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A rapid and high-quality method for total RNA isolation from *Haematococcus pluvialis*

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Genet. Mol. Res. 16 (2): gmr16029614
Received January 17, 2017
Accepted March 16, 2017
Published April 13, 2017
DOI http://dx.doi.org/10.4238/gmr16029614

ABSTRACT. *Haematococcus pluvialis*, as the most potential natural source of astaxanthin, which is a powerful antioxidant with high economic value, has attracted more and more scientific attention in recent years. An in-depth understanding of the mechanism for how *H. pluvialis* produces astaxanthin requires the intensive investigations on its genetic information. In particular, many reported studies were based on a variety of RNA analyses. However, it is difficult to extract RNA with high quality and quantity from *H. pluvialis*, because of the blockage from its thick cell wall and contamination by a large quantity of pigments, polysaccharides, and lipids. Therefore, we proposed an optimized Trizol-based RNA extraction method for *H. pluvialis* by investigating the effect of cell wall broken ways, algal strains, and cell growth status on total RNA isolation. Using this rapid, convenient, and

cost-saving method, isolated *H. pluvialis* RNA had high quantity and quality (with an RNA integrity number of 7.0 and a concentration of 1604.1 ng/μL) equivalent to that isolated by commercial kit, enabling its applications into downstream RNA analyses.

Key words: *Haematococcus pluvialis*; RNA extraction; RNA integrity; Statuses of cell growth; RT-PCR

INTRODUCTION

The unicellular alga *Haematococcus pluvialis*is is regarded as the natural source of high-valued carotenoid astaxanthin (Boussiba, 2000). Esterified astaxanthins were the predominant forms in *H. pluvialis*, exhibiting much stronger antioxidant activities than those from other organisms (Rao et al., 2013; Régnier et al., 2015). *H. pluvialis* was an ideal resource of lipid production and thus served as a suitable model organism for lipid metabolism research (Chen et al., 2015). To date, *H. pluvialis* is of economic importance and has drawn dramatic scientific attention in recent years.

Up to now, the genome of *H. pluvialis* is still unknown, which makes it difficult to investigate the molecular mechanism of secondary metabolism. With the development of metabolomics, proteomics, and transcriptomics, most key enzymes in astaxanthin synthesis have been characterized and studied (Gao et al., 2015). Through cDNA microarray, RNA-seq, real-time quantitative PCR, and other biological techniques, the gene expression patterns under stresses such as nitrogen deprivation, high light irradiation, and high salinity have been analyzed (Kim et al., 2011; Gwak et al., 2014). The premise of these molecular characterizations requires RNA samples with high purity and quality.

Because *H. pluvialis* has a large quantity of pigments, polysaccharides and lipids (Recht et al., 2012), and thick cell wall in haematocyst cells, it is difficult to isolate RNA with high quality and sufficient quantity from *H. pluvialis*. In previous transcriptome studies, RNA of *H. pluvialis* was isolated by using commercial kits or Trizol (Kim et al., 2011; Gwak et al., 2014). Using commercial kits is efficient for studying mRNA, but has limitations when applied to small RNA and other non-coding RNA. The commercial kits were merely suitable for single kind of RNA extraction and unsuitable for total RNA. The extraction of total RNA by using Trizol also has shortcomings due to high contamination of genomic DNA and low yield of RNA due to severe degradation. Here, we proposed a modified Trizol method, which is efficient to isolate total RNA from *H. pluvialis*, with few contamination and degradation. Also, the effects of cell wall broken measures, algal strains, and status of cell growth on RNA isolation were discussed.

MATERIAL AND METHODS

Algal strains and culture condition

Four strains of *H. pluvialis* including 192.80, 34-1n, 34-1a, and 34-1b were purchased from Sammlung von Algenkulturen Gottingen Culture Collection of Algae. Samples were cultured in 250-mL Erlenmeyer flasks using permeable sealing membrane, statically incubated at 22°C under a light intensity of 25 μmol photons$\cdot m^{-2}\cdot s^{-1}$ with a 12-h light/12-h dark cycle.

Three kinds of liquid media were used for cell growth, i.e., ESP, TAP, and MIX. Haematocyst cells were induced by high irradiation under 550 μmol photons·m^{-2}·s^{-1} for 6 h.

Modified RNA isolation methods

Solution for RNA extraction was incubated with 0.1% diethylpyrocarbonate (Sigma) overnight at room temperature and then autoclaved for 20 min. The plastic materials such as pipette tip and Eppendorf microcentrifuge tubes were of commercial RNase-free grade. Cylinders and flasks were baked at 180°C for 5 h before use. The sterilized work benches and centrifugation machines were wiped with RNaseZap (Ambion) to remove surface RNase. The powder-free plastic gloves were used during the entire RNA isolation procedure.

In our method, 2 x 10^5 *H. pluvialis* cells were collected by centrifugation at 10,000 *g* for 1 min. The samples were subsequently transferred to 1 mL Trizol buffer [2% (w/v) polyvinylpolypyrrolidone (PVP40) (Sigma, MW 40,000), 1% (v/v) βmercaptoethanol]. After cells were broken with a vortex at the maximum speed for 1 min, 0.2 mL chloroform was added to each tube and mixed thoroughly. The tubes were then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was collected into a 1.5-mL new RNase-Free microfuge tube and re-exacted by the equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Then, 50 U/mL DNase I (RNase free) was diluted in the supernatant, and incubated at 37°C for 10 min. The DNase I and residual proteins in supernatant were removed by the equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged. Total RNA was precipitated with 1 mL pre-chilled isopropanol for 10 min at -20°C. The RNA was washed twice with 75% ethanol, air-dried, dissolved in 60 μL RNase-free water, and stored at -80°C. Other RNA extraction methods such as CTAB-LiCl, Trizol, and commercial kits were performed as the controls.

Examination of RNA purity, yield and integrity

Once the RNA was extracted, the integrity was evaluated by visualizing the 28S rRNA and 18S rRNA bands on a 1% agarose gel. Marker DL2000 (TaKaRa) was used with the bands of 2000, 1000, 750, 500, 250, and 100 bp. The concentration of RNA was assessed with a spectrophotometer NanoDrop (BioPhotometer, Eppendorf, Hamburg, Germany) at wavelengths of 230, 260, and 280 nm. Finally, RNA extractions were run in an Agilent 2100 Bioanalyzer (Agilent Technologies) for quality measurements. The bigger RNA integrity number (RIN) the less degradation was observed.

Optimization of RNA extraction

The effects of cell wall broken ways, strains, and cell growth status on RNA quality were discussed in the study. Five ways of cell wall broken were applied: oscillation with or without 0.1 g acid washed glass beads by a vortex at the maximum speed, ultrasonic wave, oscillation using FastPrep-24 (MP Biomedicals, USA) at 4000 *g* for 1 min, frozen with liquid nitrogen, then grinded cells to the fine powder in a mortar. Four stains were used for RNA isolation. Cells at logarithmic growth stage cultured in TAP, MIX, and ESP were collected for RNA extraction.

Analysis of RNA by RT-PCR

The total RNA extracted from *H. pluvialis* vegetable and haematocyst cells was converted to cDNA according to PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa). Total RNA (1 μg) was used as template using OligodT Primer (50 μM), for 10 min at 70°C, then mixed with 200 U PrimeScript II RTase and 20 U RNase inhibitor, and incubated for 1 h at 42°C. The reaction was inactivated at 72°C for 10 min.

The housekeeping gene *β-actin* (Huang et al., 2006) was amplified using specific primers (*HpActin-F*: 5'-AGCGGGAGATAGTGCGGGACA-3', *HpActin-R*: 5'-ATGCCCACCGCCTCAATGC-3'), as the cDNA template. *Hpbkt1* gene was amplified using the primers (*Hpbkt1-F*: 5'-ACGTACATGCCCCACAAG-3', *Hpbkt1-R*: 5'-CAGGTCGAAGTGGTAGCAGGT-3'). PCRs were carried out in 20 μL containing 1 μg template (cDNA), 2 μL 10 pM primer mix (forward and reverse) and 10 μL PCR mastermix. PCR mixture was denatured at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and a final extension period at 72°C for 5 min. PCR products were separated with 2% (w/v) agarose gel, stained by ethidium bromide, and photographed by Gel Doc XR+ (BioRad, USA).

RESULTS

High-quality total RNA isolated by our method

Lanes 1 and 4 in Figure 1 show that the 28S rRNA band was equal to or brighter than that of the 18S rRNA, suggesting almost no RNA degradation. The RNA samples extracted by Trizol and CTAB-LiCl methods showed slight degradation as shown in lanes 2 and 3 (Figure 1). These suggest that high-quality RNAs from *H. pluvialis* cells were successfully isolated by our modified method as well as the commercial kit.

Spectrophotometer analysis by NanoDrop also revealed that our method as well as the commercial kit could isolate high purity and concentration RNA with A_{260}/A_{280} and A_{260}/A_{230} ratios greater than 2.0 (Table 1). The A_{260}/A_{230} ratios of total RNA isolated by Trizol and CTAB-LiClmethods were both lower than 2.0, indicating contamination by polysaccharides, polyphenols, DNA, or proteins. The highest yield (1604.1 ng/μL) of RNA was obtained by our method.

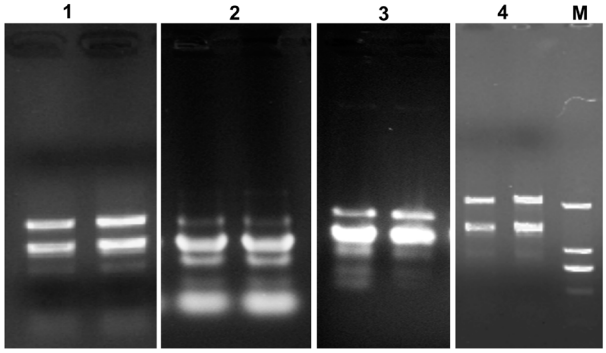

Figure 1. Total RNA isolated from *Haematococcus pluvialis* cells by four methods resolving through 1.0% agarose gel. *Lane M*: 2-kb DNA marker, *lane 1*: commercial kit, *lane 2*: Trizol method, *lane 3*: CTAB-LiCl method, *lane 4*: modified Trizol method.

Table 1. Spectrophotometer analysis of RNA isolated by four methods.

Elements	Details	A_{260}/A_{280}	A_{260}/A_{230}	RNA yields (ng/μL)
Methods	Modified method	2.09	2.07	1604.1
	CTAB-LiCl method	1.98	1.32	946.2
	Commercial kit	2.05	2.08	1537.0
	Trizol method	1.87	1.78	1408.9

Optimization of RNA extraction method

To optimize our modified RNA extraction method for *H. pluvialis*, the effects of cell wall broken ways, algal strains, and cell growth status on RNA extraction were investigated. The results are shown in Figure 2. The RNA extracted from cells treated with liquid N_2 and subsequent grind has the best quantity and quality (Figure 2B, lane 5). In contrast, the RNA extracted from cells broken by FastPrep-24 was the worst (Figure 1, lane 2). The RNA examination using NanoDrop also showed the same results (Table 2). There were no differences among RNA extracted from four *H. pluvialis* strains (Figure 2A). All the RNA has an A_{260}/A_{280} ratio over 1.8 and an A_{260}/A_{230} ratio over 2.0, and a high concentration over 1.5 μg/μL (Table 2). RNA isolated from cells cultured in Mix medium exhibited the best quality; the 28S rRNA band was brighter than that of 18S rRNA, and 5S rRNA band was weak (Figure 2C, lane 1). RNA purity and concentration were also determined by NanoDrop, showing that RNA extracted from cells grown in Mix medium has the highest A_{260}/A_{280} and A_{260}/A_{230} ratios (Table 2).

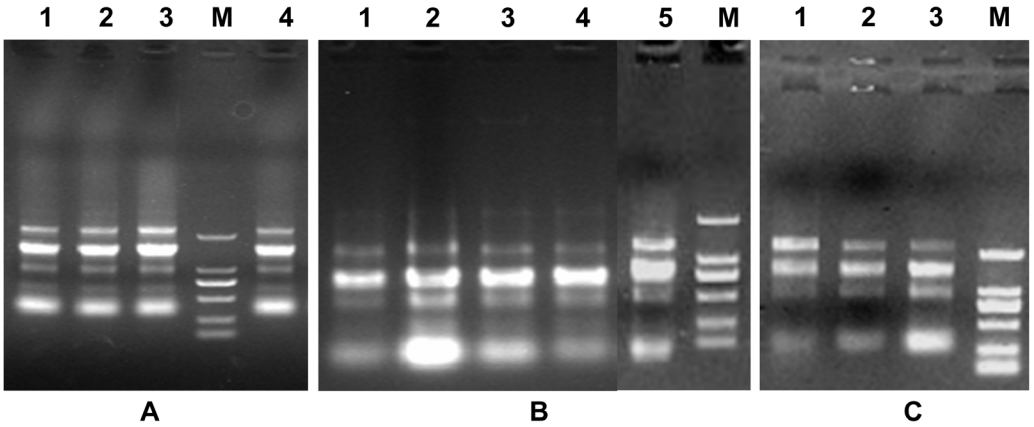

Figure 2. Electrophoretic analysis of RNA isolated from four *Haematococcus pluvialis* strains (**A**) broken by different ways (**B**) and cultured in three media (**C**). The RNA samples were separated on a 1.0% agarose gel containing ethidium bromide and photographed by Gel Doc XR+. *Lane M*: 2-kb DNA marker. **A.** RNA isolated from *H. pluvialis* strains: *lane 1*: 192.80, *lane 2*: 34-1n, *lane 3*: 34-1a, *lane 4*: 34-1b. **B.** RNA extracted was broken with ultrasonic wave (*lane 1*), FastPrep-24 (*lane 2*), oscillating on a vortexing machine with glass beads (*lane 3*), oscillating on a vortexing machine without glass beads (*lane 4*), by liquid N2 freezing and grinded to powder (*lane 5*). **C.** RNA isolated from cells cultured in media: *lane 1*: Mix, *lane 2*: TAP, *lane 3*: ESP medium.

Table 2. Spectrophotometer results of RNA in extraction optimization.

Elements	Details	A_{260}/A_{280}	A_{260}/A_{230}	RNA yields (ng/μL)
Broken ways	Ultrasonic wave	1.54	1.62	3210.2
	FastPrep-24	1.62	1.57	2637.7
	Glass beads	1.98	2.01	1847.3
	Just vortex	1.96	2.04	1525.6
	Lipid N2 frozen	2.18	2.16	1284.5
Strains	192.80	1.89	2.13	1638.3
	34-1n	1.89	2.14	1597.4
	34-1a	1.90	2.15	1610.5
	34-1b	1.90	2.13	1705.8
Medium	ESP	1.92	1.92	1490.4
	TAP	1.85	1.53	1150.2
	Mix	2.12	2.05	1508.1

RT-PCR analysis of RNA isolated by modified methods

Total RNA was isolated from *H. pluvialis* 192.80 vegetable and haematocyst cells using our modified method. Beside the electrophoretic and spectrophotometer analyses, the RNA was also detected by Agilent 2100 Bioanalyzer. RNA on 1% agarose had sharp bands (Figure 3A), A_{260}/A_{280} and A_{260}/A_{230} ratios were greater than 2.0, and RIN values was 7.0 (Figure 3B). All these results showed that RNA with high quality was successfully isolated by our modified method. After RT-PCR, the housekeeping gene *HpActin* was amplified successfully (Figure 3C). There was no difference from vegetable and haematocyst cells at the transcriptional levels of the *HpActin* gene. Also, the mRNA abundance of the *Hpbkt1* gene was analyzed by encoding key enzyme HpBKT1 (β-carotene ketolase) in astaxanthin biosynthesis in *H. pluvialis* (Meng et al., 2005). The *Hpbkt1* gene was induced in haematocyst cells, indicating astaxanthin synthesis in cells. Our optimized protocol was suitable for RNA isolated from both vegetable and haematocyst cells.

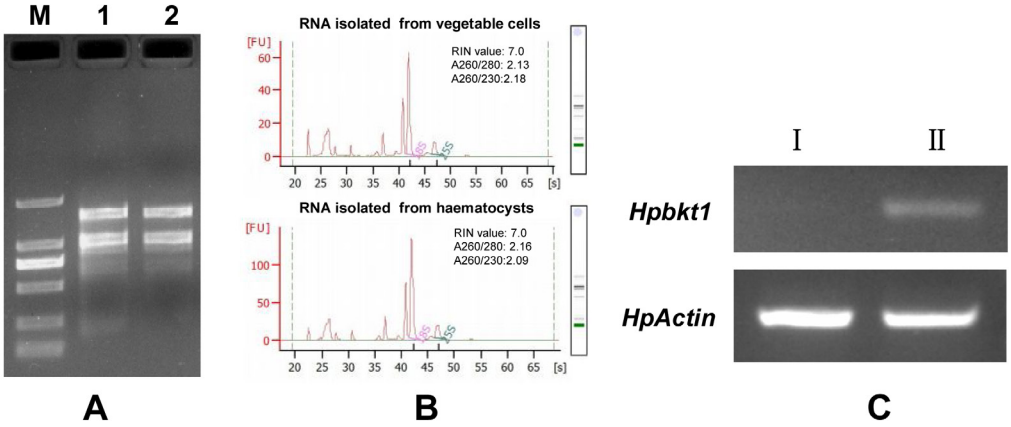

Figure 3. Electrophoretic (**A**), Bioanalyzer (**B**), and RT-PCR (**C**) analysis of RNA isolated by optimized procedure from vegetable and haematocyst cells. *Lane M*: 2-kb DNA marker, *lane 1*: RNA isolated from vegetable cells, *lane 2*: RNA isolated from haematocyst cells. RT-PCR of *Hpbkt1* and *HpActin* genes was performed using the cDNA from vegetable cells (I) and haematocyst cells (II) as templates.

DISCUSSION

PVP40 and β-mercaptoethanol were commonly applied in total RNA extraction in high plants (Lan et al., 2013), but have not been used for RNA isolation in microalgae. PVP40 and β-mercaptoethanol were utilized in our method because they could bind more phenolic compounds to phenol, and then eliminate impurities in nucleic acids. DNAse I was added before precipitation of RNA to remove the contaminated genomic DNA and to make the RNA more pure, which was revealed by the phenol/chloroform extraction protocol.

Compared with the *Chlamydomonas reinhardtii* model microalgae, the *H. pluvialis* cells have thicker cell wall, especially in cysts (Wang et al., 2004). However, our results showed no significant differences in the RNA extracted by five different broken ways except liquid N_2 (Figure 2B). Liquid N_2 maybe a best way to break cells, but it requires a very large amount of *H. pluvialis* cells (300 mg at least), making it not suitable for high throughput gene expression analysis. Other broken ways require fewer amounts of cells (more than 2 x 10^5 cells were enough). The results indicated one step of vortexing crush algae cells sufficiently, even without acid glass beads. It is simpler and quicker to use vortexing to break cells than other crush ways.

H. pluvialis cultured in Mix medium has the most biomass (Noroozi-M et al., 2012). The more cells were in a mitotic state, the more nucleic acids are synthesized. This may be a well explanation for why RNA extracted from cells in Mix medium was the best in terms of both quantity and quality. High-grade RNA could only be isolated from healthy cells.

In conclusion, we provided a rapid, convenience, efficient, and reproducible method to isolate high-quality total RNA from *H. pluvialis*. It is the first report of optimization of total RNA isolation for *H. pluvialis* cells. After comparison of effects such as cell wall broken ways, algal strains, status of cell growth, and isolation methods on quality of total RNA from *H. pluvialis* cells, we found that cell wall broken ways and algal strains did not affect RNA isolation. The status of *H. pluvialis* cell growth was the key element to determine both quantity and quality of total RNA. High RNA yield (bigger than 1.6 μg/μL) was obtained from a small biomass (more than 2 x 10^5cells). The total RNA was suitable for the subsequent molecular experiments, such as RT-PCR, RACE, RT-qPCR, and RNA Sequencing, among others. It may be used for isolating RNA from other microalgae species, which are rich in lipids, proteins, and secondary metabolites.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank the anonymous reviewers for their constructive suggestions. Research supported by the National Natural Science Foundation of China (Grants #31470389 and #31470431); the Guangdong Natural Science Foundation (#2014A030308017), the Shenzhen Grant Plan for Science & Technology (#JCYJ20160422171614147, #JCYJ20150324141711583, #JCYJ20120613112512654, #JSGG20130411160539208, #JSGG20141118114954828, and #NYSW20140327010012), and the China Postdoctoral Science Foundation (#2015M582403).

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