CHAPTER IV

DISCUSSION

1. Effect of salinity on growth of *A. halophytica*

The glycine betaine synthesis and accumulation is an important mechanism of many higher plants, mammalian tissues and microorganisms to adapt to osmotic or salinity stress. In this study, the unicellular halotolerant cyanobacterium, *A. halophytica* was chosen as a model organism to investigate the accumulation and biosynthesis of glycine betaine. The salt tolerance of *A. halophytica* was studied by following the growth rate of cells in salt stress condition. When the cells were transferred to the medium with higher NaCl concentration, the growth of of the cells was decreased. Our data showed *A. halophytica* could adapt to a broad range of salt concentrations from 0.5 to 2.0 M NaCl. This was in good agreement with the finding of Reed *et al.*(1984). They found that *A. halophytica* can be grown in high external salinity from 0.1 to 3.0 M NaCl concentration.

2. Accumulation of glycine betaine in *A. halophytica*

It has been well documented that organisms inhabiting in extreme condition such as high salinity and drought can accumulate the low molecular weight organic osmoprotectants. The most halotolerant

cyanobacteria accumulate glycine betaine to protect cells against osmotic stress. The glycine betaine accumulation is an indication that the cells are adapting to the changes of external osmolarity. In order to assess the content of glycine betaine in the cells, a suitable method for glycine betaine determination is very important. Our present study employed, two methods of glycine betaine determination (^{1}H-NMR and tri-iodide assays). It was found that the amount of glycine betaine in *A. halophytica* determined by the two methods was similar. The ^{1}H-NMR method was a suitable method for glycine betaine determination in terms of selectivity and convenience. Graham *et al.* (1986) studied the accumulation of glycine betaine in barley by ^{1}H-NMR and tri-iodide assays. They concluded that ^{1}H-NMR is a suitable method for glycine betaine determination. The ^{1}H-NMR method requires only the first step (extraction of glycine betaine) for the measurement of glycine betaine but tri-iodide assay required both steps (extraction and ion-exchange chromatography). Dowex 50-W chromatography helps remove interfering amino acid prior to glycine betaine determination. The ^{1}H-NMR technique is reliable, rapid and it can be used to detect and identify unknowns, as has been done in a study of nitrogenous compatible solutes accumulating in native South Australian flora (Poljakoff-Mayber *et al.* 1987). However, the ^{1}H-NMR method for the determination of glycine betaine is more costly than the spectrophotometric method. From ^{1}H-NMR spectrum of extract from *A. halophytica* choline peak was found at 3.20 ppm (relative to DSS). The choline peak suggested that the glycine betaine synthesis in *A. halophytica* can possibly be achieved by choline-betaine pathway.

In *A. halophytica* , the amount of glycine betaine in non salt-stressed condition was approximately 9.7 nmol/10^6 cells. The amount of glycine betaine was increased about 8 folds in salt stressed condition. The increased content of glycine betaine in response to salt stress was similarly found in spinach (Simon *et al.* 1986). The content of glycine betaine in chloroplasts isolated from salt stressed spinach leaves increased up to 11 folds as compared to that from non salt-stressed leaves.

3. BADH purification and characterization

The purification of BADH from *A. halophytica* was accomplished by ammonium sulfate precipitation and DEAE-cellulose column chromatography. The enzyme was partially purified to 18 folds and the specific activity was 290.8 μmol min^{-1} mg^{-1} with 8.6% recovery. The hydroxyapatite column step could not increase the specific activity of the BADH. The loss of BADH activity during ion-exchange chromatography and hydroxyapatite column could be due to some inactivation and aggregation of the enzyme during both steps of purification. The BADH activity staining in native polyacrylamide gel electrophoresis was not able to detect a band for BADH activity of *A. halophytica* although it could detect the activity band of spinach enzyme. This might be because the conditions used were not suitable for *A. halophytica* BADH. The optimal condition for *A. halophytica* BADH activity was at pH 7.5 and 25°C. This condition was similar to that of BADH from spinach, *E. coli* strain MC 4100(pFF423) and Horseshoe

Crab (Falkenberg and Strom 1990, Weretilnyk and Hanson 1989 and Dragolovich and Pierce 1994). The BADH purified from *A. halophytica* was specific for betaine aldehyde. The K_m for betaine aldehyde was 91 μM. This value was one order of magnitude lower than that of purified BADH from *E. coli* (K_m = 160 μM) and from Horseshoe Crab (K_m = 133 μM) (Falkenberg and Strom 1990, Dragolovich and Pierce 1994). NAD^+ was an essential coenzyme required for BADH to oxidize betaine aldehyde to glycine betaine. The BADH from *A. halophytica* seemed to prefer NAD^+ as a coenzyme rather than $NADP^+$ (K_m = 71.4 and 100 μM, respectively). The preference of NAD^+ as a coenzyme for BADH from *A. halophytica* was similarly observed for BADH from other organisms such as plant, microorganism and animal cells (Weretilnyk and Hanson 1989, Falnkenberg and Strom 1990 and Dragolovich and Pierce 1994). In this repect it is worth mentioning that the BADH from the fungus *Cylindrocarpon didymus* could use only NAD^+ (Mori *et al.* 1980).

It has been reported previously that the BADH from bacteria exhibits substrate analog inhibition (Falkenberg and Strom 1990). The BADH from *A. halophytica* was also inhibited by substrate analog. Two types of substrate analog were tested in our experiment. Aldehyde compound such as acetaldehyde was a strong enzyme inhibitor but *N*-methylated compound such as glycine betaine, choline and ethanolamine showed lesser inhibitory effect than the aldehyde compound. This suggested that the aldehyde functional group of the substrate might play an important role in binding to the active site of BADH.

The effect of salt on *A. halophytica* BADH activity was tested. It was found that the monovalent cations such as Na^+ and K^+ at low

concentration (0.05 to 0.1M) could increase BADH activity, with maximal activity at 0.1 M. At higher concentration (0.25 to 2.0 M) BADH was inhibited by both Na^+ and K^+. Inhibition of BADH by increasing Na^+ and K^+ concentrations has been reported in *E. coli* and spinach (Falkenberg and Strom 1990 and Pan *et al.* 1981). On the other hand increasing Na^+ stimulated BADH activity in *Rhizobium meliloti* (Smith *et al.*1988). *A. halophytica* BADH was more inhibited by divalent cations such as Mg^{2+} and Ca^{2+} than monovalent cations. It is therefore important to include EDTA as a chelating agent for divalent cations in the assay of BADH.

A. halophytica BADH activity was strongly inhibited by sulfhydryl-reactive compounds such as *p*-chloromercuriphenyl sulfonic acid (PCMS). Preincubation of the enzyme with reducing agent such as DTT could protect sulfhydryl group of enzyme. Thus the reducing agents are essential for BADH activity. Falkenberg and Strom (1990) studied the effect of sulfhydryl-reactive compounds and reducing agents on *E. coli* BADH. They found that the reducing agents, β - mercaptoethanol and DTT effectively protected the enzyme. The NAD^+ and betaine aldehyde provided partial protection of the enzyme when added before the sulfhydryl- reactive inhibitors. They concluded that one or more sulfhydryl groups are essential for BADH activity. The protective effect of NAD^+ and betaine aldehyde against the sulfhydryl-reactive compounds points to the existence of essential sulfhydryl groups at or near the catalytic site of *E. coli* BADH.

The results from gel filtration and SDS-PAGE indicated that the *A. halophytica* BADH constitutes a tetramer with identical 30,000 dalton

subunits. The molecular weight of *A. halophytica* BADH was 120,000 dalton as determined by gel filtration chromatography. The molecular weight of BADH reported in various organisms are different. *E. coli* BADH is a tetramer with 55,000 dalton subunits (Falkenberg and Strom 1990). The BADH of spinach chloroplasts is a dimer with subunits of 60,000 dalton (Arakawa *et al.* 1989) and 63,000 dalton (Weretilnyk *et al.* 1989).

4. The relationship between salt stress and BADH activity in *A. halophytica*

The study of the effect of external salinity on BADH activity showed that high external salinity could increase BADH specific activity (Table 10). On the other hand high concentration of NaCl was inhibitory to the activity of BADH (Figure 13). It was likely that the increase in external salinity induced the synthesis of BADH inside *A. halophytica.* It has been reported previously that in leaves and roots of barley plants grown in high salt conditions the BADH mRNA levels increased almost 8-fold and 2-fold, respectively. Barley BADH transcripts also accumulated in response to water stress or drought (Ishitani *et al.* 1995).