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DNA extraction conditions from *Porphyra perforata* using LiCl

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Abstract

A rapid and economical method of DNA extraction from a red seaweed *Porphyra perforata* J. Agardh has been developed by the use of lithium chloride. This paper describes the optimization of extraction conditions. Heat treatment of tissues in a solution (0.8 M LiCl, 0.6% Sarkosyl, 10 mM EDTA, 0.2% PVPP, 5% β-mercaptoethanol, pH 9.0) at 55 °C for 10 min extracts DNA that is of sufficient quality to be used as a template for PCR amplification. Total DNA yield was approximately 30 to 50 μg g^{-1} of partially dried tissue. Total RNA yield was approximately 400 μg g^{-1} of partially dried tissue. Carbohydrate was contained as approximately 40 to 90 mM (expressed as glucose equivalents) from 1 g tissue, and protein contamination calculated as the O.D. 260/280 ratio was in the range of 1.4 to 1.7. The DNA was characterized by high molecular weight larger than 50 kb.

Introduction

DNA extraction from seaweed tissues that are heavily embedded in viscous polysaccharides is complicated and time-consuming. Acidic polysaccharides, i.e., sulfated polysaccharides and carboxylic polysaccharides, are the major carbohydrates in red and brown algae (Bold & Wynne, 1978). They are more water-soluble than the neutral polysaccharides of land plants and their solutions are highly viscous. Most of the published references for DNA extraction from green algae (Olsen *et al.*, 1987), red algae (Roell & Morse, 1991; Patwary *et al.*, 1993) and brown algae (Mayes *et al.*, 1992) require grinding tissues in liquid nitrogen. Usually, viscous soluble polysaccharides are released by grinding *Porphyra* tissues in liquid nitrogen (Brasch *et al.*, 1981). To remove the mucilagenous cell wall substances, protoplasts were used with proteinase K and phenol extraction for the extraction of *Porphyra* DNA (Araki *et al.*, 1992).

DNA extraction by the LiCl treatment does not require grinding of tissues, protoplast release, or any method was of sufficient quality to be used as a template for PCR amplification (Hong *et al.*, 1992). This paper describes the optimization of the DNA extraction conditions such as composition of the extraction solution, heating time, shaking time, DNA precipitation, and molecular size of the LiCl-extracted DNA.

Materials and methods

Plant material

Leafy thalli of *Porphyra perforata* J. Agardh were collected during low tide at Point Piedras Blancas, San Simeon, California on October 9, 1990. Tissues were dried under forced air of an electric fan for at least 2 h to approximately 30% moisture content and stored at −70 °C. Approximately 0.6 g of drained fresh tissue resulted in 0.1 g dry weight containing 30% moisture. Moisture content was measured from oven-dried tissue that was dried for 6 days at 70 °C.

DNA extraction

For DNA extraction, 0.1 g partially dried tissue or 0.6 g fresh tissue was cut randomly with scissors to about 0.25 cm^2 and placed into a 15-ml plastic centrifuge tube containing 4 ml of extraction solution (0.8 M LiCl, 0.6% Sarcosyl, 10 mM EDTA, 0.2% polyvinylpolypyrrolidone, pH 9.0) and 0.2 ml β-mercaptoethanol. After ten min at 55 °C, the tissue was shaken gently at 4 °C for 1 h. The supernatant was collected after centrifugation (200 ***g***, 5 min, 4 °C). Total DNA in the supernatant was precipitated directly by addition of 0.1 vol of 3 M sodium acetate, pH 5.4 and 2 vol of 100% ethanol (−20 °C). The precipitate was washed twice by centrifugation (1800 ***g***, 5 min, 4 °C) with 70% ethanol. The pellet was resuspended in 300 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) stock solution and diluted ten-fold in TE again for assays. Finally, the DNA solution was spun for 10 min in a microfuge, and the supernatant was assayed for the amount of DNA and impurities.

Nucleic acid quantification

DNA was quantified using a Hoefer Mini-Fluorometer (Model TKO 100) against standard concentrations of salmon sperm DNA (Calbiochem) and calculated for one gram of partially dried tissue containing 30% moisture. RNA concentrations were calculated from total absorbance at 260 nm minus the DNA absorbance value (converted from the amount of DNA previously determined in μg by fluorimetry).

Carbohydrate quantification

Total carbohydrate was assayed by the phenol-sulfuric acid method (Kochert, 1978). A 100 μl of extract solution was mixed well with 100 μl of 5% phenol and 500 μl of 96% sulfuric acid, then agitated quickly and re-shaken after 30 min. After centrifugation for 1 min in a microfuge, the color was measured at 490 nm. The total carbohydrate was expressed with respect to a glucose standard in mM.

Protein and phenolic compound impurities

The protein impurity was represented by the ratio between absorbance readings at 260 nm and 280 nm (Sambrook *et al.*, 1989). The ratio of 260 nm and 230 nm was used for estimating phenolic compound contamination (Su & Gibor, 1988).

Table 1. Comparison of DNA extraction from dry and wet tissues. Dry tissue was prepared under forced air of an electric fan for at least 2 h to approximately 30% moisture content.

	Dry tissue	Wet tissue
DNA ($\mu g\ g^{-1}$ tissue)	45.1	29.7
RNA ($\mu g\ g^{-1}$ tissue)	417.5	125.3
Carbohydrate (mM)	77.0	22.7
A_{260}/A_{280}	1.41	1.63
A_{260}/A_{230}	0.81	0.50

Agarose gel electrophoresis

A 10 μl aliquot of the undiluted DNA stock solution was loaded on a 0.3% agarose gel and run for 1 h at 50 V in 0.5× TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.0) (Sambrook *et al.*, 1989).

Results

Tissue preparation

The DNA extraction solution softened the *Porphyra* tissue by decreasing the compressive and tensile modulus (Hong, 1993). Due to this softening, DNA was released through the cell walls of both fresh and partially dried tissues. Total DNA yields were approximately 45 and 30 $\mu g\ g^{-1}$ dry weight from dried tissue and wet tissue, respectively. Total RNA yields were approximately 418 and 125 $\mu g\ g^{-1}$ dry weight from dried tissue and wet tissue, respectively (Table 1). Dried tissue was used in this work because it gave a 1.5-fold higher yield of DNA and 3-fold higher release of RNA and carbohydrate together. The optimal size of tissue was determined to be about 0.25 cm^{-2} by consideration of DNA yield and impurities (unpubl. data). When tissue was chopped too small, the extraction solution became extremely viscous and turbid.

Extraction solution

To achieve optimal composition of extraction solution, LiCl was tested up to 1 M for the highest yield of DNA extraction. The presence of LiCl (0.8 M) in the extraction solution was one of the most critical factors for the release of DNA from tissue although protein contamination increased with the increase of LiCl con-

centration (Fig. 1). Only a small amount of DNA was released without LiCl in the extraction solution. The optimal concentration of Sarcosyl in DNA extraction solution was determined up to 1% (Fig. 2). The extraction solution containing 0.6% Sarcosyl released the highest amount of DNA. Sarcosyl also appeared to be one of the essential components for DNA extraction. As a detergent substitute for Sarcosyl, sodium dodecyl sulfate also had almost the same effect on the release of DNA, RNA, carbohydrate, protein, and phenolic compounds (unpubl. data). To chelate magnesium ion, which is required for inhibiting the activity of DNase, the optimal concentration of EDTA was determined in the extraction solution (Fig. 3). An extraction solution containing 5 mM EDTA produced the largest amount of DNA, and released the smallest amount of carbohydrate as a contaminant. When there was no EDTA in the extraction solution, almost no DNA appeared on the agarose gel in spite of much RNA release. Effect of PVPP was determined to remove phenolic compounds from the DNA extract (Fig. 4). PVPP did not have any strong effect on extracting DNA, but 0.2% of PVPP was best to reduce the contamination of phenolic compounds even though 0.05% PVPP was sufficient for obtaining high amounts of DNA and low carbohydrate. Effect of β-mercaptoethanol was determined in the DNA extraction solution (Fig. 5). The total amount of DNA was almost the same whether or not the β-mercaptoethanol was added in the extraction solution, but most of the extracted DNA without β-mercaptoethanol smeared on the agarose gel after electrophoresis, suggesting that the DNA was degraded severely during the extraction time. Thus, the β-mercaptoethanol in the extraction solution appeared to prevent such DNA degradation. The pH value of the extraction solution was compared to obtain the highest yield of DNA (Fig. 6). The extraction solution adjusted to pH 9.0 produced the highest amount of DNA. Although a small amount of carbohydrate was released at the alkaline ranges, the production of DNA also decreased. The amounts of contaminating protein and phenolic compounds were not changed at different pH ranges. Overall, it was concluded that concentrations of 0.8 M LiCl, 0.6% Sarcosyl, 10 mM EDTA, 0.2% PVPP, and 5% β-mercaptoethanol at pH 9.0 gave an optimal balance for the highest DNA yield and relatively low amount of impurities of carbohydrates, proteins, and phenolic compounds.

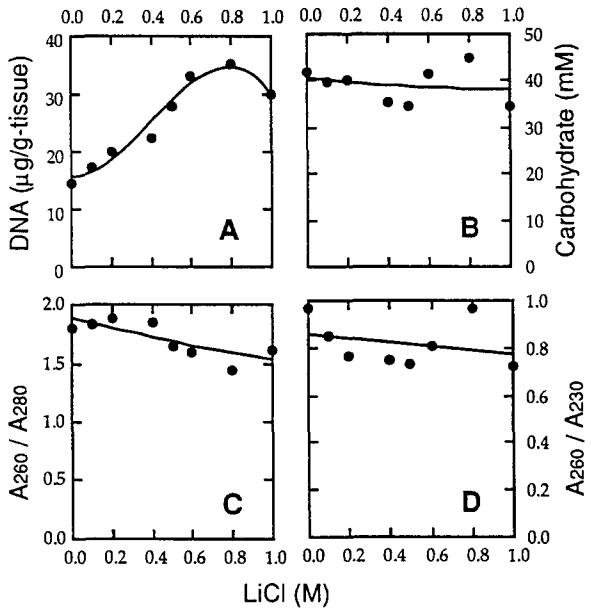

Fig. 1a-d.

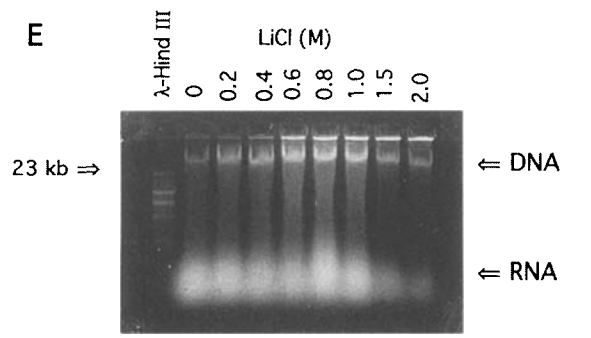

Fig. 1e. Effects of LiCl concentration in the extraction solution. A, DNA yield extracted from one gram of tissue. B, Carbohydrate amount released with DNA. C, Protein impurities in the DNA extract. D, Impurity of phenolic compounds in the DNA extract. E, Photograph of an 0.3% agarose gel stained with ethidium bromide. Lane 1: Lambda-*Hin*dIII digest. Lane 2: no LiCl. Lane 3 to 9: respective concentrations of LiCl.

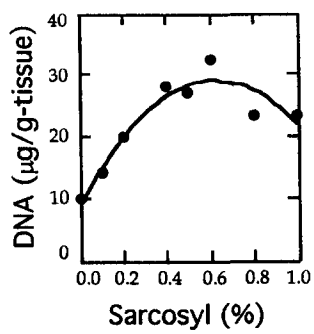

Fig. 2. Effect of Sarcosyl concentration in the extraction solution.

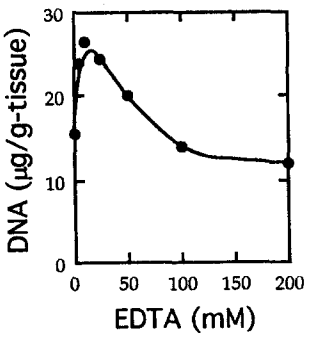

Fig. 3. Effect of EDTA concentration in the extraction solution.

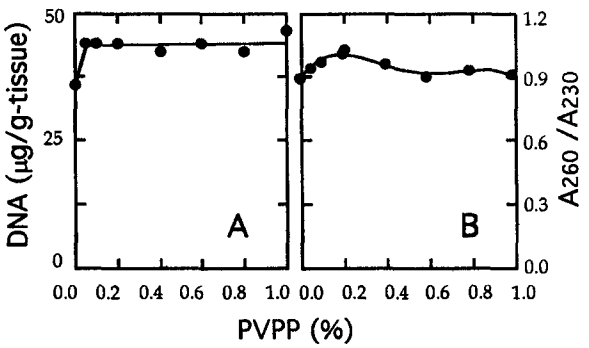

Fig. 4. Effect of PVPP concentration in the extraction solution. A, DNA yield extracted from one gram of tissue. B, Impurity of phenolic compounds in the DNA extract.

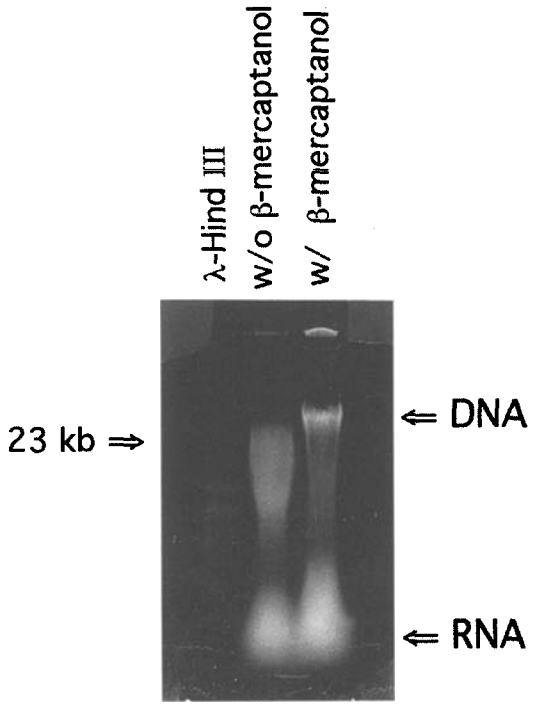

Fig. 5. Photograph of DNA extracted with or without β-mercaptoethanol in the extraction solution. An 0.3% agarose gel was stained with ethidium bromide. Lane 1: Lambda–*Hin*dIII digest. Lane 2: without β-mercaptoethanol. Lane 3: with β-mercaptoethanol.

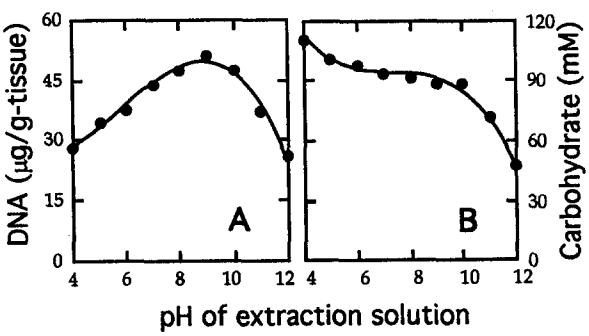

Fig. 6. Effect of pH of the extraction solution. A, DNA yield extracted from one gram of tissue. B, Carbohydrate amount released with DNA.

Heating temperature and time

The large amount and relative purity of DNA was obtained using the optimized compositions of extraction solution and heating at 55 °C for 5 to 10 min. By increasing the temperature and heating time, more DNA was released with more contamination of carbohydrate and protein (unpubl. data). With the preheated extraction solution at 55 °C, the extraction time of 5 min at 55 °C was a critical condition to get an optimal balance for the highest DNA yield and the lowest amount of impurities. Meanwhile, the heating time of 10 min at 55 °C was generally acceptable when the DNA is extracted with the extraction solution maintained at room temperature.

Shaking time

Tissues heated in extraction solution were shaken slowly to release DNA (Fig. 7). Shaking time for 1 h was good enough to obtain DNA at 4 °C, but shaking at 20 °C released a large amount of contaminating carbohydrate. Unshaken tissues were required at least 2 h to obtain a reliable amount of DNA.

Precipitation of DNA

The released DNA in extraction solution was precipitated by the addition of 0.1 volume of 3 M sodium

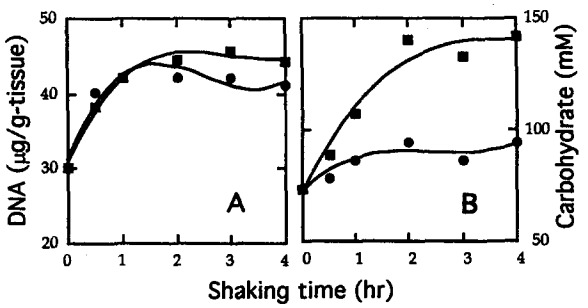

Fig. 7. Release of DNA and carbohydrate during shaking. A, DNA yield extracted from one gram of tissue. B, Carbohydrate amount released with DNA. Tissue was shaken at 4 °C (•) or 20 °C (■) in the extraction solution after heating at 55 °C for 10 min.

Table 2. Effect of sodium acetate on DNA precipitation in ethyl alcohol at −70°C.

	Na·Acetate, pH 5.2		None	
	0 min	30 min	0 min	30 min
DNA ($\mu g\ g^{-1}$ tissue)	48.2	51.2	51.0	50.8
Carbohydrate (mM)	55.8	79.8	57.9	61.6
A_{260}/A_{280}	1.63	1.63	1.43	1.43
A_{260}/A_{230}	0.81	0.81	0.64	0.64

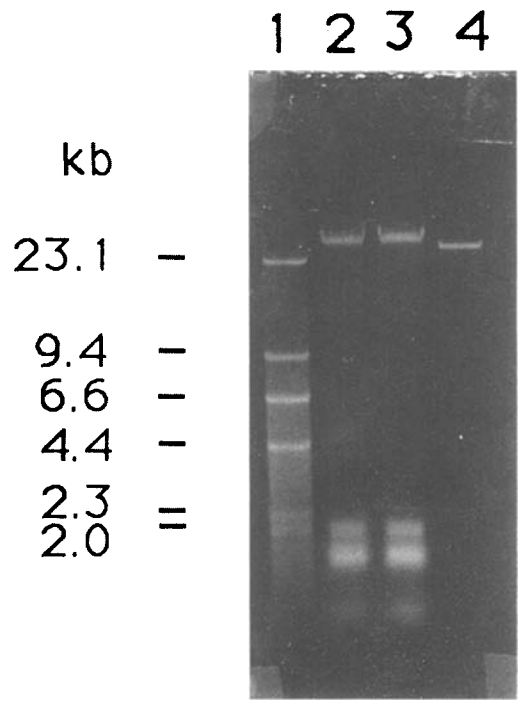

Fig. 8. Molecular size of the LiCl-extracted DNA from *P. perforata*. Lane 1, molecular weight standard of lambda DNA-*Hin*dIII fragments. Lane 2, LiCl-extracted DNA (upper band) shaken for 1 h to release DNA. Lane 3, LiCl-extracted DNA (upper band) left stationary for 1 h to release DNA. Lane 4, Molecular weight standard of lambda DNA. Electrophoresis was run on an 0.3% agarose gel for 2 h at 50 V in 0.5X TAE buffer.

acetate, pH 5.2 and 2 volumes of ethanol (Table 2). The precipitated DNA that was collected promptly by centrifugation without storage at −70 °C had almost same yield and even less contaminants such as carbohydrates, proteins, and phenolic compounds. This direct centrifugation reduced the contaminants and the time needed for the DNA extraction procedure. By addition of 0.2 volume of 10 M ammonium acetate instead of sodium acetate, more carbohydrates were precipitated together with DNA (unpubl. data).

Size of the DNA

By agarose gel electrophoresis, the LiCl-extracted DNA has been estimated to have an average size of larger than 50 kb (Fig. 8).

Discussion

In vitro, LiCl affects tissue softening in seaweeds (Evans, 1963), morphological changes of mammalian cells (Tyobeka & Becker, 1990), and release of cellular components from animal (Raha *et al.*, 1990), plant (Sato *et al.*, 1990) and bacterial cells (Fujii *et al.*, 1987). Tissues of some seaweeds are very tough and difficult to spread by squashing under a coverglass after chromosome staining. The most suitable softening technique as a pretreatment to the chromosome staining on *Fucus* was found to be immersion of small pieces of fixed tissue in a 1 M solution of a monovalent lithium chloride for 15 minutes (Evans, 1963). At first, it was suggested that the marked softening effect of monovalent metal on *Fucus* tissue resulted from the greater solubility of the alginates. Secondly, lithium chloride might swell the cellulose component of the cell wall and thereby cause a general disarticulation of cell masses and softening of individual walls. By treatment of a promyelocytic leukeamia cell line, HL-60 cells, with a concentration of 10 mM LiCl, the surface of the cells appeared like the plasma membrane that had been stripped of its outer leaflet, and at the same time holes began to show on the cell surface (Tyobeka & Becker, 1990). Also, there was a marked

lobulation of the nucleus. At 20 mM and above, there was an increase in the number of holes accompanied by shrinkage of the cells (indicating loss of cytoplasmic contents) and eventually cell death. Ribulose-1,5-biphosphate carboxylase was released from spinach thylakoid membranes with 1 M LiCl (Sato *et al.*, 1990). One molar LiCl-washed extract contained a 55-kDa polypeptide and a 14-kDa polypeptide as the major constituents. LiCl can release a nickase implicated in genetic transformation of *Streptococcus pneumoniae* (Fujii *et al.*, 1987). When competent cells for genetic transformation were treated with 1 M LiCl at 30 °C for 30 min, they released a nickase that introduced nicks into a double-stranded DNA. Furthermore, when non-competent cells were treated with LiCl, they released the putative receptors for the competence activator. For the extraction of total cellular RNA and DNA from cultured mammalian cells, a rapid procedure is described by Raha *et al.* (1990). This method combines the simultaneous disruption of cells and extraction of nucleic acids in a single step with the use of phenol and a buffer containing 100 mM LiCl. From these facts, lithium chloride is suspected to have a similar effect on the softening of *Porphyra* tissue and release of DNA through the loosened cell wall and cell membrane.

The LiCl eliminated the problems of viscous polysaccharides released by grinding tissue in liquid nitrogen. When tissues were treated with LiCl directly, they released DNA and RNA simultaneously without any severe entangling of viscous polysaccharides. The extraction procedure using LiCl appeared as a simple and efficient method to extract DNA and RNA directly from *Porphyra* tissue. This rapid and convenient procedure works on both fresh wet tissue and dried tissue stored up to two years at −70 °C. For convenient use, tissues were dried under forced air of an electric fan for at least 2 h to approximately 30% moisture content to store in a freezer. The dried tissue showed a higher release of DNA, RNA, carbohydrate and protein than the wet tissue (Table 1). It assumed that the dried tissue absorbed LiCl solution directly and affected the tissue structure to make softening more efficiently. Anionic detergents such as SDS (sodium dodecyl sulfate) and sarcosine (N-lauroyl sarcosine or Sarkosyl) have been used for the purpose of membrane dissociation, protein denaturation, and dispersion of protein aggregates (Boehringer Mannheim, 1990). For the enzymatic lysis of mammalian cells, Sarkosyl was used at 1.5% (Kendall *et al.*, 1991) or SDS at 0.2% (Laird *et al.*, 1991). For the lysis of bacterial cells, SDS was used at 4% (Nath, 1990). Meanwhile, in the case of *Porphyra*, 0.6% sarcosine or SDS yielded the highest DNA extraction. Polyvinyl pyrrolidone (PVP) is known to complex with polyphenolics through hydrogen bonding, effectively removing them from the plant homogenate before the isolation of RNA and DNA (John, 1992). Polyvinyl polypyrrolidone (PVPP) is an insoluble polymer that has same function as soluble PVP. Thus, PVPP is easier to be removed by centrifugation after complexing with polyphenolics in the tissue homogenate. For *Porphyra*, 0.2% PVPP was adequate to reduce the contamination of phenolic compounds in the DNA extract even though more carbohydrate released, for unknown reasons. Molecular size of the LiCl-extracted DNA was larger than 50 kb. When preparing DNA for PCR amplification, it is good enough if the average DNA chain length can be maintained at 20 kb or larger (Sogin, 1990). Thus, the LiCl-extracted DNA has a good enough size for PCR amplification. For a more exact size determination of the DNA, it is required to compare the DNA with the large molecular weight markers derived from lambda DNA joined by T4 DNA ligase (Ausubel *et al.*, 1987).

Normally, liquid nitrogen grinding makes it tedious at best and sometimes impossible to isolate DNA from *Porphyra* because of excess polysaccharides which entangle the high molecular weight DNA molecules. The LiCl method has no difficulties in isolating total DNA and RNA, and requires no treatment of polysaccharide removal. Usually, the amount of polysaccharides released by LiCl method was around 40–90 mM g^{-1} dry tissue. This small amount created no problems in separating DNA from polysaccharides. The major point is that whenever grinding is applied to *Porphyra*, one must use a certain method to remove excess viscous polysaccharides released by the grinding. Using the LiCl method without grinding tissue, one can save several steps: the liquid nitrogen grinding step itself and the following steps for removal of excess polysaccharides. Therefore, the principal advantages of the LiCl method are its simplicity and repeatability in isolating high yields of DNA and RNA simultaneously from *Porphyra* and several seaweeds.

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