Songklanakarin J. Sci. Technol.
37 (5), 533-537, Sep. - Oct. 2015

http://www.sjst.psu.ac.th

Original Article

Comparison of RNA extraction methods in Thai aromatic coconut water

Nopporn Jaroonchon[1,2], Wachiraya Imsabai[1,2]*, and Krisana Krisanapook[1,2]

[1] *Department of Horticulture, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Kamphaeng Saen, Nakhon Pathom, 73140 Thailand.*

[2] *Center of Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University (CASAF, NRU-KU), Chatuchak, Bangkok, 10900 Thailand.*

Received: 11 September 2014; Accepted: 2 June 2015

Abstract

Many researches have reported that nucleic acid in coconut water is in free form and at very low yields which makes it difficult to process in molecular studies. Our research attempted to compare two extraction methods to obtain a higher yield of total RNA in aromatic coconut water and monitor its change at various fruit stages. The first method used ethanol and sodium acetate as reagents; the second method used lithium chloride. We found that extraction using only lithium chloride gave a higher total RNA yield than the method using ethanol to precipitate nucleic acid. In addition, the total RNA from both methods could be used in amplification of betaine aldehyde dehydrogenase2 (*Badh2*) genes, which is involved in coconut aroma biosynthesis, and could be used to perform further study as we expected. From the molecular study, the nucleic acid found in coconut water increased with fruit age.

Keywords: nucleic acid, lithium chloride, ethanol, betaine aldehyde dehydrogenase2, liquid endosperm

1. Introduction

Coconut is native to Southeast Asia and Melanesia (Fremond *et al*., 1966), with a wide range of uses for foods, medicines and cosmetic products. Coconut growing areas and production are commonly found on islands and mainland of countries bordering the Pacific and Indian oceans (Chan and Elevitch, 2006). Coconut water from young fruit is well known as a natural drink and contains essential minerals such as sodium, potassium, calcium, magnesium, copper and phosphorus, which are beneficial to human health. In addition, coconut water contain cytokinins (e.g. kinetin and trans-zeatin) which delay premature aging on human skin cells (Yong *et al*., 2009).

Young coconut produced and consumed in Thailand is Aromatic Green Dwarf (AROD) coconut. The fragrance in AROD coconut water is mainly caused by the presence of 2 acetyl-1-pyrroline (2-AP), which can be found in many plants such as pandans, soybean and jasmine rice. This substance is involved with the *Badh2* gene (Betaine aldehyde dehydrogenase2) (Bradbury *et al*., 2005). Normally, nucleic acids are assembled with proteins, such as DNA / RNA polymerase and the histone protein (Iamtham, 2009). However, nucleic acids that are found in coconut water are in free form, dissolved in water and not associated with any cell organelles (Cutter *et al*., 1951; Cutter *et al*., 1955). The nuclei are condensed to a structure like cell, which is syncytial in form and can be found throughout the stages of fruit development (Bustamante, 2002; Cutter and Freeman, 1954; Mondal *et al*., 1970 and Siriphanich *et al*., 2011). Thus, total RNA isolation in coconut water does not require a chemical buffer or any detergent for cell breakdown which can cause denaturing or fracturing of nucleic acids. Mondal *et al*. (1970) attempted to extract nucleic acid from coconut water using centrifugation at different speeds but produced very low yields of nucleic acid and could not proceed with further

* Corresponding author.
Email address: wachiraya@gmail.com

molecular study. Cueno and Laude (2010) developed nucleic acid extraction in coconut water by centrifugation and precipitation with ethanol and produced a higher yield but it was still too low (< 10 ng/µl) to perform further mole-cular study.

Understanding the fragrance development in coconut water using a molecular method will be useful for controlling the consistency of the aroma. Since molecular studies in coconut water are still limited as mentioned above, our work aimed to develop an extraction method to produce higher amounts of total RNA. The first method developed was modified from the technique of Cueno and Laude (2010). The second method involved precipitation using lithium chloride instead of ethanol. In addition, the development of total RNA in coconut water at different stages of fruit development was also monitored.

2. Materials and Methods

2.1 Total RNA extraction procedure

Aromatic Green Dwarf fruits at ages from 7 to 8 months after the female flower had been fertilized were collected from a coconut orchard in Ratchaburi province, Thailand, since we expected the fruit at these stages to contain high volumes of nucleic acid in the water and kernel. Coconut water samples from these fruits were collected and stored at -80°C immediately to prevent total RNA degradation and were used for later analysis. Two total RNA extraction procedures were set up (Figure 1). The first method (EtOH method) was modified from Cueno and Laude (2010). Firstly, frozen coconut water was thawed on ice about 2 hours before being used, then 25 ml of coconut water were centrifuged at 17,000 x g for 20 minutes at 4°C to remove debris and cell particulates, and then 15 ml supernatant was used and the total RNA was precipitated by adding refrigerated absolute ethanol (1:1) and 3M sodium acetate (pH 5.2) 15 ml and 1.5 ml respectively, and storing at -80°C for 24 hours. Samples were then centrifuged at 17,000 x g for 20 minutes at 4°C to retrieve the pellet then the supernatant was discarded. The pellet was washed with 3 ml of 70% ethanol and centrifuged at 14,200 x g for 10 minutes at 4°C. Alcohol was discarded and the pellet was dried at room temperature under vacuum until the pellet was clear, then resuspended in 50 µl of RNase-free water and kept at -80°C for analysis.

The second method (LiCl method) started with the centrifugation of 25 ml coconut water at 17,000 x g for 20 minutes at 4°C. Then 15 ml of the supernatant was transferred to a new tube and added with 8M lithium chloride to a final concentration of 3M and stored at 4°C overnight to precipitate the total RNA. Samples were centrifuged at 14,200 x g for 20 minutes at 4°C. Each pellet was dried and resuspended in 50 µl of RNase-free water and kept at -80°C for analysis.

The total RNA concentration and purity were determined by measuring the absorbance at 230, 260 and 280 nm in water using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo scientific, USA). To determine the purity of the total RNA, the absorbance for the A260/280 and A260/230 ratios was taken in water. The integrity of the RNA sample was analyzed on 1% agarose gel electrophoresis.

2.2 Amplification with *Badh2* and *18S rRNA* gene

2.2.1 Reverse transcription polymerase chain reaction (RT-PCR)

The presence of mRNA in the coconut water was confirmed by the PCR reaction with the *Badh2* and the *18S rRNA* genes. Firstly, DNA in each sample was removed with

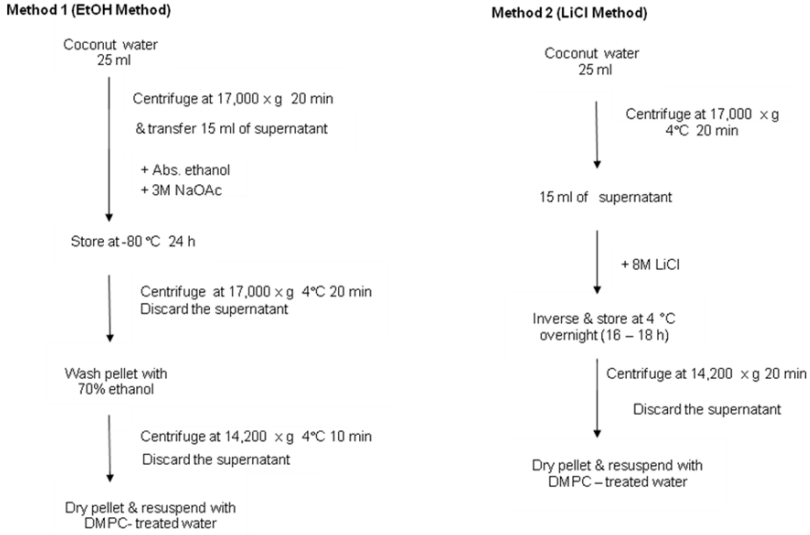

Figure 1. RNA extraction methods using aromatic coconut water. Left panel is the first method (EtOH method) and right panel is the second method (LiCl method)

DNase (Turbo DNA-free™kit, USA), an enzyme that selectively degrades DNA, then an RNA sample was transcribed to cDNA using an Omniscript® Reverse Transcription Kit (Qiagen, USA). For the cDNA synthesis, 1x Buffer RT, dNTP mix, RNase-free water, 10 µM Oligo dT primer, 10 unit/µl RNase inhibitor, Omniscript RT enzyme and the DNase-treated sample as a template were mixed in a PCR tube and then distilled water added until the total volume reached 20 µl. The solution was mixed gently, incubated for 60 minutes at 37°C and then subjected to PCR.

2.2.2 Polymerase chain reaction

The polymerase chain reaction technique was applied to the samples using HotstarTaq® DNA polymerase. Firstly, 1x PCR buffer, 200 µM of each of dNTPs, 10 µM of each of the forward and reverse primers, 2.5 unit/reaction of HotstarTaq DNA Polymerase and cDNA template were added into the sample, followed by distilled water until the total volume reached 25 µl. Primers for the PCR were of *18S rRNA*, a housekeeping gene, (Forward-CGGGGAGGTAGT GACAATAAATA, Reverse- TAATGAAAACATCCTTGG CAAAT) and *Badh2* gene (Forward-AYGTNGCNGGNTGY TTYGA, Reverse- TNGTCCARAARCANCCRAA) genes. The mixtures were placed in a T100 ™ Thermal Cycler and the reaction was set at 95°C for 15 minutes to activate enzymes, then at 94°C for 45 seconds to denature the DNA, 45°C for 45 seconds to anneal the primer with the template DNA, and finally at 72°C for 1 minute for extension. This took 35 cycles from the denaturing step, before the temperature was set to 72°C for 10 minutes to final extension. The PCR products were detected by 1% agarose gel electrophoresis.

2.3 Total RNA in coconut water from aromatic coconut fruit at different stages

To confirm that our extraction method can be used to extract total RNA for further *Badh2* gene expression study in coconut water, aromatic coconut fruit at ages from 2 to 9 months after fertilization were collected from the same location, coconut water from 3 fruits from the same spadix were mixed together and prepared in the same way as in the previous study. The extraction method that gave the highest yield and purity from previous study was chosen to extract total RNA from the coconut water. The NanoDrop 2000c UV-Vis Spectrophotometer was used to determine the total RNA concentration at various stages.

3. Results and Discussion

3.1 Comparison of total RNA extraction methods

Coconut water contains 0.08% and 0.8% of protein and fat, respectively (Jackson *et al.*, 2004), and is acidic (pH 5.62). Nucleic acids that are found in coconut water are in free form, dissolved in water and not associated with any cell organelles (Siriphanich *et al.*, 2011; Bustamante, 2002). Thus, the use of lysis buffer was not necessary in our experiment. The total RNA concentrations obtained from the samples in the first and second methods were 3.3 (±4.35) and 27.1 (±6.11) ng / µl respectively (Table 1), which were greater than in the research of Cueno and Laude (2010) which reported only 0.1-1.8 ng / µl. This may have been due to the use of DNA dipstick kit (Invitrogen) in their study, the sample's color acquired with the kit is compared with the standard chart and values were interpreted as a concentration. The precision of the standard chart was limited to a concentration range of 0.1-10 ng / µl (Anonymous, n.d.). However, where the concentration is higher than 10 ng / µl, the measured value will deviate from the actual value. In contrast, our study used a spectrophotometer (NanoDrop 2000c UV-Vis Spectrophotometer) which is capable of measuring a wide concentration range between 0.4 to 15,000 ng / µl and has higher precision. The spectrophotometer measures the maximum absorbance of samples of nucleic acid and protein at 260 nm (A260) and 280 nm (A280), respectively. In addition, we used a larger amount of coconut water (15,000 µl) than the previous research where only 600 µl was used.

The second method used lithium chloride, which precipitates only RNA, whereas the first method used ethanol that can precipitate both DNA and RNA. Lithium chloride reduces the solubility of the RNA in solution resulting in both high and low molecular weight RNA being precipitated when centrifuged while other substances including DNA remain dissolved in the solution (Sambrook and Russell, 2001). Thus, only RNA was precipitated from the coconut water. In contrast, the ethanol used in the first method could precipitate both DNA and RNA so the total RNA concentration obtained from this method may contain both DNA and

Table 1. Total RNA concentration and purity extracted using two methods from 7 months-old coconut water of Thailand Aromatic Green Dwarf (AROD).

Methods	RNA concentration (ng/µl)	RNA purity		RNA yield (ng/ml) of coconut water
		A260/280	A260/230	
EtOH Method	3.3 ± 4.35	1.48	0.28	11.0
LiCl Method	27.1 ± 6.11	1.29	0.54	90.3

RNA, which have maximum absorbance at 260 nm. The ratios of A260/280 and A260/A230 that indicated RNA purity from our study were lower than the accepted pureness (~ 1.8) (Wieczorek *et al*., 2012). However, the total RNA amount from both methods was enough to synthesize cDNA and perform PCR with the *18S rRNA* and *Badh2* genes. Furthermore, the total RNA absorbance from the second method produced a more typical spectral pattern for nucleic acid than the first one (Figure 2). Thus, we recommend that the second method is more appropriate than the first method for the extraction of total RNA from coconut water.

To confirm the presence of total RNA from the coconut water, extracted samples from both methods were subjected to gel electrophoresis. However, at least a few nanograms of RNA are required for visualization by gel electrophoresis (Wieczorek *et al*., 2012) and because of the very low amount of total RNA from our experiment, we could not detect the total RNA (*18S rRNA* and *28s rRNA*) band from either methods (Figure 3). Then, PCR amplification of *Badh2* and *18S rRNA* genes was performed using cDNA transcribed from RNA extracted from each method as template. The total RNA extracted from the coconut water from both methods was usable for PCR as suggested by the successful amplification of both genes (Figure 4).

3.2 Total RNA in coconut water at different stages of aromatic coconut fruit

The previous experiment indicated that the second method gave a higher yield of total RNA than the first method. In addition, since the first method took more steps than the second method, we considered that the LiCl method is more suitable for total RNA extraction in aromatic coconut water. Thus, the LiCl method was used to extract total RNA from coconut water at various stages of fruits development (2-9 months after fertilization). The results showed that total RNA yield increased with fruit age and was notably high when the fruit age was 7-9 months (Figure 5). This result contrasted with the research of Mondal *et al*. (1970) who reported on extracted total RNA in coconut water from fruit aged 3-12 months. In their work, the total RNA levels were almost

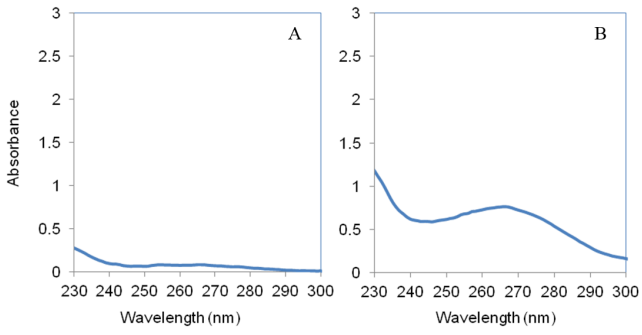

Figure 2. Nucleic acid (RNA) absorbance from EtOH method (A) and LiCl method (B).

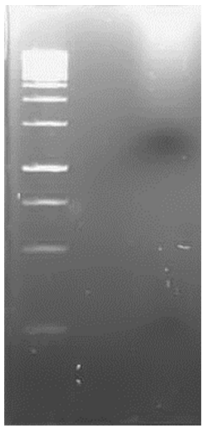

Figure 3. Total RNA (*18S rRNA* and *28S rRNA*) band from EtOH method and LiCl method

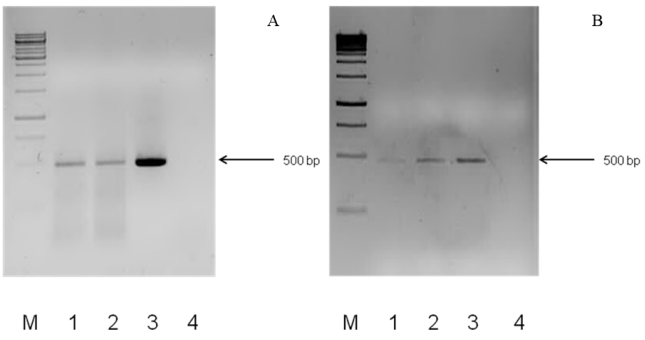

Figure 4. PCR amplification of *18S rRNA* (A) and *Badh2* gene (B) from coconut water and meat. DNA Ladder 1 kb (M), coconut water EtOH method (1), coconut water LiCl method (2), coconut meat as positive control (3) and negative control (4) (PCR product from RNA treated with DNase as a template)

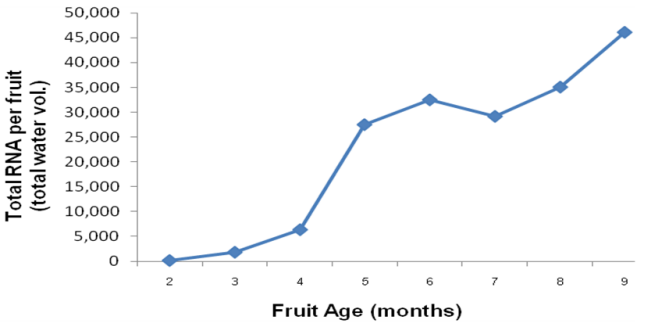

Figure 5. Total RNA per fruit in the coconut water of Aromatic Green Dwarf (AROD) at various fruit ages.

constant throughout the growth stages. This may be because of variation of total RNA yield among coconut varieties (Cueno and Laude, 2010). In addition, the RNA values from Mondal *et al*. (1970) were lower than those in our results, which is perhaps due to the different measuring methods

used as mentioned previously. On the other hand, our results were in concordant with the research of Cueno and Laude (2010), who reported that the free nucleic acid found in aromatic coconut water increased with fruit age. The increase in total RNA correlated well with the development of the solid endosperm, suggesting that RNA may be involved in the formation of the coconut endosperm and this issue needs further study.

In summary, even though total RNA yields from both methods were adequate for molecular study, the lithium chloride precipitation provide higher total RNA yield and purity than did the ethanol precipitation. We also found that the amount of nucleic acid (total RNA) in coconut water is higher with the increasing fruit age. For further study, development of extraction method for higher yield of total RNA needs to be pursued. In addition, multiple RNA quality assessment methods may be used to ensure a more precise and reliable assessment.

Acknowledgements

This research was funded by the Center of Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University (CASAF, NRU-KU, Thailand)

References

Bradbury, L.M.T., Fitzgerald, T. L., Henry, R.J., Jin, Q. and Waters, D.L.E. 2005. The gene for fragrance in rice. Plant Biotechnology Journal. 3, 363-370.

Bustamante, J.O. 2002. Nuclear pore ion channel activity in live syncytial nuclei. Pflügers Archiv - European Journal of Physiology. 444, 286-290.

Chan, E. and Elevitch, C.R. 2006. *Cocos nucifera* (coconut). Available from: http://growfledibles.com/information/TropicalFruit/documents/Cocos-coconut.pdf [May 18, 2013].

Cueno, M.E. and Laude, R.P. 2010. Molecular characterization of free nucleic acids in coconut water. The Philippine Journal of Science. 139(1), 35-41.

Cutter, V.M., Wilson, K.S., and Dube, G.R. 1951. The isolation of living nuclei from the endosperm of *Cocos nucifera*. Science. 115, 58-59.

Cutter, V.M., and Freeman, B. 1954. Development of the syncytial endosperm of *Cocos nucifera*. Nature. 173, 827-828.

Cutter, V.M., Wilson, K.S., and Dube, G.R. 1955. Nuclear behavior and cell formation in the developing endosperm of *Cocos nucifera*. American Journal of Botany. 42, 109-115.

Fremond, Y., Ziller, R., and De Nuce de Lamothe, M. 1966. The coconut palm. Berne: International Potash Institute, pp. 227.

Iamtham, S. 2009. Genetic engineering: methods and applications. Kasetsart University Publishers, Bangkok, (in Thai), Thailand, pp. 27-31.

Jackson, J.C., Andre, G., Gavin, W., Kayanne, M.C. and Rosa, R. 2004. Changes in chemical composition of coconut (*Cocos nucifera*) water during maturation of the fruit. Journal of the Science of Food and Agriculture. 84, 1049-1052.

Mondal, H., Mandal, R.K., and Biswas, B.B. 1970. Nucleic acids in coconut water. Plant and Cell Physiology. 11, 177-181.

Sambrook, J. and Russell, D.W. 2001. Molecular cloning: a laboratory manual (volume 1). Cold Spring harbor laboratory press, Cold spring harbor, New York, U.S.A., pp. 1.82-1.83

Siriphanich, J., Saradhuldhat, P., Romphophak, T., Krisanapook, K., Pathaveerat, S., and Tongchitpakdee S. 2011. Coconut, *In* Yahia, E.M.,(ed.) Postharvest biology and technology of tropical and subtropical fruits, Cambridge. pp. 8-33.

Wieczorek, D., Delauriere, L., and Schagat, T. 2012. Methods of RNA Quality Assesment. Available from: http://worldwide.promega.com/resources/pubhub/methods-of- rna-quality-assesment/ [March 13, 2015].

Yong, J.W.H., Ge, L., Ng, Y.F., and Tan, S.N. 2009. The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. Molecules. 14, 5144-5164.