Total RNA Extraction for the Red Seaweed *Gracilaria changii* (Gracilariales, Rhodophyta)

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Abstract

Five different RNA extraction methods have been tried out on the red seaweed, *Gracilaria changii* collected from the mangrove area at Morib, Selangor, Malaysia. Two methods, one utilising guanidinium thiocyanate, and another using cetyltrimethyl ammonium bromide (CTAB), were found to be potential alternatives to obtain pure RNA. By incorporating sand while grinding the tissue, the method using CTAB was found most suitable to obtain pure RNA (high $A_{260:280nm}$ ratio) with high yield (0.16μg RNA per gram of fresh tissue).

Introduction

The Rhodophyceae and Phaeophyceae are important producers of phycocolloids, *e.g.* agar, agarose, algin and carrageenan (Hoppe & Schmid 1969). These polysaccharides, credited for their gel-forming capability, are widely used as immobilising and encapsulating agents for analytical elements, especially in the emerging enzyme and biosensor technology in medical fields (Jensen 1993; Renn 1997). *Gracilaria* (Gracilariales, Rhodophyta), with its agar content ranging from 4.4% to 40.0%, is the

most common agarophytic seaweed genus in Malaysia (Doty *et al.* 1983; Phang & Maheswary 1989). It has practically replaced *Gelidium* as the most important source of agar in the world, yielding 60–70% of the world agar (Schramm 1991; Tseng & Xia 1999).

Gracilaria changii is one of the more abundant agarophytic seaweeds in Malaysia (Phang 1994). Of the eight *Gracilaria* species reported in Malaysia, *G. changii* adapts well in the mangroves fringing the west coast of Malaysia, in muddy or silty areas, ponds, and even irrigation canals (Phang *et al.* 1996). As a food, *G. changii* is high in fiber and mineral, with substantial level of lipid and protein (Norziah & Chio 2000). More importantly, this tropical seaweed could produce agar and agarose with high gel strengths (Santos & Doty 1983; Phang *et al.* 1996). The domestic demand for agar in Malaysia is high, when more than US$ 2.6 million was spent on agar imports in year 1987 alone (Jahara & Phang 1990), and the figure has never been stop increasing. With the abilities to produce good quality agar and adapt to harsh mangrove environments, *G. changii* fits well in industrial agar productions. There are a few papers reporting on molecular genetic studies on *Gracilaria chilensis* (Villemur 1990), *G. gracilis* (Lluisma & Ragan 1997; 1998a; 1998b) and *G. verrucosa* (Zhou & Ragan 1993; 1995a; 1995b; 1995c) So far, the genetic basis of *Gracilaria* is not fully understood, and *G. changii* is one of the least studied species.

To carry out molecular studies on a species with little information available, large-scale automated sequencing of partial cDNA clones as expressed sequence tags (ESTs) is essential. Similarity searches of these ESTs against public DNA and protein sequence databases can be carried out, and this approach is well-accepted for gene analysis of any

given species (Shoop *et al.* 1994; Sterky & Lundeberg 2000). Lluisma and Ragan (1997) have reported on the first project on EST from *G. gracilis*. These EST databases are a potentially valuable source of genetic markers, and they allow the construction of synthetic genome linkage maps of expressed genes among related species (Cato *et al.* 2001). Genetic mapping with ESTs would enable a more rapid transfer of linkage information between species. The EST approach had been widely applied on plants (Delseny *et al.* 1997; Asamizu *et al.* 2000), algae (Crépineau *et al.* 2000; Nikaido *et al.* 2000) and in Human Genome Project (Hillier *et al.* 1996; IHGSC 2001).

The most critical step before cDNA library construction is to extract sufficient amount of high quality total RNA from *G. changii* prior to mRNA isolation. However, RNA extraction from *G. changii* is not as easy as it seems. The difficulty of nucleic acid extraction is magnified by the release of secondary metabolites after cells disruption, in which tissues are embedded in viscous polysaccharides (Ho *et al.* 1996; Gehrig *et al.* 2000). Furthermore, samples of different species, different organ or part at different times of the year, show slight differences in their cell wall compositions (Dring 1982).

The main objective of this study is to obtain sufficient amount of good quality RNA for cDNA library construction and EST generation for *G. changii*, so as a means for large-scale gene expression analysis of this species. Here we reported five different RNA extraction methods for *G. changii*.

Materials and methods

Sample collection, preparation and maintenance

Fresh plants of *G. changii*, preferably the reproductive parts, were collected from mangrove at Morib, Selangor. The seaweeds were collected in bunches if possible to minimise thalli fragmentation or breakage, and cleansed from mud and entangling epiphytes. The samples were frozen in liquid nitrogen upon cleansing immediately and stored at -80°C.

Total RNA extraction

Method 1 (Soares & Bonaldo 1997)

Frozen tissue, ground in liquid nitrogen, was added to extraction buffer (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarkosyl, 100mM β-mercaptoethanol) in a ratio of 1:3 (w/v). The tube was gently mixed at room temperature for 15 min, before 0.1 vol of sodium salt solution (2M sodium acetate, 57% acetic acid pH 4.0) and 1.2 vol of phenol-chloroform (5:1) were added. The mixture was incubated on ice for 15 min and centrifuged (10000 rpm, 10 min, 4°C). Nucleic acid was extracted using equal vol of high-salt solution (1.2M NaCl, 0.8M sodium citrate)-isopropanol (1:1). The pellet was washed with isopropanol and then with 70% ethanol. This is followed by extraction with equal vol of chloroform, and standard ethanol precipitation with 0.1 vol 3M sodium acetate pH 5.2 and 2.5 vol ethanol. The RNA pellet was resuspended in 1 mL TES buffer (10mM Tris-HCl, 1mM EDTA, 0.5% SDS).

Method 2 (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 (10000 rpm, 20 min, 4°C). Nucleic

acid was precipitated using 0.1 vol of 3M sodium acetate pH 5.2 and 0.6 vol of isopropanol. This was followed by standard phenol-chloroform extraction and ethanol precipitation. The RNA pellet was dissolved in DEPC-treated water.

Method 3

For Method 3, extraction was carried out as stated in Method 2. After phenol-chloroform extraction, 1/5 vol of 3M potassium acetate pH 6.0 and 1/4 vol of ethanol were added, followed by extraction with equal vol of chloroform. The aqueous phase was made to 3M LiCl and left overnight at 4°C. The RNA was spun down by centrifugation (12000 rpm, 4°C, 30 min), followed by 70% ethanol washing and ethanol precipitation.

Method 4

Frozen sample, ground in liquid nitrogen, was added to extraction buffer (200mM Tris-HCl pH 9.0, 100mM NaCl, 10mM EDTA, 1% SDS, 14mM β-mercaptoethanol) in a ratio of 1:3 (w/v). The mixture was extracted once with 2 vol of phenol-chloroform (1:1), once with 1 vol of phenol:chloroform (1:1), and once with equal vol of chloroform. Ethanol precipitation were carried out using 1/30 vol of 3M sodium acetate pH 5.2 and 1/10 vol of ethanol, and again using 1/15 vol of 3M sodium acetate pH 5.2 and 2.5 vol of ethanol. Further 70% ethanol washing and standard ethanol precipitation were carried out.

Method 5 (Apt *et al.* 1995)

Ground frozen tissue was added to extraction buffer (100mM Tris-HCl, 1.5M NaCl, 20mM EDTA, 20mM DTT, 2% CTAB) in a ratio of 1:3 (w/v) and mixed gently at room temperature for 15 min. Extraction with equal vol of chloroform was carried out, and repeated after addition of 1/3 vol of ethanol. The resulting aqueous phase was made to 3M LiCl and 1% v/v β-mercaptoethanol, and left at -20°C overnight. The RNA was spun down by centrifugation (12000 rpm, 4°C, 30 min). Standard phenol-chloroform

extraction and ethanol precipitation were then carried out. In the modified protocols, autoclaved and acid-washed sand was added to the mortal in a ratio of 1:2 while grinding the tissues, and LiCl precipitation was carried out at 3M for 3 hours, instead of overnight in the original protocol.

Quantitative analysis

Quantitave analysis of RNA was done using Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech) by measuring the OD at 260nm and 280nm. One unit of absorption at 260nm represents 40μg/mL of RNA.

Formaldehyde-agarose gel electrophoresis

Formaldehyde-agarose gel (1.2%) was prepared using 1x F buffer (20mM MOPS pH 7.0, 1mM EDTA, 5mM sodium acetate) and formaldehyde 6% v/v. A total of 5-10μL sample (± 2μg RNA) was added to the sample buffer (1x F buffer, 25% v/v formamide, 6% v/v formaldehyde, trace amount of saturated bromophenol blue). The mixture was heated at 60°C for 10 min, and loaded to the gel after the addition of 0.5μL ethidium bromide (10mg/mL). The electrophoresis was run at 5 V/cm in the running buffer (1x F buffer, 6% formaldehyde).

Results and discussion

Protein denaturants, such as guanidinium thiocyanate and guanidinium hydrochloride are extensively used as chaotrophic agents for mammal and plant cell disruption in nucleic acid extraction, especially in RNA extraction (Chirgwin *et al.* 1979; Karlinsey *et al.* 1989; Dolferus 1991; Soares & Bonaldo 1997; Salzman *et al.* 1999; Wilkinson 2000). The earliest total RNA isolation from eukaryotic cells was reported by Glisin *et al.*

(1974), utilising guanidinium thiocyanate and cesium chloride centrifugation. Thirteen years later, Chomczynski and Sacchi (1987) developed a more favourable method, substituting cesium chloride ultracentrifugation with phenol-chloroform extraction, which is more time-efficient. Method 1 in this experiment is the Chomczynski and Sacchi (1987) method, with further modification by Chomczynski and Mackey (1995). The application of Method 1 on RNA extraction from *G. changii* yielded no RNA but DNA, as shown in Figure 1a.

Method 2 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 polyvinyl polypyrrolidone (PVPP) and lithium chloride in the extraction buffer (Hong *et al.* 1992; 1995a; 1995b; 1997). The insoluble PVPP, acts to bind phenolic compounds. It has been used in RNA extraction from recalcitrant tissues *e.g.* grapevine (Geuna *et al.* 1998). Some have also suggested the use of polyvinyl pyrrolidone (PVP), for it is smaller, soluble and able to bind to the phenolic compounds better (Liu *et al.* 1998; Bekesiova *et al.* 1999). 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.*, 1997). Hong *et al.* (1992; 1995a; 1995b; 1997) reported that extraction buffer containing LiCl could eliminate the entangling polysaccharides from nucleic acids in *Porphyra*, but this did not work well on *G. changii* (Figure 1b). The RNA yield and quality are very low (Table 1).

Method 3 is a modified version of Method 2, in which potassium acetate was used to precipitate the polysaccharides into potassium salts. It has been widely used for plant RNA extraction (Hughes & Galau 1988; Ainsworth 1994; Liu *et al.* 1998). Following the

addition of potassium acetate, chloroform extraction led to a compact inter-phase compound stuck strongly to the tube walls, making the transfer of aqueous phase a much easier task. LiCl precipitation was carried out at the later stage of this method to precipitate RNA specifically (Barlow et al. 1963). It is normally fixed at 2.0 – 3.0M, although 0.5M of LiCl is enough for RNA precipitation (Verwoerd *et al.* 1989; Wilkinson 2000; Ambion 2002). Furthermore, speed and duration of the centrifugation during LiCl precipitation are important to be kept at least at 12000 rpm for more than 15 minutes, and at temperature less than 4°C (Ambion 2002). As shown in Table 1 and Figure 1c, the RNA yields and quality from this method are much better than the previous two.

Extraction method 4 utilised phenol and sodium dodecyl sulphate (SDS). SDS, alike guanidinium thiocyanate, are chaotropic agents, for they are excellent hydrogen bonders that destroy the regular water structure. The destruction of the water structure decreases the hydrophobic effect thus promotes the unfolding and dissociation of protein molecules, leading to cell disruption (Pawlowski *et al.* 1994; Matthews *et al.* 2000). The ionic strength of the extraction buffer has to be maintained at certain level for effective nucleic acid extraction and cell disruption. If it is too low, the polysaccharides will co-precipitate with the RNA. If the ionic strength is too high, the protein solubility will decrease, making the cell disruption difficult. In this method, NaCl, neutral salt with monovalent ions, contributed to the ionic strength needed. Meanwhile, EDTA acted to suppress further increase of ionic strength and inhibit any enzymatic activity by chelating divalent cations, which act as enzyme cofactors, present in the solution. Nucleic acid extraction using SDS is widely used for mammalian tissues (Emmett & Petrack 1988), plant tissues (Geuna *et al.* 1998; Liu *et al.* 1998), yeasts (Schmitt *et al.* 1990; Bang *et al.*

1995) and some red algae species *e.g. Carpopeltis, Rhodymenia, Rhabdonia* (Saunders 1993), and *Gracilaria chilensis* (Meneses *et al.* 1999). Nonetheless, yield and quality of the RNA from *G. changii* resulted from this method are worse than those from Method 2 and Method 3 (Figure 1d).

Method 5 (Apt *et al.* 1995) used hexacetyltrimethyl ammonium bromide, CTAB as the cell-disrupting agent, and dithiothreitol, DTT as the reducing agent to prevent any oxidation and other unwanted reactions. DTT strengthened the function of the common anti-oxidant, β-mercaptoethanol, which is used in all five methods reported here. CTAB has been used for RNA extractions from plants (Bekesiova *et al.* 1999; Kiefer *et al.* 2000) and brown algae (Apt *et al.* 1995). This method consists of a few times of chloroform extractions, and it consumes the least time on bench-work relatively (Table 1). The *G. changii* RNA yield and quality are good, comparable to those of Method 3. Among the five methods being tried on *G. changii*, Method 3 and Method 5 were found to be the potential methods to obtain pure RNA.

This CTAB method was further modified by incorporating sand while grinding the tissue, and with a shortened period of LiCl precipitation. The result of the modified methods is shown in Table 2 and Figure 2. It is clear that the CTAB method resulted in pure, intact RNA of high quality, with the $A_{260:280nm}$ ratio ranging from 1.75 – 2.00. The incorporation of sands while grinding has significantly increased the RNA yield by four-folds. The approach of using sands, or glass beads in grinding tissues has been applied for nucleic acid extraction from recalcitrant or succulent plant tissues (Eggermaont & Goderis 1996). The sand acts as a chaotrophic agent that greatly facilitates cell disruption and the release of nucleic acids from the seaweed tissues. The three-hour LiCl

precipitation was found to give sufficiently good result as compared to the overnight LiCl precipitation in the original protocol (Table 1). This has dramatically shortened the total time required for the original CTAB method suggested by Apt *et al.* (1995). The three-hour LiCl precipitation has also recorded the better RNA yield, at approximately 0.16μg per gram of fresh tissue. The RNA from *G. changii* may be more susceptible to degradation during longer period of LiCl precipitation. Therefore, the modified CTAB method 5D (Table 2; Figure 2) was found to be the most suitable method to obtain good quality RNA from *G. changii*, credited for its simplicity and time-efficiency.

The effect of reaction surface area between the ground tissues and the extraction solution was tested, where both connical flasks (250mL) and the Nalgene centrifuge tubes (50mL) were used. There was not much difference in RNA yield between an initial extraction carried out in flasks or in tubes. Besides, the RNA quality of those extracted in tubes, are higher than those in flasks, as lysate transfer has to be carried out.

RNA extractions were carried out on both fresh and frozen samples. All extraction using fresh seaweed samples gave no RNA at all. In some cases, the RNA yields were extremely low, with high interference of DNA. The high water content of fresh samples is the main culprit causing RNA degradations, due to the containment of endogenous RNAse present virtually in all living cells (Wu *et al.* 1997). In the frozen sample preparation, samples were immersed in liquid nitrogen before storage, rather than being stored directly at -80°C. The abruptness of the cooling process, where the samples were Fimmersed into liquid nitrogen (-196°C), would 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.* 1992; 1997; Lim *et al.* 2001). However, we found that air-dried samples were good enough for DNA extraction from *G. changii*, but not RNA. The drying process in open air would be one of the main reasons why RNA is degraded before being extracted. Therefore, frozen sample is suggested to be used for RNA extraction from *G. changii*.

Conclusion

The total RNA extraction from the red seaweed, *Gracilaria changii* was optimised. The incorporation of sands while grinding the tissues and a three-hour LiCl precipitation in the CTAB method were found to give the best RNA yield with high quality from *G. changii*. This optimised CTAB method represents the best method for extracting high quality RNA from *G. changii*. The reaction surface area did not affect the RNA yield much, and frozen samples are suggested for RNA extraction from *G. changii*. The integrity of the RNA isolated will be further verified for mRNA isolation and cDNA library construction.

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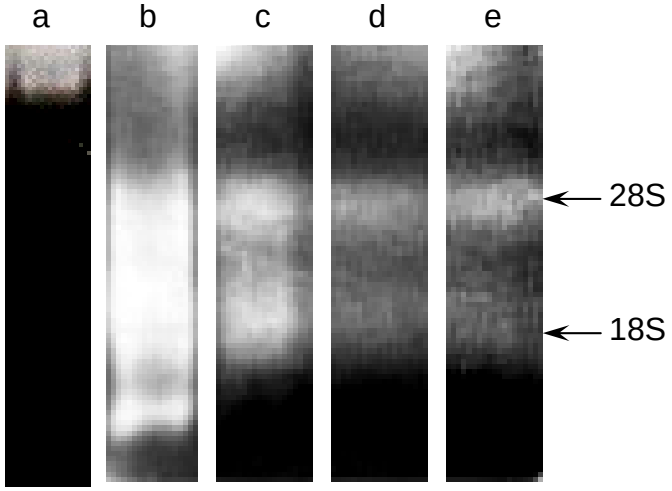

Figure 1. Formaldehyde-agarose gel electrophoresis of RNA (± 0.5 μg) obtained from (a) Method 1; (b) Method 2; (c) Method 3; (d) Method 4; and (e) Method 5.

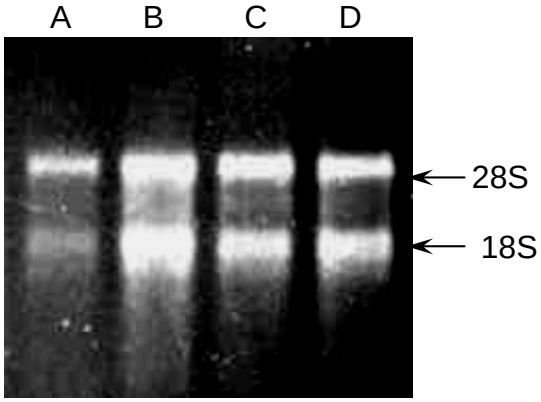

Figure 2. Formaldehyde-agarose gel electrophoresis of RNA (± 2 μg) obtained using modified protocols of the CTAB method. (A) grinding without sand, overnight LiCl precipitation; (B) grinding with sand, overnight LiCl precipitation; (C) grinding without sand; 3 h LiCl precipitation; (D) grinding with sand; 3 h LiCl precipitation.

Table 1. Different RNA extraction methods tried on *Gracilaria changii*.

Method	A_{260}:A_{280} ratio	RNA Yield (μg/g tissue)	Total time required (hour)	Time required for bench-works (hour)
1	1.54	No RNA, but DNA was detected.	7 – 8	5 – 6
2	1.60	0.03	4 – 5	3 – 4
3	1.30 – 1.80	0.01 – 0.08	25 – 26	4 – 5
4	1.33	0.02	7 – 25	5 – 6
5	1.50 – 2.00	0.10	25 – 26	2 – 3

Table 2. Different modified methods of RNA extraction using CTAB from *Gracilaria changii*.

Method	Incorporation of sand while grinding	LiCl precipitation time	A_{260}:A_{280} ratio	RNA Yield (μg/g tissue)	Total time required (hour)
5A	No	Overnight	1.80 – 2.00	0.018 – 0.021	25-26
5B	Yes	Overnight	1.75 – 1.85	0.070 – 0.073	25-26
5C	No	3 hours	2.0	0.040 – 0.043	9-10
5D	Yes	3 hours	1.9	0.161 – 0.165	9-10

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