first 30 min of labeling and reached maximum at 1 h. After 1 h slight drop of betaine aldehyde occurred in the control cells and much lower betaine aldehyde was detected in salt-stresed cells. If choline-glycinebetaine pathway via betaine aldehyde were to operate in *A. halophytica* the drop in betaine aldehyde would result in the enhanced level of glycinebetaine. This was indeed the case as shown in Fig. 4 B. Slight increase and marked increase of glycinebetaine occurred in control and salt-stressed cells respectively after 3 h. The primary aim of this study was to confirm the existence of the choline → glycinebetaine pathway in *A. halophytica*. The results in Figure 4 clearly established that this pathway was utilized by *A. halophytica*. Furthermore the increased accumulation of glycinebetaine under salt-stressed condition would be due to the increased synthesis by this pathway.

Synthesis of [^{14}C] glycinebetaine from [^{14}C] ethanolamine

When [^{14}C] ethanolamine was incubated with either the control or salt-stressed cells incorporation of radioactivity into [^{14}C] glycinebetaine was readily detectable and seemed to reach maximum after 1 h (Fig. 5). Approximately 4 fold higher level of glycinebetaine synthesis was observed in salt-stressed cells during 1 h incubation. The possible intermediate of ethanolamine → glycinebetaine pathway was shown to be choline in higher plants (50, 51). It was also shown that the potential of both the methylating reaction sequence; i.e., the conversion of ethanolamine to choline and the oxidation sequence; i.e., the conversion of choline to glycinebetaine increased upon salt stress (50, 61). For *A. halophytica*, more refined experiments are needed to determine the effect of salt stress on the methylation of ethanolamine. The experiments must take into account the possibility that the main biosynthetic sequence of the methylation reactions may involve the phosphoryl and the phosphatidyl bases which have been reported in higher plants (51, 62, 63).

Synthesis of [^{14}C] glycinebetaine from [^{14}C] glycine

In the first 30 min following incubation of *A. halophytica* with 40 μM [^{14}C] glycine the salt-stressed cells synthesized about 4-fold more glycinebetaine than the control cells (Fig. 6). Very little synthesis was observed in both control and salt-stressed cells after 30 min. The

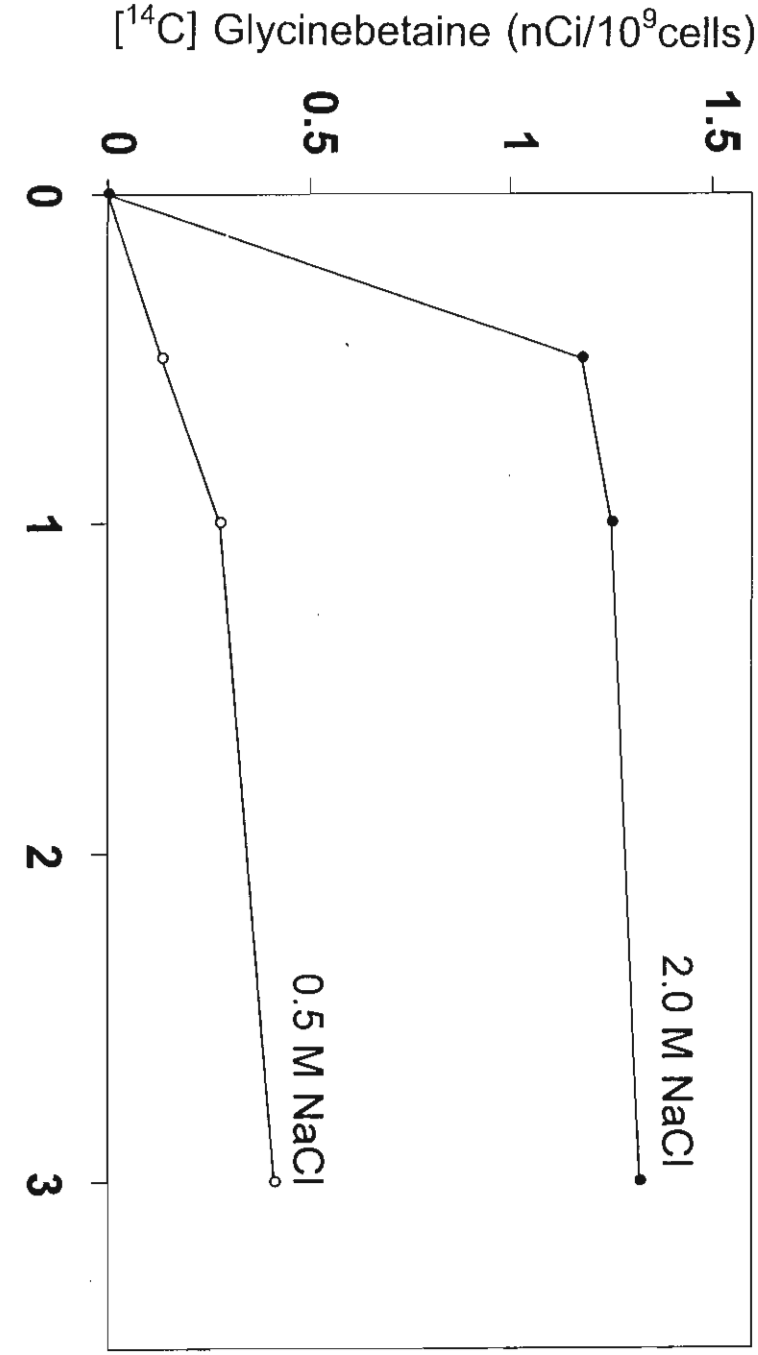


Fig. 5. Time course of the synthesis of [^{14}C] glycinebetaine from [^{14}C] ethanolamine.

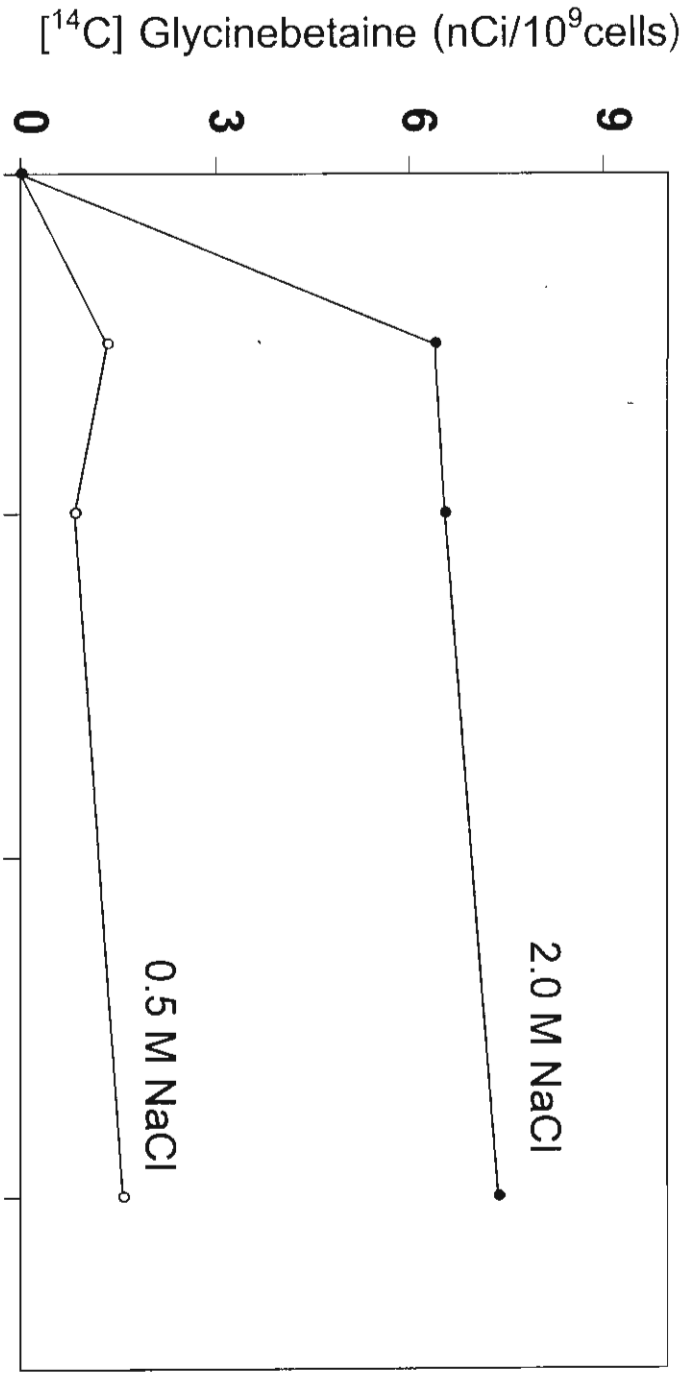


Fig. 6. Time course of the synthesis of [^{14}C] glycinebetaine from [^{14}C] glycine.

halt in glycinebetaine synthesis after 30 min was mainly due to the possibility that [^{14}C] glycine was extensively metabolized to products unrelated to glycinebetaine pathway. There are at least 2 mechanisms by which the 2-carbon moiety of glycinebetaine is derived from glycine. Glycine decarboxylase, one of the enzymes of C-2 glycolate pathway in higher plants, can decarboxylate glycine to form serine which can generate ethanolamine after decarboxylation again. Ethanolamine can then undergo a series of methylation to form choline and then glycinebetaine is formed as a result of the oxidation of choline. In the second mechanism glycinebetaine may be formed by the methylation of glycine. ^{13}C-NMR labeling study has shown that *Methanohalophilus* strain FDF 1 synthesized glycinebetaine by this mechanism (53). Glycine methyltransferase has also been reported in rat liver tissues (52). It should be mentioned here that the operable and functional biosynthetic pathway shoud be supported and substantiated by the presence or the participation of enzymes involved.

Effect of external NaCl concentrations on choline and betaine aldehyde dehydrogenase activities

At the onset of the experiment, the localization of choline and betaine aldehyde dehydrogenase was determined. It was found that the majority (about 80%) of the former localized in the membrane fraction whereas the latter (about 75%) was found in the cytoplasmic fraction. This finding was contradictory to the report by Landfald and Strom (47) where choline and betaine aldehyde dehydrogenase were exclusively localized in membrane and cytoplasmic fraction respectively in *E. coli* cells grown in the presence of choline.

Both choline and betaine aldehyde dehydrogenase activities increased with increasing NaCl concentration (Fig. 7). Detailed analysis showed that salt stress induced about 64% increase in choline dehydrogenase activity and about 125% increase in betaine aldehyde dehydrogenase activity suggesting the more sensitive response towards salt stress for the latter enzyme.

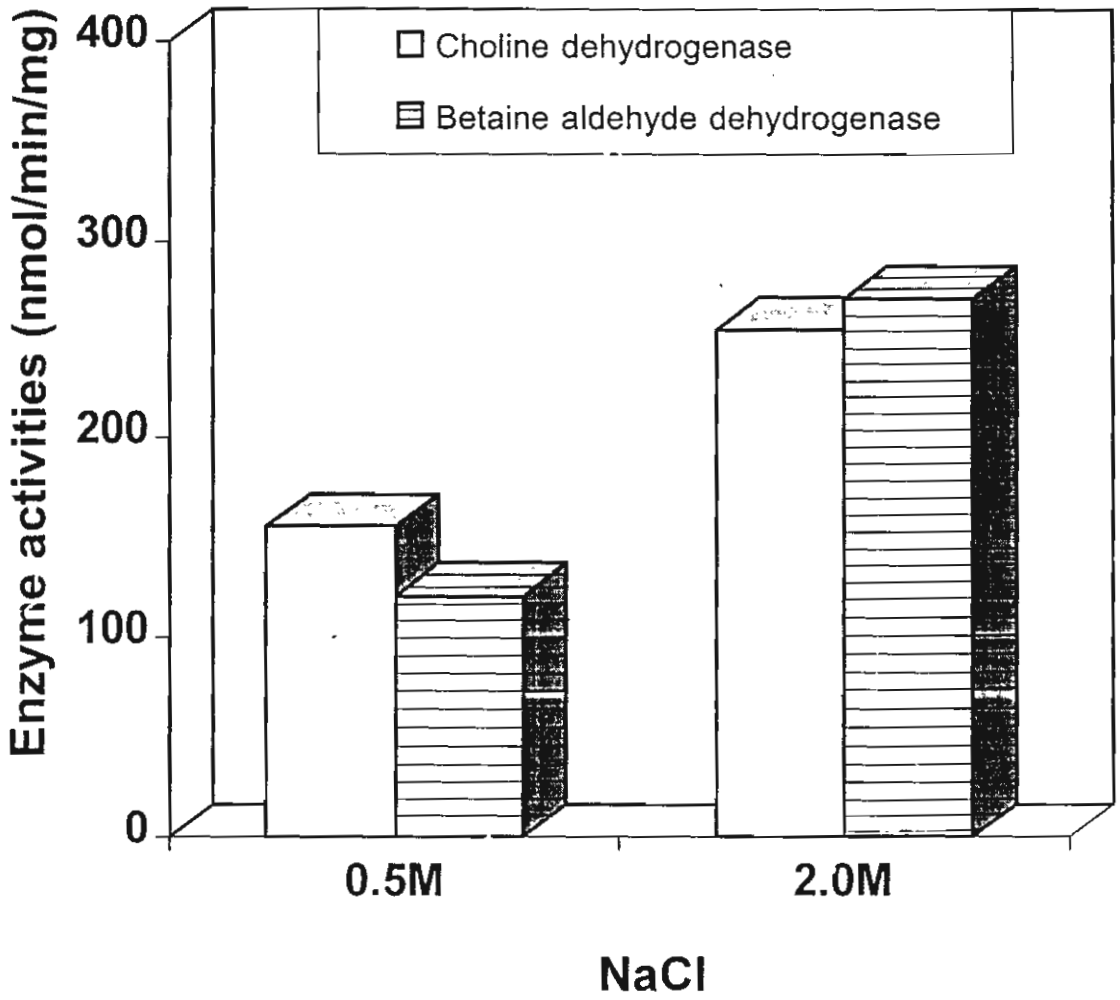


Fig. 7. Effect of external salinity on choline and betaine aldehyde dehydrogenase activities.

CHAPTER 4

Betaine Aldehyde Dehydrogenase From a Halotolerant Cyanobacterium, *Aphanothece halophytica* : Purification, Properties, and Regulation by Salinity

Summary

Betaine aldehyde dehydrogenase (EC 1.2.1.8) was purified from a halotolerant cyanobacterium *Aphanothece halophytica*. Purification was achieved by ammonium sulfate fractionation of lysozyme-disrupted cells, followed by DEAE-cellulose chromatography and hydroxyapatite chromatography. The enzyme was purified about 18-fold with a final specific activity of 298.6 μmol min^{-1} mg^{-1} protein. The enzyme was found to be a tetramer of identical 30 kDa subunits. The optima pH and temperature for the enzyme were 7.5 and 25°C respectively. Both NAD^+ and $NADP^+$ could be used as coenzyme with K_m values of 71.4 μM and 100 μM respectively. The enzyme activity was strongly inhibited by acetaldehyde. N-methylated substrate analogs could also inhibit the enzyme activity and only slight inhibition was observed for glycine betaine. Dithiothreitol enhanced enzyme activity whereas *p*-chloromercuriphenylsulfonate completely abolished the activity. The enzyme was activated by KCl and NaCl at low concentrations up to 0.1 M above which the magnitude of activation was decreased for KCl and the inhibition occurred for NaCl. The elevation of external salinity resulted in the increase of the specific activity of the enzyme.

Introduction

Aphanothece halophytica is a halotolerant cyanobacterium capable of growing at external concentration of up to 3M NaCl (7, 64). The adjustments by *A. halophytica* to counter-balance the high external salinity included the accumulation of inorganic K^+ (65) and an organic quaternary ammonium compound, namely glycine betaine (7). The accumulation of glycine betaine as an osmoregulatory compound was also reported in a marine cyanobacterium, *Spirulina subsalsa* (31). In many bacteria (11, 66, 67), plants (6) and marine animals (9), glycine betaine was also found to accumulate in response to hyperosmotic conditions.

The biosynthesis of glycine betaine in leaves of higher plant has been well studied. Glycine betaine is synthesized by a two step oxidation of choline via the intermediate betaine aldehyde with the aid of choline monooxygenase and betaine aldehyde dehydrogenase (BADH) in spinach chloroplasts (23, 24, 68). In bacteria the transformation of choline into glycine betaine is catalyzed by the membrane-bound enzyme choline dehydrogenase and the cytoplasmic enzyme BADH (47, 49).

Despite the widespread occurrence of glycine betaine its synthesis and osmotic regulation are well understood only in some bacteria (11, 66, 67), certain plant (6) and animal (16, 69) cells. To our knowledge, so far the biosynthesis of glycine betaine has never been reported in cyanobacteria. In the present study, we attempted to partially purify *A. halophytica* BADH and determine its properties as well as its response to hyperosmotic condition.

Materials and Methods

Materials

Aphanothece halophytica was originally isolated from Solar lake in Israel. The organism was kindly provided by Dr. T. Takabe, Nagoya University, Japan. Acrylamide was purchased from Merck. Coomassie brilliant blue was from BDH Laboratory Chemical. Other chemicals were of reagent grade and were from Sigma Chemical.

Organism and growth conditions

Aphanothece halophytica was grown photoautotrophically at 30°C in BG11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution as previously described (29, 64). Cotton-plugged 250 ml conical flasks containing 100 ml of medium each were used and shaken on a rotatory shaker without supplementation of CO_2 gas. The culture flasks were incubated at 30°C with illumination of about 3,000 lux provided by cool white fluorescent lamps.

Purification of betaine aldehyde dehydrogenase

All steps were carried out at 4°C. The enzyme was purified by the following procedure :

i) *Ammonium sulfate precipitation.* The cells at the late log phase (14 days) were first broken by suspending the cell pellet in 50 mM HEPES-KOH pH 7.5 containing 2 mg/ml lysozyme. The suspension was incubated at 37°C for 1 h before centrifugation at 14,000 xg for 20 min. The supernatant was fractionated with ammonium sulfate and the precipitate obtained at 35-70% saturation was dissolved in a small volume of 10 mM Tris-HCl pH 7.5 containing 1 mM DTT and 10% (v/v) glycerol before dialysis against the same buffer.

ii) *DEAE-cellulose column chromatography.* The dialysate from I) was loaded onto DEAE-cellulose column (2.5 x 17 cm) pre-equilibrated with 10 mM Tris-HCl buffer pH 7.5 containing 1 mM DTT and 10% (v/v) glycerol. The proteins were eluted with a linear gradient of 0-1.0 M NaCl prepared in the buffer at a flow rate of 0.5 ml/min. Peak enzyme activity eluted at about 0.25 M NaCl. The fractions (5 ml)

containing enzyme activity were pooled and subjected to further purification by hydroxyapatite column.

iii) *Hydroxyapatite column chromatography.* The pooled fractions from ii) were applied to a hydroxyapatite column (2.0 x 6.5 cm) pre-equilibrated with 10 mM potassium phosphate buffer pH 7.5 containing 1 mM DTT and 10% (v/v) glycerol. The proteins were eluted with a linear gradient of 10 to 200 mM potassium phosphate buffer containing 1 mM DTT and 10% (v/v) glycerol. The fractions of peak enzyme activity were pooled.

Enzyme assay

BADH activity was assayed spectrophotometrically as described by Pan *et al* (56) with slight modification. The final 1 ml reaction mixture contained 50 mM HEPES-KOH buffer pH 7.5, 10 mM DTT, 1 mM EDTA, 0.5 mM NAD^+ , 0.5 mM betaine aldehyde and an enzyme fraction. In case where the effect of various compounds were tested, they were also included in the reaction mixture. The reaction was initiated with the addition of betaine aldehyde. The slope of the increased A_{340} against time during incubation at 25°C was the measured enzyme activity.

Estimation of enzyme molecular weight

The molecular weight of native BADH was estimated by gel filtration on a Sephadex G-200 column (1.8 x 105 cm) in 10 mM Tris-HCl pH 7.5 and 1 mM DTT using thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), acid phosphatase (95 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), chymotrypsinogen A (23.2 kDa) and cytochrome c (12.3 kDa) as standards.

The molecular weight of BADH in the denatured state was determined by SDS-polyacrylamide gel electrophoresis on a 10% slab gel according to Laemmli (19). The standard proteins used were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14.4 kDa).

Other methods

The protein content was determined by a sensitive dye-binding method according to Bradford (57). The content of protein eluted from the column was monitored by measuring A_{280}. The values in the figures and tables represent the mean of 2 independent experiments.

Results

Partial purification of BADH

The results of a typical partial purification of BADH from a 10 g wet weight of *A. halophytica* is shown in Table 1. The enzyme was purified 18-fold, giving a preparation with a specific activity of 298.6 μmol min^{-1} mg^{-1}. The enzyme was essentially pure after DEAE-cellulose step, giving one single band by SDS gel electrophoresis (Fig. 1). The next step by hydroxyapatite chromatography gave no further increase in specific activity and resulted in about 3-fold loss of the yield of the enzyme. The molecular weight of the enzyme as estimated from gel filtration was 115 kDa (Fig. 2A). By the plot of the relative electrophoretic mobilities of six standard proteins and BADH versus molecular weight (Fig. 2B), the monomeric subunit molecular weight was found to be 30 kDa. The data obtained from molecular weight determinations suggest that *A. halophytica* BADH is most likely a tetramer of 30 kDa subunits.

Properties of BADH

Effects of pH and temperature on BADH activity

As shown in Fig. 3A, the activity of BADH increased sharply with the increase in pH from 6 to 7.5. Above pH 7.5 the activity decreased markedly until pH 8 and slightly decreased at pH 9. A similarly narrow range of temperature optimum for the activity was also observed (Fig. 3B). The BADH activity increased sharply from 20°C to 25°C above which a sharp decline of activity was apparent and only about 20% of maximum activity was retained at 37°C.

Enzyme kinetics

Initial reaction velocities were measured with respect to various concentrations of betaine aldehyde, NAD^+ and $NADP^+$ at either fixed concentration of NAD^+ or betaine aldehyde. The results were

Table 1. Purification of betaine aldehyde dehydrogenase from *Aphanothece halophytica*

Purification step	Total protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Yield (%)	Purification (fold)
Crude extract	420.3	6,923	16.5	100	1.0
35-70% $(NH_4)_2SO_4$	157.9	4,848	30.7	70	1.8
DEAE-cellulose	2.1	596	290.8	8.6	17.6
Hydroxyapatite	0.6	192	298.6	2.8	18.1

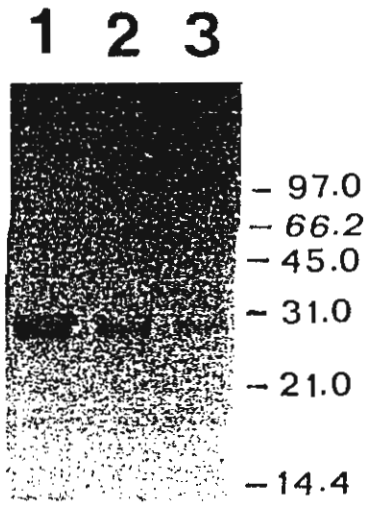
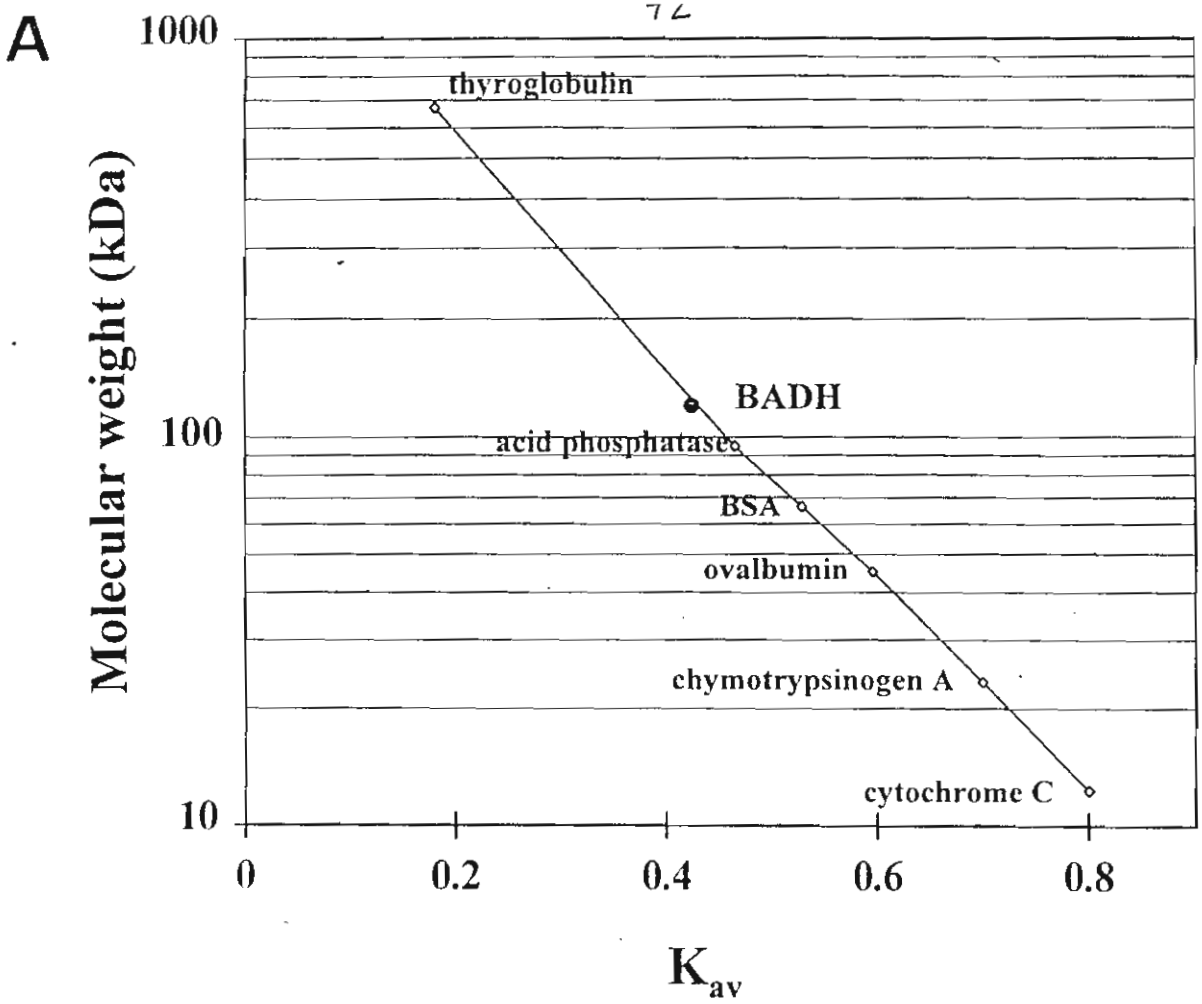


Fig. 1. SDS-polyacrylamide gel electrophoresis of purified *Aphanothece halophytica* betaine aldehyde dehydrogenase on a 10% slab gel. Lanes 1, 2 and 3 were 5, 2.5 and 1.25 μg of purified enzyme after DEAE-cellulose step. Numbers on the right indicate standard molecular weight markers in kilodalton.



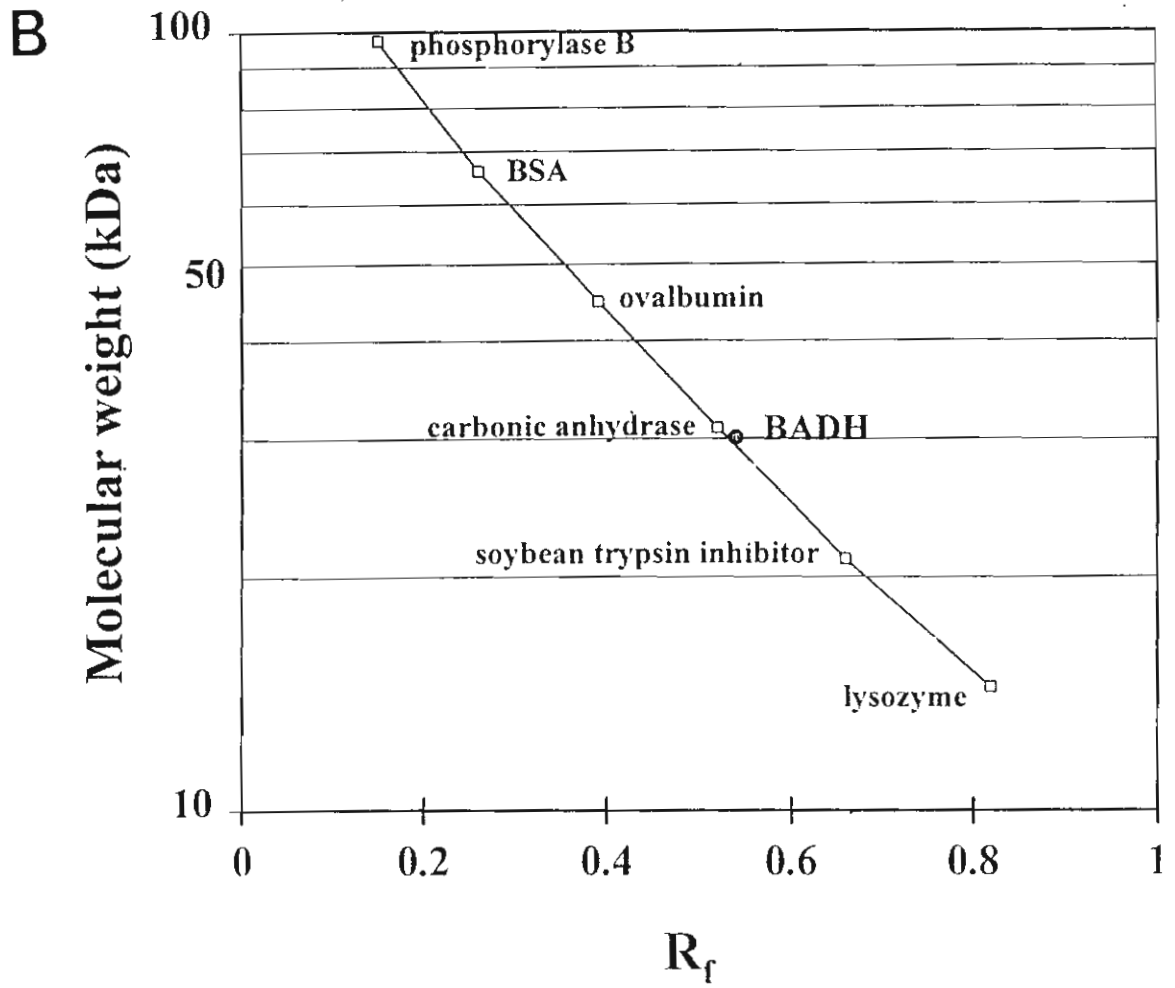


Fig. 2. Estimation of native (A) and subunit (B) molecular weight of betaine aldehyde dehydrogenase from *Aphanothece halophytica* by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively.

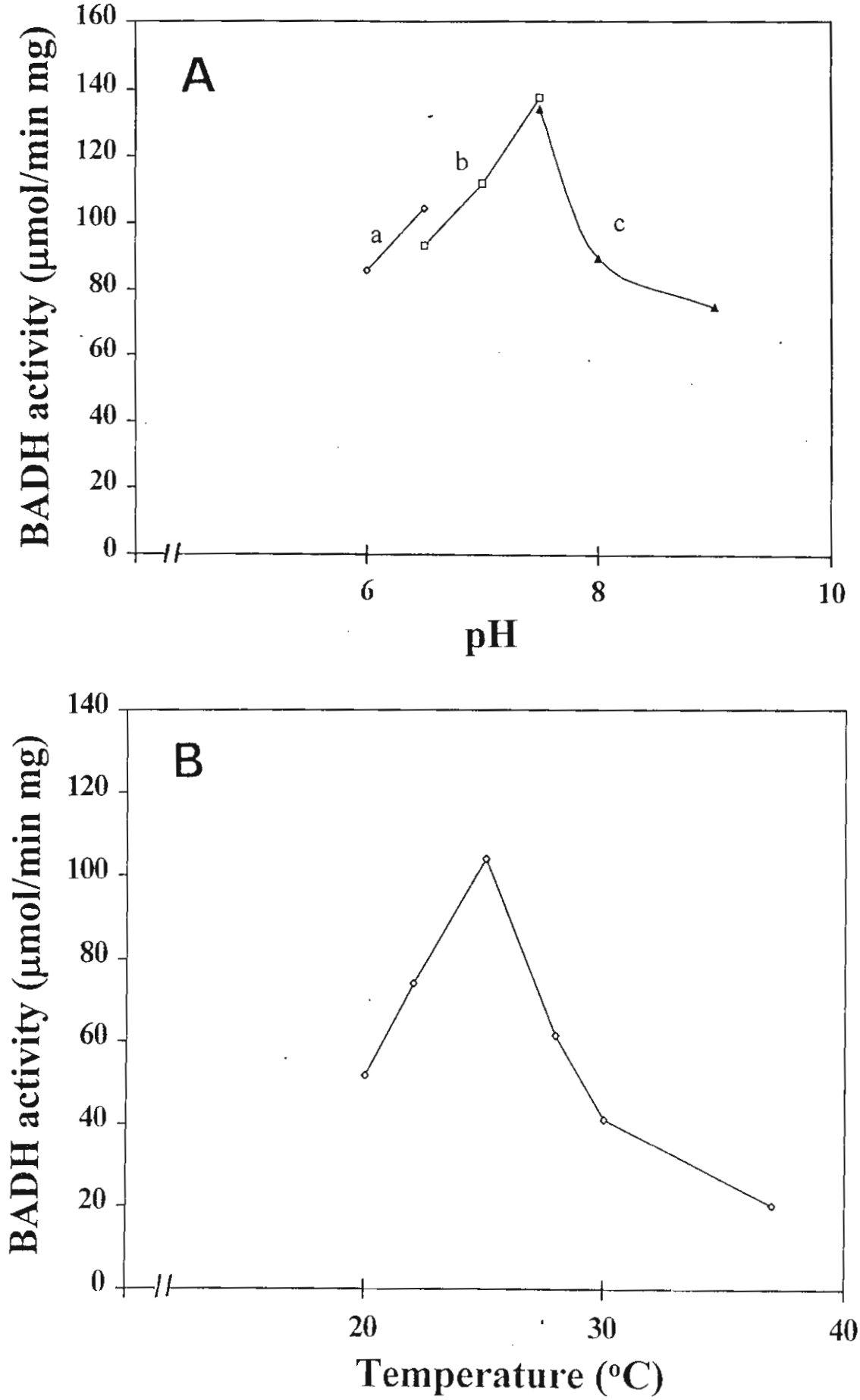
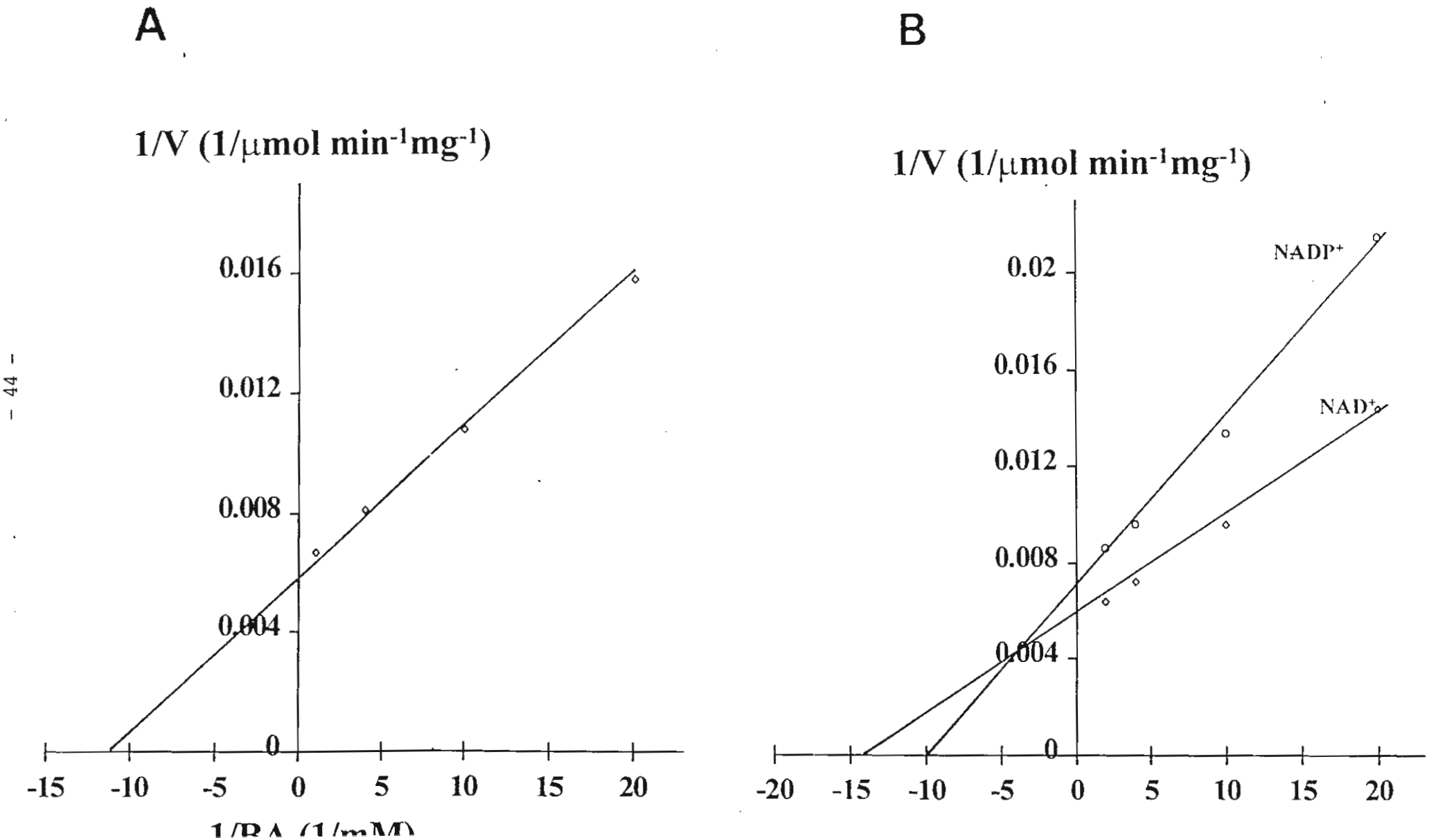


Fig. 3. Effect of pH (A) and temperature (B) on *Aphanothece halophytica* betaine aldehyde dehydrogenase activity. The buffer systems used in (A) were a) 50 mM potassium phosphate buffer, b) 50 mM HEPES-KOH buffer and c) 50 mM Tris-HCl buffer.

Fig. 4. Double reciprocal plots of activity of betaine aldehyde dehydrogenase against substrate concentration. (A) Betaine aldehyde (BA) as variable substrate at fixed 0.5 mM NAD^+. (B) NAD^+ or $NADP^+$ as variable substrate at fixed 0.5 mM betaine aldehyde.



presented in the form of Lineweaver Burk plots as shown in Fig. 4. The apparent Michaelis constants (K_m) were estimated to be 91 μM for betaine aldehyde (Fig. 4A) and 71.4 μM and 100 μM for NAD^+ and $NADP^+$ respectively (Fig. 4B). The maximal reaction velocities (Vmax) were found to be 175.4 and 138.8 μmol min^{-1} mg^{-1} when NAD^+ and $NADP^+$ were used as coenzyme respectively.

Effect of substrate-analogs on BADH activity

Four compounds bearing resemblance to betaine aldehyde substrate were tested for inhibitory effect on BADH activity. Table 2 shows that acetaldehyde was a very potent inhibitor, the BADH almost completely lost its activity in the presence of acetaldehyde. The other three compounds which were N-methylated substrate analogs showed different degree of inhibition towards BADH activity. Ethanolamine was the most effective inhibitor whereas glycine betaine, the product of the BADH-catalyzed reaction, was the least effective inhibitor.

Effect of sulfhydryl-reactive reagents on BADH activity

Sulfhydryl-reactive reagents that were used in this study were DTT and PCMS. BADH activity was strongly enhanced by DTT and severely inhibited by PCMS (Table 3). The inhibitory effect of PCMS was mostly relieved when the enzyme was preincubated with DTT. The inhibition by PCMS could be reversed about one half by the subsequent addition of DTT. Taken together, the overall results appeared to indicate that one or more sulfhydryl group at the enzyme catalytic site is essential for enzyme activity.

Effect of salts on BADH activity

Four different types of salts with respect to cations were tested for their effects on BADH activity. The control (no salt) contained 50 mM Tris-HCl pH 7.5 instead of 50 mM HEPES-KOH for buffering capacity. Salts with monovalent cations, i.e., Na^+ and K^+ at low concentrations gave an increase in enzyme activity (Fig. 5). K^+ concentration higher than 0.1 M reduced the magnitude of enzyme activation and the enzyme showed no response to K^+ at or higher than 1 M K^+. Elevated Na^+ at or higher than 0.25 M inhibited enzyme activity. Divalent cations were very inhibitory even at low concentrations. A 50%

Table 2. Inhibition of betaine aldehyde dehydrogenase by analogs of betaine aldehyde

Substrate analog at 100 mM	Remaining activity (%)
Glycine betaine	88.5
Choline	77.1
Ethanolamine	27.0
Acetaldehyde	0.9

The activity of the enzyme without substrate analog was 96.7 μmol min^{-1} mg^{-1} and was taken as 100%.

Table 3. Effect of dithiothreitol (DTT) and *p*-chloromercuriphenylsulfonate (PCMS) on the activity of betaine aldehyde dehydrogenase

Treatment	Relative activity (%)
Control	100
5 mM DTT (30 min)	274.2
0.1 mM PCMS (10 min)	0
5 mM DTT (30 min), then 0.1 mM PCMS (30 min)	90.3
0.1 mM PCMS (10 min), then 5 mM DTT (30 min)	51.6

The specific activity of the enzyme without DTT (control) was 57.8 μmol min^{-1} mg^{-1}.

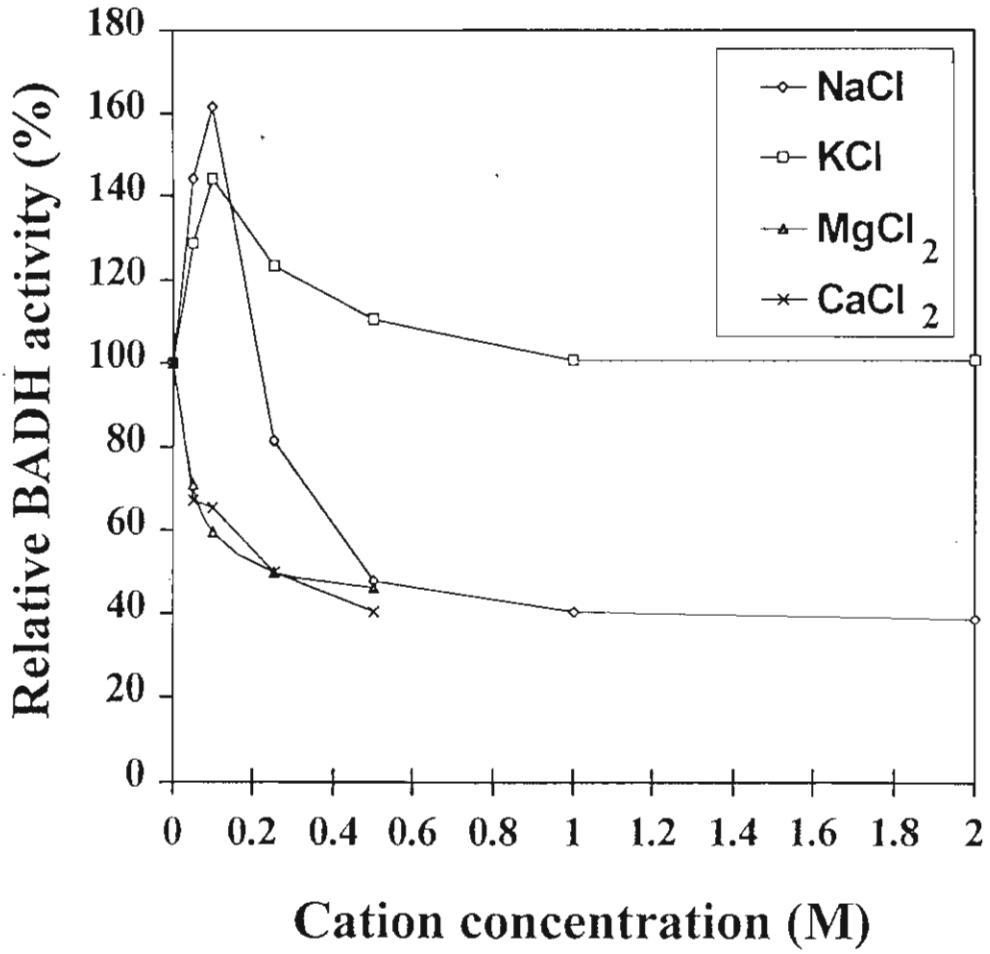


Fig. 5. Effect of salts on the relative activity of betaine aldehyde dehydrogenase from *Aphanothece halophytica*.

reduction in enzyme activity occurred in the presence of 0.25 M Mg^{2+} or Ca^{2+}.

Effect of external salinity on BADH activity

A. halophytica cells were either grown in the medium containing 0.5 M or 2.0 M NaCl. The cell extracts were subjected to 35-70% ammonium sulfate precipitation before being used for BADH assay. The activity of BADH obtained from cells grown in 2 M NaCl was about 4-fold of that from cells grown in 0.5 M NaCl (Table 4). The result appeared to be in line with the previous finding that showed the increase of glycine betaine level about 4 fold when *A. halophytica* cells were grown in 2 M NaCl as compared to those grown in 0.5 M NaCl (75).

Table 4. Betaine aldehyde dehydrogenase activity from *Aphanothece halophytica* grown in different salinities

NaCl in growth medium (M)	Enzyme activity (μmol min^{-1} mg^{-1})
0.5	1.03
2.0	4.43

Discussion

In the present study we attempted to purify the enzyme which is responsible for the synthesis of glycine betaine. Preliminary experiment by which the cells were broken by sonication failed to retrieve the activity of BADH. However, when we used lysozyme to break the cells we were able to detect the presence of BADH. An essentially pure enzyme preparation was obtained after DEAE-cellulose chromatography. The specific activity of BADH in the present study was somewhat similar to that reported for BADH of horseshoe crab (69) and about one and two orders of magnitude higher than BADH from spinach and *E. coli* respectively (56, 71).

The gel filtration and SDS-PAGE experiments indicated that BADH of *A. halophytica* appeared to be a tetramer with identical 30 kDa subunits. Previous reports on BADH molecular weight and subunit from different organisms were quite variable. BADH of spinach is a dimer with subunits of 60 (72) and 63 (55) kDa. The tetramers of subunits of 55 (71) and 58 (73) kDA were reported for BADH of *E. coli* and *C. didymum* respectively. Taken together it appears that no typical prokaryote or eukaryote BADH exists with respect to native molecular weight and subunit composition.

The BADH from *A. halophytica* preferred NAD^+ (K_m value of 71.4 μM) as a coenzyme, but could also use $NADP^+$ (K_m value of 100 μM). The specificity of BADH with regard to coenzyme is similarly observed for BADH from other sources (55, 56, 69, 71). So far only BADH from *P. aeruginosa* A-16 has been reported to prefer $NADP^+$ to NAD^+ as a coenzyme (74). *A. halophytica* BADH showed a relatively narrow pH optimum of 7.5 similar to that of BADH from horseshoe crab (69). For *E. coli* and spinach the BADH had broader pH optima spanning 2 pH units (55, 71). The V_{max} of *A. halophytica* BADH (175.4 μmol min^{-1} mg^{-1}) in the present study was highest when compared to other species, i.e., more than 100 -fold higher than that for spinach (55, 56) and about 3-times higher than the V_{max} for *E. coli* BADH (71). This suggests that *A. halophytica* is very efficient in converting betaine aldehyde to glycine betaine especially when the organism is under hyperosmotic environments.

The BADH from *A. halophytica* had a relatively high affinity for betaine aldehyde. The K_m value of 91 μM was the lowest

among other species studied so far (55, 56, 69, 71, 74). The analogs of betaine aldehyde could inhibit BADH activity with varying degree of inhibition (Table 2). Aldehyde compound such as acetaldehyde was a strong inhibitor whereas N-methylated compounds such as glycine betaine, choline and ethanolamine showed lesser inhibition than the aldehyde compound. This suggested that the aldehyde functional group of the substrate might play an important role in binding to the catalytic site of BADH. The fact that glycine betaine which is the product of BADH reaction only slightly inhibits BADH may have physiological relevance since this metabolite accumulates in *A. halophytica* at a very high level in response to salt stress (75).

One purpose of the present investigation is to study the response of BADH towards various salts with respect to the cations. It is clear that *A. halophytica* BADH is a salt tolerant enzyme (Fig. 5). Both K^+ and Na^+ activated the enzyme at low concentrations. Even at 1.0 or 2.0 M Na^+ about 40% of the original activity was retained. As for K^+, no inhibition of BADH activity was observed. This phenomenon could well explain why the accumulation of glycine betaine still occurred despite the initial massive accumulation of K^+ inside *A. halophytica* under hyperosmotic condition (7). Although both Mg^{2+} and Ca^{2+} could inhibit BADH activity in *A. halophytica*, the effect of these 2 cations on the synthesis of glycine betaine would be minimal due to their low concentrations inside the cells. It is noted that BADH from horseshoe crab could be activated by 2 fold in the presence of as low as 0.75 μM Ca^{2+} (69).

The observed salt-induced rise in *A. halophytica* BADH specific activity (Table 4) is a good circumstantial evidence in support of the existence of the choline --> betaine aldehyde --> glycine betaine pathway. The extent of the increase of BADH activity was in good agreement with the increase in the content of glycine betaine (75). Previous report by Ishitani *et al* (76) has shown that BADH mRNA of barley leaves increased 8-fold under salt stress and the level decreased upon release of the stress. Future experiments are needed to examine whether the increase of BADH activity in *A. halophytica* under salt stress is caused by an increase of BADH transcripts.

CHAPTER 5

General Discussion and Conclusion

Osmoregulation is the term used to refer to active processes carried out by living organisms during adaptation to the osmotic strength of the environment. Generally osmoregulatory phenomena are categorized into 2 types : 1) long-term or steady-state responses that are manifested during the growth of organisms at a constant osmolarity and 2) short-term or transient responses that occur soon after changes in the external osmolarity. Most studies, including the present investigation, have involved sudden shifts in osmolarity; i.e., the short-term responses. The knowledge of the osmoregulatory process occurring in different types of organisms can be beneficial for molecular geneticists as well as plant breeders to construct plant cells, especially those with economic importance, that have traits conferring salinity or drought tolerance. To reach this end basic studies on the molecular mechanism of osmoregulation at the levels of metabolism, enzymes and genes involved, are essential.

The use of cyanobacteria as a model organism is advantageous in that they are prokaryotes evolving themselves between prokaryotic bacteria and eukaryotic higher plants. The information obtained using cyanobacteria should well serve as a link between bacteria and higher plants which may be useful for comparative studies especially those related to evolution process. One such example is the endosymbiont hypothesis which states that cyanobacteria are the most likely ancestor of chloroplasts (77).

The key word for the present investigation is a compatible solute glycinebetaine which was firmly established as a major osmoticum inside *A. halophytica*. It is important that a suitable assay method must first be established for the determination of glycinebetaine. Both the ^{1}H-NMR spectrometry and the tri-iodide method were proved equally satisfactory for the determination of glycinebetaine. However each of these 2 methods has advantages and disadvantages. The former is sensitive but rather costly, not to mention the equipment cost. The tri-iodide method is much less expensive and requires only a simple spectrophotometer. Extra work is needed, however, to separate glycinebetaine from other quaternary ammonium compounds. This can

be accomplished by a small cation exchange column, i.e., a Dowex-50 from Sigma.

The finding that intracellular glycinebetaine was increased in only salt-stressed cells by $NaNO_3$ and light was interesting. The explanation for the observed phenomena may not be obvious at present. However, it remains an intriguing question as to the effect of light on an internal level of N-containing compounds especially $NaNO_3$ for both control and salt-stressed *A. halophytica.*

The major emphasis of the present investigation lies on the elucidation of the metabolic pathway of glycinebetaine synthesis. A number of problems have been encountered during the course of investigation. Initially the technique of paper chromatography was employed to separate the precursor from the intermediates and products. However, later we found that the descending paper chromatographic technique using n-butanol :ethanol : acetic acid : H_2O = 8 : 2 : 1 : 3 (v/v) was not reproducible and that the radioactivity measurement by the radioscanner machine was not sensitive enough. The problems were rectified by using the cation exchange column (Dowex-50 from Sigma) which gave clear separation and complete recovery of precursor, intermediates and products. The separated compounds containing incorporated radioactivity could be conveniently determined by liquid scintillation spectrometry.

Another point worth mentioning here is that in the preliminary radiotracer experiments we labeled the cells with appropriate precursors, i.e., [^{14}C] choline, [^{14}C] ethanolamine and [^{14}C] glycine using growth medium as incubation medium during labeling. We found that the uptake of radioactive precursor by the cells was variable and quite low for [^{14}C] ethanolamine and [^{14}C] glycine. After slight modification by replacing the growth medium as incubation medium by the aqueous buffer pH 7.5, the uptake problem was finally solved.

At this stage it appears certain that the synthesis of glycinebetaine in both control and salt-stressed *A. halophytica* is as follows : choline → betaine aldehyde → glycinebetaine. However, more work is needed in order to ascertain whether the increased synthetic rate can account for the observed increase of glycinebetaine content due to salt stress. Such work will involve the estimation of the internal pool of various metabolites and possibly the fate of glycinebetaine, i.e., can it be degraded? Equally interesting is the question whether the increased synthesis of glycinebetaine is due to salinity or osmotic effect. The

increase of glycinebetaine content has already been shown to be due to the osmotic effect.

The possibility that ethanolamine and glycine can serve as precursors for glycinebetaine synthesis is briefly presented in this study. There are more rooms to further explore this ethanolamine or glycine-derived pathway for glycinebetaine. Some examples are the series of methylation reactions from ethanolamine to choline, the involvement of free or phosphoryl or phosphatidyl form of ethanolamine and choline. Most importantly is the study on the route by which 2-carbon moiety of glycinebetaine can derive from glycine. Direct methylation of glycine to glycinebetaine has never experimentally been reported.

The investigation on the betaine aldehyde dehydrogenase was also not without problems. The main and serious problem was the unstable nature of the enzyme. It was crucial that the extraction buffer contained high content of glycerol (10%, v/v) as well as 1 mM DTT. Furthermore we had to use a very mild and gentle method of breaking the cells by the use of lysozyme. The purified enzyme from *A. halophytica* possessed properties both similar to and different from enzymes from other sources. One distinct property is its molecular structure of a tetramer of 30 kDa subunits. Also the enzyme from *A. halophytica* had higher V_{max} than those from higher plant and bacteria which may suggest that cyanobacteria are more efficient organisms to accumulate glycinebetaine and can better withstand high salinity stress.

In conclusion the investigation on the osmoregulation with respect to the synthesis of glycinebetaine has provided some important information. Nevertheless there are some points that require further experimentation so that a full description of the biosynthesis of glycinebetaine in *A. halophytica* can emerge.

References

1. Erber, W.W.A., Nitschmann, W.H., Muchl, R. and Peschek, G.A. (1986) Arch. Biochem. Biophys. 247, 28-39.
2. Fry, I.V., Huflejt, M., Erber, W.W.A., Peschek, G.A. and Packer, L. (1986) Arch. Biochem. Biophys. 244, 686-691.
3. Reed, R.H., Borowitzka, L.J., Mackary, M.A., Chudek, J.A., Foster, R., Warr, S.R.E., Moore, D.J. and Stewart, W.D.P. (1986) FEMS Microbiol. Rev. 39, 51-56.
4. Le Rudulier, D. and Bernard, T. (1986) FEMS Microbiol. Rev. 39, 67-72.
5. Riou, N. and Le Rudulier, D. (1990) J. Gen. Microbiol. 136, 1455-1461.
6. Rhodes, D. and Hanson. A.D. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 357-384.
7. Reed, R.H., Chudek, J.A., Foster, R. and Stewart, W.D.P. (1984) Arch. Microbiol. 138, 333-337.
8. Mackay, M.A., Norton, R.S. and Borowitzka, L.J. (1984) J. Gen. Microbiol. 130, 2177-2191.
9. Yancey, P.H., Clark, M.E., Hand, S.E., Bowlus, R.D. and Somero, G.N. (1982) Science 217, 1214-1222.
10. Andresen, P.A., Kaasen, I., Styrvold, O.B., Boulnois, G. and Strom, A.R. (1988) J. Gen. Microbiol. 134, 1737-1746.
11. Smith, L.T., Pocard, J.A., Bernard, T. and Le Rudulier, D. (1988) J. Bacteriol. 170, 3142-3149.
12. Galinski, E.A. and Truper, H.G. (1982) FEMS Microbiol. Lett. 13, 357-360.
13. Robertson, D.E., Noll, D., Roberts, M.F., Menaia, J.A.G.F. and Boone, D.R. (1990) Appl. Environ. Microbial, 56, 563-565.
14. Weretilnyk, E.A., Bednarek, S., McCue, K.F., Rhodes, D. and Hanson. A.D. (1989) Planta 178, 342-352.
15. Bagnasco, S., Balaban, R., Fales, H.M., Yang, Y.M. and Burg, M. (1986) J. Bio. Chem. 261, 5872-5877.
16. Dragolovich, J. (1994) J. Expt. Zool. 268, 139-144.
17. Byerrum, R.U., Sato, C.S. and Ball, C.D. (1956) Plant Physiol. 31, 374-377.
18. Skiba, W.E., Taylor, M.P., Wells , M.S., Mangum, J.H. and Awad, W.M.J. (1982) J. Biol. Chem. 257, 14944-14948.
19. White, R.F. and Demain, A.L. (1971) Biochim. Biophys. Acta 237, 112-119.

20. Korstee, G.J.J. (1970) Arch. Mikrobiol. 71, 235-244.
21. Incharoensakdi, A., Takabe, T. and Akazawa, T. (1986) Plant Physial. 81, 1044-1049.
22. Warr, S.R.C., Reed, R.H. and Stewart, W.D.P. (1984) J. Gen. Microbiol. 130, 2169-2175.
23. Weigel, P., Weretilnyk, E.A. and Hanson, A.D. (1986) Plant Physiol. 82, 753-759.
24. Weigel, P., Lerma, C. and Hanson, A.D. (1988) Plant Physiol. 86, 54-60.
25. Reed, R.H. (1988) Meth. Enzymol. 167, 528-534.
26. Jones, G.P., Naidu, B.P., Starr, R.K. and Paleg, L.G. (1986) Aust. J. Plant Physiol. 13, 649-658.
27. Storey, R. and Wyn Jones, R.G. (1977) Phytochem. 16, 447-453.
28. Blumwald, E. and Tel-Or, E. (1984) Plant Physiol. 74, 183-185.
29. Takabe, T., Incharoensakdi, A., Arakawa, K. and Yokota, S. (1988) Plant Physiol. 88, 1120-1124.
30. Wall, J.S., Christianson, D.D., Dimler, R.J. and Senti, F.R. (1960) Anal. Chem. 37, 870-874.
31. Gabbay-Azaria, R., Tel-Or, E. and Schonfeld, M. (1988) Arch. Biochem. Biophys. 264, 333-339.
32. Incharoensakdi, A. and Takabe, T. (1988) Plant Cell Physiol. 29, 1073-1075.
33. Singh, N.K., Handa, A.K., Hasegawa, P.M. and Bresan, R.A. (1985) Plant Physiol. 79, 126-137.
34. Csonka, L.N. and Hanson, A.D. (1991) Annu. Rev. Microbiol 45, 569-606.
35. Le Rudulier, D., Strom, A.R., Dandekar, A.M., Smith, L.T. and Valentine R.C. (1984) Science. 224, 1064-1068.
36. Itai, C. and Paleg, L.G. (1982) Plant Sci. Leth. 25, 329-335.
37. Lone, M.I., Kueh, J.S.H., Wyn Jones, R.G. and Bright, S.W.J. (1987) J. Exp. Bot. 38, 479-490.
38. Flowers, T.J., Hall, J.L. and Ward, M.E. (1978) Ann. Bot. 42, 1065-1074.
39. Manetas, Y., Petropoulou, Y., Karabourniotis, G. (1986) Plant Cell Envir. 9, 145-151.
40. Matoh, T., Yasuoka, S., Ishikawa, T. and Takahashi. E. (1988) Physiol. Plant. 74, 675-678.
41. Schwab, K.B. and Gaff. D.F. (1990) J. Plant Physiol. 137, 208-215.
42. Lerma, C., Hanson, A.D. and Rhodes. D. (1988) Plant Physiol. 88, 695-702.

43. Weretilnyk, E.A., Bednarek, S., Mc Cue, K. F., Rhodes. D. and Hanson, A.D. (1989) Planta 178, 342-352.
44. Brouquisse, R., Weigel. P., Rhodes, D., Yocum, C.F. and Hanson, A.D. (1989) Plant Physiol. 90, 322-329.
45. Lin, C.S. and Wu, R.D. (1986) J. Prot. Chem. 5, 193-200.
46. Ameyama, M., Shinagawa, E., Matsushita, K., Takimoto, K., Nakashima, K. and Adachi, O. (1985) Agri. Biol. Chem. 49, 3623-3627.
47. Landfald, B. and Strom, A.R. (1986) J. Bacteriol. 165, 849-855.
48. Russell, R. and Scopes, R.K. (1994) Bioseparation 4, 279-284.
49. Choquet, C.G., Ahonkai, I., Klein, M. and Kushner, D.J. (1991) Arch. Microbiol. 155, 153-158.
50. Hanson, A.D. and Scot, N.A. (1980) Plant Physiol. 66, 342-348.
51. Conghlan, S.J. and Wyn Jones, R.G. (1982) Planta 154, 6-17.
52. Konishi, K. and Fujioka, M. (1987) Biochemistry 26, 8496-8502.
53. Roberts, M.F., Lai, M-C. and Gunsalus, R.P. (1992) J. Bacteriol. 174, 6688-6693.
54. Fan, L.L. and Masters, B.S.S. (1974) Arch. Biochem. Biophys. 165, 665-671.
55. Weretilnyk, E.A. and Hanson, A.D. (1989) Arch, Biochem. Biophys. 271, 56-63.
56. Pan, S.M., Moreau, R.A., Yu, C. and Huang, A.H.C. (1981) Plant Physiol. 67, 1105-1108.
57. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
58. Reed, R.H., Richardson, D.L. and Stewast, W.D.P. (1985) Biochem. Biophys. Acta. 814, 347-355.
59. Lamark, T., Styrvold, O.B. and Strom, A.R. (1992) FEMS Microbiol. Lett. 96, 149-154.
60. Hanson, A.D. and Hitz, W.D. (1982) Annu. Rev. Plant Physiol. 33, 163-203.
61. Hanson, A.D. and Nelson, C.E. (1978) Plant Physiol. 62, 305-312.
62. Hanson, A.D. and Rhodes, D. (1983) Plant Physiol. 71, 692-700.
63. Hitz, W.D., Rhodes, D. and Hanson, A.D. (1981) Plant Physiol. 68, 814-822.
64. Garlic, S., Oren, A. and Padan, A. (1977) J. Bacteriol. 129, 623-629.
65. Miller, D.M., Jones, J.H., Yopp, J.H., Tindall, D.R. and Schmid, W.E. (1976) Arch. Microbiol. 111, 145-149.
66. Imhoff, J.F. and Rodriguez-Valera, F. (1984) J. Bacteriol. 160, 478-479.

67. Strom, A.R., Falkenberg, P.and Landfald, B. (1986) FEMS Microbiol. Rev. 39, 79-86.
68. Hanson, A.D., May, A.M, Grumet, R., Bode, J., Jamieson, G.C. and Rhodes, D. (1988) Proc. Natl. Acad. Sci. 82, 3678-3682.
69. Dragolovich, J. and Pierce, S.K. (1994) J. Expt. Zool. 270, 417-425.
70. Laemmli, U.K. (1970) Nature 227, 680-685.
71. Falkenberg, P. and Strom, A.R. (1990) Biochimica et Biophysica Acta 1034, 253-259.
72. Arakawa, K., Takabe, T., Sugiyama, T. and Akazawa, T. (1987) J. Biochem. 101, 1485-1488.
73. Mori, N., Kawakami, B., Hyakutome, K., Tani, Y. and Yamada, H. (1980) Agric. Biol. Chem. 44, 3015-3016.
74. Nagasawa, T., Kawabata, Y., Tani, Y. and Ogata, K. (1976) Argic. Biol. Chem. 40, 1743-1749.
75. Incharoensakdi, A. and Kum-arb, U. (1996) Proceedings of the Second Thai-French Symposium on Plant Molecular Biology, Bordeaux, France (October 6-10), pp. 282-292.
76. Ishitani, M., Nakamura, T., Han, S.Y. and Takabe, T. (1995) Plant Mol. Biol. 27, 307-315.
77. Margulis, L, (1970) Origin of eukaryotic cells. Yale University Press, New Haven.

Output

Proceedings

1. Incharoensakdi, A. and Kum-arb, U. (1996) Osmoregulation in a halophilic cyanobacterium, *Aphanothece halophytica.* In "Proceedings of the 2nd Thai-French Symposium on Plant Molecular Biology", Bordeaux, France, October 6-10, 1996, (Bove, J.M., ed.) pp. 283-292.

2. Incharoensakdi, A. and Kum-arb, U. and Wutipraditkul, N. (1997) Biosynthesis and accumulation of glycinebetaine confer salt tolerance in the cyanobacterium, , *Aphanothece halophytica.* In "Proceedings of Chulalongkorn University 80th Anniversary Research Conference", Chulalongkorn University, October 15-17, 1997, pp. 677-684.

Publications

1. Kum-arb, U. and Incharoensakdi, A. "Betaine aldehyde dehydrogenase from a halotolerant cyanobacterium *Aphanothece halophytica* : Purification, properties and regulation by salinity". (Submitted to J. Sci. Soc. Thailand)

2. Incharoensakdi, A. and Wutipraditkul, N. "Accumulation of glycinebetaine and its synthesis from radioactive precursors in salt-stressed cyanobacterium *Aphanothece halophytica*". (Submitted to J. Appl. Phycology)

3. Wutipraditkul, N., Kum-arb, U. and Incharoensakdi, A. "Factors affecting the accumulation of glycinebetaine in a halophilic cyanobacterium, *Aphanothece halophytica*". [Submitted to J. Sci. Res. (Chula. Univ.)]

Publication (Not related to the project)

1. Incharoensakdi, A. and Kitjaharn, P. (1998) Removal of lead from aqueous solution by filamentous cyanobacterium, *Spirulina platensis.* J. Sci. Res. (Chula. Univ.) 23, 37-44.

Appendix 1

Growth Medium for *A halophytica*

1). Turks Island Salt Solution

A solution of 5 l (made with distilled water) of Turks Island Salt Solution consists of the following components :

A). KCl	3.33 g
B). $MgCl_2 . 6H_2O$	2.75 g
C). $CaCl_2 . 2H_2O$	7.33 g
D). $MgSO_4 . 7H_2O$	34.70 g
E). NaCl	140.80 g

2). BG 11 plus NO_3 solution

The components of BG 11 plus NO_3 solution are as follows :

A). $NaNO_3$	150.0 g/l
B). KH_2PO_4	4.0 g/l
C). $MgSO_4 . 7H_2O$	75.0 g/l
D). $CaCl_2 . 2H_2O$	36.0 g/l
E). Na_2CO_3	20.0 g/l
F). Citric acid	6.0 g/l
G). EDTA . Na_2	1.0 g/l
H). $FeSO_4 . 7H_2O$	6.0 g/l

I). Trace element A_5 solution consisting of the following components in 1 l solution

H_3BO_3	2.68 g
$MnCl_2 . 4H_2O$	1.81 g
$ZnSO_4 . 7H_2O$	0.22 g
$Na_2MoO_4 . 2H_2O$	0.39 g
$CuSO_4 . 5H_2O$	0.079 g
$Co(NO_3)_2 . 6H_2O$	0.049 g

The growth medium is prepared by adding 50 ml of item 2.A and 5 ml each of items 2.B. to 2.I. to 5 l of Turks Island Salt Solution.