Optimisation of RNA Extraction from *Gracilaria changii* (Gracilariales, Rhodophyta)

Cheong-Xin Chan[1], Swee-Sen Teo[2], Chai-Ling Ho[2,*], Rofina Yasmin Othman[3] and Siew-Moi Phang[3]

[1]*Institute of Postgraduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia;* [2]*Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia;* [3]*Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia.*

[*]*Author for correspondence (e-mail: clho@biotech.upm.edu.my; phone: +60-3-89468404; fax: +60-3-89423552)*

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Abstract

RNA extraction from seaweed tissues is problematic due to the presence of polysaccharides and polyphenolic compounds upon cell disruption. Besides, a successful RNA isolation from seaweed tissues can sometimes be strain- and species-specific. Four different methods were used to extract RNA from *Gracilaria changii* (Gracilariales, Rhodophyta), collected from the mangrove area at Morib, Selangor, Malaysia. An optimised and modified total RNA extraction method was developed for this recalcitrant species. The use of sand in tissue grinding, and the incorporation of phenol extraction at the initial stage resulted in the highest RNA yield (0.65 – 1.14 μg.g^{-1} fresh weight) with high quality (A$_{260:280}$ ratio 1.80 – 2.05). The RNA obtained is suitable for cDNA synthesis and future functional genomic studies.

Introduction

Gracilaria changii is an indigenous agarophytic seaweed in Malaysia (Phang, 1994). It grows abundantly in the mangroves fringing the west coast of Malaysia (Phang et al., 1996). *G. changii* when used as food, provides substantial amounts of fiber, minerals, lipids and protein (Norziah and Chio, 2000). More importantly, this tropical seaweed produces agar and agarose with high gel strengths (Phang et al., 1996), and is a potential resource for industrial development in Malaysia. Information on the genetics of *Gracilaria changii* is therefore important for better utilization of this seaweed.

Clean and intact RNA is important for functional genomic studies. Unfortunately, the task of extracting intact RNA is difficult due to the susceptibility of RNA molecules to enzymatic degradation by RNase. The problem of isolating RNA from seaweeds is magnified by the release of secondary metabolites after disruption of the cells which are embedded in viscous polysaccharides (Ho et al., 1996). Furthermore, samples from different species, different organs or parts at different times of the year, show slight differences in their cell wall composition (Dring, 1982). The difficulties of RNA extraction from seaweed and plant tissues rich in polysaccharides have been reported by numerous publications, which demonstrated the different conditions required for successful RNA isolation for different species and also for the same species grown under different environments (Hong et al., 1997; Gehrig et al., 2000).

Anionic surfactants (e.g. sodium dodecyl sulphate), and chaotropic agents (e.g. guanidinium isothiocyanate) are widely used in RNA isolation to inhibit the RNase activity. Organic solvents like phenol and chloroform are used to dissociate RNA from proteins, separating them into two different phases. Lithium chloride and cesium chloride are used to precipitate RNA at certain concentrations, while potassium acetate is introduced to remove polysaccharides from the solution. Here we report the

comparison of four RNA extraction methods with modifications, on *G. changii*. The objective of this study was to develop an efficient RNA extraction method for *G. changii*.

Materials and methods

Sample collection and preparation

Fresh plants of *Gracilaria changii* Xia *et* Abbott (Zhang, Abbott *et* Xia) were collected from mangroves at Morib, Selangor, Malaysia. The seaweeds were collected in whole bunches where possible to minimise thalli fragmentation or breakage, and cleansed from mud and epiphytes. Samples were randomly checked for epiphytes, under the dissecting microscope. The samples were frozen in liquid nitrogen immediately upon cleansing and stored at –80 °C.

Total RNA extraction

The frozen tissues were ground in the presence of acid-washed sand at a ratio of 2:1, in liquid nitrogen, and used in the following RNA extraction methods.

Method 1 (modified from Hong et al., 1997)

After grinding in liquid nitrogen, the frozen tissue was added to the extraction buffer (4M guanidinium thiocyanate, 0.8M LiCl, 0.6% sarkosyl, 10mM EDTA, 0.2% PVPP, 2% β-mercaptoethanol) in a ratio of 1:3 (w/v). The mixture was heated at 55 °C for 10 min, shaken gently on ice for 1 h, and centrifuged (12,000 *g*, 20 min, 4 °C). Nucleic acid was precipitated by adding 0.1 volume of 3M sodium acetate pH 5.2 and 0.6 volume of isopropanol. Phenol-chloroform extraction was carried out, followed by the addition of 1/5 volume of 3M potassium acetate pH 6.0 and 1/4 volume of ethanol. Extraction with equal volume of chloroform was then carried out, before the aqueous phase was made to

3M LiCl and left overnight at 4 °C. The RNA was collected by centrifugation (16,000 *g*, 4 °C, 30 min), followed by 70% ethanol washing and ethanol precipitation.

Method 2 (modified from Apt et al., 1995)

Ground frozen tissue was added to extraction buffer (100mM Tris-HCl, 1.5M NaCl, 20mM EDTA, 20mM DTT, 2% CTAB) at a ratio of 1:3 (w/v) and mixed gently at room temperature for 15 min. Extraction with equal volume of chloroform was carried out, and repeated after the addition of 1/3 volume of ethanol. The resulting aqueous phase was made to 3M LiCl and 1% (v/v) β-mercaptoethanol, and left at –20 °C for 3 h. The RNA was spun down by centrifugation (16,000 *g*, 4 °C, 30 min). Standard phenol-chloroform extraction and ethanol precipitation were then carried out.

Method 3 (modified from Kim et al. 1997)

Ground frozen tissue (7-8 g of fresh weight) was added to the extraction buffer (24 mL) prepared by mixing phenol (8 mL) with lysis buffer (16 mL of 25mM Tris pH 8.0, 50mM LiCl, 35mM EDTA, 35mM EGTA and 0.5% SDS). The mixture was vortexed, and again after the addition of chloroform (8 mL). After centrifugation (7,700 *g*, 5 min, 20 °C), the aqueous phase was made to the final concentration of 2M LiCl, and kept at –20 °C for 3 h. The RNA was collected by centrifugation (16,000 *g*, 15 min, 4 °C). The pellet was dissolved in DEPC-treated water and transferred into a microfuge tube. Standard phenol-chloroform extraction was carried out before the LiCl precipitation at 2M was repeated at –20 °C for 3 h. Upon RNA collection by centrifugation (16,000 *g*, 15 min, 4 °C), the RNA pellet was washed with 70% ethanol and followed by ethanol precipitation.

Method 4 (modified from Glišin et al., 1974)

Ground frozen tissue was added into the extraction buffer [4M guanidine hydrochloride, 100mM Tris-Cl pH 7.5, 1% (v/v) β-mercaptoethanol, 10% (w/v) sodium laurylsarcosine and 10% (w/v) Triton X-100] at a ratio of 1:2. The mixture was incubated on ice for 3 h and centrifuged (21,000 g, 4 $^{\circ}$C, 20 min). Solid CsCl was added to the supernatant at a ratio of 2:5 (w/v). The lysate with CsCl was added carefully onto the CsCl pad solution (5.7M CsCl; 200 μL) pre-loaded in one-mililiter Beckman centrifuge tube. The RNA pellet was collected by ultracentrifugation (100,000 rpm, 20 $^{\circ}$C, 20 h), followed by 70% ethanol washing, a standard phenol-chloroform extraction, and ethanol precipitation.

Quantitative and qualitative analysis

Quantitative analysis of RNA was done using Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK) by measuring the OD at 260nm and 280nm. One unit of absorption at 260nm represents 40μg.mL^{-1} of RNA. Polysaccharide contamination was determined by their maximum absorbance measurement at 230nm. The ratio measurements at wavelengths 230, 260 and 280 indicate the degree of purity of the RNA. A pure RNA sample is indicated by the ratio 1:2:1 at 230, 260 and 280 nm, respectively. RNA was visualised by running formaldehyde-agarose gel electrophoresis.

Results and Discussion

The results of the four RNA extraction methods used on the frozen samples of *G. changii* are shown in Table 1. Ethylenediaminetetraacetic acid (EDTA) that functioned as a chelating agent for cations, were used in all extraction buffers to prevent enzymatic activity.

Method 1 is a modified method of Hong et al. (1997) for RNA extraction from seaweed tissues. In addition to guanidinium thiocyanate, Hong and his co-workers

introduced the use of polyvinylpolypyrrolidone (PVPP) and lithium chloride in the extraction buffer. The insoluble PVPP, which has been used for RNA extraction from recalcitrant tissues like grapevine (Geuna et al., 1998), binds and removes phenolic compounds. The use of LiCl in the extraction buffer is mainly to soften seaweed tissues, helping the release of nucleic acids through the loosened cell wall and cell membrane (Evans 1963). Hong et al. (1995) reported that extraction buffer containing LiCl could eliminate the entangling polysaccharides from nucleic acids in *Porphyra*, but this did not work well in the RNA extraction from *G. changii*. LiCl precipitation at a final concentration of 2M was carried out to precipitate RNA. Higher LiCl concentration resulted an increased amount of RNA impurities, despite higher RNA yield. This method was further modified by using potassium acetate to precipitate polysaccharides into potassium salts. This approach was used in many plant RNA extraction methods to remove polysaccharides (Ainsworth 1994; Liu et al. 1998). However, the low $A_{260:280}$ ratio obtained from Method 1 (Table 1) suggested that potassium acetate is not suitable for removing polysaccharides from *G. changii*.

In Method 2, hexacetyltrimethyl ammonium bromide (CTAB) was used as the cell-disrupting agent, while dithiothreitol (DTT) was included in the extraction buffer as a reducing agent to prevent any oxidation reactions (Apt et al. 1995). This method consists of a few steps of chloroform extraction, and it consumes the least time for bench-work. The three-hour LiCl precipitation was found to give sufficiently good results as compared to the overnight LiCl precipitation in the original protocol. This has dramatically shortened the total time required for the original CTAB method suggested by Apt et al. (1995). The three-hour LiCl precipitation gave better RNA yield, ranging from 0.16 to 0.17 μg.g^{-1} fresh tissue (Table 1). This suggested that the RNA from *G. changii* may be more susceptible to degradation during longer period of LiCl precipitation.

In Method 3, the phenol extraction at the initial stage ensured the inhibition of RNase activity, thus minimizing RNA degradation (Kim et al., 1997). Nevertheless, the use of phenol is limited to low-salt extraction buffer. The aqueous layer would be trapped at the bottom of the tube upon centrifugation under high salt concentration. Most of the RNA extraction buffers for seaweeds contain high concentration of salts like guanidinium thiocyanate (Hong et al., 1997) and CTAB (Apt et al., 1995), in which the incorporation of phenol extraction at the very initial stage is not practical. In addition to EDTA, ethyleneglycol-bis(2-aminoethoxy)-tetraacetic acid or EGTA, was used in the extraction buffer. EDTA acts primarily on both Mg^{2+} and Ca^{2+}, whereas EGTA chelates Ca^{2+} only. EGTA in this case acts to chelate the remaining Ca^{2+} from the EDTA chelation. The use of both EDTA and EGTA improved the RNA yield and quality from *G. changii*, as compared to when only EDTA was used. The main advantage of this method is its simplicity, time-efficiency, and it does not require expensive machineries. The highest yield of RNA (0.65 – 1.14 μg.g^{-1} fresh weight) with the best quality was successfully obtained from *G. changii* using this method (Table 1), and cDNA was successfully synthesised subsequently. From formaldehyde agarose gel electrophoresis, the total RNA extracted using Method 3 showed two distinct bands of 18S and 28S ribosomal RNAs (Figure 1), indicating that the RNA was intact and undegraded.

Method 4 is a modified version of the conventional cesium chloride ultracentrifugation protocol for RNA extraction developed by Glišin et al. (1974). The CsCl density gradient centrifugation was proved to be an effective approach in separating polysaccharides, protein and DNA from RNA, depending on their buoyant densities upon centrifugation at ultrahigh speed. The proteins will float at the top of the tube, RNA will be pelleted, whilst DNA and polysaccharides will migrate between them. As shown in Table 1, the negligible content of polysaccharides in the RNA suggested that the CsCl density gradient had efficiently removed the polysaccharides

from the sample. The major drawbacks of this method are, the longer time required to complete RNA extraction, and the dependence on the availability of the expensive ultracentrifuge. This method is not practical for a small laboratory, despite the relatively high RNA yield obtained.

The incorporation of sand while grinding, significantly increased the RNA yield. The sand, as a physical chaotropic agent, greatly facilitates cell disruption and the release of nucleic acids from the seaweed tissues. RNA extractions from both fresh and frozen samples were compared. In the cases of fresh samples, the RNA yields were extremely low, with high contamination of DNA. The high water content of fresh samples is the main culprit causing RNA degradation, due to the containment of endogenous RNAse. In the frozen sample preparation, the abruptness of the cooling process tremendously shortened the time needed for ice formation, thereby diminishing the RNA degradation, which is a problem in a gradual cooling process. Before this, air-dried samples have been suggested for nucleic acid extraction from seaweed samples (Hong et al. 1997; Lim et al. 2001). It was found that air-dried samples were good enough for DNA extraction from *G. changii*, but not RNA. The drying process in open air is one of the main reasons why RNA is degraded before being extracted. Therefore, freezing of samples is recommended for RNA extraction from *G. changii*.

This study shows that *G. changii* is an extremely recalcitrant seaweed for RNA isolation. Method 3, a newly adapted and modified method from Kim et al. (1997), was found to be the best method for isolating high quality RNA from *G. changii*.

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Legend

Table 1. Results from different RNA extraction methods used on the frozen samples of *Gracilaria changii*.

Figure 1. Formaldehyde agarose gel electrophoresis [1.2% (w/v)] of the total RNA extracted using Method 3. The two distinct bands, the 18S and 28S ribosomal RNA show that the RNA is intact.

Figure 1
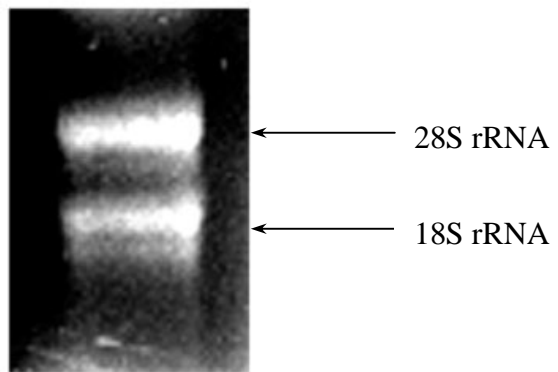
28S rRNA

18S rRNA

Table 1 Results from different RNA extraction methods used on the frozen samples of *Gracilaria changii*

Method	RNA Yield (μg.g^{-1} tissue)	$A_{260}:A_{280}$ ratio	$A_{260}:A_{230}$ ratio	Total time required (h)	Time required for bench-work (h)
1	0.01 – 0.06	1.30 – 1.80	N/A	25 – 26	4 – 5
2	0.16 – 0.17	1.90	N/A	9 – 10	2 – 3
3	0.65 – 1.14	1.80 – 2.05	> 2.00	10 – 12	3 – 5
4	1.00	1.80 – 1.90	> 2.00	30 – 32	6 – 8