermidine and 2% β-mercaptoethanol. The mixture was shaken vigorously for 15 sec every 1 min for 5-6 times and then cooled down to room temperature. The 1 ml chloroform was added to it and mixed well by invert mixing. The content was centrifuged at 12,000 x g for 20 min at 4ºC. To carefully collect the upper aqueous phase, equal volume of chloroform was added and mixed well, followed by centrifugation at 12,000 x g for 20 min at 4ºC. Exactly 0.25 volumes of 12 M LiCl was added in the upper aqueous phase and stored at -20ºC for one night after slightly mixed. The mixture was centrifuged at 14,000 x g for 20 min at 4ºC and the RNA pellet was re-suspended in 500 µl water treated with DEPC for 5 min and additional 500 µl chloroform was added, followed by centrifugation 14,000 x g for 10 min. The two volumes of ethanol were added in the collected upper phase. The pellet was collected by centrifugation (12,000 x g for 20 min at 4ºC) after incubating the tubes at -80ºC for 2 hrs and then washed twice with 75% ethanol, and centrifuged at 12,000 x g for 10 min at 4ºC. The pellet was re-suspended in 30 µl DEPC treated water after evaporation of ethanol. The steps of combined Trizol Plus + RNAiso-mate for Plant Tissue method are: 0.1 g mycelium was added in a pre-chilled mortar and pestle and ground to fine powder using liquid nitrogen, and quickly transferred to 2 ml RNAiso-mate for Plant Tissue buffer to eliminate polysaccharide. The mixture was ground again till that the solution became transparent after the powder completely dissolved in the kit buffer. The following procedure of RNA isolation was carried according to the Trizol Plus kit's instructions, followed by the mixture was centrifuged at 12,000 x g for 5 min at 4ºC.

Total RNA analysis

The quality and quantity of total RNA were determined by monitoring both A260/A280 and A260/A230 absorbance ratios using the UV-2802H spectrophotometer (Unico, Shanghai) and running 1.2% of agarose gel electrophoresis.

Reverse transcription PCR

The first strand cDNA was prepared using Tianscript RT Kit (Tiangen, Beijing) in a one-tube protocol according to the manufacturer's instructions. Five cDNAs obtained from five strains were amplified by employing sequence-related amplified polymorphism (SRAP) primer Me4-Em4 (forward: 5'-TGAGTCCAAACCGGACC-3'; reverse: 5'-GACTGCGTACGAATTTGA-3'). The 1251 bp cDNA of GDP-D-mannose pyrophosphorylase (GMP) gene was transcript from Shaanxi strain using the specific primers designed from PGM cDNA as follows: GMP1 (sense) (5'- ACCGSCAGCCABAAYCCG-3') and GMP2 (antisense) (5'-BSCGCTCATYTCGCC-3'). The SRAP PCR amplification was performed with 20 ng of first-strand cDNA, 1x Taq MasterMix (Cwbio, Beijing), 0.4 μM of primer pair. The amplification was as follows: 94ºC for 5 min, followed by 5 cycles of 94ºC 1 min, 33ºC for 1 min, 72ºC for 1 min and subsequent 35 cycles of 94ºC 1 min, 50ºC for 1 min, 72ºC for 1 min, and a final elongation step of 72ºC for 10 min. The PGM gene fragment amplification was performed with 20 ng of first-strand cDNA, 1x Taq MasterMix (Cwbio, Beijing), 0.4 μM of primer pair. The amplification was as follows: 94ºC for 5 min, followed by 40 cycles of 94ºC 1 min, 56.5ºC for 30 sec, 72ºC for 1 min, and a final elongation step of 72ºC for 7 min. The PCR product of the expected size was obtained for the desired PGM cDNA as confirmed later by sequencing.

RESULTS AND DISCUSSION

The mycelium samples of five *P. umbellatus* strains collected from four provinces of China were used to isolate RNA and to compare their quality and quantity by employing five methods. The commonly used Trizol method isolated good quality RNA but slightly lower than that of Trizol Plus + RNAiso-mate for Plant Tissue in both purity and yield (Table 1). The other two commercial kits and improved CTAB were not efficient, which was also reported in other fungal species, *i.e. Rhodosporidium toruloides* (Yang et al. 2010). The improved CTAB-LiCl described here efficiently eliminated most of the secondary metabolites in lotus (*Nelumbo nucifera* Gaertn. ssp. *nucifera*) and produced white and water-soluble RNA precipitates with considerable yield (ranging from 59.87 to 163.75 μg g^{-1} fresh weight) (unpublished data). Although this method can eliminate polyphenols, polysaccharides and proteins from plant species, the effect on RNA isolation from fungi with high polysaccharide is limited.

Table 1. Absorbance ratios and yield of total RNA isolated from five mycelium samples of *Polyporus umbellatus* collected from four provinces of China by using five isolation methods.

Methods	A260/A280 ratio	A260/A230 ratio	Yield (ng/μl)
CTAB-LiCl	1.59 ± 0.09	0.56 ± 0.04	17.95 ± 0.04
Trizol	1.97 ± 0.03	1.85 ± 0.02	469.60 ± 0.03
Trizol Plus	1.73 ± 0.04	1.60 ± 0.02	501.00 ± 0.02
Trizol Plus + RNAiso-mate for Plant Tissue	2.01 ± 0.08	2.07 ± 0.06	1122.00 ± 0.21
Trizol Reagent	1.70 ± 0.03	0.70 ± 0.04	51.75 ± 0.03

Our results showed the combined method of Trizol Plus + RNAiso-mate for Plant Tissue exhibited high efficiency for RNA isolation by using two commercial kits and the brightness of 28 S were approximately twice that of 18 S (Figure 1), which was considered of good intactness. Both A260/A280 and A260/A230 ratios are often used to indicate RNA purity because RNA absorbs UV light maximally at 260 nm, whereas protein absorbs at 280 nm and other contaminants including polysaccharides, phenol and aromatic compounds generally absorb around 230 nm (Wang et al. 2011). The A260/A280 and A260/A230 ratios obtained with this combined method were 2.01 ± 0.08 and 2.07 ± 0.06, respectively. In general, both ratios range from 1.8 to 2.1 indicate high purity RNA and free of polysaccharide/polyphenol and protein contamination.

Due to high sensitivity of reverse transcription to impurities, it is most important to produce pure RNA for downstream work, such as construction of full-length cDNA library and gene expression. To evaluate the suitability of our isolated RNA in downstream molecular procedures, RNA obtained from five origins reversely transcribed into cDNAs, which was used for SRAP PCR amplification and PGM gene cloning. The SRAP PCR amplified clear and polymorphic bands in all five samples by using

primer Me4-Em4 (Figure 1). The GMP gene fragment (1251 bp) was also successfully amplified (Figure 1) by using Shaanxi sample and compared with GenBank data after sequenced. High homology through alignment confirmed the identity of the RT-PCR product after that full-length cDNA was sequenced by RACE (data not shown).

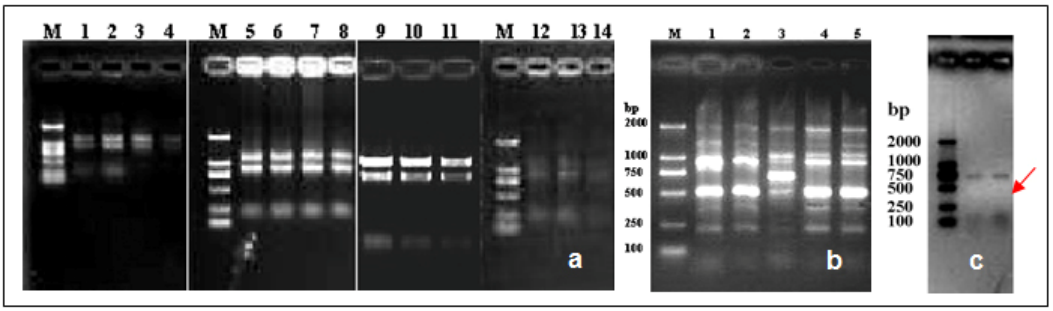

Fig. 1 (a) Five methods for RNA extraction from mycelium of *Polyporus umbellatus* collected from Shaanxi Province. Lane 1-4: CTAB-LiCl; Lane 5-6: Trizol; Lane 7-8: Trizol Plus; Lane 9-11: Trizol Plus + RNAiso-mate for Plant Tissue; Lane 12-14: Trizol Reagent. **(b)** RNA examples were extracted by Trizol Plus + RNAsio-mate for Plant Tissue. Lane 1-5 represented five mycelium strains that collected from five regions, namely, Taibai Mountain (Shaanxi), Qinling Mountain (Shaanxi), Henan, Heilongjiang and Sichuan Province. **(c)** The GDP-D-mannose pyrophosphorylase (GMP) gene fragment was successfully amplified from mycelium strain of *Polyporus umbellatus* that collected from Shaanxi Province, China.

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