Bot. Bull. Acad. Sin. (1999) **40:** 289-294

High temperature-induced free proline accumulation in *Gracilaria tenuistipitata* (Rhodophyta)

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(Received November 23, 1998; Accepted February 12, 1999)

Abstract. The effects of high temperature (35°C) on free proline biosynthesis were studied in a marine red macroalga *Gracilaria tenuistipitata* var. *liui* Zhang et Xia. Exposure to 35°C decreased the specific growth rate after 3 days and increased the free proline levels after 2 days, but did not affect the free amino acid levels. Soluble protein levels were constant on exposure to 35°C while those of 25°C-treated thalli increased steadily through the treatment periods. The specific activities of both γ-glutamyl kinase (γ-GK; EC 2.7.2.11) and glutamate-5-semialdehyde dehydrogenase (GSAd; EC 1.4.1.3) remained unchanged after exposure to 35°C. Upon exposure to 35°C, the specific activity of ornithine δ-aminotransferase (δ-OAT; EC 2.6.11.3) increased rapidly after 2 days and decreased at Day 5, while that of Δ^1-pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2) also increased after 1 day and decreased at Day 4. The increase in δ-OAT and P5CR specific activities coincides with the increase in free proline levels. The 35°C induction of free proline accumulation and δ-OAT and P5CR activity increase is a light-dependent process. These results suggest that the stimulation of both synthesis (δ-OAT and P5CR) and protein proteolysis contributes to the 35°C-induced free proline accumulation in *G. tenuistipitata*.

Keywords: *Gracilaria tenuistipitata*; GSAd; High temperature; P5CR; Proline; Protein proteolysis; Red alga; δ-OAT; γ-GK.

Abbreviations: **γ-GK,** γ-glutamyl kinase; **GSAd,** glutamate-5-semialdehyde dehydrogenase; **δ-OAT,** ornithine δ-aminotransferase; **P5CR,** Δ^1-pyrroline-5-carboxylate reductase.

Introduction

In higher plants, proline accumulates under stress and shows an association with stress adaptation (Aspinall and Paleg, 1981; Lalk and Dorffling, 1985). Proline also accumulates in algae under stress (Bartels and Nelson, 1994; Kalinkina and Naumova, 1992; Singh et al., 1996; Wu et al., 1995). It has been shown in higher plants that proline is synthesized from either the glutamate pathway or the ornithine pathway (Delauney and Verma, 1993). The glutamate pathway is initiated by phosphorylating L-glutamate to L-glutamyl-γ-phosphate by γ-glutamyl kinase (γ-GK; EC 2.7.2.11). Then, γ-GK is converted to glutamate-γ-semialdehyde by GSA dehydrogenase (GSAd; EC 1.4.1.3). Finally GSA spontaneously forms Δ^1-pyrroline-5-carboxylate (P5C), and P5C is reduced to proline by P5C reductase (P5CR; EC 1.5.1.2). In the ornithine pathway, ornithine is converted to GSA by ornithine δ-aminotransferase (δ-OAT; EC 2.6.11.3) and then follows the same route to form P5C as in the glutamate pathway.

A stimulation of synthesis is a factor contributing to the stress-induced proline accumulation, but which pathway becomes responsible for proline synthesis is dependent on the plant systems involved or the kind of stress (Delauney and Verma, 1993). Under osmotic stress, the glutamate pathway is generally considered the primary route for proline synthesis (Delauney et al., 1993). However, in the case of the NaCl-tolerant *Brassica juncea* L., the NaCl-induced proline synthesis is suggested to be derived from the ornithine pathway with δ-OAT as the principle enzyme (Madan et al., 1995). In addition, the excess nitrogen supply switches the synthesis of proline from the glutamate to the ornithine pathway (Delauney et al., 1993).

The economically important marine red alga *Gracilaria tenuistipitata* var. *liui* Zhang et Xia, which is farmed in brackish water ponds as a main food source for the cultivation of sea abalone *Haliotis diversiolor supertexta* Lischke in Taiwan, frequently suffers from high water temperatures in summer (Chiang, 1981). It was recently found that proline significantly accumulated in *G. tenuistipitata* at 35°C, its upper survival limit (Lee et al., 1999). This study attempted to investigate whether synthesis was stimulated by the 35°C-induced free proline accumulation in *G. tenuistipitata*. The time course of changes in the levels of soluble protein, free amino acids, and free proline were compared to discover whether protein proteolysis is involved in the free proline accumulation. The relationships between proline accumulation and specific growth rate were also compared to understand their possible roles in *G. tenuistipitata* exposed to high temperature. The time-

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course changes in the specific activities of proline biosynthetic enzymes, that is, γ-GK, GSAd, P5CR and δ-OAT, were also determined. Since illumination has been known to be required for the heavy metal-induced free proline accumulation in several microalgae (Wu et al., 1995), the effects of 35°C on free proline accumulation and biosynthetic enzyme activity were compared between light and dark conditions.

Materials and Methods

Algal Culture and Temperature Treatments

Gracilaria tenuistipitata was collected in July from the brackish water ponds located at Yunlin, Taiwan, Republic of China (ROC). Following collection, thalli were pre-incubated in autoclaved natural seawater (pH 7.8, 30‰ adjusted by NaCl or distilled water) enriched with full strength Provasoli nutrient solution (Provasoli, 1968) under conditions of 25°C and 100 μE m^{-2} s^{-1} (12 h photoperiod) for 7 days before treatments. For the temperature treatment, a branch tip of approximately 5 cm (1.8~2.1 g fresh weight of each branch tip) in a polycarbonate vessel (Magenta GA-7 vessel, Sigma, St. Louis, MO., USA) containing 300 mL of autoclaved natural seawater (pH 7.8, 30‰ adjusted by NaCl or distilled water) enriched with full strength Provasoli nutrient solution (Provasoli, 1968) was kept at a constant temperature of 35°C. An inorganic carbon source was provided by adding $NaHCO_3$ to a final concentration of 3 mM. The photoperiod was 12/12 h L/D cycle and the photon irradiance was 100 μE m^{-2} s^{-1} in the absence of algae, achieved by a mixture of six 40W cool-fluorescent (FL40D, China Electric Apparatus Ltd., Tao-yuan, Taiwan, ROC) and two 60W incandescent (I60, China Electric Apparatus Ltd., Tao-yuan, Taiwan, ROC) lamps. Seawater was changed every day. After treatments, thalli were lyophilized and stored at -70°C for further analysis.

Determination of Specific Growth Rate

Fresh weight of discs was determined at the beginning (W_0) and after 4 days of incubation (W_4). The specific growth rate was expressed as the percentage of fresh weight increase (expressed as the natural log value) per day, $\%\bullet d^{-1} = \ln(W_4-W_0)/4d \times 100$ (Lee and Chen, 1998). The specific growth rate was the average of five replicates.

Determination of Soluble Protein, Free Amino Acid and Free Proline

Lyophilized thalli were homogenized into powder in liquid nitrogen, and then 50 mM phosphate buffer (pH 7.0) was added to extract soluble protein and free amino acid. Soluble proteins were determined according to the Bradford dye binding method (Bradford, 1976) using bovine serum albumin as a standard, and total free amino acids were analyzed according to Moore and Stein (1948) using L-lysine as a standard. Free proline was extracted and determined according to Bates et al. (1973) using L-proline as a standard.

Enzyme Extraction and Activity Determination

The lyophilized thalli were homogenized into powder in liquid nitrogen and mixed with extraction buffer in a ratio of plant material : extraction buffer = 1 g dry weight : 10 mL. The extraction buffer for γ-GK and GSAd was 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulphonylfluoride, 10 mM β-mercaptoethanol, and 1 mM Na_2EDTA while that for δ-OAT and P5CR was 50 mM potassium phosphate buffer (pH 8.0) containing 10 mM $MgCl_2$, 1 mM phenylmethylsulphonylfluoride, 10 mM β-mercaptoethanol, and 1 mM pyridoxal phosphate. After centrifugation at 15,000 *g* for 10 min under 4°C, the supernatant was collected and fractionated with ammonium sulfate to obtain partial purified enzymes. The ammonium sulfate saturated fraction ranging from 15~35% was used for GSAd assay; the fraction above 30% was used for γ-GK assay; the fraction ranged from 30~60% was used for P5CR assay, and the fraction above 60% was used for δ-OAT assay. The precipitates containing γ-GK or P5CR were dissolved in their respective extraction buffers. These fractionated enzyme solutions were desalted by dialysis against the extraction buffer within 2 h with three changes at 4°C. It was found that the specific activities of both γ-GK and δ-OAT fell to about 23% of their initial values after 6 h of dialysis while those of both GSAd and P5CR showed a faster loss within 3 h. The enzyme assay in this study was completed within 2 h after dialysis.

The activities of both γ-GK and GSAd were measured according to Hayzer and Leisinger (1980). The γ-GK reaction was started by adding L-glutamate, and the reaction solution contained 50 mM Tris (pH 7.0), 50 mM glutamate, 20 mM $MgCl_2$, 10 mM ATP, 200 mM hydroxylamine HCl, and 100 μL of desalted extract. After incubation at 25°C for 5 min in darkness, 1 mL of solution containing $FeCl_3\bullet 3H_2O$ (2.5%, w/v) and trichloroacetic acid (6%, w/v) in 2.5 N HCl was added to terminate the reaction. After centrifugation for 20 min at 16,000 *g*, the absorbance of the supernatant was measured at 534 nm, and the amount of γ-glutamyl hydroxamate was estimated from the standard curve of authentic γ-glutamyl hydroxamate. The GSAd activity was measured by detecting the increase of A_{340} at 25°C for 10 min in the reaction mixture containing 50 mM imidazal base (pH 7.0), 20 mM potassium phosphate, 0.75 mM P5C, 1 mM β-$NADP^+$, and 50 μL of desalted extract. One unit (U) of GSAd is equal to 1 nmol NADPH min^{-1} as calculated from the molar extinction coefficient for NADPH, 6.2 mM cm^{-1}, while one U of γ-GK is defined as 1 nmol γ-glutamyl hydroxamate min^{-1}.

The activity of δ-OAT was determined according to the method of Vogel and Kopac (1960). The reaction mixture contained 100 mM potassium phosphate (pH 8.5), 40 mM ornithine, 1 mM pyridoxal phosphate, 20 mM α-ketoglutarate, and 100 μL of desalted extract in a final volume of 0.8 mL. It was incubated at 35°C for 5 min in darkness. At the end of the reaction, 0.2 mL of 10% trichloroacetic acid was added to terminate the reaction, and then 0.2 mL of 0.5% *o*-aminobenzaldehyde was added. After shaking at 25°C for 30 min in darkness, the absorbance of

the supernatant was measured at 440 nm. One U of δ-OAT is defined as 1 nmol P5C min^{-1}, in which the molar extinction coefficient is 2.71 mM cm^{-1}.

The P5CR activity was determined by recording the decrease of A_{340} (Madan et al., 1995). The reaction mixture contained 50 mM potassium phosphate (pH 8.0), 1 mM DTT, 0.125 mM P5C, 200 μM β-NADH, and 50 μL of desalted extract in a final volume of 1 mL. One U of P5CR is 1 nmol β-NAD^+ min^{-1}, and the molar extinction coefficient of NADH is 6.2 mM cm^{-1}.

Statistical Analysis

Each treatment was performed at least four times. Since a similar trend was observed each time, the data shown here is from a single treatment. Each value was the mean of 3 replicates (n=3). The significance of the difference in the time course of changes in soluble protein, free amino acid, and free proline between 25°C and 35°C was analyzed by covariance on regression (SAS, SAS Ltd., NC, USA). Differences in other data were tested by using t-tests or 95% confidence intervals (SAS, SAS Ltd., NC, USA).

Results

Specific Growth Rate, Soluble Protein, Free Amino Acids and Free Proline

Figure 1 shows the changes in the specific growth rate and the levels of soluble protein, free amino acid, and free proline between 25°C- and 35°C-treated thalli. The specific growth rate significantly decreased 3 days after exposure to 35°C (ANCOVA, day, F=6.19, p=0.0031). The levels of soluble protein in 25°C-treated thalli increased gradually as time increased while those in 35°C-treated thalli remained unchanged as the initial value. As compared to the 25°C control, the effects of 35°C on the levels of soluble protein were significant as time increased (ANCOVA, temperature • day, F=20.67, p=0.0001). The levels of free amino acid in both 25°C- and 35°C-treated thalli increased as time increased (day, F=9.15, p=0.0049) but were similar between both temperature treatments (temperature • day, F=0.06, p=0.8075). The levels of free proline in 25°C-treated thalli raised from the initial value of 0.73 mg/g DW to a final value of 1.81 mg/g DW. The levels of free proline in 35°C-treated thalli significantly increased at Day 2 (t-test, p<0.05) and were approximately three times the 25°C control levels at Day 6. As time advanced, the 35°C-induced increase of free proline was more significant (temperature • day, F=18.0, p=0.0002).

Enzyme Activities

As shown in Figure 2, the specific activities of both γ-GK and GSAd in 25°C-treated thalli were not statistically different from those of 35°C-treated thalli. On exposure to 35°C, the specific activity of δ-OAT in 35°C-treated thalli increased to a plateau after 2 days, decreased at Day 5 and then dropped to the 25°C level at Day 6 (Figure 3A). The specific activity of P5CR in 35°C-treated thalli in-

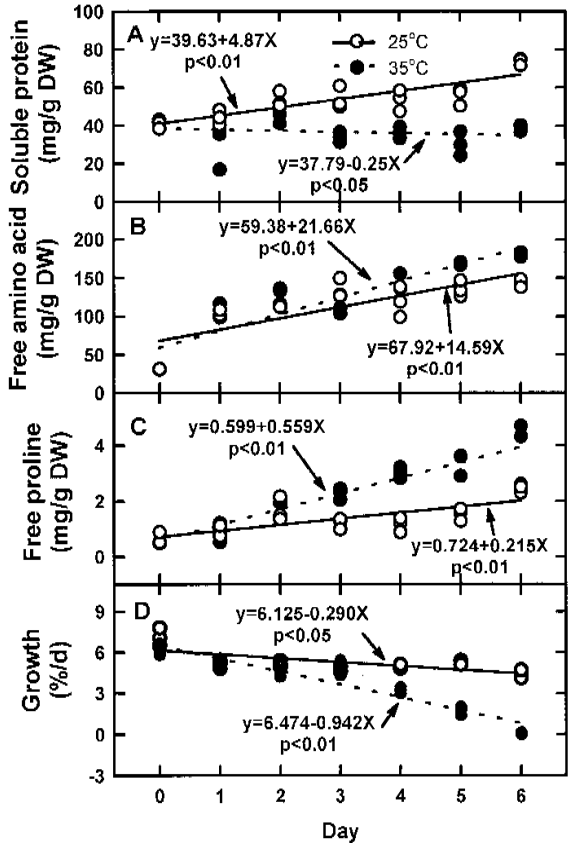

Figure 1. Time-course changes in the levels (n=3) of soluble protein (A), free amino acid (B) and free proline (C) and the specific growth rate (D) (n=3) in *Gracilaria tenuistipitata* grown at 25°C (❍) or 35°C (●).

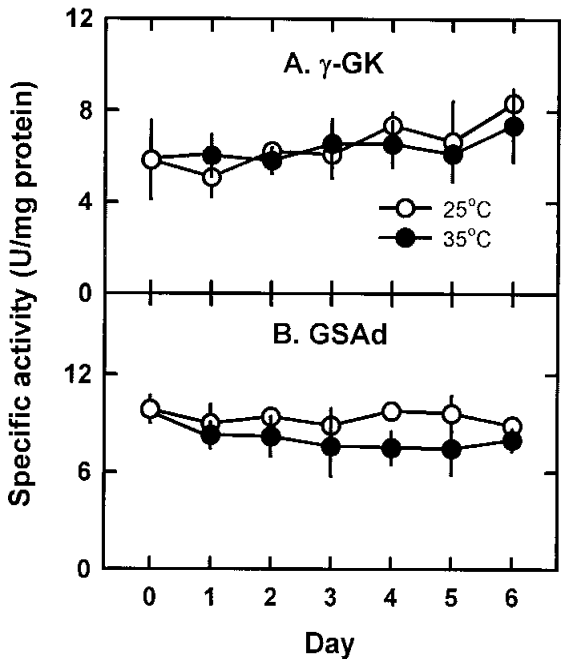

Figure 2. Time-course changes in the specific activities of γ-GK (A) and GSAd (B) in *Gracilaria tenuistipitata* grown at 25°C (❍) or 35°C (●). Means ± 95% confidence intervals (n=3) are indicated.

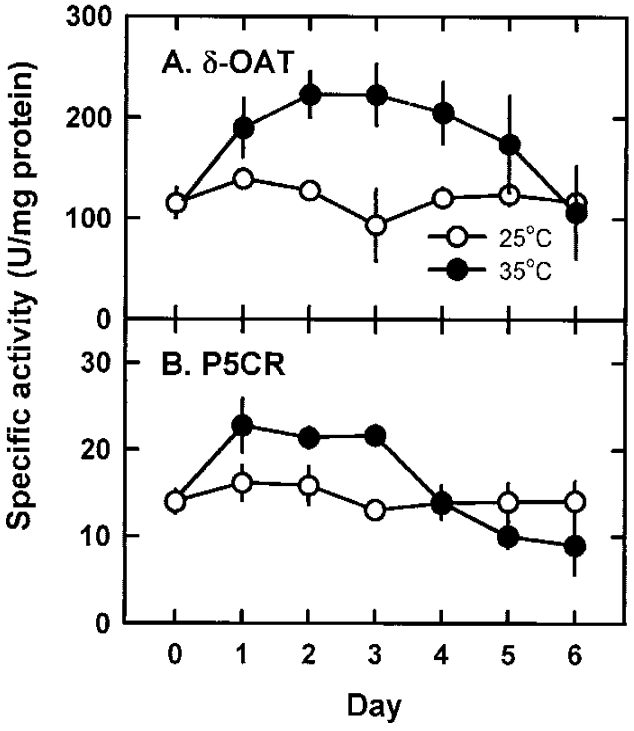

Figure 3. Time-course changes in the specific activities of δ-OAT (A) and P5CR (B) in *Gracilaria tenuistipitata* grown at 25°C (○) or 35°C (●). Means ± 95% confidence intervals (n=3) are indicated.

creased to a plateau 1 day after exposure to 35°C and decreased to the 25°C level after 4 days (Figure 3B).

Effect of Darkness on Free Proline Accumulation and Enzyme Activities

After incubation in darkness for 3 days, the free proline accumulation in 35°C-treated thalli was inhibited (Figure 4C). The 35°C-induced increase in δ-OAT (Figure 4A) and P5CR (Figure 4B) specific activities was also inhibited by darkness. The specific activities of both γ-GK and GSAd were not influenced by darkness (data not shown).

Discussion

A decrease in growth rate in *G. tenuistipitata* on prolonged exposure to 35°C shows that 35°C is a stressful temperature for this red alga. This is similar to the results reported by Lee et al. (1999).

The present study shows that the activities of proline biosynthetic enzymes can be detected in cell-free extract of the marine red macroalga *G. tenuistipitata*. These proline biosynthetic enzyme activities were also detected in another *Gracilaria* species *G. salicornia* (Lee, unpublished data). It is evident that both the glutamate and the ornithine pathways for proline synthesis co-exist in *Gracilaria* spp.

Compared to the linear increase in 25°C-treated thalli, the constant level of soluble protein in 35°C-treated thalli indicates that the proteolysis of protein might be involved in the proline accumulation in *G. tenuistipitata* in response to 35°C.

The consistency in the time-course changes of δ-OAT and P5CR activities and free proline levels during the early periods of 35°C treatment suggests that the high temperature-induced free proline accumulation may be due to a stimulation of synthesis via the ornithine pathway in *G. tenuistipitata*. Because the specific activities of both γ-GK and GSAd were similar in the 35°C and 25°C treatments, the glutamate pathway may not contribute to the 35°C-induced free proline accumulation. It has been found that

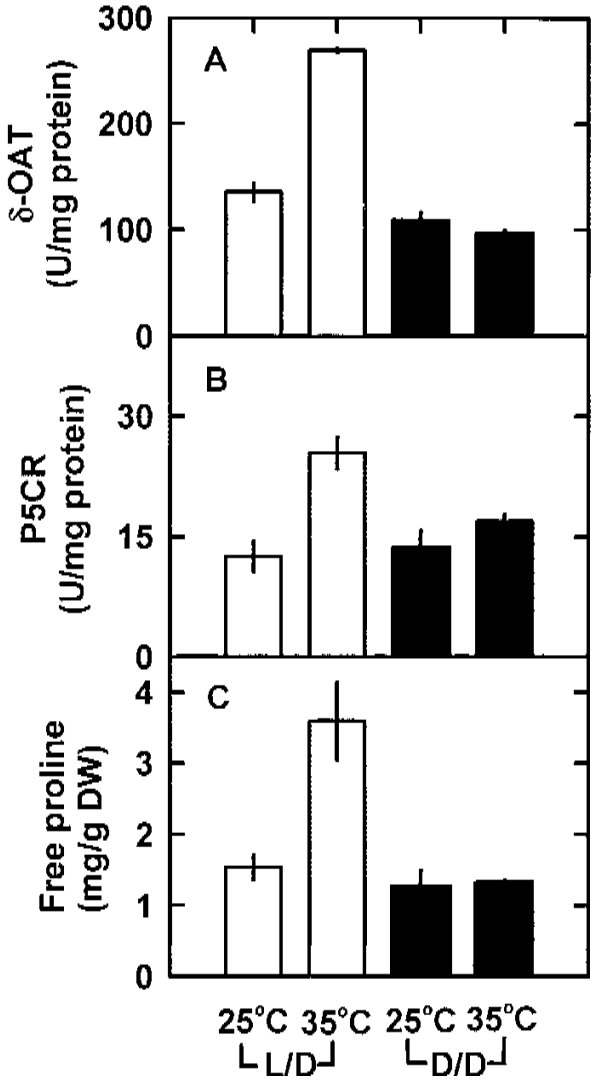

Figure 4. The effects of darkness on the specific activities of δ-OAT (A) and P5CR (B) and the levels of proline (C) in *Gracilaria tenuistipitata* grown at 25°C or 35°C for 3 days. After 3 days of treatment, thalli were sampled for enzyme activity assay and proline level determination. Means ± 95% confidence intervals (n=3) are indicated. L/D, light 12 h/dark 12 h; D/D, dark 12 h/dark 12 h.

an extremely high temperature (40°C) inhibited the gene expression of P5CS, a bifunctional enzyme in higher plants that possesses both γ-GK and GSAd activities (Hu et al., 1992), in both *Arabidopsis thaliana* (Yoshiba et al., 1995) and rice (Igarashi et al., 1997). However, more work is needed to clarify whether the glutamate pathway (γ-GK and GSAd) is involved in the 35°C-induced free proline accumulation in *G. tenuistipitata*.

According to the time-course results, a decrease in δ-OAT and P5CR specific activities was apparent after 5 days of exposure to 35°C. However, the levels of free proline continued to increase after the 5-day exposure. It is likely that factors other than synthesis stimulation may be involved in the regulation of proline accumulation during the prolonged periods of 35°C treatment. In addition to protein proteolysis, we recently found that the specific activity of proline dehydrogenase, an oxidation enzyme (Steward, 1992), significantly decreased after exposure to 35°C (Lee, 1998). This seems to suggest that the inhibition of proline oxidation is also involved in the long-term effect of high temperature on the accumulation of free proline in *G. tenuistipitata*.

The accumulation of imino acid proline in plants in response to salinity- or water stress-mediated osmotic stress has been extensively studied. A variety of studies also shows that proline accumulates in algae under hypersaline conditions to counteract the osmotic stress (Greenway and Setter, 1979; Schobert, 1980; Edwards et al., 1987; Ahmad and Hellebust, 1988; Kalinkina and Naumova, 1992; Bartels and Nelson, 1994; Singh et al., 1996). Proline is considered to be involved in osmotic adjustment. That is, it acts as a compatible osmolyte, and functions as a protector of macromolecules such as proteins and membranes and as a nitrogen-storage compound and energy source after stress release (Aspinall et al., 1981; Delauney and Verma, 1993). According to our current data in Figure 1, which indicates that the decrease in growth rate was accompanied by an increase in proline levels, the accumulated proline seems related to heat injury in *G. tenuistipitata*. However, more investigation is needed.

In conclusion, the stimulation of synthesis (δ-OAT and P5CR) in combination with an increase of protein proteolysis and an inhibition of oxidation are factors contributing to the high temperature-induced free proline accumulation in *G. tenuistipitata*. This accumulation is a light-dependent process. Research into the role of proline in high temperature responses in *G. tenuistipitata* is now in progress.

Acknowledgments. We are grateful to Dr. Y. Yoshiba and one anonymous reviewer for their critical reading of our manuscript, and to Prof. C. H. Kao in the Department of Agronomy, National Taiwan University, Taipei, ROC, for valuable discussion on the experiments. We also thank Dr. S. C. Fong in the Institute of Marine Biology, National Sun Yat-sen University, Kaohsiung, Taiwan, ROC, for comments on the statistical analysis of results, and the Institute of Marine Biology, National Sun Yat-sen University, Kaohsiung, Taiwan, ROC, for providing facilities. The financial support of the National Science Council of the Republic of China (NSC 87-2611-B-110-008) to T. M. Lee is acknowledged.

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高溫誘導龍鬚菜 (*Gracilaria tenuistipitata*) 脯氨酸累積

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本研究探討高溫 (35°C) 對屬於大型海洋紅藻的龍鬚菜 (*Gracilaria tenuistipitata* var. liui Zhang et Xia) 自由態脯氨酸累積之影響。在 35°C 下，自由態脯氨酸在 2 天後隨時間持續累積，自由態氨基酸含量不受影響。可溶性蛋白質含量在 35°C 下保持不變，25°C 下則持續增高累積之影響。與 25°C 的對照組比較，γ-glutamyl kinase (γGK; EC 2.7.2.11) 及 glutamate-5-semialdehyde dehydrogenase (GSAd; EC 1.4.1.3) 的比活性不受 35°C 的影響。Ornithine δ-aminotransferase (δ-OAT; EC 2.6.11.3) 的比活性在 35°C 下的 2 天後隨時間增加而增高，第 5 天後下降。Δ^1-Pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2) 的比活性在 35°C 下的 1 天後隨時間增加而增高，第 4 天後下降。δ-OAT 及 P5CR 比活性的增加趨勢與自由態脯氨酸累積的趨勢相符。35°C 所誘導的自由態脯氨酸累積是需光的反應。所以，本結果顯示合成途徑 (δ-OAT 及 P5CR) 及蛋白質水解可能參與 35°C 高溫誘導龍鬚菜自由態脯氨酸的累積。

關鍵詞：紅藻；高溫；脯氨酸；蛋白質水解；γGK；龍鬚菜；GSAd；δOAT；P5CR。