CHAPTER III

RESULTS

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

A. halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane as shown in Figure 3. The cells were grown photoautotrophically at 30°C in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt solution + modified BG_{11} medium at different NaCl concentrations, i.e., 0.5, 1.0 and 2.0 M NaCl. Determination of cell growth was performed by counting the cells using the haemocytometer. Figure 4 shows that optimal growth occurred in the medium containing 0.5M NaCl. At either 1.0 or 2.0 M NaCl a reduction in growth was observed.

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

2.1 Determination of glycine betaine by ^{1}H-NMR measurements

2.1.1 The ^{1}H-NMR spectra of glycine betaine in *A. halophytica*

A.halophytica cells grown in medium containing 0.5 M NaCl were extracted as described in Materials and Methods. The content of glycine

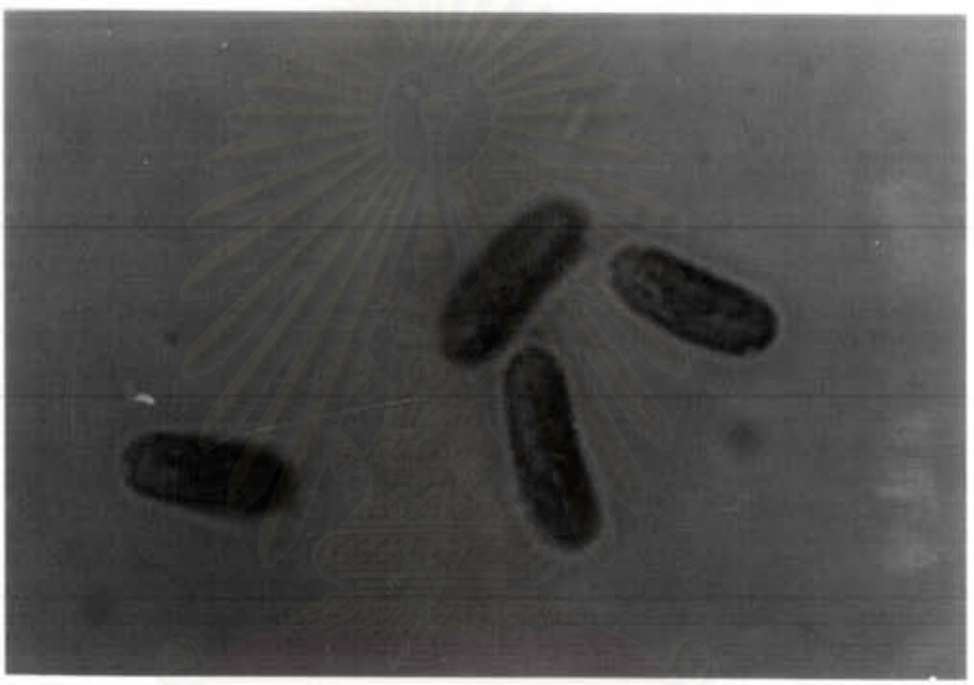

Figure 3 **Microscopic picture of *A. halophytica* grown in Turk Island Salt solution + modified BG_{11} containing 0.5 M NaCl at day 14 (×2250).**

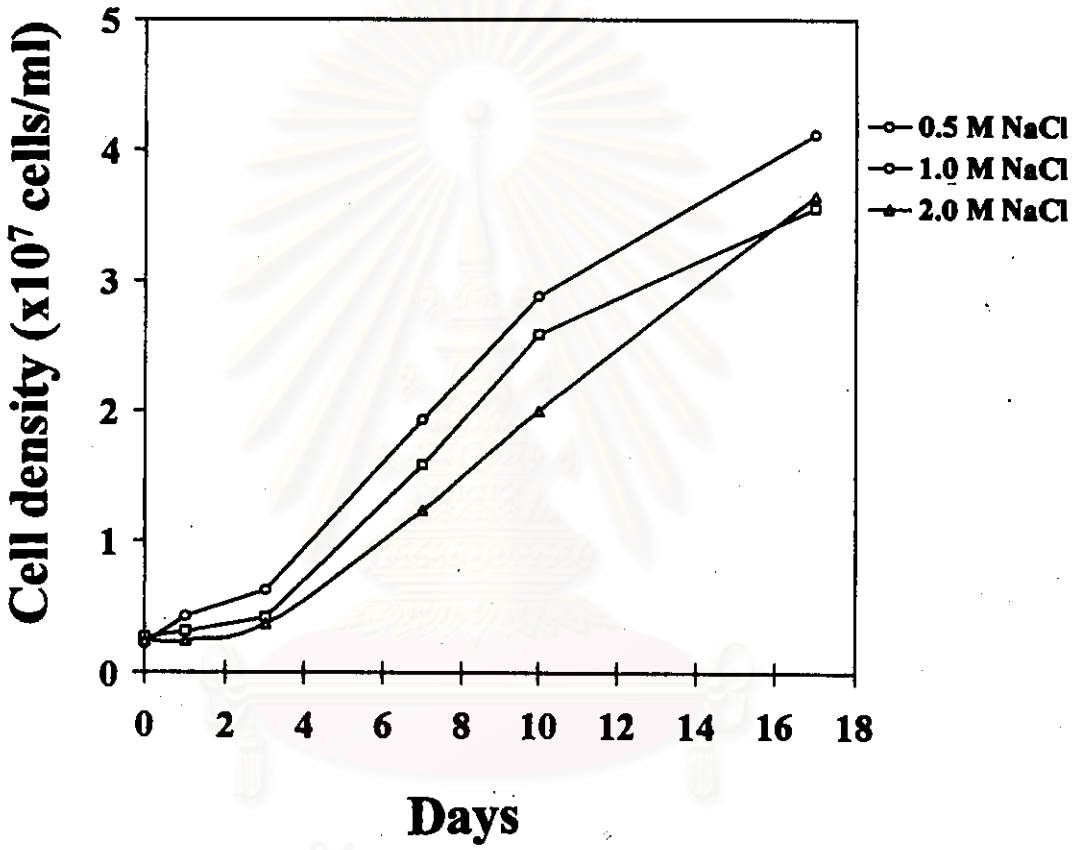

Figure 4 Growth of *A. halophytica* under different salinities.

betaine was estimated by ^{1}H-NMR analysis. Figure 5 shows the ^{1}H-NMR spectrum of an extract from *A. halophytica* without salt stress. The peaks at 3.25 ppm and 3.19 ppm (relative to DSS) are due to *N*-methyl protons of glycine betaine and choline,respectively.

2.1.2 The recovery of glycine betaine extracted from the cells as analyzed by ^{1}H-NMR

A.halophytica cells grown in medium under non-salt stressed condition were extracted twice by 80% (v/v) ethanol. The overall recovery of glycine betaine from cells was evaluated by addition of known amount of glycine betaine to the cells prior to extraction. One sample represented the control (without added glycine betaine) and the other sample was added with 3.5 mg glycine betaine. The two samples were analyzed by the ^{1}H NMR. Table 3 shows that the method of extraction was satisfactory to recover glycine betaine and the recovery level was 100%.

2.2 Determination of glycine betaine by spectrophotometric mesurements

2.2.1 The recovery of glycine betaine from column as analyzed by spectrophotometric method

A known amount of commercial glycine betaine was loaded onto a Dowex-50W column. The column was washed by 10 ml distilled water

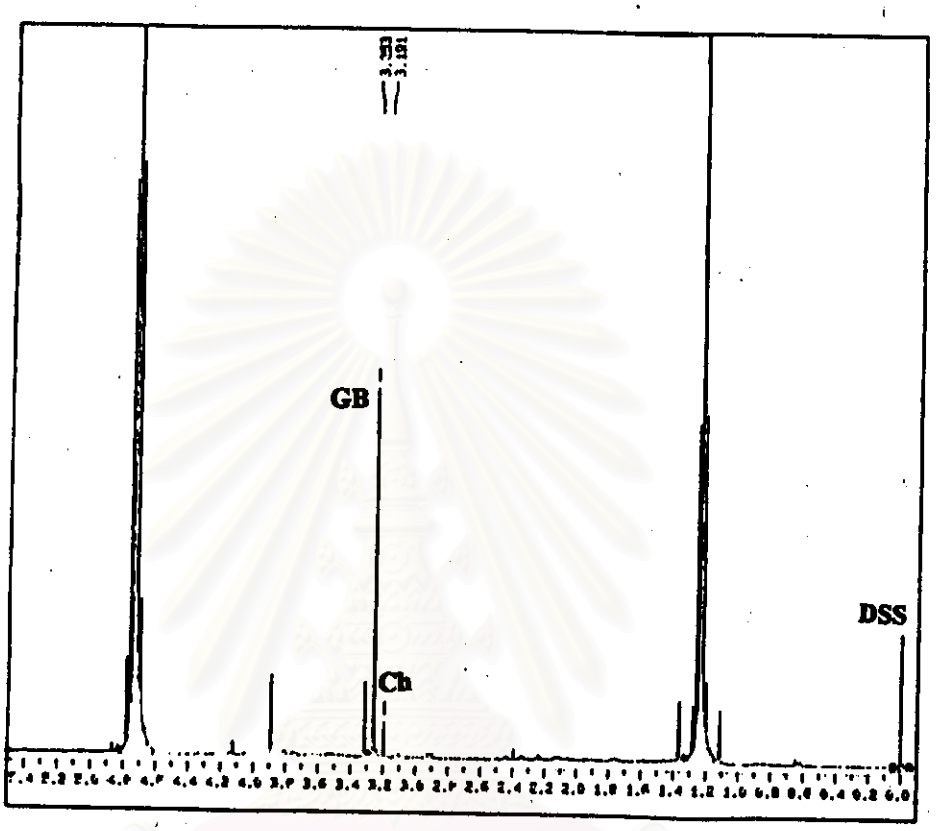

Figure 5 **^{1}H-NMR spectrum of extract from *A. halophytica* grown in culture medium without salt stress.** The peaks at 3.25 ppm and 3.19 ppm (relative to DSS) are glycine betaine (GB) and choline (Ch), respectively.

and glycine betaine was eluted by 2 M NH_4OH (0-5, 5-10, 10-15 and 15-20 ml). Table 4 shows that the washing step by water did not elute glycine betaine. Glycine betaine was eluted completely by 15 ml of 2 M NH_4OH. The recovery of glycine betaine from an ion-exchange column was 100%.

2.2.2 The recovery of glycine betaine after extraction and ion-exchange column as analyzed by spectrophotometric method

A. halophytica cells grown in medium under non-stressed condition were extracted twice by 80%(v/v) ethanol. The overall recovery of glycine betaine from cells was evaluated by addition of known amounts of glycine betaine to the cells prior to the extraction. The pellet after the extraction was loaded onto an ion-exchange column. The amount of glycine betaine was analyzed by spectrophotometric method. Table 5 shows that the method of extraction and ion-exchange column almost completely recovered glycine betaine. The recovery level was 96.6%.

2.3 The comparision of ^{1}H-NMR and spectrophotometric assay methods for glycine betaine

A. halophytica cells were extracted and glycine betaine was determined by two methods, which were ^{1}H-NMR assay and tri-iodide assay with the use of a spectrophotometer. The tri-iodide assays were run in duplicate. The results show that the values obtained from two assay methods were comparable (Table 6).

Table 3 The recovery of glycine betaine extracted from *A.halophytica* as analyzed by ^{1}H-NMR.

	Sample	
	Without added glycine betaine	With added 3.5 mg glycine betaine
Glycine betaine in cells after extraction (mg)	0.78	4.29
(%)recovery of added glycine betaine	-	100

Table 4 The recovery of glycine betaine from ion-exchange column as analyzed by spectrophotometric method.

	Condition						
	Before elution	Water-eluent fraction	2 M-NH_4OH eluent fraction				After elution
			0-5 ml	5-10 ml	10-15 ml	15-20 ml	
Amount of glycine betaine eluted from the column (mg)	4.0	0	3.73	0.19	0.15	0	4.07
(%)recovery of glycine betaine from the column	-	-	-	-	-	-	100

2.4 Effect of NaCl on glycine betaine content in A. *halophytica*

A. halophytica cells were transferred from culture medium containing 0.5 M NaCl to either culture medium containing 1.0 M or 2.0 M NaCl. Extraction of cells was done with 80%(v/v) ethanol and glycine betaine was determined by tri-iodide and ^{1}H NMR assays. The data show that the glycine betaine content of *A. halophytica* increased in response to an increased in salinity of culture medium. Approximately 8 fold increase of glycine betaine was observed when salinity was raised from 0.5 M NaCl to 2.0 M NaCl (Table 6). On the other hand when NaCl concentration was increased the cell number was decreased (Table 6).

3. Purification of BADH from *A. halophytica*

A. halophytica cells (Approx. 10 g), were disrupted by lysozyme. The enzyme was purified by using 35-70% ammonium sulfate precipitation, DEAE-cellulose column and hydroxyapatite column. The BADH protein was eluted from DEAE-cellulose column by 0.2-0.3 M NaCl gradient (Figure 6). For hydroxyapatite column, the BADH protein was eluted by 500 ml linear gradient of 0.01 M to 0.2 M potassium phosphate buffer pH 7.5 and it was eluted at about 20 ml. The results of a typical purification of BADH from *A. halophytica* are shown in Table 7. The enzyme was purified 18-fold giving a preparation with a specific activity of 298.6 μmol $min^{-1}mg^{-1}$. The hydroxyapatite column gave little increase in specific activity. The purity of the enzyme was determined by native and SDS polyacrylamide gel electrophoresis (Figure 7,8). The

Table 5 **The recovery of glycine betaine after extraction and ion-exchange column as analyzed by spectrophotometric method.**

	Sample	
	Without added glycine betaine	With added 3.7 mg glycine betaine
Glycine betaine in cells after extraction (mg)	0.79	4.36
(%) recovery of added glycine betaine	-	96.6

Table 6 **Effects of salinity on intracellular content of glycine betaine and number of cells.**

NaCl concentration in medium (M)	Cell number ($\times 10^6$cells/ml)	Glycine betaine content (nmol / 10^6cells)	
		^{1}H NMR	Tri-iodide
0.5	23.4	9.7	9.1
1.0	16.3	26.9	20.4
2.0	4.3	76.0	78.7

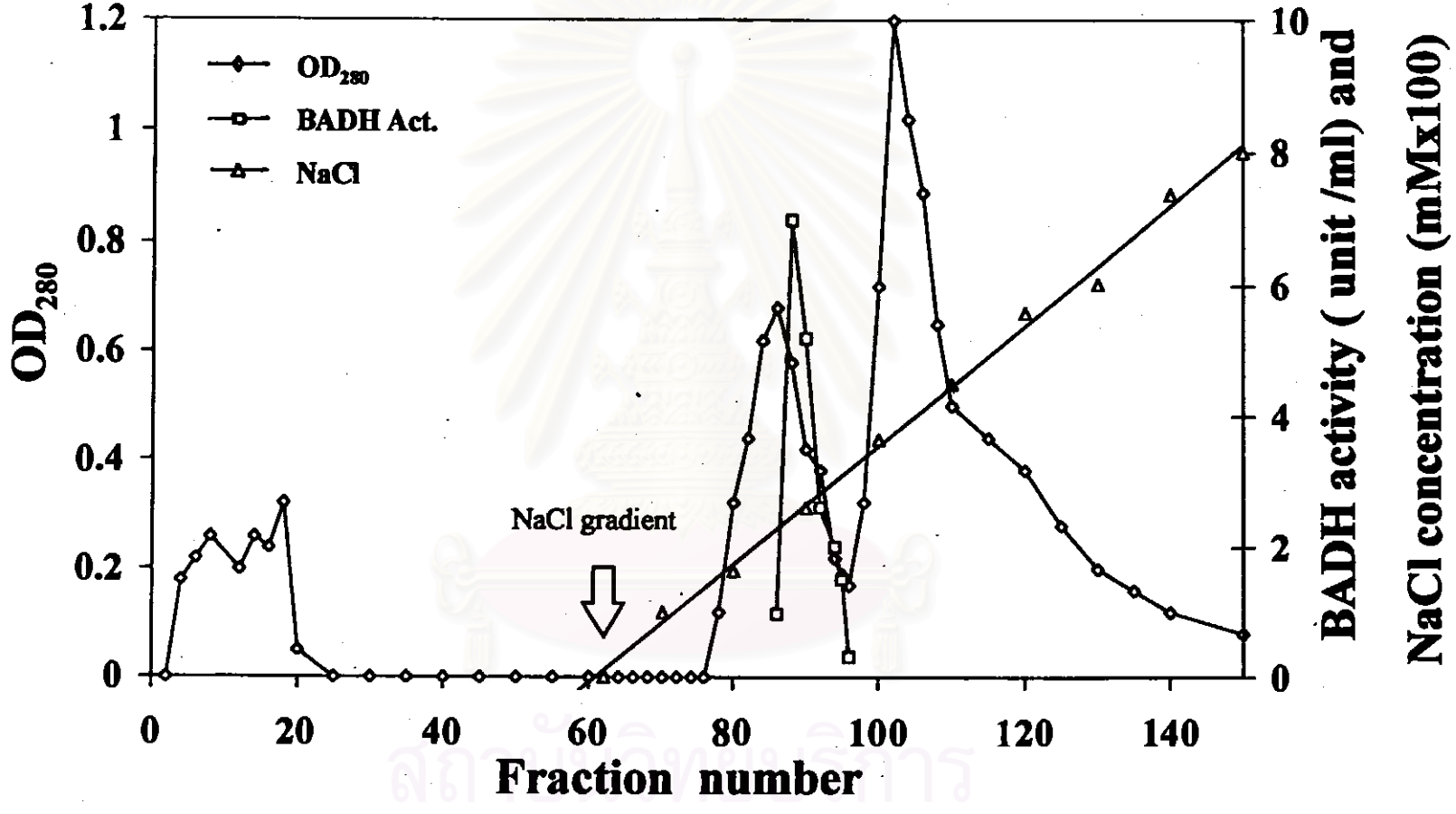

Figure 6 Chromatographic profile of DEAE-cellulose column.

Table 7 **The purification of BADH from *A. halophytica*.**

Step	Total prot. (mg)	Total act. (μmol min^{-1})	Specific act. (μmol min^{-1} mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	420.3	6923	16.5	100	1.0
35-70% $(NH_4)_2SO_4$	157.9	4848	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

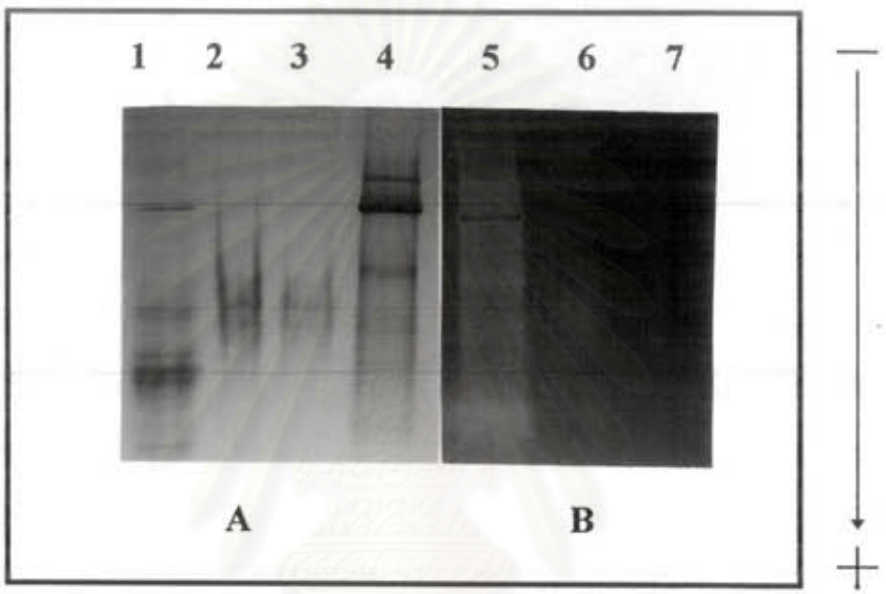

Figure 7 Non-denaturing PAGE pattern of proteins obtained from different steps of purification. Coomassie blue staining was used to detect protein (A). The native protein was stained for enzyme activity (B).

Lane 1,6 = 35-70% $(NH_4)_2SO_4$	20 μg
2,7 = DEAE-cellulose pool	10 μg
3 = DEAE-cellulose pool	5 μg
4,5 = crude spinach leaves extract	20 μg

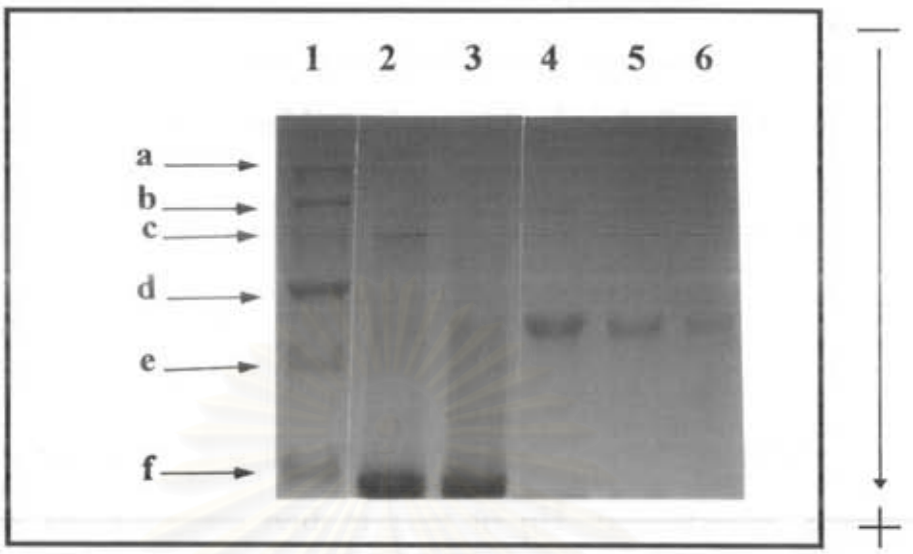

Figure 8 **SDS-PAGE pattern of proteins obtained from different steps of purification.**

Lane 1= standard molecular weight proteins;
a: phosphorylase B (97,000 dalton)
b: bovine serum albomin (66,200 dalton)
c: ovalbumin (45,000 dalton)
d: carbonic anhydrase (31,000 dalton)
e: soybean trypsin inhibitor (21,000 dalton)
f: lysozyme (14,400 dalton)

2= crude enzyme 20 µg
3= 35-70% $(NH_4)_2SO_4$ 20 µg
4= DEAE-cellulose pool 5 µg
5= DEAE-cellulose pool 2.5 µg
6= DEAE-cellulose pool 1.25 µg

results of gel electrophoresis show that the enzyme was mostly purified after DEAE-cellulose column. The BADH activity staining in native gel electrophoresis did not yield a detectable band for BADH of *A. halophytica* (Figure 7).

4. The properties of BADH

The partially purified BADH obtained from DEAE-cellulose column was characterized with respect to pH, temperature, coenzyme requirements, substrate analog inhibition and salt response.

4.1 Effect of pH on BADH activity

The partially purified BADH was tested for the effect of pH. The enzyme was tested at various pHs (potassium phosphate buffer pH 6.0 and 6.5, HEPES-KOH buffer pH 6.5, 7.0 and 7.5 and Tris-HCl pH 7.5, 8.0 and 9.0). The enzyme was incubated at 25°C. Protein (6.88 μg) was used in each assay. Figure 9 shows that the enzyme exhibited maximal activity at pH 7.5. Therefore, in the subsequent experiments all enzyme assays were carried out at pH 7.5.

4.2 Effect of temperature on BADH activity

The partially purified BADH was assayed in 50 mM HEPES-KOH buffer pH 7.5. The enzyme was incubated at various temperatures,i.e., 20, 22, 25, 28, 30 and 37°C. Protein (6.88 μg) was used in each assay. The

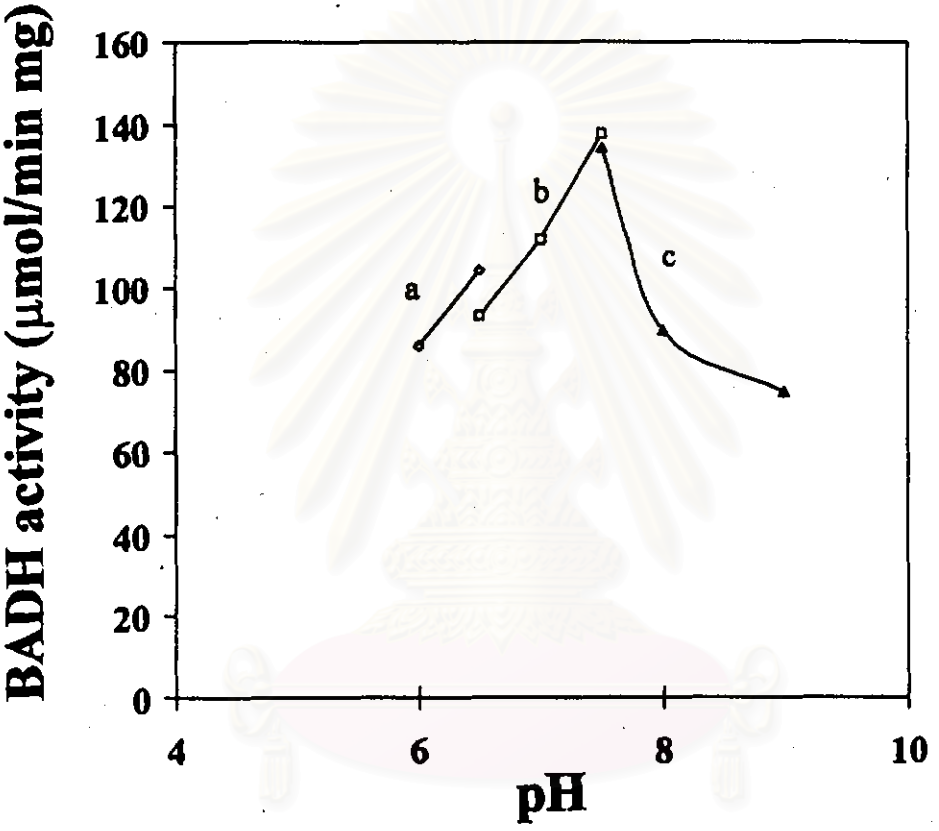

Figure 9 **Effect of pH on BADH activity.**
a= 50 mM potassium phosphate buffer
b= 50 mM HEPES-KOH buffer
c= 50 mM Tris-HCl buffer

Enzyme showed maximal activity at 25°C (Figure 10). Therefore, in the subsequent experiments all enzyme assays were carried out at pH 7.5 and 25 °C.

4.3 The kinetics of BADH

The K_m and V_{max} of the enzyme were determined. The enzyme was assayed in 50 mM HEPES-KOH buffer pH 7.5 at 25°C. Protein (6.88 μg) was used in each assay. The K_m and V_{max} were determined by assaying enzyme at varing concentration of betaine aldehyde (0.05, 0.1, 0.25 and 0.5 mM) and fixed concentration of coenzyme (NAD^+) at 0.5 mM. Figure 11 shows that the BADH activity was specific for betaine aldehyde with an apparent K_m of 91 μM. Two coenzymes (NAD^+ and $NADP^+$) were tested for their requirements by fixing the concentration of betaine aldehyde at 0.5 mM for each assay and varying coenzyme concentration (0.05, 0.1, 0.25 and 0.5 mM). Figure 12 shows that, the presence of NAD^+ was essential to the oxidation of betaine aldehyde to glycine betaine. The apparent K_m for NAD^+ was 71.4 μM. The higher K_m (100 μM) for $NADP^+$ indicated that the enzyme preferred NAD^+ as a coenzyme. V_{max} as determined by the double reciprocal plot was found to be 175.44 μmol min^{-1} mg^{-1} (when $NADP^+$ was a coenzyme, V_{max} was 138.8 μmol min^{-1} mg^{-1}).

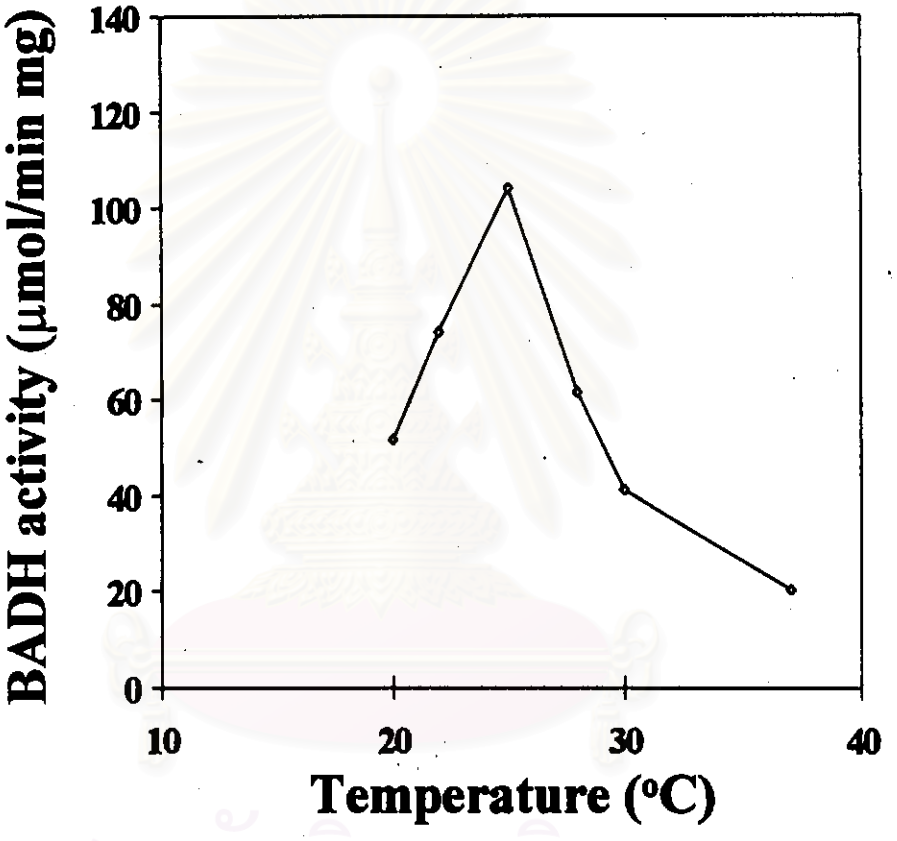

Figure 10 **Effect of incubation temperature on BADH activity.**

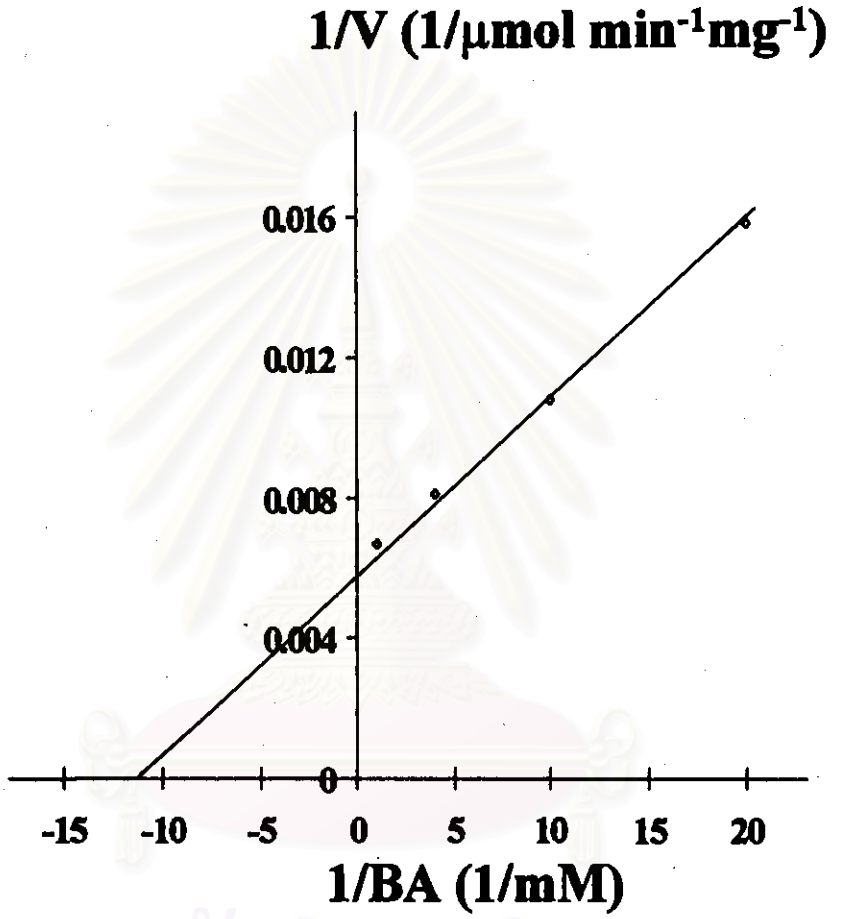

Figure 11 **Double reciprocal plot of activity of partially purified BADH as a function of the concentration of the substrate, betaine aldehyde.** The K_m and V_{max} were 91 μM, 175.4 μmol $min^{-1}mg^{-1}$.

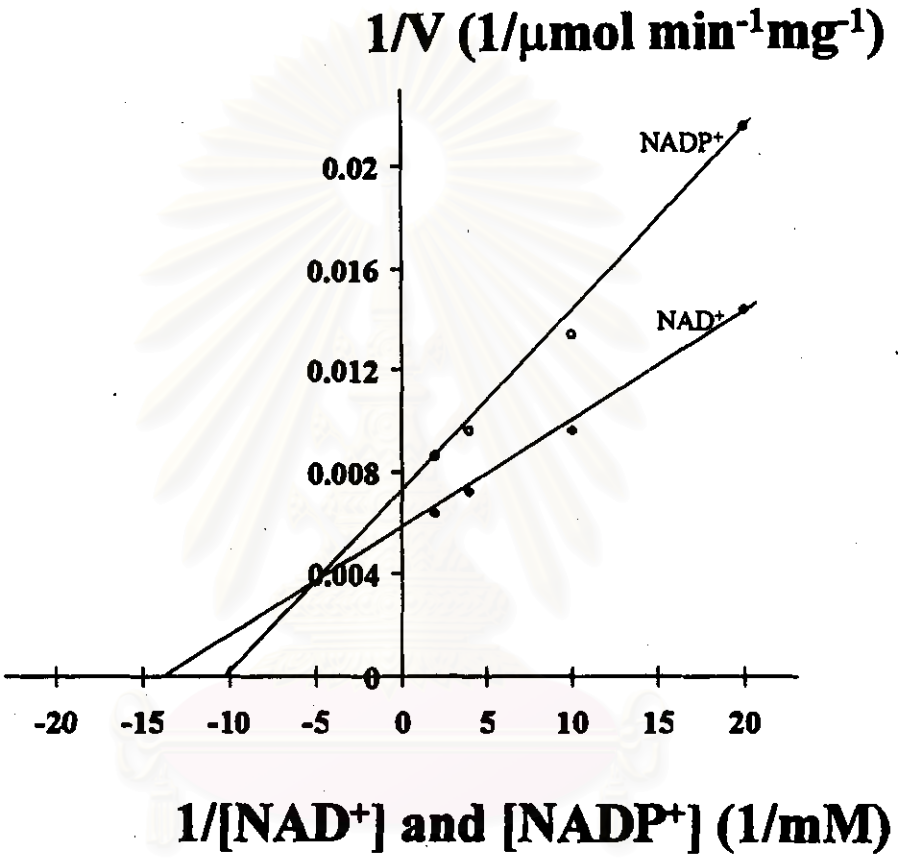

Figure 12 **Double reciprocal plot of activity of partially purified BADH as a function of the concentration of the coenzymes, NAD^+ and $NADP^+$.** The K_m for NAD^+ and $NADP^+$ were 71.4 μM and 100 μM, respectively.

4.4 The substrate analog inhibition

For the substrate analog inhibition test, the enzyme was assayed in 50 mM HEPES-KOH buffer pH 7.5 at 25 °C. Protein (6.88 μg) was used in each assay. The reaction was started by adding 0.5 mM betaine aldehyde. Four substrate analoges (glycine betaine, choline, ethanolamine and acetaldehyde) were tested. Table 8 shows that glycine betaine and choline were slightly inhibitory to the enzyme. Ethanolamine was a potent inhibitor whereas acetaldehyde completely inhibited the enzyme activity.

4.5 Effect of cation on BADH activity

In this experiment Tris-HCl buffer pH 7.5 was used instead of HEPES-KOH pH 7.5 for BADH assay. BADH activity was measured in the presence of varying concentration of Na^+, K^+, Ca^{2+} and Mg^{2+}. Protein (6.88 μg) was used in each assay. The enzyme activity was initially stimulated by increasing concentration of $Na^+$and K^+ from 0 to 0.1 M above which the BADH activity was decreased. Ca^{2+} and Mg^{2+} appeared to have a particularly strong influence on BADH activity. Increasing concentration of Ca^{2+} and Mg^{2+} resulted in a marked decrease in BADH activity (Figure 13).

4.6 Effect of DTT and PCMS on BADH activity

The partially purified BADH from DEAE-cellulose column was assayed in 50 mM HEPES-KOH buffer pH 7.5 containing 1 mM EDTA,

Table 8 **Inhibition of BADH activity by analogs of betaine aldehyde.**

Substrate analog	Concentration of analog (mM)	Remaining activity (%)
Glycine betaine	100	88.5
Choline	100	77.1
Ethanolamine	100	27.0
Acetaldehyde	100	0

:The activity of the enzyme without substrate analog was 96.67 μmol min^{-1} mg^{-1} and was taken as 100%.

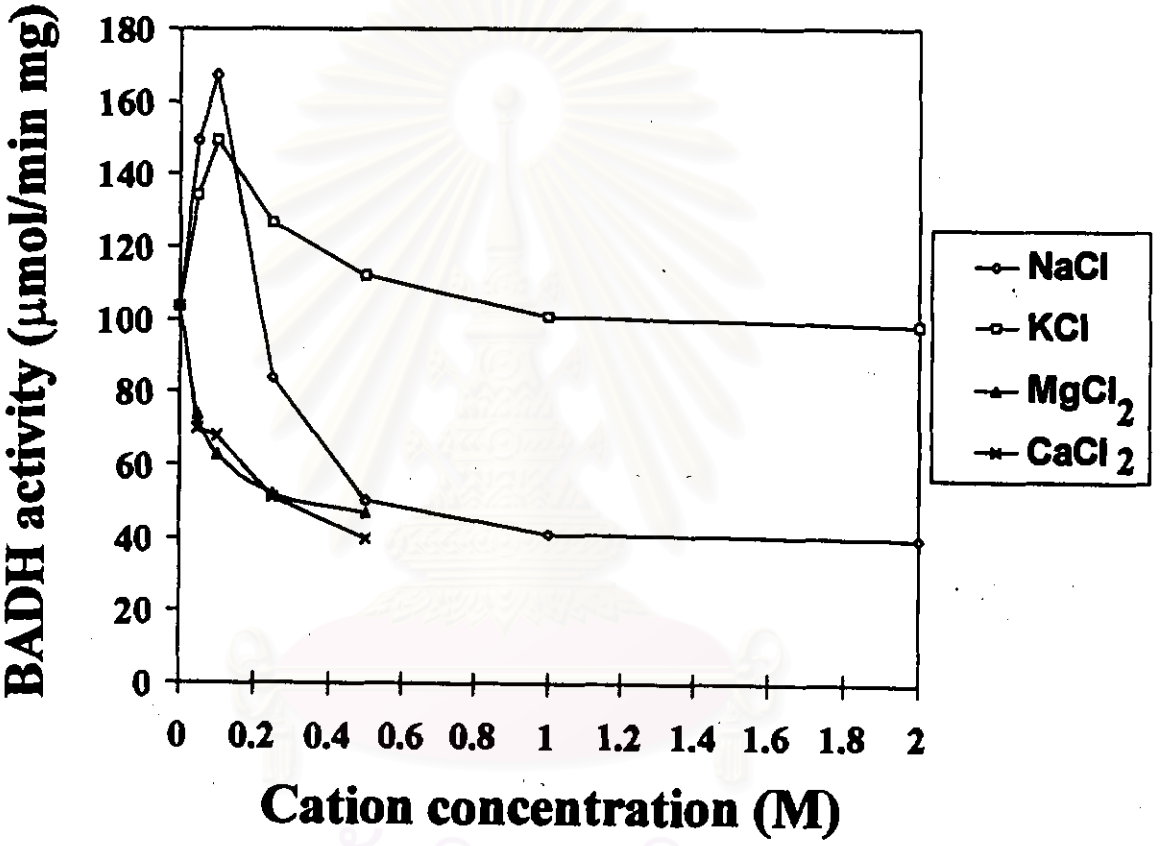

Figure 13 Effect of cations on BADH activity.

0.5 mM NAD^+, 0.5 mM betaine aldehyde and 6.88 μg enzyme per each assay. The reaction was started by the final addition of betaine aldehyde. Table 9 shows that enzyme activity was strongly stimulated by DTT and completely inhibited by PCMS. On the other hand, preincubation of the enzyme with DTT protected the enzyme against the inhibition by PCMS, i.e., only 10% of the activity was lost by this treatment. Although complete loss of enzyme activity occurred after PCMS treatment, about half of the total activity could be restored when the enzyme was further activated with DTT.

5. The determination of molecular weight of BADH

Gel filtration of BADH on Sephadex G-200 column was used for the determination of the molecular weight of the native enzyme. K_{av} value of BADH was used to estimate its molecular weight using the standard curve of K_{av} values of known proteins standard versus their respective molecular weight (Figure 14). The gel filtration data indicated that the BADH molecular weight was 120,000 dalton. Likewise, SDS-PAGE gave one single band with an apparent molecular weight of 30,000 dalton (Figure 15). The results of gel filtration and SDS-PAGE indicated that *A.halophytica* BADH constituted a tetramer with identical 30,000 dalton subunits.

Table 9 **Effect of DTT and PCMS on the activity of partially purified BADH.**

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 0.1mM PCMS(30 min)	90.3
0.1 mM PCMS(10 min), then 5mM DTT(30 min)	51.6

: The specific activity of the enzyme without DTT (the control) was 57.81 μmol min^{-1} mg^{-1}.

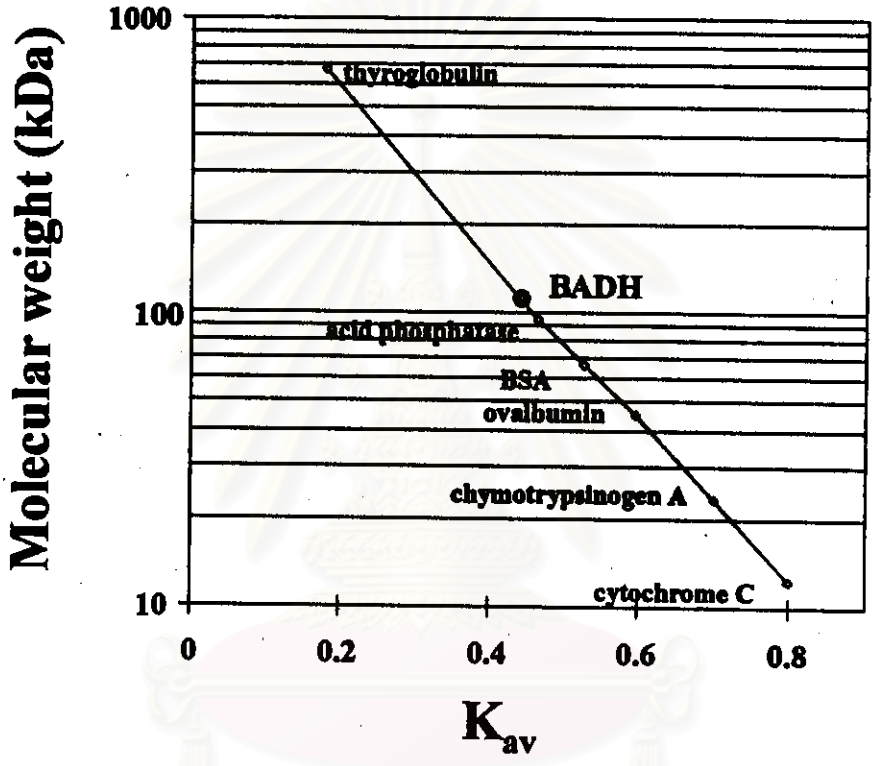

Figure 14 Molecular weight calibration curve of standard proteins on Sephadex G-200 column.

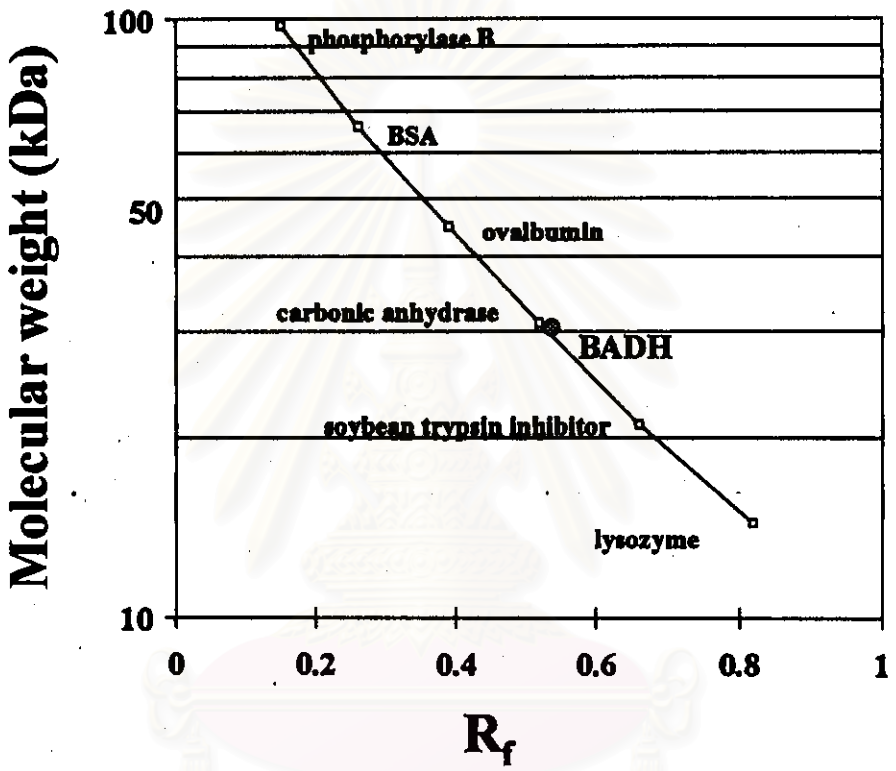

Figure 15 **Molecular weight calibration curve of standard proteins on 10% SDS-PAGE.**

6. Effect of external salinity on BADH activity

A. halophytica cell pellet from 400 ml culture containing either 0.5 M NaCl or 2.0 M NaCl was disrupted in 50 mM HEPES-KOH buffer pH 7.5 containing 2.0 mg/ml lysozyme. The suspension was centrifuged and the supernatant was subjected to 35-70% ammonium sulfate precipitation. The final pellet was suspended in a small volume of 50 mM HEPES-KOH buffer pH 7.5 before desalting through a Sephadex G-25 column. The blue extract obtained was used for enzyme assay. Table 10 shows the BADH activity of the extract from *A. halophytica* cells grown in medium containing 0.5 M NaCl and 2.0 M NaCl. Cells grown in 2 M NaCl exhibited 4-fold increase in BADH activity when compared to those grown in 0.5 M NaCl.

Table 10 BADH activity from *A. halophytica* grown in different salinites.

NaCl concentration in growth medium (M)	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)
0.5	1.03
2.0	4.43