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Factors Affecting PGE2 Production in Seaweed *Gracilaria tenuistipitata*

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ABSTRACT

Gracilaria tenuistipitata is an edible red alga and can be utilized as feed for small abalone and foodstuff (ex. agar extract). It is the major species of *Gracilaria* that is commonly cultivated and consumed in Taiwan. Effects of environmental factors on prostaglandin E_2 (PGE_2) production in the seaweed *G. tenuistipitata* were investigated in this study. PGE_2 amount increased nearly 2-fold and 47% when the seaweed was exposed to two ways of fluctuation of seawater salinity from 2% to 1% and 3% for 12 hr, respectively. The stresses of low temperature and irradiance promoted the seaweed to produce PGE_2. When the seaweeds were exposed to air for 2 hr and 4 hr, the PGE_2 levels were elevated by 25% and 31%, respectively. When the seaweed was soaked in freshwater, PGE_2 amount gradually decreased. Cu^{2+} and Zn^{2+} inhibited PGE_2 production at the concentrations of 3 and 50 mg/L, respectively. Mg^{2+} slightly inhibited PGE2 production about 20% at 2,025 mg/L, while Ca^{2+} boosted PGE_2 production about 59% at 600 mg/L. Hence, environmental factors significantly affect the PGE_2 production in the seaweed *G. tenuistipitata*. Prostaglandins might be associated with processes permitting the algae tissue to survive in unfavorable conditions. To lower the amount of PGE_2 in the seaweed and ensure the food safety, it is suggested to minimize the variation of growth condition to avoid PGE_2 increase in the seaweed during cultivation period. In addition, consumers should avoid eating raw seaweed since PGE_2 could be destroyed by heating.

Key words: prostaglandin, arachidonic acid, *Gracilaria tenuistipitata*, environmental factors

INTRODUCTION

Gracilaria sp. is one kind of edible red algae (Japanese name, "ogonori). They are generally utilized in foodstuff, food additive and cosmetic. People are accustomed to take raw fish with *Gracilaria* sp. in Japan[1,2]. Raw fish and red algae are rich in polyunsaturated fatty acids (PUFA), mainly arachidonic acid (AA) and eicosapentaenoic acids which are precursors of prostaglandins (PGs)[3,4]. However, some poisoning cases occurred due to ingesting seaweed *G. verrucosa*, known as ogonori, or similar species in Japan[1-3]. Those victims ate raw fish and raw seaweed. The common symptoms in the patients are nausea, vomiting and diarrhea appearing 30-60 min after ingestion. In extreme cases, they developed very low blood pressure, followed by shock and death. In addition, the symptoms in female were more serious than those in male. The possible reason of seaweed poison cases was that raw seaweed contained cyclooxygenase (COX) which could transform AA (precursor of prostaglandin E_2, PGE_2) or other highly unsaturated fatty acids to prostaglandins, especially PGE_2. The enzyme in the seaweed and/or the body tissues of the victim may be acting on the highly unsaturated fatty acids in the raw fish or in the seaweed producing a great amount of PGE_2 at short time in the body[1]. PGE_2 has many physiological effects on mammals including hyperthermia, hypotension, smooth muscle dilatation, gastric secretion inhibition, hyperalgesia, womb shrink and relating to cancer[5-10]. Noguchi *et al*. reported that 15.8-102 ppm of PGE_2 and small amounts of other PGs (A_1, E_1 and $F_{2\alpha}$) could be detected from poison sample. They assumed that AA from raw tuna slices and ogonori were catalyzed by the COX in ogonori and victim to form PGs which caused the poisoning symptoms[1].

Prostaglandins were also found in different species of *Gracilaria*, such as *G. asiatica* and *G. lichenoids* containing PGE_2[2,11]. Some studies reported that prostaglandin amounts were various with seasons and species of *Gracilaria*. For example, PGE_2 amounts in *G. asiatica* were much higher in winter than in summer[2,3]. There are several factors that can influence on prostaglandin amount in the different seasons, including stage of life cycle, activity of enzymes of related prostaglandins synthesis, environmental conditions (seawater temperature, irradiance, contents of dissolved components, etc.) and others[3]. Sajiki reported PGE_2 amount in *G. asiatica* was much higher than that in *G. rhodocaudata*. There-

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fore PGE_2 concentrations in ogonori may depend on species and environmental conditions[2].

G. tenuistipitata is one of ogonori and the major cultivated and utilized species in Taiwan. As described above, PGE_2 was the possible causative agent for "ogonori poison". Therefore the present study was aimed to investigate effects of environmental factors on PGE_2 production in *G. tenuistipitata*. The PGE_2 amount in the seaweed *G. tenuistipitata* was investigated when it was exposed to different environments, including the stress of salinity, temperature, irradiance, metal ions, tide exposure, and freshwater exposure. The change of growing conditions may exert influence on the PGs production in *G. tenuistipitata*.

MATERIALS AND METHODS

I. *Chemicals and Solvents*

Prostaglandin E_2 and arachidonic acid sodium salt (90% of purity) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Acetonitrile was from Mallinckrodt Baker, Inc. (NJ, USA). Methanol, ethyl acetate, citric acid, sodium citrate, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $CaCl_2$ and $MgCl_2$ were from Panreac Co. (Barcelona, Spain).

II. *Materials*

Raw seaweed (*G. tenuistipitata*) samples were collected from a seaweed farm in Hukou, Yunlin County, Taiwan. These samples were collected all year long.

III. *Standards*

Standard stock solutions of PGE_2 were made in acetonitrile. Suitable amount of standard PGE_2 was dissolved in acetonitrile to make l mg/mL stock solution of standard. Various concentrations (1, 5, 10, 25, 50 and 100 μg/mL) of standard solutions were prepared by serial dilution of the stock solution (1 mg/mL) with acetonitrile. The standard solutions were stored at -18°C because PGE_2 is unstable at room temperature.

IV. *Sample Preparation for Analysis*

Ten grams wet raw seaweed sample with ~90% moisture content was finely sliced and ground in a mortar. The centrifugation was performed at 13,700 ×g, 0°C for 30 min and the supernatant was collected. To the supernatant was added 2 mg of arachidonic acid sodium salt and incubated at 37°C for 30 min. The pH was adjusted to 3-4 with 1.5 M citric acid. The mixture was incubated in boiling water for 10 min and then cooled on ice. The solution was purified by solid phase extraction (SPE). The C-18 SPE cartridge (Mallinckrodt Baker, Inc. NJ, USA) was activated by rinsing with 5 mL methanol and then with 5 mL distilled water. The sample was loaded over the C18 SPE cartridge and the cartridge was rinsed with 5 mL distilled water. The cartridge was then eluted with 2 mL methanol followed by 5 mL ethyl acetate[12]. The ethyl acetate extracts were evaporated to dryness under reduced pressure and the residue was dissolved in 1 mL acetonitrile as test solution. Test solution (10 μL) was submitted to HPLC analysis.

V. *HPLC Analysis*

The PGE_2 contents were determined by using a Hitachi Liquid Chromatography system (Hitachi, Ltd, Tokyo) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-7455 diode array detector set at 196 nm and a Model D-7000 Chromato-integrator. Analysis of PGE_2 was performed on a YMC-Pack ODS-AQ AQ 312 (150 × 6.0 mm I.D., S-5 μm, 120 A) reversed phase column[1].

The mobile phase is composed of 17 mM phosphoric acid and acetonitrile gradient from 35% to 60% in 30 min. The flow rate was 1.0 mL/min[13].

VI. *Standard Curve and Sample Quantification*

Standard curve was obtained in the range of 1~100 μg/mL for PGE_2. The curve was plotted by standard peak area versus standard concentration in μg/mL. Amount of PGE_2 in the sample was calculated out of the standard curve.

VII. *Effect of Environmental Factors on PGE_2 Production*

(I) *Effect of Salinity on PGE_2 Production*

The seaweed *G. tenuistipitata* was acclimated in seawater with salinity 2% at least 3 days, moved to salinity 1% and 3% seawater and then incubated for 12 hr. All samples were then submitted to the extraction and PGE_2 analysis by HPLC.

(II) *Effect of Temperature on PGE2 Production*

The seaweed was acclimated in seawater (25°C) at least 3 days, moved to 4, 15 and 35°C seawater, and incubated for 12 hr. All samples were then submitted to the extraction and analysis.

(III) *Effect of Irradiance on PGE_2 Production*

The seaweed was acclimated in seawater with irradiance 330 μE m^{-2} s^{-1} for 3 days, moved to seawater with different irradiance including 0 and 640 μE m^{-2} s^{-1}, and then incubated for 12 hr. Light meter was used to monitor the irradiance (Lutron LX-102) in the range of 0 to 1,000 μE m^{-2} s^{-1}. All samples were submitted to the extraction and analysis.

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(IV) *Effect of Air Exposure on PGE_2 Production*

The seaweed was acclimated in seawater at least 3 days and then exposed to air for 2 and 4 hr. All samples were submitted to the extraction and analysis.

(V) *Effect of Freshwater Exposure on PGE_2 Production*

The seaweed was acclimated in seawater at least 3 days and then soaked in freshwater for 2 and 4 hr. All samples were submitted to the extraction and analysis.

(VI) *Effects of Metal Ions on PGE_2 Production*

According to the standard amounts of various metal ions in ocean from Environmental Protection Administration, Executive Yuan, Taiwan, ROC, the standard levels of Zn^{2+}, Cu^{2+} and Mn^{2+} are 0.5, 0.03 and 0.05 mg/L (control concentration), respectively. The concentrations of Zn^{2+}, Cu^{2+} and Mn^{2+} in this study were 10 and 100 folds higher than the standards. The common levels of Ca^{2+} and Mg^{2+} in the ocean are 400 and 1350 mg/L (control concentration), respectively. The concentrations of Ca^{2+} and Mg^{2+} in this study were 50% higher than average concentration in seawater. The seaweed was acclimated in seawater at least 3 days and cultivated in seawater with respectively containing Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} for 12 hr. All samples were submitted to the extraction and analysis.

VIII. *Statistical Analysis*

All measurements were conducted in triplicate, and experimental results were evaluated by analysis of variance (ANOVA) (SAS Version 9.0, SAS Inst. Inc., Cary, NC, USA) and Duncan's multiple range tests was applied to compare the mean values at $P < 0.05$.

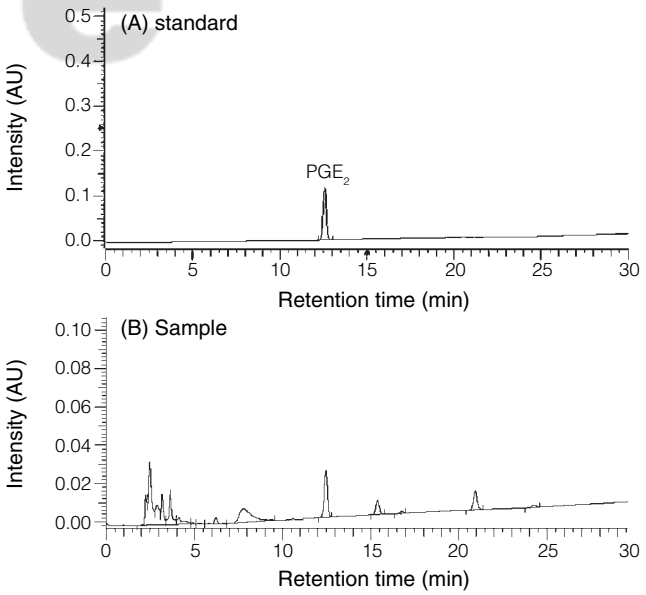

Figure 1. HPLC chromatograms of PGE_2 standard (A), along with sample (B). Acetonitrile concentration increased from 35 to 60% in 30 min and the flow rate was 1.0 mL/min.

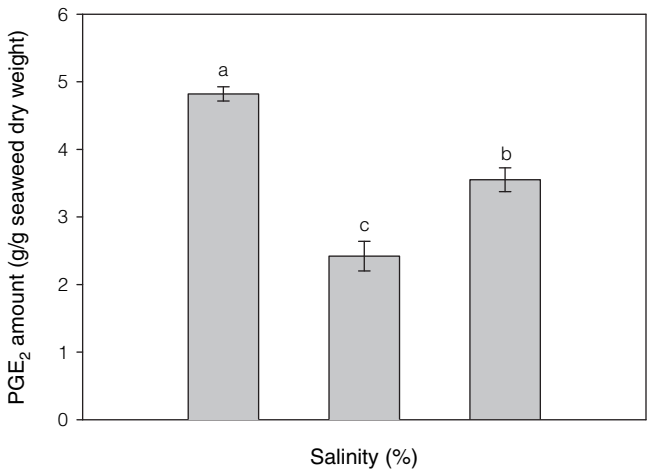

Figure 2. PGE_2 amount of *Gracilaria tenuistipitata* in different salinities. The seaweed was cultivated in 2% salinity seawater and then transferred to salinity 1% and 3% for 12 hr, respectively. Values are mean ± S.D. (N = 3). a, b, c: Values with different superscripts are significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

The mobile phase of HPLC for PGE_2 was as follows: the ratio of acetonitrile and 17 mM phosphoric acid changed from 35/65 to 60/40 in 30 min. The flow rate was 1.0 mL/min[13]. Typical chromatograms are shown in Figure 1. The retention time of PGE_2 was 12.82 ± 0.20 min. The peak in the sample at retention time 21 min was suggested to be PGA_2 by comparing to previous datum[13]. However, the other two peaks in the sample at retention time 8 min and 15.5 min were unknown peaks.

The effect of salinity fluctuation on PGE_2 amount in *G. tenuistipitata* is shown in Figure 2. The optimal growth salinity of *G. tenuistipitata* is 1.5-2.5%. Therefore the seaweed is cultivated in 2% salinity seawater (control condition). When the seaweed was cultivated in 2% salinity seawater and then transferred to 1% and 3% salinity seawaters after 12 hr, PGE_2 amount in the seaweed increased, resulting in the highest production in 1% salinity seawater, followed by 3% salinity seawater and control (2% salinity seawater). PGE_2 amount increased nearly 2-folds and 47%, respectively, when the seaweed was transferred from 2% to 1% and 3% salinity seawaters. Dawes *et al*. reported low salinity (1.5%) increased the level of AA in *G. tikvahiae* sporelings[14]. The levels of PGE_2 and AA were increasing at the same time in lower salinity; therefore there could be positive relationship between AA and prostaglandins due to AA being a precursor of prostaglandins. The result indi-

cated that conversion of salinity caused the seaweed to response to the stress from environmental change the due to the role of PGE_2 in homeostasis of seaweed.

PGE_2 amounts in *G. tenuistipitata* were different when the seaweed was transferred to different temperatures of seawater after 12 hr. The optimal growth temperature of *G. tenuistipitata* is 20-25°C. Therefore seaweed is cultivated in 25°C (control condition). PGE_2amount increased 49% and 76% when the seaweed was transferred from 25°C to 4 and 15°C, respectively after 12 hr as shown in Figure 3. In addition, PGE_2 amount reduced 45% when the seaweed was transferred from 25°C to 35°C after 12 hr. Imbs *et al*. reported that there were different prostaglandin contents in *G. verrucosa* during June-November. The levels of PGE_2 and $PGF_{2\alpha}$ did not show significant difference in June-September, but showed the highest amounts in November when water temperature is relatively low[3]. Sajiki analyzed the levels of PGE_2 of *G. asiatica* in June, November and February. The results showed the levels of PGE_2 were much higher in November and February than June[2]. Therefore lower temperature seemed to promote prostaglandin production. There are several factors that can be attributed to the accumulation of prostaglandins in the cold season, including stage of life cycle, activity of enzymes involved in prostaglandins synthesis, environmental conditions (seawater temperature, irradiance, contents of dissolved components) and others. Prostaglandins may be related to processes permitting the algae tissue to survive in cold water[3]. In addition, PGE_2 amount reduced when seawater temperature rose from 25°C to 35°C. The optimal growth temperature of *G. tenuistipitata* is 20-25°C. The result indicated that high temperature is not favorable for *G. tenuistipitata* and lowers the PGE_2 production.

PGE_2 amount in *G. tenuistipitata* also changed in different irradiance (Figure 4). *G. tenuistipitata* was cultivated in 330 μE m^{-2} s^{-1} of irradiance (control condition) and then transferred to 640 μE m^{-2} s^{-1} and dark for 12 hr. PGE_2 amount in the seaweed increased about 38% when the seaweed was transferred from 330 μE m^{-2} s^{-1} to 640 μE m^{-2} s^{-1} of irradiance. PGE_2 amount increased about 37% when the seaweed was transferred to dark condition. According to Imbs's report, the content of prostaglandins in *G. verrucosa* was influenced by light intensity. *G. verrucosa* grew at various levels of photosynthetic active radiation (PAR_0) including 95%, 50% and 5% PAR_0. The amount of PGE_2 and $PGF_{2\alpha}$ in the seaweed exposed to 50% of PAR_0 was about half of that of the control sample (95% PAR_0). But the levels of PGE_2 and $PGF_{2\alpha}$ were higher in extremely low (5% PAR_0) than normal illumination condition (50% PAR0)[3]. This result was similar to that reported by Imbs *et al*. (2001).

The seaweed in this study was collected from a seaweed farm and normally fully submerged under water. PGE_2 amounts in *G. tenuistipitata* increased 25% and 31% when the seaweeds were exposed to air for 2 hr and 4 hr, respectively (Figure 5). The result was presumed that the seaweed was under stress when exposed to air. The increase of PGE_2 is responsible to adapt desiccation condition during the period of emergence.

PGE_2 amount decreased in *G. tenuistipitata* when the seaweeds were soaked into freshwater, as shown in Figure 6. PGE_2 decreased 56%, 81% and 100% when the seaweed was soaked into freshwater for 2, 4, and 8 hr, respectively. It is harmful for *G. tenuistipitata* to be soaked into freshwater. PGE_2 amount decreased gradually with increasing of the soaking time in freshwater.

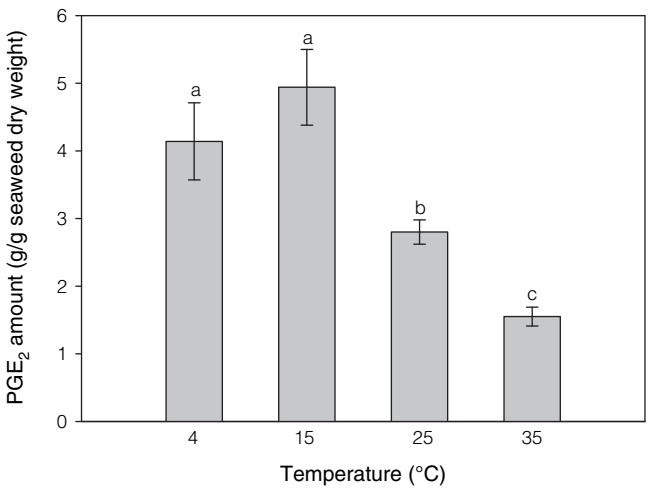

Figure 3. PGE_2 amount of *Gracilaria tenuistipitata* in different temperatures. The seaweed was cultivated in 25°C seawater and then transferred to temperature 4°C, 15°C and 35°C for 12 hr, respectively. Values are mean ± S.D. (N = 3). a, b, c: Values with different superscripts are significantly different at $P < 0.05$.

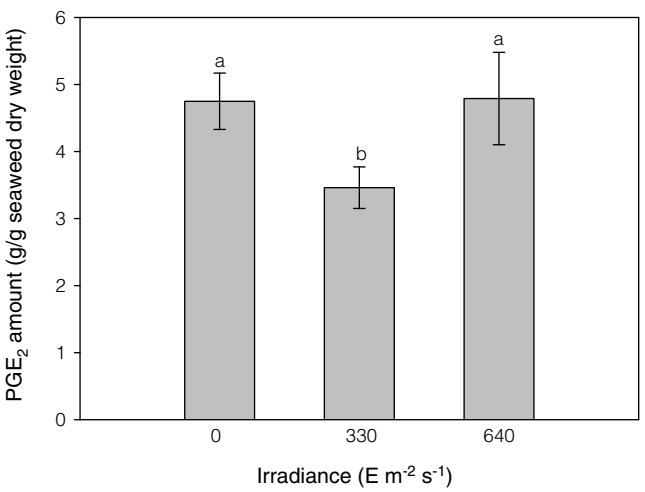

Figure 4. PGE_2 amount of *Gracilaria tenuistipitata* in different irradiance. The seaweed was cultivated in seawater with irradiance 330 μE m^{-2} s^{-1} and then transferred to 640 μE m^{-2} s^{-1} of irradiance and dark (0 μE m^{-2} s^{-1}) for 12 hr, respectively. Values are mean ± S.D. (N = 3). a, b: Values with different superscripts are significantly different at $P < 0.05$.

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The level of PGE_2 was not detected when the seaweed was soaked for 8 hr in freshwater.

Effects of metal ions on PGE_2 level of *G. tenuistipitata* were shown in Figure 7. The common amount of various metal ions in the ocean from Environmental Protection Administration, Executive Yuan, Taiwan, ROC., are 0.5, 0.03 and 0.05 mg/L (control concentration) in terms of Zn^{2+}, Cu^{2+} and Mn^{2+}, respectively. The concentrations of Zn^{2+}, Cu^{2+} and Mn^{2+} in this study were 10 and 100-fold higher than the control concentrations. PGE_2 level did not change significantly when the concentration of Cu^{2+} in seawater was 0.3 mg/L, but was not detected when exposed into seawater with 3 mg/L of Cu^{2+} (Figure 7a). Cu^{2+} has been pointed to decrease PGE_2 synthesis. However, Cu^{2+} has no direct effect upon the transformation of AA to prostaglandin[15-17]. In this study, Cu^{2+} was found to block PGE_2 synthesis in *G. tenuistipitata* at higher concentration. Similarly, PGE_2 amount did not change when the concentration of Zn^{2+} in seawater was 5 mg/L, but decreased about 76% when the concentration of Zn^{2+} rose to 50 mg/L (Figure 7a). Zn^{2+}may affect prostaglandins synthesis and metabolism through regulation of enzymes related to prostaglandins synthesis[18]. In *in vitro* studies, Zn^{2+} has been reported to completely inhibit the conversion of AA to prostaglandins in peritoneal polymorphonuclear cells from rabbits[17,19]. Concentrations of Mn^{2+} had no effect on PGE_2 level (Figure 7b) as well. The common levels of Ca^{2+} and Mg^{2+} in the ocean are 400 and 1350 mg/L (control concentration), respectively. The concentration

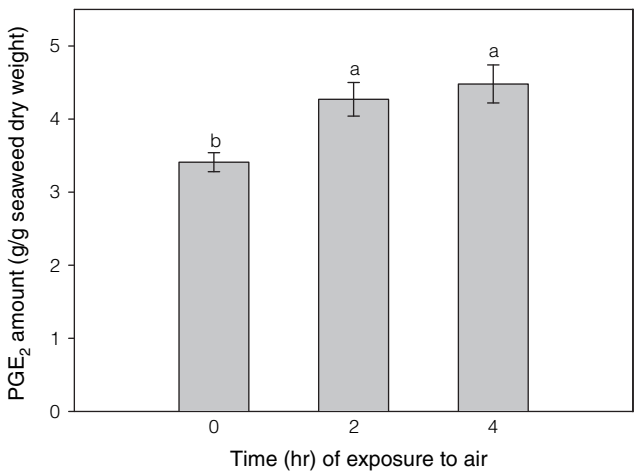


Figure 5. PGE_2 amount of *Gracilaria tenuistipitata* in different exposure times to air. The seaweed was cultivated in seawater and then exposed to air for 2 and 4 hr, respectively. Values are mean ± S.D. (N = 3). a, b: Values with different superscripts are significantly different at $P < 0.05$.

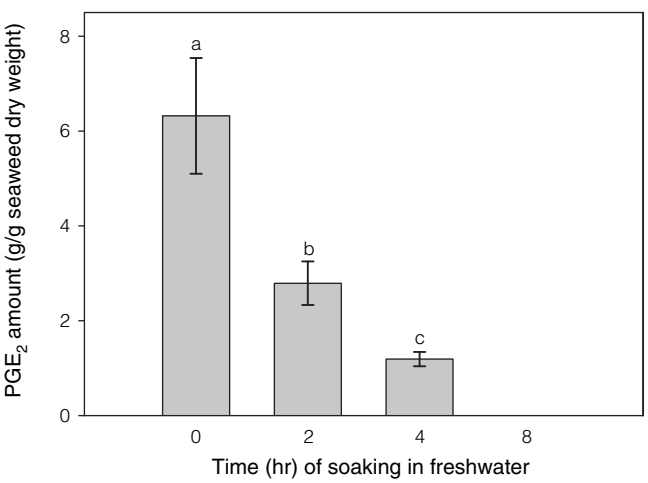
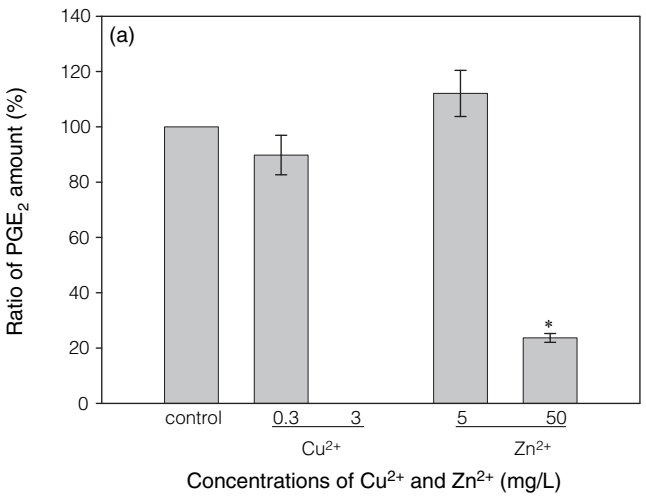


Figure 6. PGE_2 amount of *Gracilaria tenuistipitata* in different soaking times in freshwater. The seaweed was cultivated in seawater and then soaking in freshwater for 2, 4 and 8 hr, respectively. Values are mean ± S.D. (N = 3). a, b, c: Values with different superscripts are significantly different at $P < 0.05$.



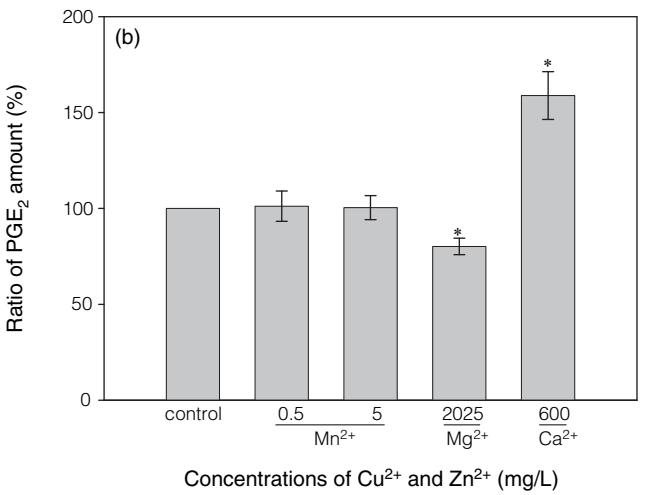


Figure 7. Ratio of PGE_2 amount of *Gracilaria tenuistipitata* cultivated in seawater with various concentrations of metal ions. The seaweed was cultivated in seawater with various concentrations of metal ions for 12 hr. *Value is significantly different at $P < 0.05$ when compared to that of control.

of Ca^{2+} and Mg^{2+} in this study was 50% higher than the control. The result indicated that Ca^{2+} promoted PGE_2 production in *G. tenuistipitata*. PGE_2 level increased about 59% when the concentration of calcium in the seawater was 600 mg/L. We supposed that Ca^{2+} induced enzyme activity related to prostaglandins synthesis to produce more PGE_2 in *G. tenuistipitata*. However Mg^{2+} slightly decreased PGE_2 level in *G. tenuistipitata* with the concentration 2025 mg/L in the seawater (Figure 7b). The result showed that high concentration of Mg^{2+} may inhibit PGE_2 production.

G. tenuistipitata is the major species of *Gracilaria* and is commonly cultivated in Taiwan. According to the results, the environmental factors significantly affect PGE_2 production in *G. tenuistipitata*. These results can also be found in another popular seaweed (*G. verrucosa*) in Japan[2,3], but the amount of PGE_2 was different. The PGE_2 amount in *G. verrucosa* was much higher than *G. tenuistipitata*. Furthermore, the variation of PGE_2 production in *G. verrucosa* was also greater than *G. tenuistipitata* when the seaweed grew in different environmental conditions. Therefore the PGE_2 amounts in the seaweed may also depend on species.

PGE_2 has many physiological functions which are necessary for human body, but excess level of PGE_2 is harmful. The side effects of PGE_2 included hypotension, nausea, vomiting, diarrhea etc. *G. tenuistipitata* contains low quantity of PGE_2 therefore it was not toxic in general condition. However, the environmental conditions affect the PGE_2 production in *G. tenuistipitata*, which might lead to the increase of the amount of PGE_2 in the seaweed. Hence, we suggested that minimizing the variation of growth condition could avoid PGE_2 increase in the seaweed during the cultivation period. In addition, consumers should avoid eating raw seaweed, because PGE_2 can be destroyed by heating, to reduce the amount of PGE_2 in the seaweed and may ensure the edible safety.

CONCLUSIONS

The PGE_2 production in *G. tenuistipitata* depended on environment conditions. The environmental conditions may exert effects on the PGE_2 production in the seaweed. The change in the amount of PGE_2 indicates that the seaweed adapts to different growth conditions during the period of emergence. Some special environment factors, such as low temperature, salinity, irradiance, air and Ca^{2+}, could promote PGE_2 production in the seaweed.

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