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A Simple and High Quality Method for Isolation and Extraction of Total RNA of *Pholiota adipose*

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Abstract Analyzing functional values of RNA using RNA-seq technology is a hot spot of researches. In order to study the medicinal value of *Pholiota adipose* from the transcriptome level, it is necessary to extract and isolate RNA samples of high purity and high quality *P. adipose*. In this study, liquid nitrogen grinding Trizol one-step method was used to extract the total RNA of *P. adipose*. Quality test and statistical comparative analysis were carried out for RNA extract of liquid nitrogen grinding treated and untreated *P. adipose*. The results showed that the concentration of RNA in the samples treated with liquid nitrogen was much higher than that of the samples without grinding treatment. The $OD_{260/280}$ of both was about 2, indicating that the purity of RNA was very high. Besides, the ratio of fluorescence intensity of 25S and 18S subunit strips of three replicate samples was 1.8, 1.9, and 1.9, close to 2, indicating RNA integrity is good. RIN test results of Agilent 2100 were 9.1, 8.7, and 9.3, higher than the standard value 6.8, further proving the integrity. In sum, liquid nitrogen grinding Trizol one-step method is a very simple and efficient method for extracting high quality total RNA of *P. adipose*.

Key words RNA isolation, *Pholiota adipose*, RNA-seq, Antineoplastic

1 Introduction

Pholiota adipose has wide distribution in China, mainly including Heilongjiang, Jilin, Guangxi, Shandong, and Hebei[1,2]. It belongs to Basidiomycota, Agaricomycetes, Agaricales, Strophariaceae, *Pholiota*[3]. Sporophore and fermented mycelium of *P. adipose* are rich in amino acids[4], vitamins[5], mineral elements[5], and proteins[6]. Through comprehensive evaluation of nutritional value of *P. adipose* by internationally general nutritional evaluation method, the fermented mycelium and sporophore of *P. adipose* have higher score of amino acids and chemicals than other edible fungi, thus *P. adipose* is an edible fungus with high nutritional value and broad development prospect[7-9]. Besides, *P. adipose* has huge medicinal value[10]. Extensive studies have shown that *P. adipose* polysaccharides can play a great role in inhibiting bacteria, resisting cancer[11], resisting tumor[12], activating macrophage of mice[13], reducing blood fat[14], adjusting immunity[15], anti-oxidization[16], and antimicrobial activity[17]. Therefore, research of edible and medicinal values of *P. adipose* has become a hot spot of *P. adipose*.

As high-throughput sequencing technology for research of transcriptomics developed in recent years[18], transcriptome sequencing (RNA-seq) technology provides a new and effective method for studying edible and medicinal values of *P. adipose* from transcriptome level. With five major unique advantages (high accuracy[19], high sensitivity, and transcriptome analysis of any species[20-22], wide detection range and high reproducibility[23,24], and few initial samples[25]), RNA-seq technology has gradually become mainstream of study of transcriptomics. On this basis, a complete set of transcriptome research process has been established in the field. The technology is mainly applied in researches of mRNA, microRNA and lncRNA; in addition, researches focusing on lncRNA have become the hot spot of transcriptome research[26,27]. RNA-seq technology has also been applied in cancer diagnosis, analysis of microbial metabolic pathways, and drug research and development, but its application in the study of *P. adipose* has not been reported at both home and abroad. The application of RNA-seq technology has a great significance for carrying out the study of *P. adipose* transcriptomics and exploring its edible and medicinal value, anti-cancer mechanism, and drug development potential, and has become an inevitable trend of the study of *P. adipose*. However, without the high-purity and high-quality *P. adipose* RNA, RNA-seq study will not be carried out. Thus, it is crucial to explore a suitable method for the simple and efficient extraction of total RNA from *P. adipose*.

At present, RNA extraction methods mainly include CTAB, Trizol, and CTAB isopropanol method[28]. Although many biospecific RNA isolation and extraction methods have been established[29-34], and several RNA isolation and extraction kits have been developed, there are differences in extraction effect, reagent composition and operation steps between different methods. *P. adipose* is rich in polysaccharides and other substances interfering with the extraction of RNA, thus the establishment of fully suitable for high-quality *P. adipose* RNA extraction method is of great significance for study of transcriptomics, and whether the extracted RNA can be used for RNA-seq analysis of *P. adipose* is the purpose of this study.

In this study, we applied liquid nitrogen grinding Trizol single-step method to extract the total RNA of *P. adipose*, and carried out quality test and statistical comparative analysis for RNA

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extract of liquid nitrogen grinding treated and untreated *P. adipose*. The results are of great value to obtain high quality and high purity of total RNA of *P. adipose* and of great significance for the study of transcriptomics of *P. adipose* based on RNA-seq.

2 Materials and methods

2.1 Experimental materials

2.1.1 Bacterial species. Sporophores of *P. adipose* were collected from the North Campus of Dezhou University. Golden yellow, uniform umbrella shape, and cap just expanding sporophores of *P. adipose* were selected. Through tissue separation, fresh tissue in the junction of the cap and the stipe were taken, to obtain the species.

2.1.2 Reagent. Trizol reagent kit, Diethy pyrocarbonate (DEPC), chloroform and isopropanol were bought from Sigma Biotech; liquid nitrogen was bought from Dezhou Dairy Technology Center; other biochemical reagents were domestic analytical reagent (AR).

PDA liquid culture medium formula: 100 g peeled potato, 100 g carrot, 50 g willow sawdust, 20 g glucose, 2 g potassium hydrogen phosphate (K_2HPO_4), 2 g potassium dihydrogen phosphate (KH_2PO_4), 1 g magnesium sulfate, and 1 000 mL water.

2.2 Experiment methods

2.2.1 Culture and collection of *P. adipose* mycelium pellet. The species obtained through extraction were poured into 3 autoclaved PDA liquid culture media for inoculation. After completion of inoculation, first placed the conical flask on the sterile console to culture for 48 h. Later, put the conical flask into the shaker, kept the pH at 5.5, set the temperature of shaker at 25℃, speed at 130 r/min, cultured for 5 d, and finally obtained *P. adipose* mycelium pellet culture medium.

Transferred the *P. adipose* mycelium pellet culture medium to EP tube, centrifuged at 5 000 rpm for 10 min, poured out the supernatant, and obtained *P. adipose* mycelium pellet for the next step of total RNA extraction of *P. adipose*.

2.2.2 Total RNA extraction. First, using the liquid nitrogen grinding Trizol single-step method, in the process of RNA extraction, all instruments and reagents were RNA-free treated using DEPC water. During the extraction, aseptic operation is needed. (i) Homogenization: took 100 mg *P. adipose* mycelium pellet and put in precooled ceramic grinder, added liquid nitrogen several times to grind (generally 5 times); after the liquid nitrogen was consumed completely, scrapped the ground tissue samples with a spoon, poured into 1.5 mL EP tube, rapidly added 1 mL Trizol reagent, inverted and mixed up 10 – 20 times, and placed at room temperature for 5 min. (ii) Isolation stage: added 0.2 mL chloroform to the above EP tube containing 1 mL of Trizol reagent, tightly closed the EP tube cover, shook with strength for 15 s to mix up, placed at room temperature for 5 min and then centrifuged at 12 000 rpm for 15 min. (iii) Precipitation and elution of RNA: The upper aqueous phase was transferred to a new 1.5 mL EP tube (about 400 – 500 uL), added 0.5 mL isopropanol, mixed up and placed at – 20℃ for 1 h, then centrifuged at 12 000 rpm for 10 min, carefully drained the supernatant, then collected the pellet, added 1 mL of 75% ethanol (precooled) to the tube, shook and washed the RNA pellet, and centrifuged at 7 500 rpm for 5 min. (iv) Redissolution of RNA: carefully poured the supernatant, placed the precipitation in a clean bench and blowed the pellet dry (for about 30 minutes, at which time the RNA pellet became clear, taking care not to allow the RNA pellet to completely dry, to avoid reducing its solubility), added 20 μL DEPC in water for dissolution and incubated at 55 – 60℃ for 10 minutes.

RNA was further extracted by Trizol single-step without liquid nitrogen grinding. Only the homogenization procedure was not added with liquid nitrogen grinding, other steps were the same as the above.

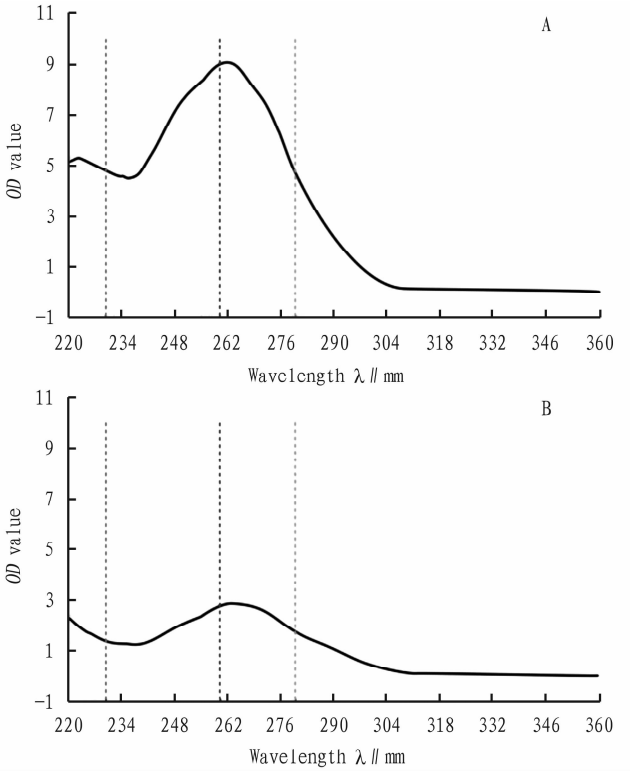
2.2.3 RNA quantitative analysis and quality evaluation. According to the requirements of RNA-seq technology, high-quality RNA products must meet the following requirements: (i) the sample should have good integrity of RNA, and the RNA integrity number (RIN) must reach 6.8 or above; (ii) the total content should be greater than 10 μg (to be calculated using the Gibo green dye method); (iii) the $OD_{260/280}$ value should be in the range of 1.8 – 2.2; (iv) the 25S/18S RNA concentration by agarose gel electrophoresis (AGE) is about 2, no genomic pollution, no protein or impurity pollution, and no color abnormalities in the strip observation. Based on this standard, we performed the following RNA quantitative analysis and quality evaluation.

Using 1% agarose gel electrophoresis, we monitored the degree of degradation and contamination of RNA; we measured RNA purity using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA); we measured RNA concentration using Qubit® 2.0 fluorometer (Life Technologies, CA, USA); we measured RNA integrity using Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) and carried out analysis and evaluation using RNA Nano6000 reagent kit.

3 Results and analyses

3.1 Comparative experiment In order to analyze the quality of total RNA extracted from two *P. adipose* samples with and without liquid nitrogen grinding, we divided three *P. adipose* mycelium pellet culture medium samples into two parts, and measured the absorbance (*OD*) value at the wavelength of 230 nm, 260 nm, and 280 nm. The results (Fig. 1) showed that the *OD* value of total RNA extracted from liquid nitrogen ground *P. adipose* was 9.021 at the characteristic absorption wavelength of 260 nm (the dotted line in the center of Fig. 1-A), much higher than the *OD* value of that without liquid nitrogen grinding (2.758, the dotted line in the center of Fig. 1-B), indicating that RNA content was abundant in RNA extracted from samples with liquid nitrogen grinding. From Fig. 1, there was a significant difference in the optical density absorption values at the wavelength of 280 nm. Compared with the absorption value at the wavelength of 260 nm, the $OD_{260/280}$ value of samples with liquid nitrogen grinding was

nearly equal to 2 (2.032), while $OD_{260/280}$ value of samples without liquid nitrogen grinding was much lower than 2 (1.572), lower than the standard required in RNA-seq technology ($OD_{260/280}$ 1.8 – 2.0), indicating its serious degradation of RNA. In addition, the absorption peak at the wavelength of 230 nm of the two samples was also high. At this wavelength, it was characteristic absorption peak of carbohydrates and sugars, proving the high sugar content in samples of *P. adipose*. Therefore, it can be concluded that the total RNA content extracted from samples of *P. adipose* with the liquid nitrogen grinding was high and the degree of degradation significantly declined.



Note: A. samples with liquid nitrogen grinding; B. samples without liquid nitrogen grinding; dotted lines in the left, center, and right denote the wavelength of 230 nm, 260 nm, and 280 nm respectively.

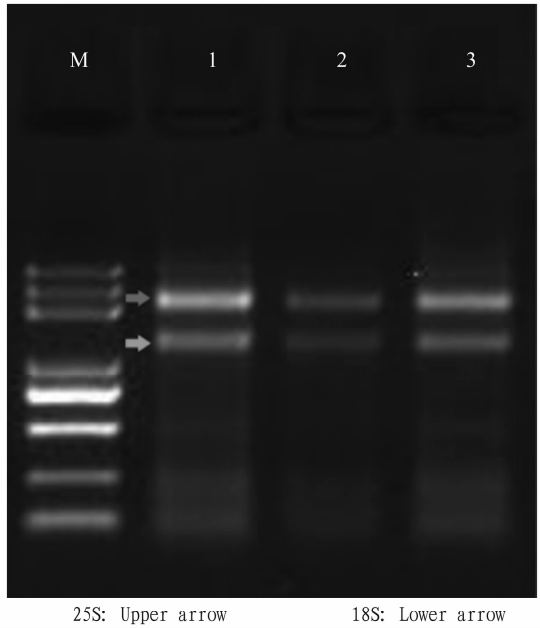
Fig. 1 Comparison of absorbance of RNA extracted from samples with and without liquid nitrogen grinding

3.2 Analysis of difference significance In the experiment, we divided three bottles of *P. adipose* mycelium pellet culture medium into two groups, three samples in each group, one group adoptedTrizol single-step method with liquid nitrogen grinding to extract RNA, the other group without liquid nitrogen grinding. Then, we measured OD_{260} value of the RNA of these two groups of samples, and analyzed the significance of the difference (Table 1). According to the data in Table 1, RNA OD_{260} value of three samples with liquid nitrogen grinding was 10.08, 9.021 and 9.847, respectively, while that without liquid nitrogen grinding was 3.346, 2.758 and 2.941, respectively, all were smaller than the former, indicating that through liquid nitrogen grinding, the extracted RNA content was high and the degradation was low, consistent with the results of comparative experiment. The *P* value (*T*-test) of the two groups with or without liquid nitrogen grinding was 0.000 4 (lower than 0.01), indicating that the two groups of data had extremely significant differences. Therefore, the liquid nitrogen ground samples were very poor for extracting high quality total RNA of *P. adipose* products.

Table 1 Difference significance of RNA absorbance of samples with and without liquid nitrogen grinding

Sample No.	OD_{260} value		*P* value (*T* test)
	Liquid nitrogen grinding	No liquid nitrogen grinding	
1	10.08	3.346	0.000 4
2	9.021	2.758	
3	9.847	2.941	

3.3 RNA quality evaluation The *OD* value of RNA at the wavelength of 260 nm and 280 nm did not fully reflect the quality. We further tested the RNA quality using agarose gel electrophoresis and bioanalyzer. The results are as follows.



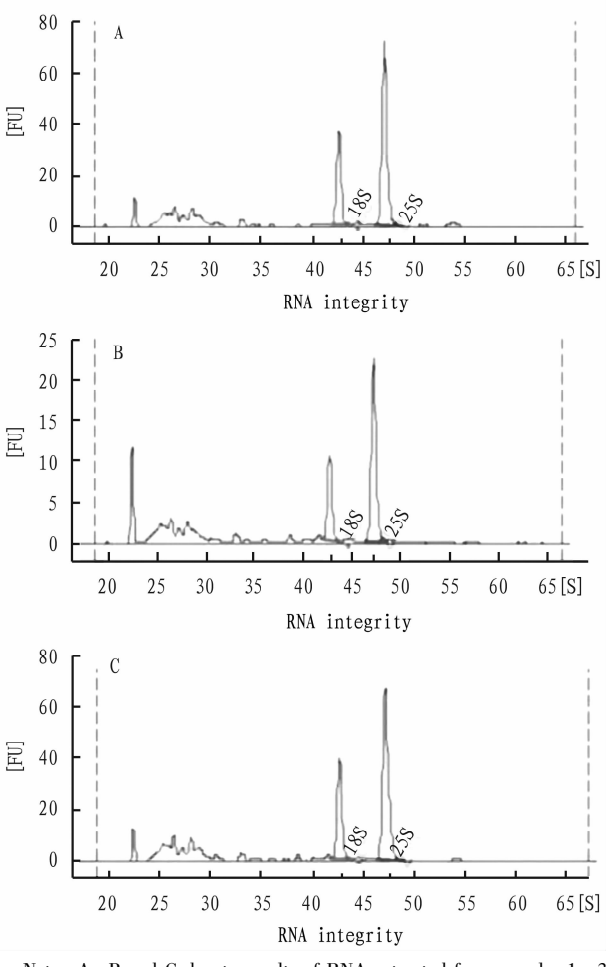
Note: M denotes Trans 2K Plus marker; lanes 1 – 3 are RNA samples extracted through liquid nitrogen grinding (loading 1 μL sample with dilution of 10 times of original solution)

Fig. 2 Agarose gel electrophoresis pattern of RNA samples extracted through liquid nitrogen grinding

3.3.1 Agarose gel electrophoresis. Fig. 2 is an RNA electrophoresis pattern of three samples extracted by Trizol single-step method with liquid nitrogen grinding. There were two clear strips in lanes 1 – 3, with a molecular weight of 1 500 bp (indicated by arrows above) and 2 500 bp (indicated by arrows below), 18S RNA and 25S RNA, respectively. As can be seen, except these two RNA strips, there was basically no other strip, indicating that the extracted RNA integrity was good, the quality was high, and there was no degradation. Besides, using Image J software, we carried out a quantitative analysis of 25S and 18S bands in three

lanes, the grayscale ratio of 25S and 18S bands in three lanes was 1.85, 1.88 and 1.80 respectively, all were close to 2, basically conforming to the non-degradable eukaryotic content ratio of 25S and 18S RNA. Thus, the electrophoresis results proved that liquid nitrogen grinding extraction method meets the high quality standard of RNA-seq technology, and RNA quality is high.

3.3.2 RNA integrity analysis. It is known that 25S and 18S are the two major subunits of eukaryotic ribosomal RNA, which are abundant in eukaryotes. In the process of RNA extraction, there may be various forms of RNA degradation. For the nondegraded total RNA, after formaldehyde denaturing agarose gel electrophoresis, the 25S/18S RNA content ratio was about 2/1. In case of partial degradation, 25S will degrade to 18S, causing changes in the ratio of the two. In case of complete degradation, the RNA is diffused without any strip[35]. Therefore, the 25S/18S RNA fluorescence intensity ratio is an important indicator of integrity of the extracted RNA.



Note: A, B and C denote results of RNA extracted from samples 1 – 3.

Fig. 3 RNA integrity of samples extracted by Trizol single-step method with liquid nitrogen grinding

In order to accurately analyze and evaluate the quality of RNA samples extracted by liquid nitrogen grinding, we tested the integrity of extracted RNA using the Agilent 2100 Bioanalyzer (Fig. 3). From Fig. 3, RNA 18s subunit absorption peak of three samples appeared in 41 – 43 s, 25s subunit absorption peak appeared in 46 – 49 s. In Fig. 3B, peak fluorescence intensity was lower than that in Fig. 3A and Fig. 3C, and the total peak area was also lower (107.2), the peak area in Fig. 3A and Fig. 3C was 288.9 and 259.9 respectively, indicating that the RNA concentration in sample 1 and sample 3 was higher than that in sample 2, and the ratio of 25s and 18s subunit area of three samples was 1.8, 1.9 and 1.9 respectively, which was nearly completely consistent with the results of agarose electrophoresis, and further proved the above results. Based on this, we obtained the RIN value of RNA. The RIN values of three samples were 9.1, 8.7 and 9.3, respectively, all higher than the quality standard 6.8. Therefore, through comprehensive analysis, the quality of RNA extracted by Trizol single-step method with liquid nitrogen grinding meets the quality requirement of RNA-seq technology, and it is the product of high quality RNA.

4 Conclusions

Based on the urgent need of researches about antitumor and other medicinal value of *P. adipose* from the transcriptome level, we carried out an in-depth analysis on the difficulty in RNA extraction using RNA-seq hot spot technologies. We compared the differences in RNA extraction with and without liquid nitrogen trituration of Trizol, and analyzed the significance of the difference. The results revealed that after the liquid nitrogen grinding, the OD_{260} value of the RNA product was much higher than that without liquid nitrogen grinding. The higher the amount of RNA products, the lower the degree of degradation. These results are further confirmed by the difference significance test. Then, using RNA agarose gel electrophoresis and bioanalyzer, we evaluated the quality of RNA extracted by liquid nitrogen grinding. The results showed that: (i) RNA products were less degraded and free of impurities. Agarose gel electrophoresis results showed that there was no diffusion in 25s and 18s RNA strip in all three samples, and no other strip appeared in the lanes. RIN values in the integrity analysis are much larger than the standard requirement (6.8). Besides, 25s and 18s subunits obtained by two methods are close to 2, consistent with the content ratio of 25S and 18S RNA in eukaryotes without degradation, and also reaching the standard value of RNA-seq technology. (ii) RNA products have high purity. In results of Nanodrop analyzer, OD_{260} and OD_{280} value of three replicate samples were close to 2, indicating high purity of RNA products.

In sum, the purity and integrity of the total RNA extracted by Trizol single-step method with liquid nitrogen grinding are high, fully meet the requirements of RNA-seq technology. Also, this method clears away the difficult problem of RNA extraction from the perspective of transcriptome studies, and promotes the in-depth edible and medicinal study of *P. adipose*.

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