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The Capability of the Red Seaweed *Gracilaria vermiculophylla* in Producing Prostaglandins

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Abstract

The red seaweed *G. vermiculophylla* is rich in polyunsaturated fatty acids with 20 carbon atoms, mainly arachidonic acid (AA) and eicosapentaenoic acid, which are precursors of prostaglandins (PGs). The present study aimed to elucidate the capability of the seaweed in releasing PGs using acetone powder as the crude enzyme. Crude enzyme was prepared using cold acetone. The crude enzyme was incubated with AA at different concentrations (0.1– 4 mg). For determination of PG contents, 5 µL of sample as the test solution corresponding to 0.2 g wet mass of the seaweed was injected into the HPLC. For mass spectrometer analysis, an HPLC system connected with mass spectrometer was used. Results of the study showed that the released PGs from incubation of acetone powder and AA analyzed by HPLC consisted of PGE_2, 15-keto-PGE_2, 15-hydroperoxy-PGE_2, PGA_2, and AA while PGs detected by LC-MS were $PGF_{2\alpha}$, PGE_2, 15-keto-PGE_2, 15-hydroperoxy-PGE_2, and PGA_2. The capability of the red algae in producing PGs was affected by available oxygen, aspirin, a cyclooxygenase inhibitor, and AA concentration. The crude enzyme of the red alga (250 mg) was capable to produce 1.63 µg and 1.32 µg of PG_2 and 15-keto-PGE_2 from incubation with 0.25 mg of AA. This method could be the one way to provide PGs *in vitro* to fulfill demands of PGs in the pharmaceutical industry.

Keywords: Gracilaria, Chromatography, Arachidonic acid, Enzyme, HPLC, Acetone powder.

1. Introduction

The red algae *G. vermiculophylla* is rich in polyunsaturated fatty acids (PUFA) with 20 carbon atoms, mainly arachidonic acid (AA) and eicosapentaenoic acid (EPA), which are precursors of prostaglandins. The algae contain a high amount of PGE_2, 15-keto-PGE_2, and PGA_2 (Hammann et al., 2016; Imbs, Vologodskaya, Nevshupova, Khotimchenko, & Titlyanov, 2001; Rybin, Svetashev, & Imbs, 2013). The mechanism of production of those prostaglandins has already been determined that it oxidized by prostaglandin H synthase (cyclooxygenase) (Külliki Varvas et al., 2013). However, the capability of the seaweed in producing prostaglandin need to be calculated in order to use the seaweed in producing prostaglandin *in vitro* for pharmaceutical and clinical applications. Prostaglandin compounds commonly applicated in human and animal reproduction (Bartlewski & Candappa, 2015) but also used in handling disease in livestock (Caldas, Freitas, Azevedo, & de Souza, 2018) and human health (Lawrence et al., 2018; Lee, Choe, Heo, & Je ong, 2018).

In the red alga *G. vermiculophylla*, AA, the precursor of PG synthesis is hydrolized from lipid membranes, mainly monogalactosyldiacylglycerol (MGDG) and phosphatidylcholine (PC) by glycerolipid acyl hydrolase (Illijas et al., 2008). The AA is then converted to PGs through the cyclooxygenase pathway (Külliki Varvas et al., 2013). PGs are confirmed to be synthesized via the novel intermediate 15-hydroperoxy-PGE_2 (Nakajima et al. 1998), while in mammals, 15-hydroperoxy-PGE_2 is formed from endoperoxide PGG_2 when peroxidase is limited (Eling, Glasgow, Curtis, Hubbard, & Handler, 1991). The present study attempted to elucidate the capability of prostaglandin production of the red alga *G. vermiculophylla* using acetone powder as the crude enzyme. Preparation of acetone powder as a crude enzyme for analysis of prostaglandin production has been demonstrated successfully in coral (Kulliki Varvas et al., 1999). The acetone powder is able to be stored frozen for a quite a long time. In *G. verrucosa*, prostaglandin contents are affected by storage conditions and seasons (Imbs et al., 2001). Therefore, prostaglandin analysis of this alga using acetone powder as the crude enzyme is a capable method to eliminate the effect of storage and seasonality.

Materials and Methods

Chemicals Prostaglandin (PG) E_2, PGE_3, PGA_2, $PGF_{2\alpha}$, and 15-keto-PGE_2 were obtained from Cayman Chemical Co. (Michigan, USA). Arachidonic acid sodium salt (99 % of purity), ethyl acetate, acetylsalicylic acid (aspirin) and HPLC-grade acetic acid were purchased from Wako Pure Chemicals (Osaka, Japan). Thin-layer chromatography plates, Silica gel 60 F_{254} (20 x 20 cm) were obtained from Merck, Darmstadt, Germany. HPLC-grade solvents, acetonitrile, and water were purchased from Kanto Chemicals (Tokyo, Japan).

Algae The red seaweed *G. vermiculophyla* was collected from Sinori Beach, Hakodate, Japan in June and stored under frozen at –30 ºC until use.

Preparation of crude enzyme Crude enzyme was prepared using cold acetone. Briefly, the alga sample (1 kg of wet weight) was cut into small pieces (3-4 mm length), lyophilized for 36 h and then milled to obtain a fine powder. Lipid was removed by rinsing with cold acetone (1000 mL) at –30ºC and vortex overnight. The solution was then filtered and the residue was dried under reduced pressure to obtain acetone powder as the crude enzyme and stored at –30ºC until use.

HPLC and MS conditions PG contents were determined using an HPLC (Model LaChrom D-7000 with a LaChrom model L-7455 diode array detector, L-7100 pump and L-7610 degasser,

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Hitachi Ltd, Tokyo, Japan) equipped with Mightysil column, RP-18 GP (250 mm x 4.6 mm, 5 µm). Sample analysis of PG was performed at 40 ºC with acetonitrile/water (40:60, v/v) containing 0.02% acetic acid (solvent A) and 100 % acetonitrile (solvent B) as a mobile phase. The mobile phase system was as follows: 0 – 20 min (solvent A), 20 – 60 min (gradient of solvent A and B) and 60 – 80 min (solvent B). The flow rate was 0.5 mL/min. A 5 µL volume of the methanol of the test solution corresponding to 0.2 g wet mass of *G. vermiculophylla* was injected into the HPLC. The PG peaks were monitored by diode array detector set at 196 nm. For mass spectrometer analysis, an HPLC system, Model SpetraSYSTEM and SpectraSERIES Autosamplers with Mightysil column, RP-18 GP (250 x 4.6 mm, 5 µm) and connected with mass spectrometer Finnigan LCQ^{TM} LC/MS^{n} System (Finnigan MAT, USA) was used. Analytical conditions in negative ion scanning modes of ESI were as follows: capillary temperature and voltage, 170 ºC and -4 V.

Sample Preparation for PG Analysis The crude enzyme was incubated with arachidonic acid at different concentrations (0.1 – 4 mg) at 20ºC for 1 h in 5 mL of water. The mixtures were submitted to the extraction procedure as described above. The reaction mixtures in 5 mL of water were transferred to a single test tube for centrifugation at 3,000 rpm for 5 min. The resulting supernatant was collected and adjusted to pH 3-4 by 1 M HCl. Prostaglandins were extracted twice by 20 mL of ethyl acetate. The solvent was removed at 25ºC under reduced pressure using a rotary evaporator, and then the residue was concentrated in 10 µg/µL methanol as a test solution. For confirmation of PG composition, the test solution (5µL) was submitted to HPLC analysis.

Identification and content determination of PGs For identification of PGs, the alga (10 g) mixed with 20 ml of water were incubated for 1 h at 20 C. The reaction mixtures were centrifuged at 3000 rpm for 5 min. The supernatant was collected and adjusted pH to 3-4 by HCl in methanol. Extraction of PG was carried out with ethyl acetate (30 ml x 2). Identification of PG was conducted by comparing the HPLC peak retention times and their mass spectra with those of authentic standards. For complete identification of the PG, co-chromatography using authentic compounds was employed.

Determination of PG contents was carried out using a standard curve (Figure 1). The standard curves were obtained in the range of 5 – 100 µg/mL for the all standards of PGs and arachidonic acid (AA). The curves were plotted by standard peak height versus standard concentration in µg. The amount of PGs in the samples was calculated from the standard curve of PG standards. The actual PG contents were determined from PGs produced from the samples and reduced by PG contents produced from controls (acetone powder only).

Effect of Oxygen on PG Production The reaction mixtures in 5 mL of water, which consisted of 250 mg acetone powder and 1 mg arachidonic acid, were incubated at 20ºC for 1 h in anaerobic condition by displaced oxygen with N_2 gas. Furthermore, other reaction mixtures were incubated in aerobic condition. All samples were extracted by the above extraction procedure and determination of PG content was carried out as described previously.

Effect of Aspirin on PG Production Aspirin, a cyclooxygenase inhibitor at different concentrations (1.1 mM, 5.6 mM, and 10.1 mM) was added to the reaction mixture, which consisted of 250 mg acetone powder and 100 µg of arachidonic acid in 5 mL water. The mixture was incubated at 20ºC for 1 h. After extraction of PG as described above, the sample was subjected to HPLC.

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Isolation and Reduction of 15-hydroperoxy-PGE$_2$ Isolation of 15-hydroperoxy-PGE$_2$ was carried out using TLC (silica gel 60 F254, 20 x 20cm, Merck, Darmstadt, Germany) with solvent chloroform-ethyl acetate-ethanol-acetic acid (20:20:4:1, v/v/v/v). A part of the TLC plate (3 cm), which contained PG standard and the test solution bands, was cut. The small part of the plate was sprayed with orcinol reagent and heated at 120ºC until the bands of prostaglandin appeared. By comparing the bands with those bands on the heated plate it was possible to detect 15-hydroperoxy-PGE$_2$ band, which was scrapped off from the plate with a spatula. The 15-hydroperoxy-PGE$_2$ was then extracted by chloroform. After removal of the solvent, the residue was dissolved in methanol. For confirmation of 15-hydroperoxy-PGE$_2$, the sample was injected to HPLC.

15-hydroperoxy-PGE$_2$ was reduced by mild and strong reductions using $SnCl_2$ and $NaBH_4$. The reduction was carried out as described by Hamberg *et al*. (1974)(Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974). Briefly, 0.05 mg of 15-hydroperoxide-PGE$_2$ was treated with 25 mg of $SnCl_2$ or $NaBH_4$ in 5 mL ethanol at room temperature for 2 min. Analysis of reduction products was carried out by HPLC.

Results and Discussion

Identification of PG produced by AA metabolism The released PGs from incubation of acetone powder and AA in 5 mL of water for 1 h at 20ºC were identified by HPLC and LC-MS. HPLC chromatograms of the sample and authentic PGs showed 4 significant peaks were detected in the sample (**Fig. 2)**. The retention time of peak 1 corresponded to that of the authentic PGE$_2$, peak 2 to 15-keto-PGE$_2$, peak 3 to PGA$_2$ and peak 4 to AA. Further confirmation and identification of AA metabolites were carried out using LC-MS. The total negative ion chromatograms showed 6 significant peaks (**Fig. 3)**. The ion associated with peak 1 is PGF$_{2\alpha}$, which gave a prominent ion at *m/z* 353 in the mass spectrum (**Fig. 4**). HPLC peak 2 is PGE$_2$, which has a prominent ion at *m/z* 351 (**Fig. 5**). HPLC peak 3 is 15-keto-PGE$_2$, which has a prominent ion at *m/z* 349 (**Fig. 6**). HPLC peak 4 has a prominent ion at *m/z* 367 (**Fig. 7**). This compound is suggested as 15-hydroperoxy-PGE$_2$, the PGE$_2$ intermediate (Nakajima et al., 1998). HPLC peak 5 is PGA$_2$, which has a prominent ion at *m/z* 333 (**Fig. 8**). HPLC peak 6 is unknown compounds. The mass spectrum pattern of all PGs is agreed well with the mass spectrum of PGs found in *G. asiatica* (Sajiki & Kakimi, 1998). The prominent ion at *m/z* 353, *m/z* 351, *m/z* 349, and *m/z* 333 detected in *G. vermiculophylla* are $[M\text{-}H]^-$ ion of PGF$_{2\alpha}$, PGE$_2$, 15-keto-PGE$_2$, and PGA$_2$, respectively.

Effect of oxygen and arachidonic acid on PG production The effect of oxygen on PG productions in the incubation of the crude enzyme of *G. vermiculophylla* and AA showed different amounts of PGs (**Fig. 9)**. Amounts of PGE$_2$, 15-keto-PGE$_2$, and 15-hydroperoxy-PGE$_2$ were higher in aerobic condition than in anaerobic condition. The production of 15-hydroperoxy-PGE$_2$ was blocked in an anaerobic condition indicating that remaining 15-hydroperoxy-PGE$_2$ in the crude enzyme was almost all converted to PGE$_2$ and 15-keto-PGE$_2$ and no 15-hydroperoxy-PGE$_2$ production during 1-h incubation occurred. As described earlier cyclooxygenase is a bi-functional enzyme containing a site that converts AA to PGG$_2$ and contains another site that reduces PGG$_2$ to PGH$_2$. The conversion step of AA to PGG$_2$ is oxygenation (Hussain, Gupta, & Mukhtar, 2003). This chemical reaction is stimulated by oxygen (Michael Garavito, Malkowski, & DeWitt, 2002). The result of this study is consistent with the decrease of PGE$_2$ production under anaerobic condition in the red alga *Gracilaria gigas* (Hsu, Tsao, Chiou, Hwang, & Hwang, 2007).

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The effect of AA on PG production in *G. vermiculophylla* showed a different amount of PGs at different AA concentrations (**Fig. 10)**. The maximum amounts of PGE_2 (1.63 µg), 15-keto-PGE_2 (1.32 µg), and 15-hydroperoxy-PGE_2 (1.36 µg) were found in addition of 0.25 mg of AA. Addition of AA above 0.25 mg (0.5 – 4 mg) decreased the production of PGs and still remaining high amounts of unmetabolized AA. The control of treatment (only acetone powder) still produced PGs and AA indicating that during the preparation of crude enzyme, lipids were not completely discarded by cool acetone. Hence, production of AA, the precursor of PG synthesis, from enzymatic hydrolysis of the lipids still occurred during the incubation.

Effect of aspirin on prostaglandin production To confirm cyclooxygenase involved in the prostaglandin synthesis in *G. vermiculophylla*, aspirin, a cyclooxygenase inhibitor, was added to the reaction mixtures. Addition of 1.1 mM of aspirin to the reaction mixtures showed no effect on the content of PGE_2, 15-keto-PGE_2, and 15-hydroperoxy-PGE_2. However, the addition of 5.6 mM and 10.1 mM aspirin significantly decreased the contents of those prostaglandins (**Fig. 11**). The contents of 15-hydroperoxy-PGE_2 decreased drastically by adding at least 5.6 mM of aspirin indicating that cyclooxygenase is involved in the synthesis of 15-hydroperoxy-PGE_2 and also indicating that 15-hydroperoxy-PGE2 is an intermediate compound for PG synthesis. Inhibition of 15-hydroperoxy-PGE_2 formation by addition of aspirin has also been demonstrated in chick spinal cord (Vesin, 1992). Slightly inhibition of PG production by non-specific NSAIDs have also been reported from *G. vermiculophylla* and it suggested that the active site of the algal PGHS might be very different from that of the mammalian PGHS (Külliki Varvas et al., 2013).

The data presented here indicate that 15-hydroperoxy-PGE_2 is the precursor of prostaglandin synthesis in the red alga *G. vermiculophylla*. The contents of PGE_2 and 15-keto-PGE_2 decreased when 15-hydroperoxy-PGE_2 was blocked by limited oxygen and addition of aspirin. Furthermore, 15-hydroperoxy-PGE_2 conversion into PGE_2 was confirmed by strong and mild reduction of the hydroperoxide using $NaBH_4$ and $SnCl_2$. The results showed that with mild and strong reduction, 15-hydroperoxy-PGE_2 was converted into PGE_2 and $PGF_{2\alpha}$ and PGE_2, respectively (**Fig. 12**). Conversion of 15-hydroperoxy-PGE_2 into PGE_2 and 15-keto-PGE_2 in this alga might involve hydroperoxidase, which is similar to the conversion of 15-hydroperoxy-PGE_2 into PGE_2 in homogenates of chick spinal cord (Vesin, 1992).

Four types of prostaglandins, ($PGF_{2\alpha}$, PGE_2, 15-keto-PGE_2, and PGA_2) and only one unknown compound were observed in the incubation of crude enzyme and AA sodium salt. However, extraction of PGs from fresh *G. vermiculophylla* exhibited large numbers of peaks appeared in HPLC chromatogram (chromatogram not shown) and it needed sequentially TLC solvents to purify the PGs. Purification of the PGs easily conducted using preparative TLC as the PGs are stable compounds (Stehle & Oesterling, 1977). Therefore, *in vitro* production of PGs using a crude enzyme prepared from *G. vermiculophylla* offers several advantages either a simple chemical synthesis or efficient purification of PGs. This will encourage the provision of PGs easily and sustainably on a large scale for the pharmaceutical industries.

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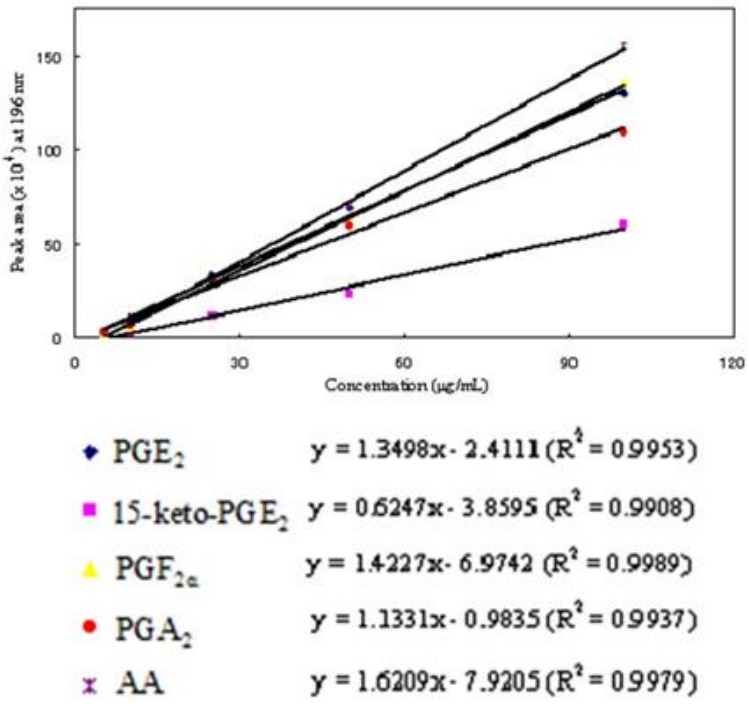

Figure 1. Standard curves of PGE_3, PGE_2, 15-keto-PGE_2, $PGF_{2\alpha}$, PGA_2, and AA. The prostaglandin and arachidonic acid standards were injected to HPLC with an ODS column (Mightysil, RP-18 GP, 250 mm x 4.6, 5 μm). Solvent system: ACN:H_2O (40:60, v/v) containing 0.02% acetic acid (solvent A) and 100% ACN (solvent B). 0-20 min (100% solvent A), 20-60 min (gradient of solvent A and B), and 60-80 min (100% solvent B).

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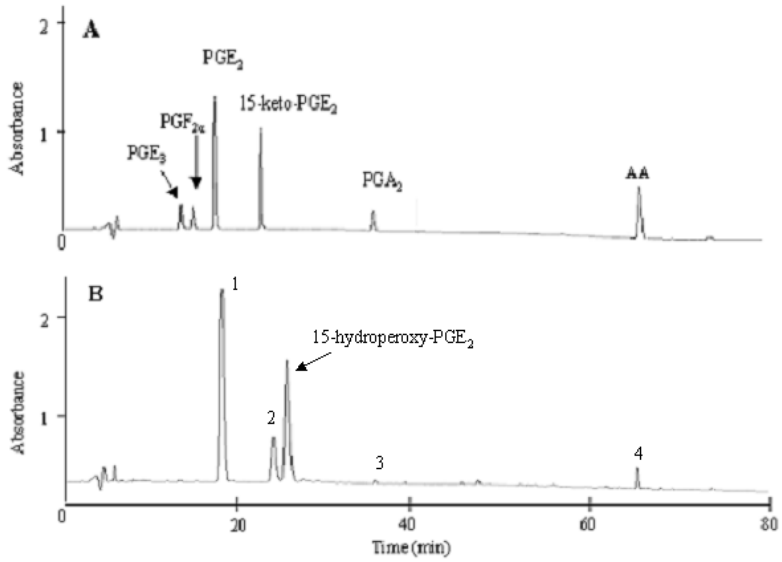

Figure 2. HPLC chromatograms of the authentic standard of prostaglandins (A) and prostaglandins released by incubation of crude enzyme prepared *G. vermiculophylla* from and arachidonic acid for 1 h at 20 ºC (B). Solvent system: ACN:H_2O (40:60, v/v) containing 0.02% acetic acid (solvent A) and 100% ACN (solvent B). 0-20 min (100% solvent A), 20-60 min (gradient of solvent A and B), and 60-80 min (100% solvent B). Detection: 196 nm. Peak 1: PGE_2, Peak 2: 15-keto-PGE_2, Peak 3: PGA_2, Peak 4: AA

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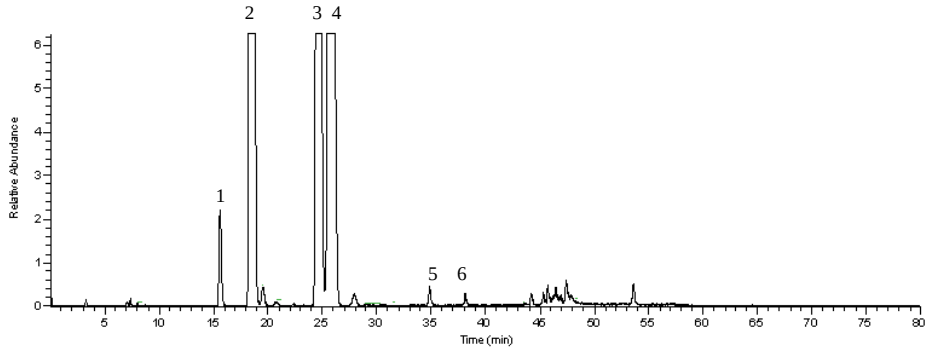

Figure 3. LC-MS chromatogram of prostaglandins released from incubation of crude *G. vermiculophylla* enzyme and AA for 1 h at 20 ºC. Solvent system: ACN:H_2O (40:60, v/v) containing 0.02% acetic acid (solvent A) and 100% ACN (solvent B). 0-20 min (100% solvent A), 20-60 min (gradient of solvent A and B), and 60-80 min (100% solvent B). Peak 1: $PGF_{2\alpha}$, Peak 2: PGE_2, Peak 3: 15-keto-PGE_2, Peak 4: 15-hydroperoxy-PGE_2, Peak 5: PGA_2, Peak 6: Unknown.

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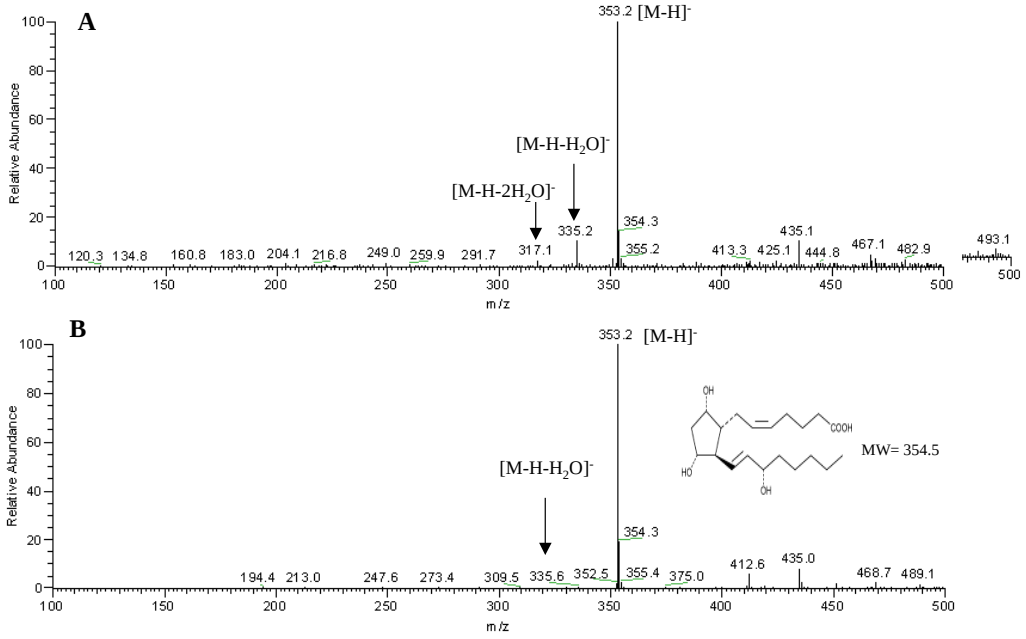

Figure 4. Mass spectra of HPLC peak 2 from the red alga *G. vermiculophylla* (A) and authentic standard of $PGF_{2\alpha}$ (B).

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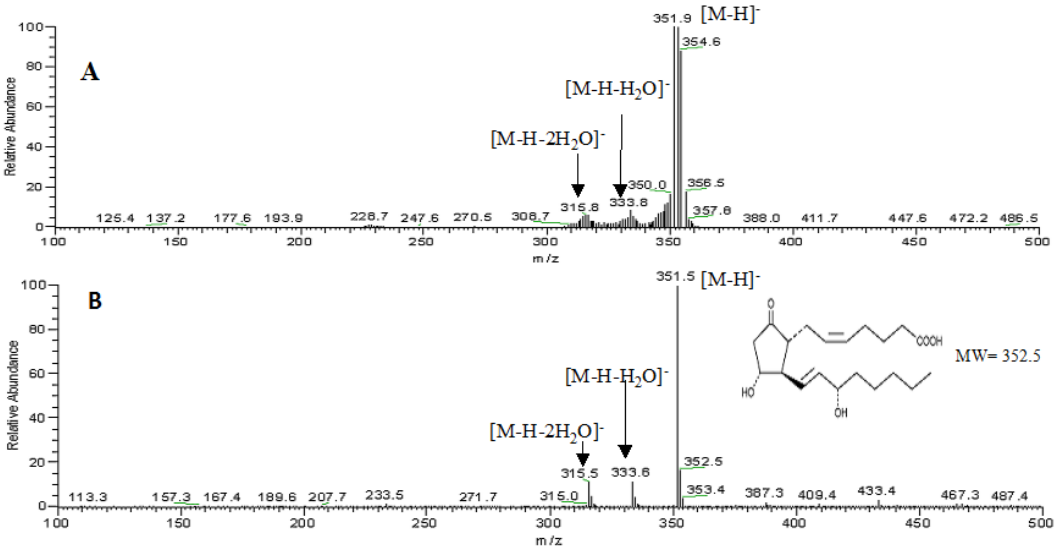

Figure 5. Mass spectra of HPLC peak 3 from the red alga *G. vermiculophylla* (A) and authentic standard of PGE_2 (C).

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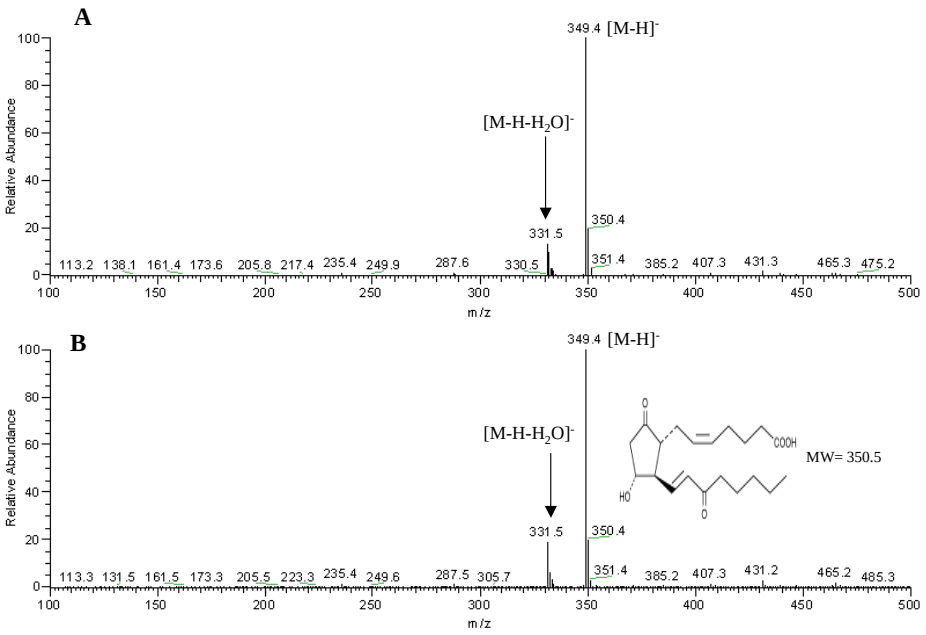

Figure 6. Mass spectra of HPLC peak 5 from the red alga *G. vermiculophylla* (A) and authentic standard 15-keto-PGE_2 (B).

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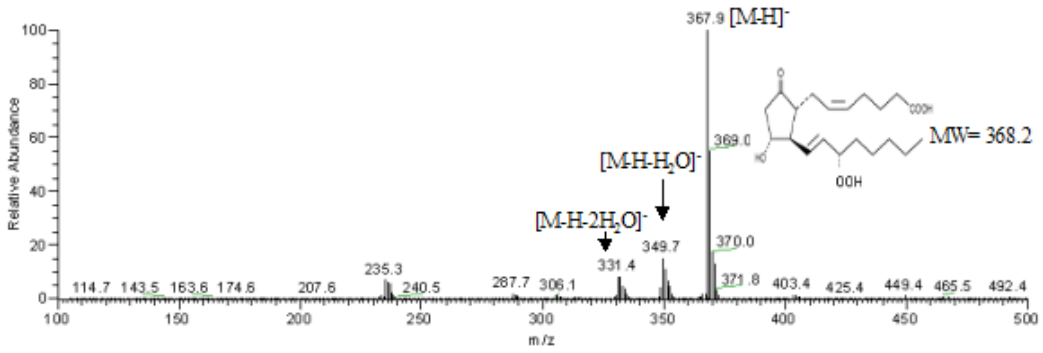

Figure 7. Mass spectrum of HPLC peak 6 from the red alga *G. vermiculophylla*. The molecule is suggested as 15-hydroperoxy-PGE_2.

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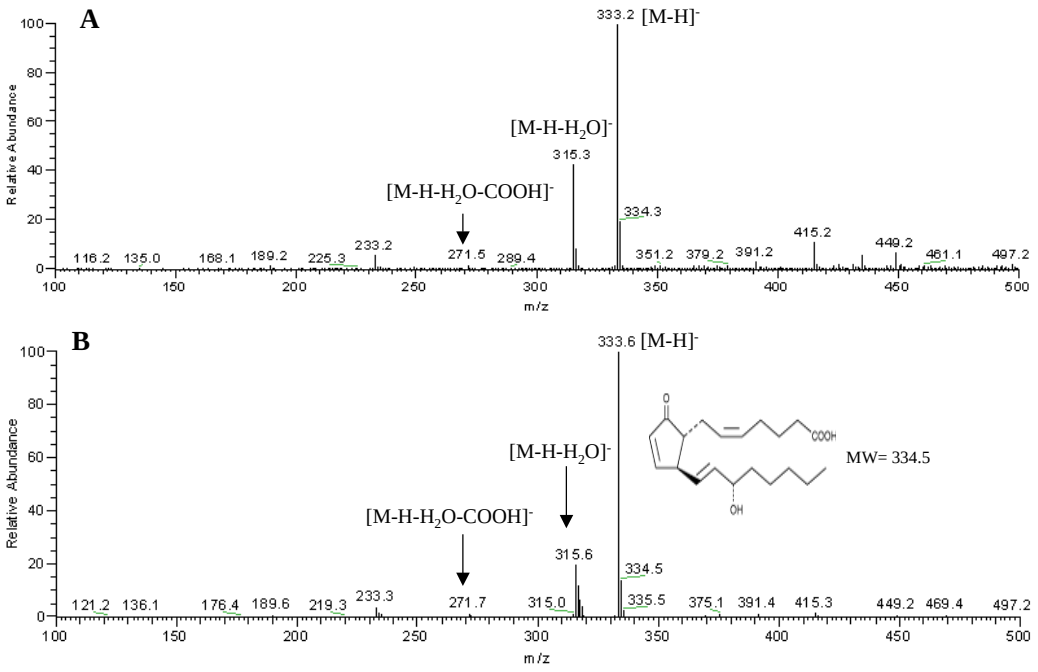

Figure 8. Mass spectra of HPLC peak 8 from the red alga *G. vermiculophylla* (A) and authentic standard PGA_2 (B).

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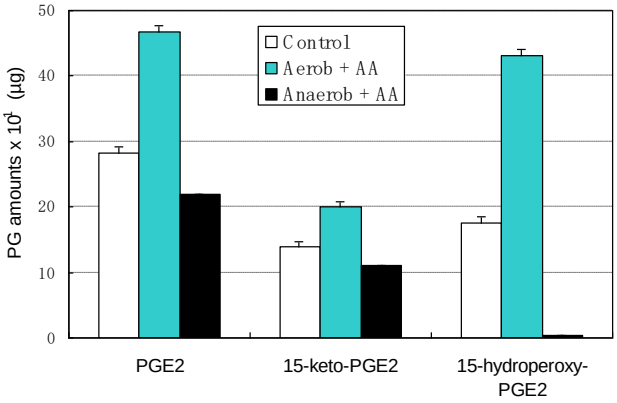

Figure 9. Effect of oxygen on prostaglandin production in *G. vermiculophylla*

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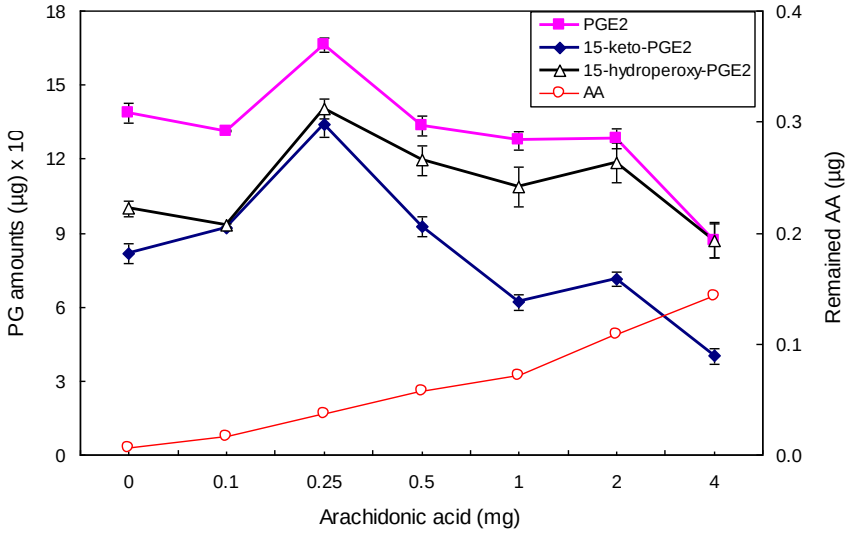

Figure 10. Effect of arachidonic acid on prostaglandin production in *G. vermiculophylla*

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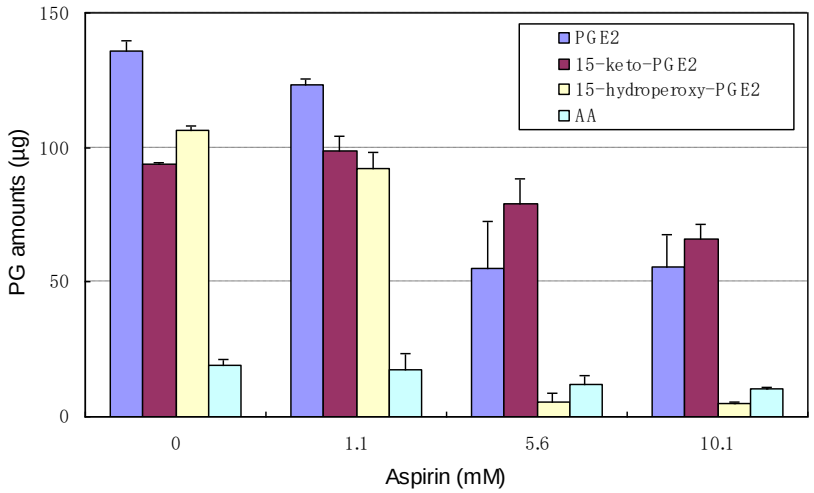

Figure 11. Effect of aspirin on prostaglandin production in *G. vermiculophylla*

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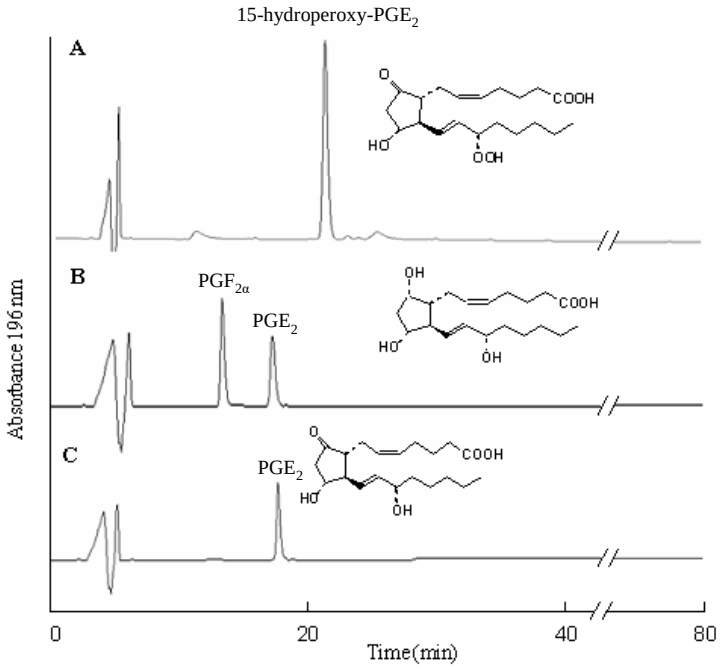

Figure 12. HPLC chromatograms of 15-hydroperoxy-PGE_2 and its reduction products. 15-hydroperoxy-PGE_2 (A), strong reduction by $NaBH_4$ (B), mild reduction by $SnCl_2$ (C).

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