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Identification of a *Taq* DNA polymerase inhibitor from the red seaweed *Symphyocladia latiuscula*

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Abstract: *Two inhibitors of Taq DNA polymerase were isolated from the marine red alga Symphyocladia latiuscula. The inhibitors were purified by methanol extraction, molecular fractionation below 3,000 MW, and reverse-phase HPLC. The purified compound SL-1 containing three bromines was identified as 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol ($C_7H_5Br_3O_3$: MW 374) by NMR and MS analyses. The purified compound SL-2 was identified as 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether ($C_8H_7Br_3O_3$: MW 388). In a 25-µl reaction mixture containing 1.5 units of Taq DNA polymerase, the enzyme was completely inhibited by 0.5 µg SL-1 or 5 µg SL-2.*

Key words: *DNA polymerase inhibitor, Symphyocladia latiuscula, 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol, 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether*
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Introduction

The polymerase chain reaction (PCR) catalyzed by *Taq* DNA polymerase is a very powerful and sensitive analytical technique, with applications in many diverse fields, including molecular biology and population genetics (Innis *et al.*, 1990). Since Hong *et al.* (1992; 1995) established a simple method for the rapid extraction of nucleic acids from seaweed using lithium chloride, PCR has also been applied to the field of seaweed molecular biology. However, attempts to use PCR to analyze DNAs extracted from some seaweed species have been unsuccessful (Hong *et al.*, 1997). We previously confirmed the presence of DNA polymerase inhibitors in 11 species of marine macrophytes, among 59 species tested (Jin *et al.,* 1997). From this result, we found that the methanol-soluble extract from the red seaweed *Symphyocladia latiuscula* was the strongest inhibitor of *Taq* DNA polymerase activity.

S. latiuscula is known to have antiviral activity against the *Herpes simplex* DNA virus (Kim *et al.*, 1997; Park *et al.*, 2005). Several bromophenolic compounds from *S. latiuscula* are also known to inhibit α-glucosidase activity (Kurihara *et al.*, 1999), and to have antioxidant (Park *et al.*, 1999; Chung *et al.*, 2001), feeding-deterrent (Kurata *et al.*, 1997), anti-inflammatory (Wiemer *et al.*, 1991), and anti-microbial (Lim *et al.*, 2000) properties. The goal of the present study was to purify *Taq* DNA polymerase inhibitors from *S. latiuscula* and identify the structures of these compounds (Watson and Blackwell., 2000; Mizushina *et al.*, 2004; Pungitore *et al.*, 2007; Kim *et al.*, 2007).

Materials and Methods

Plant materials: Leafy thalli of the red seaweed *S. latiuscula* (Harvey) Yamada were collected from the coast of Busan, Korea (35°09'34''N, 129°11'50''E). For convenience of use, the sample was dried completely at room temperature in the shade and then ground to a powder using a coffee grinder (Jin *et al.*, 1997).

Isolation and identification of inhibitors: To extract the methanol-soluble fraction of the seaweed powder, 20 g of the powder were mixed with 1 l of 100% methanol and incubated at room temperature for 1 day. The methanol extract (40 mg ml^{-1}) was passed through an ultra-filtration membrane with a cutoff of MW 3,000 (Millipore Corp., Billerica, MA, USA), and the filtrate containing active compounds of MW 3,000 or less was then fractionated by three rounds of reverse-phase high-performance liquid chromatography (RP-HPLC). First, 300-µl (120 mg) aliquots of filtrate were applied to a Unisil Q C_8 column (10.7 mm i.d. x 25 cm; GL Sciences, Tokyo, Japan) connected to a Waters 600 gradient liquid chromatography system (Waters Corp., Milford, MA, USA) with monitoring at 214 nm wavelength Elution was performed with a linear gradient of 0 to 75% (v/v) acetonitrile over 50 min at a flow rate of 2 ml min^{-1}.

The active peak, which eluted at 51% acetonitrile (40.6–44.2 min), was applied to the same Unisil Q C_8 column, which was then isocratically eluted with 50% acetonitrile at a flow rate of 2 ml min^{-1}. Finally, the resulting active peaks II and III were combined and applied to a Nova-pak C_8 column (3.9 mm i.d. x 15 cm; Waters Corp Milford, MA, USA.) The column was eluted with a linear gradient from 0 to 65% acetonitrile over 40 min at a flow rate of 1 ml min^{-1} to yield purified compounds SL-1 and SL-2.

The purified compounds were analyzed on a GC-MS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, and the spectral data were compared to those in the database of the GC-MS. ^{1}H-NMR spectra of the compounds were taken on a JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan) using acetone-d (CD_3COCD_3). From these data, we determined the

structures of SL-1 and SL-2 and confirmed that their structures were identical to those described by Lim *et al.* (2000).

PCR inhibition assay: For template DNA, total DNA containing the β-globin gene was isolated from mouse liver by the alkali-boiling method (Ivinson and Taylor, 1991). The isolated DNA was quantified using a Hoefer Mini Fluorometer (Model TKO 100; San Francisco, CA, USA) with a standard of calf thymus DNA. The GH20 and PC04 primers used for PCR were 20-base oligonucleotides consisting of the β-globin gene sequences 5,-GAAGAGCCAAGGACAGGTAC-3' and 5'-CAACTTCATCCACGTTCACC-3', respectively (Bauer *et al.*, 1991). PCR of a mouse genomic DNA template using these usually produces an approximately 200-bp β-globin gene fragment.

PCR amplification was carried out using a Perkin-Elmer DNA thermal cycler (Waltham, MA, USA). The 25-µl PCR reaction mixtures contained 1 µl of template DNA (0.5 µg μl^{-1}), 1 µl of each β-globin primer (100 pM μl^{-1}), 1 µl of dNTP mix (consisting of 0.1 mM

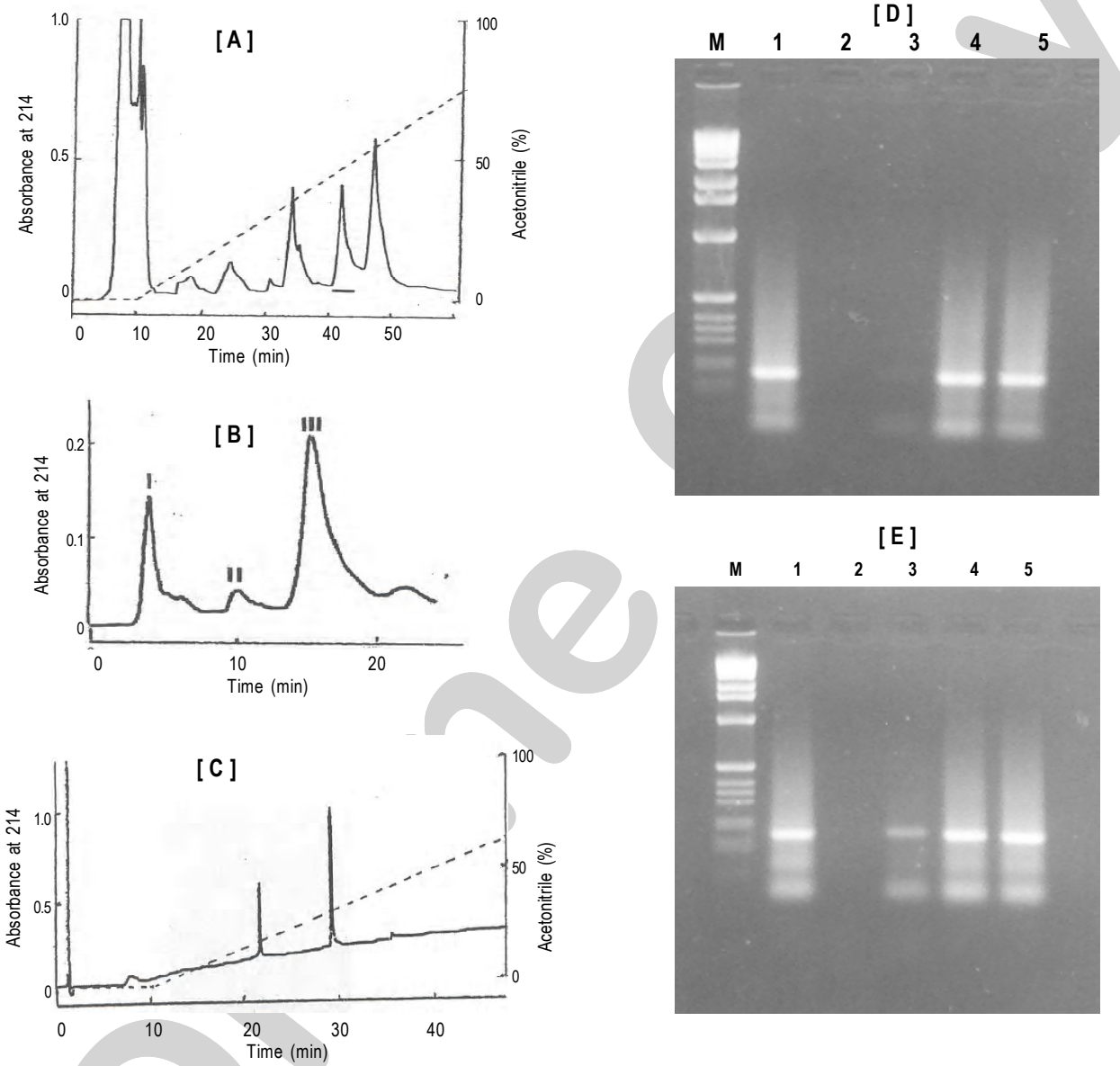

Fig. 1: HPLC profiles and inhibition activities of DNA polymerase inhibitors from *Symphyocladia latiuscula*. (A) First HPLC: the low-MW fraction of *S. latiuscula* methanol extract was subjected to RP-HPLC on a Unisil Q C_8 column. The bar indicates active fractions. (B) Second HPLC: the active peak from (A) was subjected to RP-HPLC on the same column as in (A), but this column was eluted isocratically with 50% acetonitrile. (C) Third HPLC: active peaks II and III from (B) were purified with a linear 20-41% acetonitrile gradient on a Nova-pak C_8 column. (D) Inhibition of DNA polymerase by purified SL-1. M, molecular size markers (1-kb DNA ladder; BRL/Gibco); lane 1, no SL-1; lane 2, 5 µg SL-1; lane 3, 0.5 µg SL-1; lane 4, 0.05 µg SL-1; lane 5, 0.005 µg SL-1. (E) Inhibition of DNA polymerase by purified SL-2. M, molecular size markers; lane 1, no SL-2; lane 2, 5 µg SL-2; lane 3, 0.5 µg SL-2; lane 4, 0.05 µg SL-2; lane 5, 0.005 µg SL-2

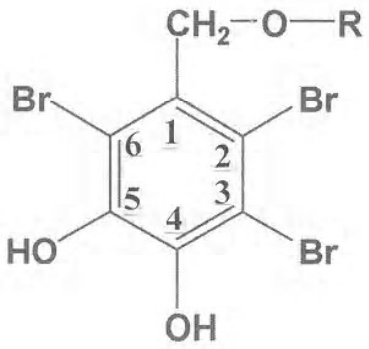

Fig. 2: Basic structure of inhibitors SL-1 and SL-2 purified from *S. latiscula*. SL-1 ($C_7H_5Br_3O_3$) has a hydrogen atom at position R, whereas SL-2 ($C_8H_7Br_3O_3$) has a methyl group at position R

each dATP, dCTP, dGTP, and dTTP), 2.5 µl of PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 µl of 25 mM $MgCl_2$, 1 µl of 12.5% Tween 20, 0.3 µl of *Taq* DNA polymerase (5 U μl^{-1}), 12.5 µl (0.005–5 µg) of inhibitor compound, and 2.7 µl of distilled water. The cycling parameters consisted of an initial incubation at 94°C for 30 s; 30 cycles of 1-min denaturation at 94°C, 2-min annealing at 60°C, and 3-min extension at 72°C; and a final 10-min extension step at 72°C to ensure complete extension of the amplified products.

Agarose gel electrophoresis: Ten µl of PCR product, mixed with 0.1 vol of loading dye (0.4% bromophenol blue, 0.4% xylene cyanol, 10% glycerol), were loaded on a 3% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and run for 1.5 h at 50 V in 0.5 x TAE buffer (20 mM Tris-acetate, pH 8.0, 0.5 mM EDTA) (Sambrook *et al.*, 1989).

Results and Discussion

The methanol extract of *S. latiuscula* was the strongest inhibitor of *Taq* DNA polymerase activity among the 59 seaweed species tested (Jin *et al.,* 1997). To isolate the major inhibitor from the methanol extract, we separated it into three MW fractions (≥10,000; 10,000–3,000; ≥ 3,000) using ultra-filtration and tested them for inhibitory activity. No PCR product was produced when the ≤ 3,000 MW fraction was used in the PCR, because it inhibited *Taq* DNA polymerase (data not shown). Therefore, this fraction was chosen for fractionation by RP-HPLC. The first HPLC run was performed on a Unisil Q C_8 column, which was eluted with a gradient of acetonitrile and water over 60 min, generating eight major peaks. The most active portion eluted at 40.6 min or 51% acetonitrile (Fig. 1A). The second HPLC run, performed using the same column but with isocratic 50% acetonitrile elution, generated three major peaks (Fig. 1B). Two peaks (II and III) showed potent inhibition against the DNA polymerase. These active peaks II and III were combined and applied to a third HPLC column (Nova-pak C_8 column), which was eluted with a gradient of acetonitrile. The purified compounds SL-1 and SL-2 eluted at 17 and 32% acetonitrile, respectively (Fig. 1C).

The mass spectrum of SL-1 revealed that three bromines were present in the structure, which exhibited typical signal strength ratios for M^+: $(M+2)^+$: $(M+4)^+$: $(M+6)^+$ of 17: 51: 49: 16 at m/z 374, 376, 378, and 380, respectively. The molecular weight was therefore assumed to be 374 ($C_7H_5Br_3O_3$). The ^{1}H-NMR spectrum revealed three singlet signals (d_H 4.19, 5.00, and 8.80) with ratio of 2:1:2, corresponding to five protons. From these spectral data, we identified SL-1 as 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol (Fig. 2).

The mass spectrum of SL-2 also revealed three bromines in the structure, which showed the same signal-strength ratios as those of SL-1, but at m/z 388, 390, 392, and 394. The molecular weight was therefore assumed to be 388 ($C_8H_7Br_3O_3$). The ^{1}H-NMR spectrum revealed three singlet signals (d_H 3.39, 4.80, and 8.80) with a ratio of 3:2:2, corresponding to seven protons. From these spectral data, we identified SL-2 as 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (Fig. 2). The spectra of the purified compounds were confirmed to be identical to the spectral data shown in Lim *et al.* (2000).

To determine the effectiveness of SL-1 and SL-2 at inhibiting DNA polymerase, we added various amounts (5–0.005 µg) of purified SL-1 or SL-2 to 25-µl PCR reactions. At 5 or 0.5 µg, SL-1 completely inhibited the enzyme (Fig. 1D), whereas SL-2 inhibited the enzyme at 5 µg (Fig. 1E). Thus, SL-1 is a more potent inhibitor than SL-2. Some portion of SL-1 was converted to SL-2 by heating for 1 hr in methanol (data not shown).

PCR technique has been applied to the field of seaweed molecular biology. However, some seaweed species did not amplify target DNA because they contained certain inhibitors of the PCR reaction (Jin *et al.*, 1997). Many compounds are known to inhibit PCR amplification (Demeke and Adams, 1992; Kreader, 1996; Watson and Blackwell, 2000). In this study, we found that 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol and 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether, isolated from the marine red alga *S. latiuscula*, have potent inhibitory activity against *Taq* DNA polymerase used in PCR. These chemicals were reported to have an aldose reductase inhibition (Wang *et al.*, 2005), radical-scavenging activity (Chung *et al.*, 2001; Duan *et al.*, 2007), and anti-virus effect against *Herpes simplex* (Kim *et al.*, 1997; Park *et al.* 2005). Recently, the methanol extract of *S. latiuscula* also showed anti-tumor activity against human colon cancer cell (Kim *et al.*, 2007). We suggest that the PCR inhibition assay may be used as a simple alternative tool in screening for DNA polymerase inhibitors, targeting for antiviral, antimicrobial, and/or anti-tumor effects.

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