Correlation of decreased calcium contents with proline accumulation in the marine green macroalga *Ulva fasciata* exposed to elevated NaCl contents in seawater

Tse-Min Lee¹ and Chia-Hsiung Liu

Institute of Marine Biology, National Sun Yat-sen University, Kaohsiung, Taiwan 80424, Republic of China

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**Abstract**

The involvement of Na⁺, K⁺, Cl⁻ or Ca²⁺ in the regulation of salinity stress-induced proline accumulation via the inhibition of the activity of proline dehydrogenase (PDH; EC 1.4.3.1), a catabolic enzyme of proline, was investigated in the marine green macroalga *Ulva fasciata* Delile. After 6 h of exposure to elevated artificial seawater (ASW) salinity, adjusted either by increasing the NaCl content in 30‰ ASW (a change in ion ratio) or by concentrating ASW (a constant ion ratio), the contents of Na⁺, K⁺ and Cl⁻ linearly accumulated with increasing salinity from 30–90‰ (parts per thousand); the accumulation pattern of each ion was similar between the two treatments. An increase in NaCl content in ASW induced proline accumulation, but decreased both the PDH activity and the total and water-soluble Ca²⁺ contents, while concentrated ASW had no effect. As compared to a constant value at 30‰, both the contents of total and water-soluble Ca²⁺ increased, while proline accumulated after 3 h. The addition of 15 mM ethylene glycol-bis-(2-aminoethyl ether) N,N,N',N''-tetraacetic acid (EGTA) in 60‰ ASW (adjusted by increasing the NaCl content in 30‰ ASW) enhanced both the proline accumulation and the decrease in the content of total and water-soluble cellular Ca²⁺ and the activity of PDH; the effects of EGTA were reversed by 10 mM CaCl₂ or 10 mM CaSO₄. These results indicate that a loss of cellular Ca²⁺ is associated with the NaCl induction of proline accumulation via an inhibition of PDH activity in *U. fasciata*.

**Key words:** Calcium, NaCl, PDH, proline, *Ulva fasciata*.

**Introduction**

Salinity fluctuation significantly affects the growth and distribution of macroalgae living in the rocky intertidal zone and estuaries. It is considered that the control of constant cell turgor by regulating osmotic potentials, through the adjustment of ion and organic osmolyte contents, is a typical tolerance mechanism observed in most marine algae (Kirst, 1990). When algae are exposed to salinity changes, the movement of water occurs first, followed by ion fluxes for the maintenance of constant cell turgor by regulating osmotic potential; monovalent ions including Na⁺, K⁺ and Cl⁻ are the main ionic osmolytes contributing to the osmotic adjustment (Kirst, 1990). Several organic osmolytes such as mannitol, glycerol, betaine, and dimethylsulphoniopropionate (DMSP) may also be accumulated under prolonged hypersalinity treatment to counteract a long-term osmotic stress (Hellebust, 1976; Kirst, 1990; Reed, 1990). Numerous studies have shown that the amino acid proline accumulated in microalgae (Greenway and Setter, 1979; Ahmad and Hellebust, 1988; Kalinkina and Naumova, 1992; Bartels and Nelson, 1994; Singh et al., 1996) and macroalgae (Edward et al., 1987, 1988) in response to elevated salinity. Evidence shows that the accumulated proline functions as a compatible organic osmolyte, a protector of macromolecules such as proteins and membranes, via the interaction between proline and macromolecules, and also as a nitrogen-storage compound and energy source after the release of stress (Aspinall and Paleg, 1981; Delauney and Verma, 1993).

¹ To whom correspondence should be addressed. Fax: +886 7 525 5100; E-mail: tmlee@mail.nsysu.edu.tw

Abbreviations: DW, dry weight; EGTA, ethylene glycol-bis-(2-aminoethyl ether) N,N,N',N''-tetraacetic acid; P5C, Δ¹-pyrroline-5-carboxylate; PDH, proline dehydrogenase.

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Ca^{2+} is not only relevant to plant metabolism and growth (Hepler and Wayne, 1985; Kauss, 1987) but also acts as a second messenger in the perception of plant responses to environmental changes (Gilroy et al., 1993; Poovath and Reddy, 1993; Bush, 1995; Webb et al., 1996). It is well known that hypo-osmotic and hyper-osmotic stresses trigger a change in intracellular Ca^{2+} content. Measurement of Ca^{2+}-dependent luminescence output in the cells transformed with aequorin, a Ca^{2+}-dependent photoprotein, showed that hypo-osmotic shock increased the content of cytosolic Ca^{2+} in *Nicotiana tabacum* suspension-culture cells (Takahashi et al., 1997). Hyper-osmotic stress caused by high salinity (NaCl) also has an impact on the uptake and translocation of Ca^{2+} in higher plants. When exposed to NaCl stress, the content of Ca^{2+} in shoots decreased due to an inhibition of the Ca^{2+} loading into xylem vessels in *Hordeum vulgare* L. roots (Lynch and Lauchli, 1985; Halperin et al., 1997). By using protoplasts of *Zea mays* L. roots, it was found that NaCl stress increased the intracellular Ca^{2+} contents via a stimulation of phosphoinositide-mediated release of Ca^{2+} from intracellular pools (Lynch and Lauchli, 1988; Lynch et al., 1989). It is thought that the increase in intracellular Ca^{2+} activity will lead to an efflux of Ca^{2+}.

It was recently shown that the exposure to NaCl stress decreased the intracellular Ca^{2+} contents in the meristematic regions of *Arabidopsis thaliana* (Cramer, 1996).

There is growing evidence that the intracellular Ca^{2+} contents also show a significant change in algae exposed to salinity changes. In the giant-celled Charophyte alga, *Chara corallina*, a salt-sensitive freshwater alga, NaCl stress (hyper-osmotic stress) stimulated the Ca^{2+} influx at the plasmalemma of the internodal cells (Reid et al., 1993). The brackish Charophyte, *Lamprothamnum succintum*, a salt-tolerant alga, showed an increase in intracellular Ca^{2+} content in the internodal cells upon exposure to hypo-osmotic shock; the transient increase in intracellular Ca^{2+} was suggested to play a role in turgor regulation (Okazaki et al., 1987; Okazaki and Tazawa, 1987). However, in the marine green macroalga, *Codium decorticatum*, the intracellular Ca^{2+} content remained unchanged over a salinity range of 23–37‰ (Bisson and Gutnkecht, 1975). It has been suggested that the change in intracellular Ca^{2+} contents following salinity stress is a signal that triggers algal responses to stressful conditions. For example, Ca^{2+} has been proposed to be a factor in the regulation of the activity of isofloridoside phosphate synthase (IFP-synthase; EC 2.4.1.96) and, in turn, controls the content of isofloridoside phosphate, an organic osmolyte, in the wall-less flagellate, *Poteriococchromonas malhamensis*, in response to external salinity changes (Kauss and Thomson, 1982). Recently, Knight et al. have shown in a higher plant, *A. thaliana*, that treatment with both La^{3+}, a Ca^{2+} channel blocker, and ethylene glycol-bis-(2-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), a Ca^{2+} chelator, caused an inhibition of the NaCl stress-induced expression of the gene for Δ-pyrroline-5-carboxylate synthetase, a principal enzyme in the glutamate pathway for proline synthesis (Knight et al., 1997). This provides evidence indicating that Ca^{2+} signalling is involved in the regulation of proline synthesis in higher plants exposed to salinity stress. However, the possible involvement of Ca^{2+} in salinity stress-induced proline accumulation has not been studied in algae.

In this study, the role of Ca^{2+} or other ions (Na^{+}, K^{+} and Cl−) associated with the salinity stress-induced proline accumulation was studied in the marine green macroalga *Ulva fasciata* Delile living in the upper intertidal region. The changes in the content of Na^{+}, K^{+}, Cl−, Ca^{2+} and proline in response to varying ASW salinity (from 30‰ to 90‰), adjusted by increasing the NaCl content in 30‰ ASW or by concentrating 30‰ ASW (increasing the concentration of every ASW component in the same ratio in 30‰ ASW), were determined, together with the content of water-soluble Ca^{2+}. Since it has been shown that the salinity stress-induced proline accumulation is partly due to a decrease in the activity of a catalytic enzyme, proline dehydrogenase (PDH; EC 1.4.3.1) (Madan et al., 1995), the activity of PDH was also determined. The time-course of changes in the content of ions and proline and the activity of PDH was compared between 30‰ and 60‰ (adjusted by increasing NaCl contents in 30‰ ASW) treatments. The effects of 15 mM EGTA, 10 mM CaCl_{2} or 10 mM CaSO_{4} on total and water-soluble Ca^{2+} and proline contents and PDH activity in *U. fasciata* exposed to 60‰ ASW (adjusted by increasing NaCl contents in 30‰ ASW) were also determined.

**Materials and methods**

**Algal culture and treatments**

The algae were collected during May 1998 from the intertidal region of Hsitzu Bay, Kaohsiung, Taiwan, Republic of China. Following collection, thalli were incubated at 25 °C for 120 h in the 30‰ Provasoli nutrient solution (Provasoli, 1968)-enriched artificial seawater (ASW; 408 mM NaCl, 10 mM KCl, 10 mM CaCl_{2}, 30 mM MgSO_{4}, and 10 mM TRIS-HCl, pH 8.0). An inorganic carbon source was provided by adding NaHCO_{3} at a final concentration of 3 mM. The photoperiod was a 12/12 h light/dark cycle and the photon irradiance (400–700 nm) ranged from 500 μE m^{-2} s^{-1} in the absence of algae, achieved by 20 × 60 W cool-fluorescent lamps (FL60D, China Electric Apparatus Ltd., Tao-yuan, Taiwan, Republic of China). It was found that both the growth rate and the ion contents of *U. fasciata* reached a steady state around 96 h after incubation; thus the materials used in this study were incubated in enriched ASW for at least 96 h. After pre-incubation, thallus discs of 1 cm diameter were cultured in 25 ml flask containing 20 ml ASW with varying salinity; the elevation of ASW salinity was
adjusted by increasing the NaCl contents in 30% ASW or by increasing the concentration of every ASW component in the same ratio in 30% ASW. The inorganic carbon source was provided by NaHCO₃ at a final concentration of 3 mM and the concentration of Provasoli nutrient solution was the same in all salinity treatments. ASW was changed every day. The salinity treatment was performed in a growth chamber with a 12:12 h light/dark cycle and the photon irradiance of 500 µmol m⁻² s⁻¹, achieved by 20 × 60 W cool-fluorescent lamps (FL60D, China Electric Apparatus Ltd., Tai-yuan, Taiwan, Republic of China). After treatments, the thallus discs for Na⁺, K⁺ and Cl⁻ determination were washed three times (1 min each time) by isotonic Ca(NO₃)₂ (in TRIS-HCl, pH 8.0) to remove the residual ions in the free space and the cell wall of the discs (Ritchie, 1988) and blotted dry with tissue paper. The materials for the determination of Ca²⁺ were washed three times with MQ H₂O. These thallus discs were lyophilized and stored at −80 °C for further analysis. In this study, each treatment has five replicates (a flask as a replicate). Each experiment was repeated three times and, because of the similar results, only one has been shown in this paper.

**Determination of ion and proline**

For the determination of total ion contents, lyophilized thallus discs of 0.05 g DW were ashed at 500 °C overnight and dissolved in 10 ml of 0.5 N HCl. Water-soluble Ca²⁺ was extracted by grinding the lyophilized discs of 0.05 g DW in liquid nitrogen and subsequently extraction in 10 ml of MQ H₂O at 95 °C for 20 min. After filtration through the ash-free Whatman No.1 filter paper, the filtrate was stored at −20 °C for ion analysis. For the determination of ions in ASW, the ASW was filtered through 0.45 µm filter and stored at −20 °C. Na⁺, K⁺ and Ca²⁺ were detected by the atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, USA) and Cl⁻ using a chloride titrator. Proline was extracted and determined spectrophotometrically (according to Bates et al., 1973) using l-proline as standard.

**Enzyme extraction, partially purification and activity determination**

Lyophilized thallus discs were homogenized into powder in liquid nitrogen and mixed with extraction buffer in a ratio of 1 g DW plant material:10 ml extraction buffer. The extraction buffer consisted of 100 mM TRIS-HCl (pH 7.8) containing 10 mM MgCl₂, 1 mM phenylmethylsulphonylfluoride, 10 mM β-mercaptoethanol, and 1 mM pyridoxal phosphate. After centrifugation at 15,000 g for 20 min at 4 °C, the supernatant was subjected to ammonium sulphate fractionation on ice. The ammonium-sulphate-saturated fraction, ranging from 10 to 40%, was collected and the precipitates were dissolved in the extraction buffer and centrifuged at 350,000 g for 30 min at 4 °C. The supernatant was desalted by dialysis against 10 mM TRIS-HCl (pH 7.8) containing 10 mM MgCl₂, 1 mM phenylmethylsulphonylfluoride, 10 mM β-mercaptoethanol, and 1 mM pyridoxal phosphate) for 6 h, with three changes of buffer, at 4 °C. Then the dialysed enzyme was applied to a DEAE Sepharose CL-6B column (1.2 × 30 cm; Bio-Rad) and eluted by a linear gradient of NaCl from 0 to 1.2 M in 10 mM TRIS-HCl buffer (pH 7.8) containing 10 mM MgCl₂, 1 mM phenylmethylsulphonylfluoride, 10 mM β-mercaptoethanol, and 1 mM pyridoxal phosphate) with a flow rate of 0.4 ml min⁻¹ in the cool room (4 °C). Fractions containing enzyme activity were collected, concentrated by Millipore Ultrafree-15 centrifugal filter (Millipore, MA, USA) and desalted by dialysis against extraction buffer at 4 °C. Enzyme activity was determined by detecting the amount of Δ⁻pyrroline-5-carboxylate (PSC) (according to the method of Lee, 1998). One unit (U) of PDH is equal to 1 nmol PSC formation min⁻¹ as calculated from the molar extinction coefficient for PSC, 2.71 × 10³ M⁻¹ cm⁻¹. Protein was determined by the Bradford method (Bradford, 1976).

**Statistics**

Significance of ion content and PDH activity between increased NaCl contents and concentrated ASW treatments was assayed by using t-test (n=5) at P<0.05 (SAS, SAS Ltd., NC, USA). Effects of varying ASW salinity on proline and ion contents and the time-course changes in proline and ion contents were compared by the 95% confidence interval (SAS, SAS Ltd., NC, USA). Effects of EGTA, CaCl₂ and CaSO₄ on the proline and Ca²⁺ contents and the PDH activity were compared by using Duncan’s new multiple range test from significant ANOVA test (SAS, SAS Ltd., NC, USA).

**Results**

**Changes in Na⁺, K⁺, Cl⁻, Ca²⁺, and proline contents and PDH activity in response to varying ASW salinity**

The initial contents of Na⁺, K⁺ and Cl⁻ were 231.49 ± 10.30, 306.79 ± 28.97 and 328.68 ± 45.63 μmol g⁻¹ DW (mean ± 95% confidence interval, n=5), respectively, in U. fasciata grown in 30% ASW. Figure 1 shows the effects of varying ASW salinity (6 h) on Na⁺, K⁺ and Cl⁻ contents. Upon exposure to elevated ASW salinity (from 30 to 90%) adjusted by increasing NaCl content, the contents of Na⁺, K⁺ and Cl⁻ increased linearly with increasing ASW salinity. The changes in the
contents of Na⁺, K⁺ and Cl⁻ in response to concentrated ASW showed a similar trend as those exposed to elevated ASW salinity adjusted by increasing the NaCl content in ASW.

To understand the relationship between Ca²⁺ and NaCl-induced proline accumulation, the changes in the total and water-soluble Ca²⁺ contents in response to varying ASW salinity were examined. The contents of both total and water-soluble Ca²⁺ decreased on exposure to elevated ASW salinity adjusted by increasing NaCl content and in contrast, the content of proline increased (Fig. 2). The NaCl-induced proline accumulation had an inverse relationship with total (\( y = 118-11.30x, r^2 = 0.64 \)) or water-soluble (\( y = 34.25-3.77x, r^2 = 0.54 \)) Ca²⁺ content. However, elevated ASW salinity produced by concentrating ASW did not affect the contents of total and water-soluble Ca²⁺ and proline as compared with the initial control (Fig. 3). The initial total and water-soluble Ca²⁺ and proline contents were 121.09 ± 28.96 and 39.56 ± 4.12 µmol g⁻¹ DW and 0.66 ± 0.15 mg g⁻¹ DW, respectively.

The activity of PDH decreased when exposed to elevated ASW salinity by increasing NaCl content (Fig. 4); the extent of decreased activity increased with increasing ASW salinity. But, elevated ASW salinity achieved by concentrating ASW did not affect the PDH activity (Fig. 4).

**Time-course changes of Na⁺, K⁺, Cl⁻, total and water-soluble Ca²⁺, net seawater Ca²⁺ and proline contents, and PDH activity**

Figure 5 shows the time course of changes in the contents of proline, total and water-soluble Ca²⁺ and net seawater Ca²⁺ (a difference between treatment and initial seawater) in response to elevated ASW salinity (by increasing NaCl content). As compared to a constant value at 30‰, the content of proline increased 3 h after exposure to 60‰ and reached a plateau after 6 h. The total and water-soluble Ca²⁺ contents decreased after 0.5 h and concomitantly those of seawater Ca²⁺ increased. The activity of PDH decreased rapidly to a plateau 1 h after exposure to elevated ASW salinity adjusted by increasing NaCl content in ASW, but remained unchanged when exposed to concentrated ASW (Fig. 6). The contents of Na⁺, K⁺ and Cl⁻ significantly increased by 1 h after exposure to 60‰ and reached a plateau after 3 h (Fig. 7).

**Effects of EGTA, CaCl₂ and CaSO₄**

The effects of EGTA, CaCl₂ or CaSO₄ are shown in Fig. 8. EGTA, CaCl₂ or CaSO₄ were added at the start of 60‰ (adjusted by increasing NaCl content in ASW)
Ca$^{2+}$ and proline in Ulva

Fig. 5. The time-course changes in the contents of proline (A), total Ca$^{2+}$ (B), water-soluble Ca$^{2+}$ (C), net seawater Ca$^{2+}$ (the difference in seawater Ca$^{2+}$ between treatment and initial value) and the activity of PDH (D) in Ulva fasciata Delile in response to 30‰ and 60‰ (adjusted by increasing NaCl content in 30‰ ASW). Vertical bars indicate the 95% confidence interval (n = 5).

Fig. 6. The time-course changes in the activity of PDH in Ulva fasciata Delile in response to 30‰ and 60‰ (adjusted by increasing NaCl content in 30‰ ASW). Vertical bars indicate the 95% confidence interval (n = 5).

Fig. 7. The time-course changes in the contents of Na$^+$ (A), K$^+$ (B) and Cl$^-$ (C) in Ulva fasciata Delile in response to 30‰ and 60‰ (adjusted by increasing NaCl content in 30‰ ASW). Vertical bars indicate the 95% confidence interval (n = 5).

Fig. 8. Effects of EGTA (15 mM), CaCl$_2$ (10 mM) or CaSO$_4$ (10 mM) on the contents of proline (A), total Ca$^{2+}$ (B) and water-soluble Ca$^{2+}$ (C), and the activity of PDH (D) in Ulva fasciata Delile exposed to 60‰ ASW (adjusted by increasing NaCl content in ASW) for 6 h. EGTA, CaCl$_2$ or CaSO$_4$ was added at the start of 60‰ treatment. Bars indicate the 95% confidence interval (n = 5). Different symbol represents significant difference at P < 0.05.

The addition of 15 mM EGTA resulted in a more pronounced decrease in the contents of total and water-soluble Ca$^{2+}$ and the activity of PDH at 60‰, and a more significant accumulation of proline. The effects of EGTA were reversed by 10 mM CaCl$_2$ or 10 mM CaSO$_4$.

The effect of EGTA was also found at 30‰, but the effective concentration of EGTA was higher than that used for 60‰ ASW and its effect at 30‰ was less than the effect of EGTA at 60‰. The addition of 25 mM EGTA at 30‰ (6 h) induced an increase in proline content, 0.91 ± 0.13 mg g$^{-1}$ DW (mean ± 95% confidence interval, n = 5), and a slight decrease in total and water-soluble Ca$^{2+}$ contents, 87.30 ± 9.11 and 24.76 ± 2.98 µmol g$^{-1}$ DW (mean ± 95% confidence interval, n = 5). In the presence of 25 mM CaCl$_2$, the effects of EGTA were reversed. The effect of EGTA was maximal at 25 mM; however, the maximal amount of EGTA-
induced proline accumulation at 30‰ was smaller than the hypersalinity-induced proline accumulation.

**Discussion**

Similar to other marine algae (Kirst and Bisson, 1979; Dickson et al., 1982), Na⁺, K⁺ and Cl⁻ are the main ions in the marine green macroalga *U. fasciata* Delile responsible for the increase in internal osmotic pressure following elevated seawater salinity. It has been reported that the pattern of cation accumulation under elevated salinity is different among algae and can be divided into three categories: K⁺ type, Na⁺ type and K⁺/Na⁺ type (Kirst and Bisson, 1979). Current data indicate that *U. fasciata* is a K⁺/Na⁺ type alga.

In this work, the increase in seawater salinity was adjusted either by increasing NaCl contents in seawater, so keeping the other ion content constants, or by concentrating seawater, that is, maintaining a constant ion ratio. Since there is a similar accumulation pattern of Na⁺, K⁺ and Cl⁻ in *U. fasciata* in response to the two systems, it seems that the accumulation of Na⁺, K⁺ and Cl⁻ is independent of the ion composition or ion ratio in the external media. It is possible that these ions accumulated to restore the difference in osmotic potential between the medium and the algal cell.

The responses of cellular Ca²⁺ to elevated seawater salinity are different to those for Na⁺, K⁺ and Cl⁻. Only elevating ASW salinity by increasing NaCl content in 30‰ ASW induced a fast decrease in the contents of both total and water-soluble Ca²⁺. It is known that intracellular Ca²⁺ content is controlled by the action of pumps and channels in the plasma membrane and subcellular organelles. So, the change in Ca²⁺ content in *U. fasciata* in response to increasing NaCl content in ASW is likely to be due to an alteration in the characteristics of Ca²⁺ channels caused by an imbalance in external seawater ionic composition. In addition, it has been shown that Na⁺ could displace the Ca²⁺ associated in the plasma membrane of cotton (*Gossypium hirsutum* L. cv. Acala SJ-2) root cells in response to salinity stress (Cramer et al., 1985). Salinity stress was also found to increase cytoplasmic Ca²⁺ activity via an induction of Ca²⁺ release from intracellular pools and in turn leads to Ca²⁺ efflux (Lynch et al., 1987; Lynch and Lauchli, 1988). It has been shown in several studies using higher plants that the excess Na⁺ reduces Ca²⁺ availability (Zid and Grignon, 1985; Maas and Grieve, 1987). According to the above observations, it is proposed that, in the case of *U. fasciata*, an increase in NaCl content in seawater leads to a decrease in the availability of Ca²⁺ via both the displacement of membrane-associated Ca²⁺ and the enhancement of Ca²⁺ efflux by Na⁺.

In algae, the cell wall has been shown to adsorb cations (Ritchie and Larkum, 1982, 1984). It has been recently reported that in the internodal cells of freshwater Chara, the cell wall-bound Ca²⁺ will be released in aequous NaCl solution (Kiyosawa, 1999). Thus, Na⁺ might replace the cell wall-associated Ca²⁺ and, in turn, decreases the availability of Ca²⁺ in *U. fasciata* exposed to high NaCl conditions.

Evidence shows that the imbalance in seawater ion composition resulting from the supply of NaCl in ASW is a cue for a decrease in Ca²⁺ content and, subsequently, the induction of proline accumulation. Proline accumulated only under a condition of elevated ASW salinity adjusted by increasing NaCl content in ASW, but not under concentrated ASW. Similarly, it has been shown that elevating seawater salinity by concentrating seawater did not affect the content of proline in *U. lactuca* L. (Dickson et al., 1982). Current data showed that there is an inverse relationship between Ca²⁺ and proline contents in *U. fasciata*. In addition, the enhancement of proline accumulation by EGTA was accompanied by a decrease in total and water-soluble Ca²⁺ contents, indicating a reduction of external Ca²⁺ availability has an effect on internal cellular Ca²⁺ content. The above observations indicate that Ca²⁺ is negatively associated with the NaCl induction of proline accumulation in *U. fasciata*. In contrast, it has been reported in *Sorghum bicolor* roots that the Ca²⁺ supplement enhanced the NaCl (150 mM NaCl) stress-induced proline accumulation (Colmer et al., 1996). It seems that the relationship between Ca²⁺ content and proline accumulation under salinity stress is different among plant species.

The effect of EGTA and its reversal by 25 mM CaCl₂ were also found at 30‰. Was the effect of EGTA caused by an increase in osmolality in external ASW after the addition of 25 mM EGTA? Figure 2C shows that the threshold salinity for the induction of proline accumulation was 50‰, that is, 1447 mOsmol kg⁻¹. Since the osmolality of 30‰ ASW containing 25 mM EGTA was 787 mOsmol kg⁻¹, the effect of EGTA was unlikely to be due to an increase in external osmolality. The effect of EGTA at 30‰ was less than that at 60‰ and the maximal effect of EGTA on proline and Ca²⁺ contents at 30‰ was less than the hypersalinity-induced proline accumulation, indicating that factors other than external Ca²⁺ are involved in the NaCl-induced proline accumulation.

To maintain a constant turgor pressure, algae usually accumulate several ions including Na⁺, K⁺ and Cl⁻, and organic metabolites under salinity conditions. In general, it is considered that the significant accumulation of these ions is an efficient way for marine algae to counteract osmotic changes when suffering changed salinity in natural habitats such as rocky shores and estuaries. This work shows there was a fast accumulation of cations in *U. fasciata* in response to elevated seawater salinity. With regard to the significant accumulation of Na⁺, K⁺ and Cl⁻, the small amount of proline accumulated under
elevated seawater salinity by increasing NaCl content implies that proline was not of major significance in the turgor regulation in *U. fasciata* exposed to hypersalinity. It is possible that one of the functions of accumulated proline is to compensate the NaCl-induced Ca\(^{2+}\) loss. In Charophyte algae, there are a variety of studies showing that Ca\(^{2+}\) has a role in turgor regulation in euryhaline species (e.g. *Chara longifolia*) exposed to salt stress (Tazawa *et al.*, 1987; Hoffmann and Bisson, 1988) and in the prevention of Na\(^{+}\) influx in freshwater species (e.g. *Nitellopsis* spp.) (Katsuhara and Tazawa, 1988). Alternatively, the accumulation of proline is a result of excessive ion accumulation in *U. fasciata*. In several cases, the proline accumulation has been also interpreted as being symptomatic of salt-stress induced damage (Hanson *et al.*, 1977; Hasegawa *et al.*, 1986). By using gabaculine, the inhibitor of ornithine \(\delta\)-aminotransferase (\(\delta\)-OAT; EC 2.6.11.3), it has also been found in the red macroalga, *G. tenuestipitata*, that the \(\delta\)-OAT-mediated accumulation of proline under high temperature may be associated with the high-temperature injury (Lee and Chang, 1999). However, whether the accumulated proline has a positive role in *U. fasciata* confronting NaCl stress or is a result of impeding metabolism is still an open question.

The 60‰-treated thallus discs showed a time-dependent increase of proline content with an accompanying decrease in PDH activity as salinity treatment progressed. The results from EGTA, CaCl\(_2\) or CaSO\(_4\) treatment suggest that Ca\(^{2+}\) plays a negative role in the regulation of PDH activity in *U. fasciata* under NaCl stress. These results, together with an inverse relationship of proline content with PDH activity on exposure to varying seawater salinity, suggest that the inhibition of catabolism via an inhibition of PDH activity is involved in the regulation of NaCl-induced proline accumulation in *U. fasciata*. In the case of the red macroalga, *G. tenuestipitata var. liui* Zhang et Xia, a decrease in PDH activity contributed to high temperature induction of proline accumulation (Lee, 1998). In a higher plant, *B. juncea* L., the salinity stress-induced proline accumulation was also accompanied by a decrease in the activity of PDH (Madan *et al.*, 1995).

*U. fasciata*, which lives in the intertidal pools, occasionally suffers an elevated seawater salinity resulting from evaporation in the summer, especially during low tide. This leads to concentrated seawater. According to the present study, the accumulation of proline and a decrease in Ca\(^{2+}\) contents will not be observed in *U. fasciata* in the natural condition.

In conclusion, the changes in Na\(^{+}\), K\(^{+}\) and Cl\(^{-}\) contents in *U. fasciata* in response to elevated seawater salinity were similar between salinity adjusted by increasing the NaCl content in seawater and salinity adjusted by concentrating seawater. An efflux of cellular Ca\(^{2+}\) may have a role in the induction of proline accumulation in the presence of elevated ASW salinity (by increasing NaCl contents) via the inhibition of PDH activity in *U. fasciata*.

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**References**


