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Efficient Isolation of Intact RNA from the Soft Coral *Scleronephthya gracillimum* (Kükenthal) for Gene Expression Analyses

Seonock Woo, Seungshic Yum, Moongeun Yoon[1], Sa Heung Kim[2], Jongrak Lee[2], Jeong Ha Kim[1]* and Taek-Kyun Lee*

Southern Coastal Environment Research Division, Korea Ocean Research and Development Institute, Geoje 656-830, Korea; [1]Department of Biological Science, Sungkyunkwan University, Suwon 440-746, Korea; [2]Laboratory of Marine Biodiversity, In The Sea Korea Co., Ltd., Jeju 697-816, Korea

Abstract: A rapid, simple and efficient method to extract RNA from the adult polyps of a soft coral, marine cnidarian, *Scleronephthya gracillimum* (Kükenthal), was developed in this study. The highest yield and purity of RNA was obtained with the lysis solution containing 35 mM EDTA, 0.7 M LiCl, 7.0% SDS, and 200 mM Tris-Cl (pH 9.0). Approximately 40 μg of total RNA was extracted from 200 mg of liquid nitrogen-pulverized polyp tissue. The ratio of absorbance at 260 nm and 280 nm ranged from 1.8 to 2.0. The results of the reverse transcription polymerase chain reaction (RT-PCR) with β-actin gene specific primers and Northern blot analysis using the same gene probe revealed that the RNA extracted by our method had high quality, and was sufficient for subsequent molecular biological analyses. This method was effective for RNA extraction from other soft coral species which belong to the genus *Dendronephthya*.

Key words: *Scleronephthya gracillimum*, RNA isolation, northern blot, gene expression, soft coral

Successful isolation of RNA molecules is essential for studying gene expression, regulation and function. The acid guanidine thiocyanate-phenol-chloroform (AGPC) protocol developed by Chomczynski and Sacchi (1987) and its improved versions (Chomczynski, 1993; Chomczynski and Mackey, 1995) are known to be one of the most frequent and convenient methods to extract RNA from cells and tissue samples. The more specialized protocols have been developed for various types of tissues, such as tissues rich in fat (Tavangar et al., 1990) and rich in proteoglycan and ribonuclease (Chirgwin et al., 1979; Gropper and Morse, 1993). However, most of these methods are time-consuming, tedious and costly as well as requiring sophisticated equipments, such as ultracentrifuge. More serious problems for RNA isolation are found in marine organisms. For instance, polyphenolic compounds, dopa-containing proteins and pigments act as obstacles in RNA purification of marine animals (Groppe and Morse, 1993). Problems are also caused by high contents of fluid and connective tissue in ctenophore *Beroe ovata*, and in this case, the use of 8 M LiCl in the extraction buffer was the solution (Witchel et al., 1996). Thus, there are no general rules for RNA isolation from marine animals.

A soft coral *Scleronephthya gracillimum* (Alcyonacea, Octocorallia, Anthozoa) inhabits the ocean at depths from 15 m to 40 m near Jeju Island, Korea and is one of the dominant species contributing to the rich species diversity of this area. In addition to its ecological importance, this soft coral species has revealed biochemical and pharmaceutical importance as well because isolated bioactive steroids have shown cytotoxic and antiviral activity (Seo et al., 1995). In order to carry out cDNA analysis in this organism, we tried to purify RNA using several conventional methods, including the classical AGPC method (Chomczynski and Sacchi, 1987), a TRI reagent method (Chomczynski and Mackey, 1995) and a CTAB buffer method (Warner, 1996), without success. In the present study, we developed a simple low cost method to purify RNA from this ecologically and biochemically important species and determined the quantitative and qualitative adequacy of the extracted RNA for further analyses.

*To whom correspondence should be addressed.
Tel: 82-55-639-8530, Fax: 82-55-639-8539
E-mail: tklee@kordi.re.kr, jhkimbio@skku.ac.kr

MATERIALS AND METHODS

Animal

Scleronephthya gracillimum colonies were collected at a depth of 15-25 m near Seogwipo, Jeju, Korea on April 26, 2003. The samples were put in seawater and transported to the laboratory. Polyps of the soft coral were cut into pieces about 5×5×5 mm in size, and then these pieces were quickly frozen in liquid nitrogen and stored at –80°C.

Total RNA isolation and optimal condition of RNA extraction

Frozen tissues of the soft coral were mortar-pulverized in liquid nitrogen. Approximately 200 mg of polyp powder was homogenized in 750 μl of lysis solution [25 mM EDTA, 0.4 M LiCl, 1% SDS and 200 mM Tris-Cl (pH 9.0), as a trial version] and 750 μl of water-saturated phenol was added and then mixed. The mixture was centrifuged at 14,000 rpm for 10 min. The aqueous phase was retained and mixed with the same volume of water-saturated phenol and then recentrifuged. One third volume of 8 M LiCl was added to the retained aqueous phase and this was kept at 4°C for 2 h. RNA was precipitated by centrifugation at 14,000 rpm for 30 min and the precipitate was resuspended in 300 μl of DEPC-treated water. RNA was precipitated with a 1/10 volume of 3 M sodium acetate (pH 5.2) and the same volume of isopropanol. The precipitated RNA was rinsed with 70% ethanol (diluted in DEPC-treated water) and dissolved in an appropriate volume of DEPC-treated water (30~40 μl) and kept at -20°C.

To find an optimal condition of RNA extraction, the different composition and concentrations of chemicals (EDTA: 1, 5, 15, 25, 35, 50, 100 mM; LiCl: 0.1, 0.3, 0.5, 0.7, 1.0, 2.0 M; SDS: 1.0, 5.0, 7.0, 8.0 9.0, 10.0%) in lysis solution were evaluated.

Gel electrophoresis

The total RNA was separated and resolved on a 1.0% formaldehyde-denatured agarose gel (Sambrook and Russell, 2001). After electrophoresis, the gel was stained with methylene blue (0.04% in 0.5 M sodium acetate, pH 5.2) or ethidium bromide (0.5 μg/ml) and then destained to detect 28S and 18S RNA. An alternative way to visualize the two major ribosomal RNAs was done by adding 1 μl of ethidium bromide to the RNA sample prior to loading on the agarose gel and observing on a transilluminator.

RNA quantification and qualification

An ultraviolet spectrophotometer (Gene Quant pro, Pharmacia) was used to determine the quantity and quality of the isolated RNA: The RNA concentration was estimated by measuring the absorbance at 260 nm. Protein contamination was estimated by the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}, Sambrook and Russell, 2001).

Reverse transcription polymerase chain reaction (RT-PCR), cloning and sequencing

The total RNA extracted was used as a template for first strand cDNA synthesis (Powerscript Reverse transcriptase, BD Biosciences), and it was primed with G-anchored oligo $(dT)_{18}$. For 5'-rapid amplification of the cDNA end (RACE) an oligonucleotide primer (5'-GTA AAA GGT GTG ATG CCA GA-3') that corresponds to the nucleotide numbers 354 to 335 of the β-actin sequence of *S. gracillimum* (the DDBJ/EMBL/GenBank database under the accession number AY672569) was used. In the upstream area corresponding to the SMART IV oligonucleotide (BD Biosciences), a newly designed primer (5-AAG CAG TGG TAT CAA CGC AGA GT-3') was used. PCR was carried out using the first strand cDNA, primers described above and *Taq* DNA polymerase (Super-Bio, Korea) for 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The amplified DNA was separated on a 1% agarose gel. A single band of amplified DNA fragment was cut out and purified using a gel extraction kit (LaboPass, Korea), and then this was ligated to the pGEM-T Easy vector (Promega). Sequencing of positive clones was carried out with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Northern blot analysis

The total RNA (10 μg) was fractionated on a formaldehyde-agarose gel and blotted to a Hybond N^+ nylon membrane (Amersham). A β-actin cDNA (962 bp long), obtained by random sequencing of the *S. gracillimum* cDNA library, was labeled with [α-^{32}P]dCTP (Amersham) using a random primed labeling kit (Roche). Rapid hybridization buffer (Amersham) was used in this procedure. Hybridization and washing were carried out according to manufacturer's instructions. The membrane was exposed for 3 h to X-ray film (Agfa) and then the film was developed.

RESULTS AND DISCUSSION

Comparison of various RNA purification methods

Even though a number of RNA isolation methods have been developed for various kinds of organisms (Witchel et al., 1996; Mukhtar et al., 1998; Nuyts et al., 2001) and tissues (Michel et al., 1997; Hipfel et al., 1998; Vicient and Delseny, 1999), there seems to be no universal protocols that are applicable for a wide range of marine animals. In order to isolate RNA from an important species of soft coral, *S. gracillimum*, we used three commonly used methods; the original AGPC method (Chomczynski and Sacchi, 1987), a method with the TRI reagent (MRC, Ohio, USA) (Chomczynski and Mackey, 1995), a commercial

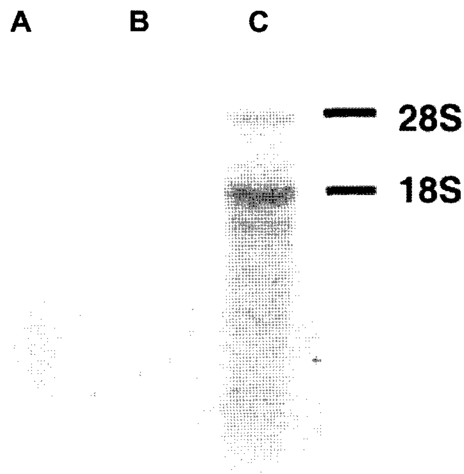

Fig. 1. Comparison of RNA isolation methods. A, Extracted by TRI reagent. B, Extracted by CTAB buffer. C, Extracted by the method described in this report. The same volume of extracts were loaded onto the gel.

reagent based on the AGPC method, and a method with the CTAB buffer [2% CTAB, 20 mM EDTA, 1.4 M NaCl, 1% PVP, 100 mM Tris-Cl (pH 9.0) and 0.2% β-mercaptoethanol] (Warner, 1996). However, we could not successfully isolate RNA from *S. gracillimum*. The results of the latter two methods are shown in Fig. 1A, B. Thus, we developed a trial version of RNA extraction method (see Materials and Method) and applied it to *S. gracillimum*. The result is shown in Fig. 1C. Compared to the conventional methods, the trial version worked well. Moreover, the water-saturated phenol extraction showed adequate quantitative and qualitative results better than the usage of a phenol/chloroform/isoamyl-alcohol (25:24:1) solution (data not shown). We applied this method as well as the conventional TRI reagent method to other soft coral species, *Dendronephthya gigantea* and *D. castanea*. Unlike *S. gracillimum*, both methods worked well for these dendronephthyan species (data not shown). This probably indicates that species belonging to the genus *Scleronephthya* and *Dendronephthya* have different or unique contents of specialized proteins, lipids and/or carbohydrates that affect RNA purification, although the two genera are closely related (Lee and Song, 2000; Fabricius and Alderslade, 2001). These results indicated that a method to isolate RNA must be refined depending on the organism. In addition, since the yield of RNA with our trial version was not high enough, it was necessary to optimize the method.

Optimal condition of the RNA purification

Various concentrations of each component (i.e. EDTA, LiCl and SDS) of the lysis solution were examined to what extent they affect the purity and yield of RNA. First, EDTA was tested at various concentrations of 1, 5, 15, 25, 35, 50 and 100 mM (Fig. 2). Purity of RNA in terms of protein contamination was judged by the ratio of absorbance at 260 nm and 280 nm wavelengths (A_{260}/A_{280}). The ratio with the high between 0 and 35 mM of EDTA (1.86-1.90), with the highest at 5 mM of EDTA (Fig. 2A). The purity drastically decreased as the EDTA concentration became higher. On the other hand, a sharp peak of the RNA yield was observed at 35 mM of EDTA, being approximately 1.28 μg/μl (Fig. 2B). Considering the purity and the yield of RNA, we adopted 35 mM of EDTA as an optimal concentration.

Lithium chloride concentrations ranging between 0.1 and 2.0 M (0.1, 0.3, 0.5, 0.7, 1.0 and 2.0 M) were investigated (Fig. 3). The high purity of RNA was achieved between 0.1 and 0.7 M, with the highest at 0.5 M (A_{260}/A_{280} = 1.91) and the purity decreased at higher LiCl concentration (Fig. 3A). High yield of RNA was obtained with broad concentrations of LiCl between 0.1 and 1.0 M, with the highest at 0.7 M (approximately 1.48 μg/l) (Fig. 3B). LiCl at 2.0 M gave a poor yield. From these results, we chose 0.7 M LiCl as the optimal concentration.

Finally, six different concentrations of SDS (1.0, 5.0, 7.0, 8.0, 9.0 and 10.0%) were examined (Fig. 4). As shown in Fig. 4A, the highest purity of RNA was obtained at 7.0% SDS (A_{260}/A_{280} = 1.99). The highest yield of RNA was also obtained at the same concentration (approx. 1.68 μg/μl; Fig. 4B). Lower or higher concentrations than 7% gave less favorable results. Thus, the optimal concentration of SDS was taken as 7%.

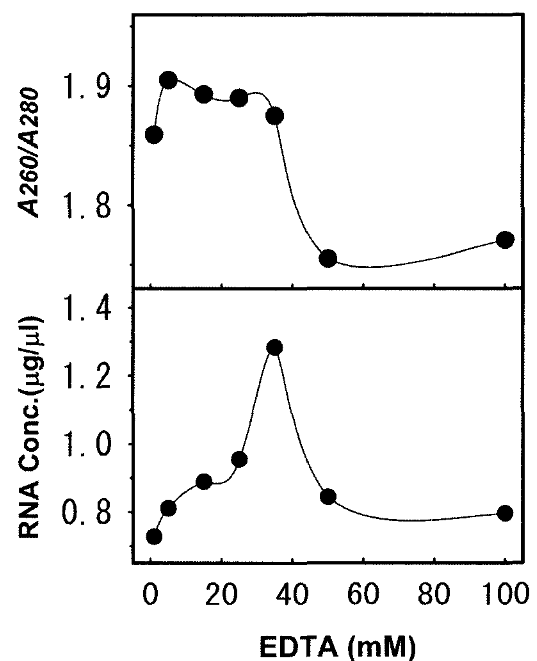

Fig. 2. Effect of EDTA concentration in the lysis solution on RNA purification. A, The ratio of 260 nm and 280 nm. B, The yield of RNA (μg/μl).

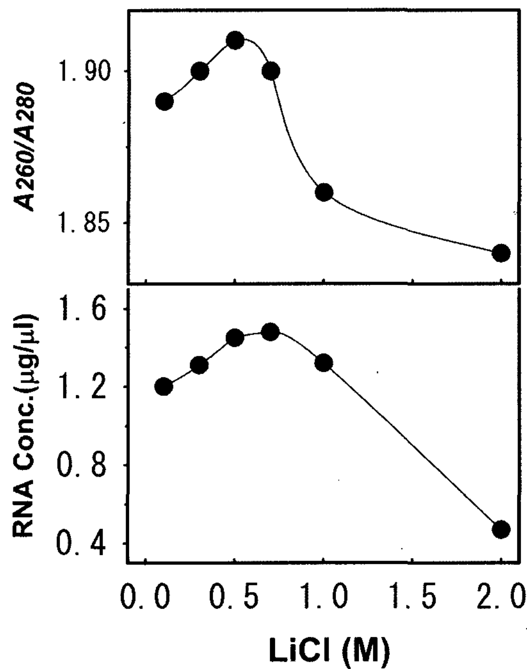

Fig. 3. Effect of LiCl concentration in the lysis solution on RNA purification. A, The ratio of 260 nm and 280 nm. B, The yield of RNA (μg/μl).

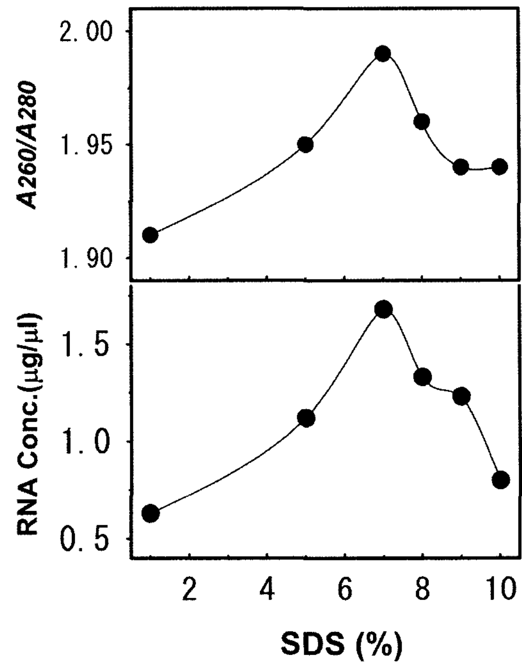

Fig. 4. Effect of SDS concentration in the lysis solution on RNA purification. A, The ratio of 260 nm and 280 nm. B, The yield of RNA (μg/μl).

Quality of purified RNA examined by 5'-rapid amplification of cDNA end (RACE) and Northern blot analysis

Quality of the purified RNA was assessed by both RT-PCR and Northern blot analysis. During random sequencing of the cDNA library, we obtained a clone of 962 bp β-actin gene but missed a few hundred bases at 5'-flanking region. In order to obtain a full-length sequence, RT-PCR was performed using primers described in Materials and Method and first strand cDNA as a template. A single band of about 400 bp was clearly amplified (Fig. 5). After extraction from agarose gel, the DNA fragment was cloned and sequenced. The nucleotide sequence of a clone isolated from cDNA library and that of this fragment were assembled into a full-length cDNA of *S. gracillimum* β-actin gene. The nucleotide sequence of 1,258 bp with poly $(A)^+$ stretch was deposited in the DDBJ/EMBL/GenBank database with the accession number AY672569. Northern blot analysis showed a single transcript of about 1,300 bases (Fig. 6) corresponding to the intact β-actin mRNA of this species. The results indicate that RNA extracted by the present method contains reasonably long mRNAs and possibly many full-length messages.

In summary, we have developed a new method by which total RNA from *S. gracillimum* was first purified in quick and efficient ways. RNA obtained had high purity and was long enough for full-length cDNA amplification. The method was applicable to other genera of soft corals and possibly other marine organisms. Our newly developed method was much quicker and more efficient for the total RNA purification from *S. gracillimum* than conventional RNA extraction methods (Chomczynski, 1993; Chomczynski and Mackey, 1995). Furthermore, RNA obtained by the present method was used without any problems for routine molecular techniques such as reverse transcription and Northern blot analysis. This extraction method would be particularly suitable for the large scale screening of differential gene expressions, including cDNA microarrays.

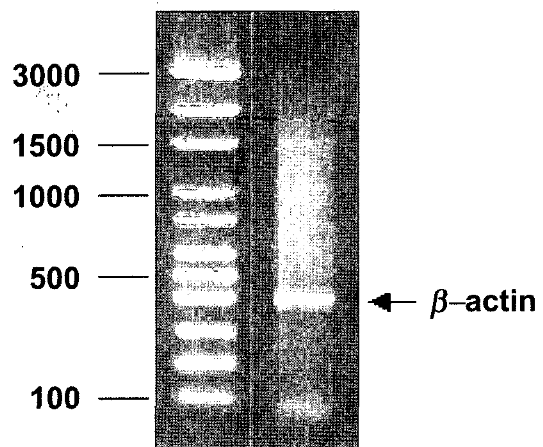

Fig. 5. Agarose gel image of RT-PCR product. Lane 1, 100 bp molecular size marker; lane 2, an amplified fragment of β-actin gene.

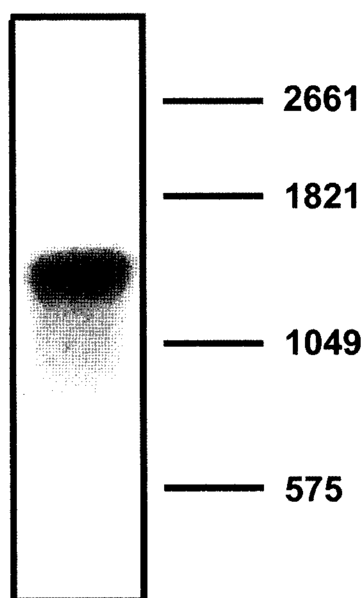

Fig. 6. Northern blot analysis of β-actin transcript. The sizes of the markers in bases are indicated at the right.

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