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A Simple Method for Extraction of High Molecular Weight DNA from *Porphyra Tenera* (Rhodophyta) Using Diatomaceous Earth

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The innate soluble polysaccharides and phenolic compounds of marine macroalgae are serious contaminants which interfere with experimental procedures such as restriction enzyme digestion, polymerase chain reaction (PCR) and other enzymatic reactions using extracted DNA samples. The viscous polysaccharides are co-precipitated with DNA samples by isopropanol or ethanol precipitation in conventional experiment. To overcome the problem, a method for the isolation of high molecular weight DNA from *Porphyra tenera* is developed with the application of diatomaceous earth column. The isolated DNAs by this method were about 50-100 kb in size and could be digested well with restriction enzymes. The nuclease activity seemed to be minimal, and high reproducibility in the arbitrary primed PCR for RAPD analyses was a distinctive feature. These results suggest that this method is very efficient in isolating nucleic acid from macroalgae including *Porphyra*.

Key Words: Diatomaceous earth, DNA preparation, *Porphyra tenera*, RAPD pattern, Rhodophyta

INTRODUCTION

Porphyra species have been distinguished mainly on the microscopic or macroscopic characteristics of their blade-morphology. However, their morphological characteristics are very similar and they are frequently interspersed in the overlapped natural habitat. The identification of species is important at the point of preservation of genetic resources, breeding and further researches. In addition, these are also related with commercial interest. Molecular genetic markers which can identify the genetic characteristics were suggested as important keys in molecular breeding such as somatic hybrid (Mizukami *et al.* 1995). The DNA analysis of biological organisms which is difficult to distinguish them from each other on the basis of only morphological characteristics is expected to be both convenient and reliable means.

However, DNA analysis of *Porphyra* species has not been a popular topic, due to the difficulties in nucleic acid isolation methods and in their applications (Hong *et al.* 1997; Mizukami *et al.* 1998; Lee and Lee 2003). Major problems in the isolation of DNA and further experimental procedures with DNA isolated from marine macroalgae were caused by high nuclease activity, RNA contamination or soluble polysaccharide etc. (Hong *et al.* 1997; Kitade *et al.* 1996; Mizukami *et al.* 1998). In plant, acidic polysaccharides contaminated in extracted DNA have been shown to give inhibitory effects on restriction enzyme digestion (Do and Adams 1991) and polymerase chain reaction (PCR) amplification (Demeke and Adams 1992). Sulfated and carboxylic polysaccharides are major carbohydrates in red and brown algae (Bold and Wynne 1978; Ragan 1981). These are more water-soluble than the neutral polysaccharides of land plants, and their solutions are highly viscous (Hong *et al.* 1997). The soluble polysaccharides like porphyrans which are major component in lavers' cell wall can interfere with enzymatic treatments (Kitade *et al.* 1996).

It is also reported that the reliability of random amplified polymorphic DNA (RAPD) analysis for the identifying genetic characteristics of each species could be affect-

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ed by contaminants of template DNA. The decrease of PCR yield or unreliable DNA amplification in RAPD analysis can be caused from contaminating polysaccharide for other organisms (Pandey *et al.*,1996). An excess of RNA extracted concomitantly with DNA also can cause these problems in RAPD analysis (Pikaart and Villeponteau 1993). Mizukami *et al.* (1998) reported that reproducibility of RAPD pattern was very different by the isolation methods of DNA from *Porphyra* due to the contaminants. Thus, the effective purification of DNA is very important for the genomic applications in macroalgae.

To isolate pure DNA from macroalgae, *Porphyra*, for PCR or genomic DNA library construction, several DNA isolation methods were reported in previous works (Alberto *et al.* 1997; Araki *et al.* 1992; Hong *et al.* 1997; Kitade *et al.* 1996; Mayes *et al.* 1992; Nakajima *et al.* 2000; Roell and Morse 1991). A method for extraction of high molecular weight DNA from *Porphyra yezoensis* using ultracentrifugation was reported by Kitade *et al.* (1996), and Mizukami *et al.* (1998) reported that only purified DNA with CsCl-gradient ultracentrifugation was showed reproducibility of RAPD pattern among several common DNA isolation methods. However, these methods have CsCl-gradient ultracentrifugation procedure which is very expensive and time-consuming as a conventional method.

The extraction of nucleic acid using diatomaceous earth which has a binding affinity to DNA molecule was applied in several extraction procedures to isolate plasmid (Kim and Pallaghy 1996) or DNA in microorganisms (Atyeo *et al.* 1998, Kamenetzky *et al.* 2000; Parrish and Greenberg 1995). It was reported that this method using diatomaceous earth was effective in isolating DNA from small amount of samples with various contaminants and the isolated DNA showed good results indicating the removal of PCR inhibitor when compared with conventional methods (Kamenetzky *et al.* 2000).

In this paper, we explore the application of the diatomaceous earth column as a routine experimental procedure for isolating the pure DNA from *Porphyra* tissue and the reproducibility of RAPD pattern of extracted DNA.

MATERIALS AND METHODS

Materials

Fresh thalli of *Porphyra tenera* were collected from an aquacultural field at Jindo, Jeollanamdo. The thalli alive transferred to laboratory and thoroughly washed with filtered fresh seawater. After drying in the dim light for 12 h, samples were stored at -20°C in sealed plastic bags.

DNA extraction

Stored samples were ground to fine powder in liquid nitrogen using a mortar and pestle. The powder with 0.02 g of dried weight was transferred to a 1.5 ml microtube, and 550 μL extraction buffer (100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% polyvinylpyrrolidone Mr. 40,000) with 30 μL of 2-mercaptoethanol and 50 μL of 10% sodium dodecylsulfate was added. The homogenate was mixed well with gentle inverting and incubated for 15 min. The homogenate was extracted with equal volume of phenol/chloroform/isoamylalcohol (25:24:1) solution. After centrifugation the recovered aqueous solution was treated with 5 unit of RNase A at 37°C for 1h and extracted with equal volume of phenol/chloroform/isoamylalcohol (25:24:1) solution. Otherwise RNase A was treated at the finally purified DNA solution. After centrifugation, the supernatant was used for further purification by diatomaceous earth column.

Binding and elution of DNA from diatomaceous earth binding matrix

Diatomaceous earth solution: Diatomaceous earth solution for DNA binding matrix was prepared as reported by Kim and Pallaghy (1996). The diatomaceous earth (Sigma D-5384) was suspended at 50 mg mL^{-1} in deionised distilled water and left to sediment for more than 3 h. The milky suspension was discarded carefully and the intact sediment was resuspended with original volume of deionised distilled water. This procedure was repeated at least 3 times, and 600 μL of the diatomaceous earth solution was packed into minicolumn using syringe. The Promega Wizard^{TM} minicolumn was used and the disposable plastic syringe (5 mL volume) was linked to minicolumn to manipulate a pressure.

DNA binding and elution: About 500 μL supernatant prepared at the above DNA extraction procedure was transferred to syringe assembled with a minicolumn freshly made using diatomaceous earth and applied by pushing plug. For washing the unbound material, 1 mL of 80% isopropanol was applied, and all trace of isopropanol was completely removed by repetitive pushing plug or short spin by microfuge. The washed and drained column was placed into a new microtube and 50 μL of preheated TE buffer or deionised distilled water was applied to elute the DNA. After 10 min incubation,

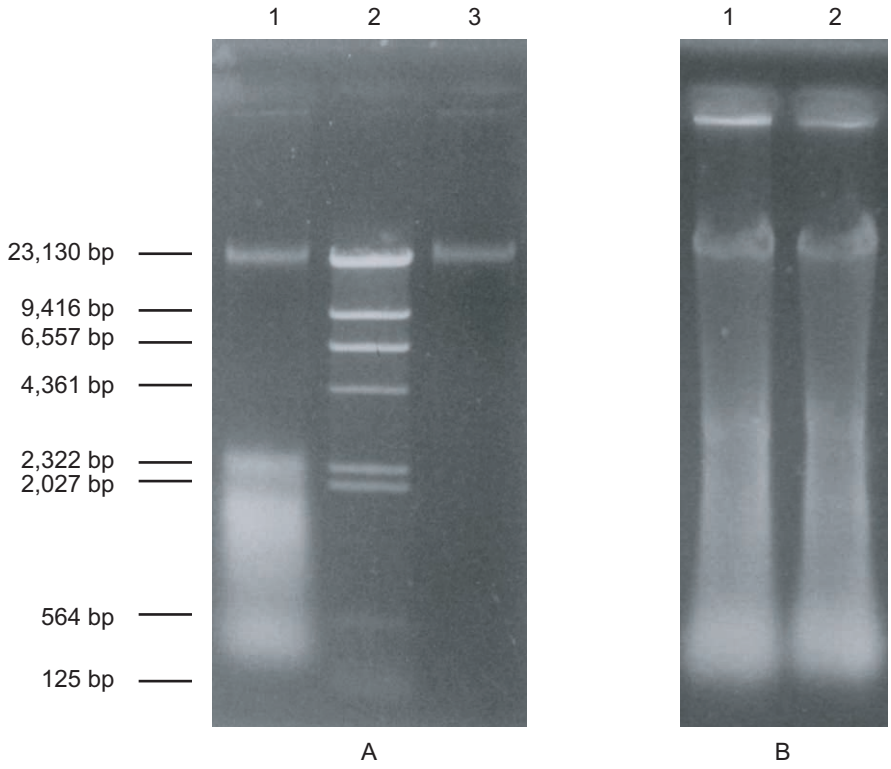

Fig. 1. DNA extraction of *P. tenera* on 0.7% agarose gel in 0.5× TAE buffer. (A) Extracted DNA using diatomaceous earth; Lane 1, extracted DNA without RNase treatment; Lane 2, size standards (*Hind*III-digested lambda DNA); Lane 3, after RNase treatment. (B) DNA extraction using alcohol precipitation; Lane 1, DNA precipitated with isopropanol; Lane 2, DNA precipitated with ethanol.

the DNA solution was harvested by centrifugation at maximum speed using microfuge for 1 min.

Restriction enzyme digestion and electrophoresis for size determination

The purified DNA (1 μg) was digested with a couple of restriction enzymes (5 U each), *Pst*I and *Eco*RI, at 37°C for 3 h, and residual enzymes were removed by phenol extraction. To verify the digestion of purified DNA, 0.7% agarose gel (Agarose LE, Promega) electrophoresis was conducted. To determine the size range of purified DNA, 0.3% agarose gel (SeaKem Gold, BMA) electrophoresis was performed at 0.85 $V \cdot cm^{-1}$ with 0.5× TAE buffer.

PCR amplification for RAPD pattern

Ten-base oligonucleotide primers purchased from Operon Technologies (Alameda, California) were used for RAPD PCR reactions. The sequences of these primers were as follows: OPA-1, CAGGCCCTTC; OPA-2, TGCCGAGCTG; OPA-12, TCGGCGATAG. The reaction components included 0.25 mM dNTP 5 pM primer, 1 unit of Taq polymerase and 100 ng of genomic DNA in Taq polymerase buffer. The final volume of the amplification reaction was 25 μL. All of the reaction components were purchased from Bioneer (Republic of Korea). The amplification was performed in a Perkin Elmer GeneAmp PCR system 2400 programmed for 45 cycles at 94°C (1 min), 36°C (1 min), 72°C (2 min) with initial incubation at 94°C (5 min). The products were electrophoresed in 1.2% agarose gels.

RESULTS

When the diatomaceous earth column was used as a harvesting method, high molecular weight DNA with averaged yield about 3 μg per 0.02 g of *Porphyra tenera* dried tissue was successfully obtained as shown in Fig. 1A. The protein contamination in the isolated DNA was estimated by the absorbance ratio of A260/A280. A sharp band of isolated DNA was separated on a 0.7% of agarose gel electrophoresis (Fig. 1A, lane 1), and no RNA was shown after RNase treatment (Fig. 1A, lane 3). There is no warping of the loading well in agarose gel electrophoresis and samples were not shown the gelatinous pellets which were considered as viscous polysaccharides (Manning 1991; Kim *et al*. 1997). However, the DNA precipitated with isopropanol (Fig. 1B, lane 1) or ethanol (Fig. 1B, lane 2) from the same cell extract used

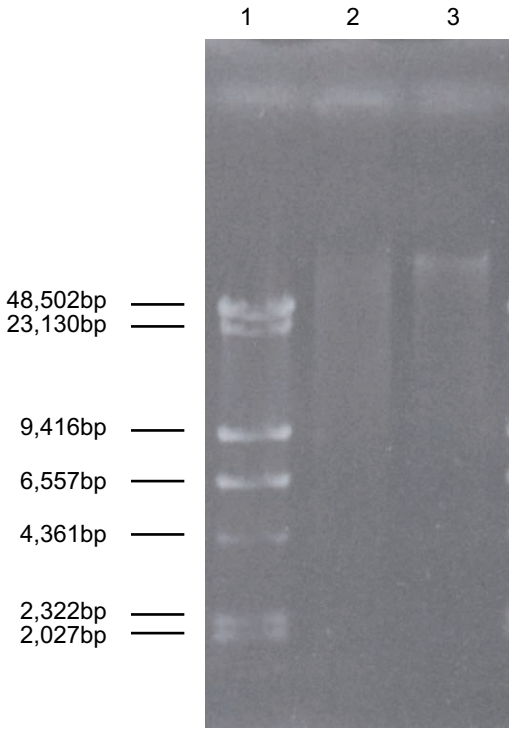

Fig. 2. The size of extracted DNA on 0.3% agarose gel electrophoresis in 1× TAE buffer. Lane 1, size standards (undigested lambda DNA and *Hind*III-digested lambda DNA); Lane 2-3, different DNA samples extracted by different batches.

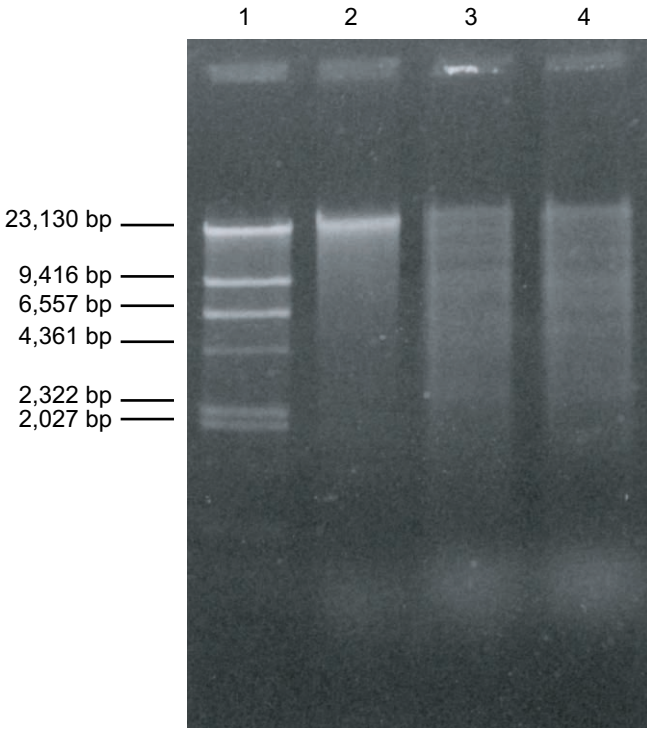

Fig. 3. The restriction enzyme digestion of extracted DNA sample. Lane 1, size standards (*Hind*III-digested lambda DNA); Lane 2, 3 h incubation of sample DNA without restriction enzyme; Lane 3, 5 U *Eco*RI digests; Lane 4, 5 U *Pst*I digests.

in Fig. 1A showed gelatinous DNA pellet and it showed smear band in electrophoresis as shown in Fig. 1B.

The size of isolated DNA using this method was estimated with a range of 50-100 kb when electrophoresed in 0.3% agarose gel electrophoresis (Fig. 2). The size marker was prepared by mixture of undigested lambda DNA and *Hind*III-digested Lambda DNA. The undigested lambda DNA was used as a size marker of 48.502 kb. The lanes 2 and 3 showed the size of isolated DNA is over 50 kb when compared with size markers. The maximum size of the isolated DNA with our method could be postulated to 100 kb, because the maximum bound DNA size of the diatomaceous earth is reported to be about 100 kb (Kim and Pallaghy 1996). This recovering method using diatomaceous earth has been reported that it was successfully employed to recover plasmid DNA ranging 3-100 kb in size (Kim and Pallaghy 1996). A reference reported by Kitade *et al.* (1996) referred that about 25-166 kb of DNA was extracted when total DNA was isolated by CsCl gradient ultracentrifugation.

The isolated DNA was successfully digested with only 5 units of selected restriction enzymes (*Pst*I, *Eco*RI) as shown in the Fig. 3. There were no intense band in the original size of isolated DNA after digestion, and good

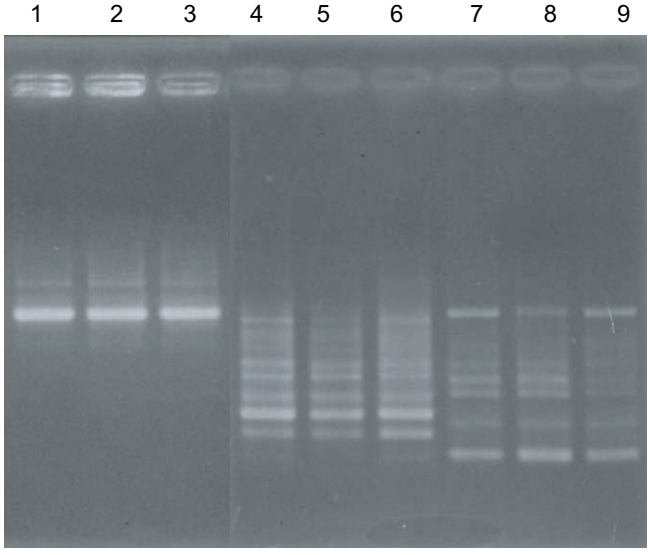

Fig. 4. Effect of independent DNA preparations using diatomaceous earth on the reproducibility of RAPD banding patterns. An algal sample was divided into three portions and the extracted DNAs independently from three portions were amplified with three random PCR primers (OPA-1, OPA-2, OPA-12). The products were electrophoresed for comparison of RAPD patterns. Lane 1-3, DNAs amplified with OPA-1 primer, Lane 4-6, DNAs amplified with OPA-2 primer, Lane 7-9, DNAs amplified with OPA-12 primer.

restriction fragment patterns were appeared in the electrophoresed restriction enzyme digests. Also least amount of the nuclease activity of isolated DNA was detected when the isolated DNA was incubated in the same condition without the restriction enzyme (Fig. 3. lane 1).

Fig. 4 showed the reproducibility of RAPD band pattern using three arbitrary 10-mer primers. The template DNA was isolated independently from three parts of mixed crude samples. When the isolated DNA was amplified with arbitrary 10-mer primers for RAPD, multiple bands over 10 bands were obtained with most primers among 20 tested primers and the reproducibility of RAPD band pattern could be obtained with the isolated DNAs regardless of most primer used (data not shown).

DISCUSSION

Generally, organic solvents such as ethanol or isopropanol were widely used in common nucleic acid isolation procedure for precipitating or concentrating nucleic acid. However, when isopropanol or ethanol was used in some specified ionic environments to precipitate the DNA extracted from plant tissue, viscous polysaccharides were frequently resulted in the precipitation of DNA (Manning 1991) and nucleic acid could be precipitated selectively (Manning 1991; Xing and Gibor 1988). These specified conditions also could be different to each variant of samples, because macroalgal tissue has different contents and components of these contaminants according to its developmental stages or distributions (Hong *et al.* 1997).

In contrast, using the diatomaceous earth column to bind nucleic acids is a comparatively simple condition for harvesting DNA from cell extracts. In addition, the isolation of nucleic acid using diatomaceous earth was effectively applied in several extraction procedures with several tricky samples of microorganisms from such as dental plaque, feces of pigs and germinal layers (Atyeo *et al.* 1998, Kamenetzky *et al.* 2000; Parrish and Greenberg 1995). Kamenetzky *et al*. (2000) reported that DNA extracted with diatomaceous earth showed reliable PCR amplification and subsequent sequencing of amplification products when compared with conventional method. They assumed that their success was possibly due to the removal of PCR inhibitors such as polysaccharides in germinal layer samples.

In RAPD study of *Porphyra*, Mizukami *et al*. (1998) also showed that only pure DNA isolated using CsCl gradient ultracentrifugation could be obtained with high reproducibility in PCR amplification for RAPD when compared with several conventional methods. In this paper, we could also obtain highly reproducible RAPD band patterns and fairly good digestion patterns with restriction enzyme using DNAs isolated according to the procedures employing diatomaceous earth column. Four base (Kitade *et al.* 1996) or five base (Mizukami *et al.* 1998) recognition restriction enzymes was relatively nonspecific compared with the six base recognition enzymes. Kitade *et al.* (1996) also reported that the extracted DNA without CTAB purification was successfully digested with four base recognition enzymes, but not digested when the DNA was digested with six base recognition enzymes. The successful digestion with two of six base recognition enzymes (*Pst*I and *Eco*RI) in our results represent that the isolated DNA was very pure, and no further purification treatment was needed such a CTAB treatment or CsCl gradient ultracentrifugation.

These results represent this method using diatomaceous earth can isolate pure DNA which shows reliable PCR for RAPD and restriction enzyme digestion without CsCl gradient ultracentrifugation and it means diatomaceous earth can be a effective method for these purposes without time-consuming and expensive procedures when compared with previous work.

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