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BETAINE ALDEHYDE DEHYDROGENASE FROM A HALOTOLERANT CYANOBACTERIUM *APHANOTHECE HALOPHYTICA* : PURIFICATION, PROPERTIES, AND REGULATION BY SALINITY

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ABSTRACT

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.[1,2] The adjustments by *A. halophytica* to counter-balance the high external salinity included the accumulation of inorganic K^{+3} and an organic quaternary ammonium compound, namely glycine betaine.[2] The accumulation of glycine betaine as an osmoregulatory compound was also reported in a marine cyanobacterium, *Spirulina subsalsa*.[4] In many bacteria,[5-7] plants[8] 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.[10-12] In bacteria the transformation of choline into glycine betaine is catalyzed by the membrane-bound enzyme choline dehydrogenase and the cytoplasmic enzyme BADH.[13,14]

Despite the widespread occurrence of glycine betaine its synthesis and osmotic regulation are well understood only in some bacteria,[5-7] certain plant[8] and animal[15,16] 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 including hydroxyapatite (Type I) 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.[1,17] 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.*[18] 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).

Protein determination

The protein content was determined by a sensitive dye-binding method according to Bradford.[20] The content of protein eluted from the column was monitored by measuring A_{280}.

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 a slight increase in specific activity. However, the use of hydroxyapatite chromatography resulted in about 3-fold loss of the yield of the enzyme. The molecular weight of the enzyme as estimated from gel filtration under non-denaturing conditions was 115 kDa. By the plot of the relative electrophoretic mobilities of six standard proteins and BADH (under SDS-denaturing conditions) versus molecular weight, 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.

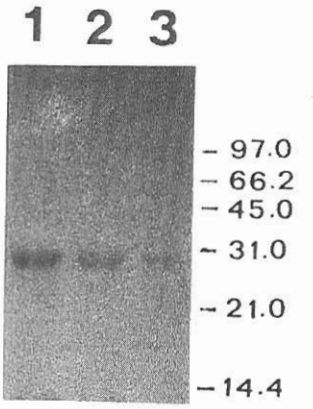

Fig.1 SDS-polyacrylamide gel electrophoresis of purified *Aphanothece halophytica* betaine aldehyde dehydrogenase on a 10% slab gel. Lanes 1, 2 and 3 were 5, 2.5 and 1.25 μg of purified enzyme after DEAE-cellulose step. Numbers on the right indicate standard molecular weight markers in kilodalton.

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 n (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

Properties of BADH

Effects of pH and temperature on BADH activity

As shown in Fig. 2A, 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. 2B). 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 presented in the form of Lineweaver Burk plots as shown in Fig. 3. The apparent Michaelis constants (K_m) were estimated to be 91 μM for betaine aldehyde (Fig. 3A) and 71.4 μM and 100 μM for NAD^+ and $NADP^+$ respectively (Fig. 3B). 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.

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%.

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.

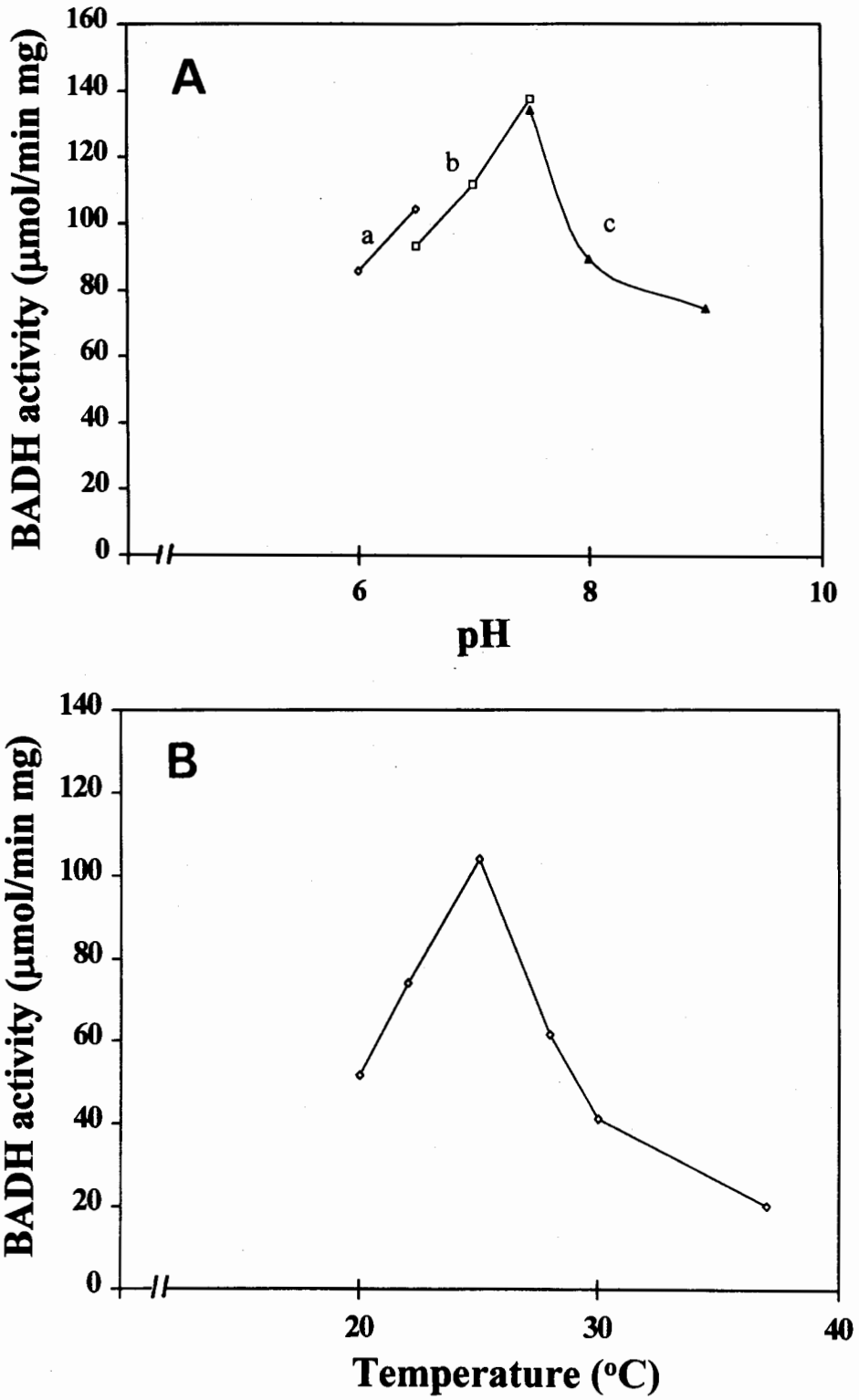

Fig.2 Effect of pH (A) and temperature (B) on *Aphanothece halophytica* betaine aldehyde dehydrogenase activity. The buffer systems used in (A) were a) 50 mM potassium phosphate buffer, b) 50 mM HEPES-KOH buffer and c) 50 mM Tris-HCl buffer.

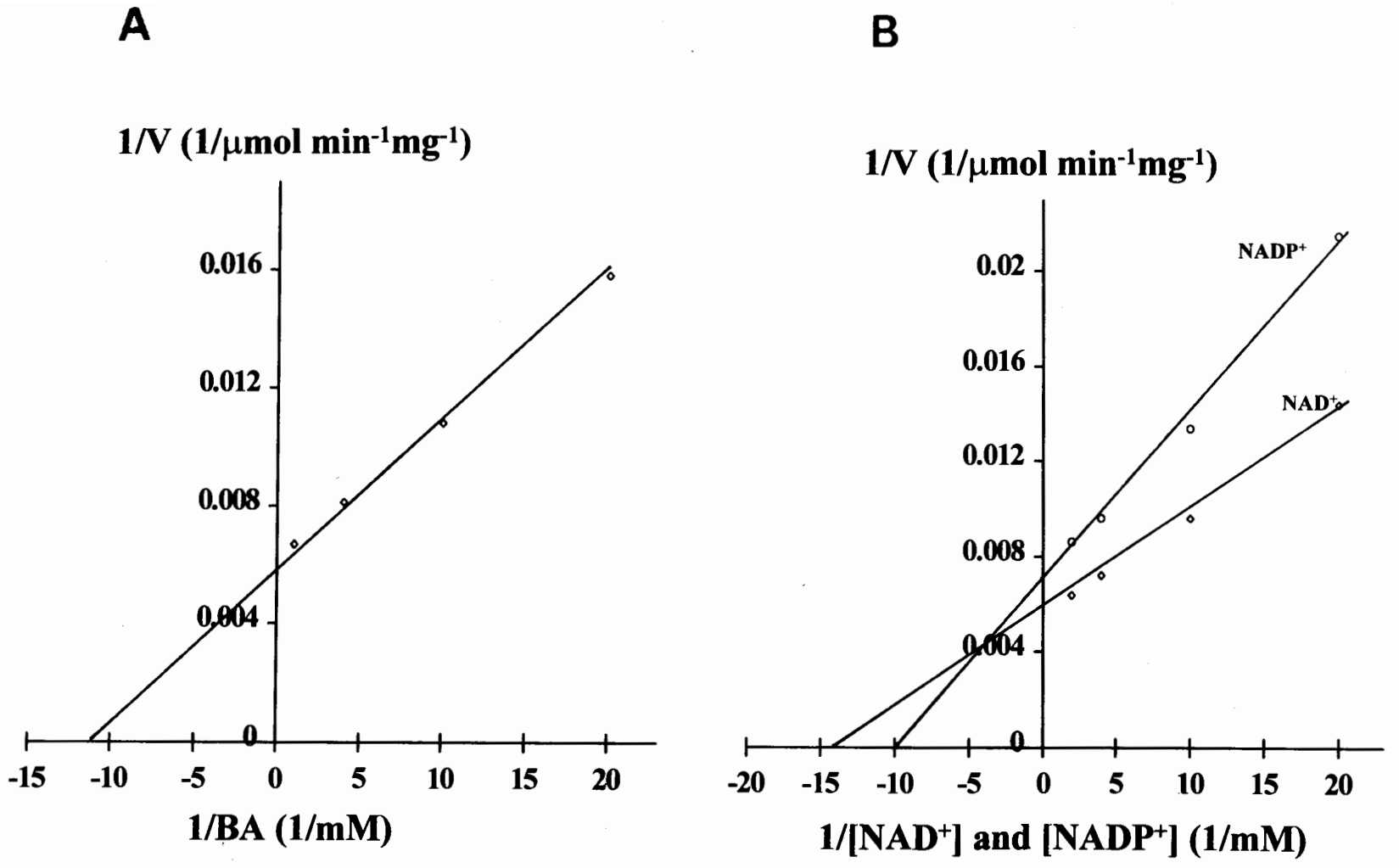

Fig.3 Double reciprocal plots of activity of betaine aldehyde dehydrogenase against substrate concentration. (A) Betaine aldehyde (BA) as variable substrate at fixed 0.5 mM NAD^+. (B) NAD^+ or $NADP^+$ as variable substrate at fixed 0.5 mM betaine aldehyde.

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}.

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

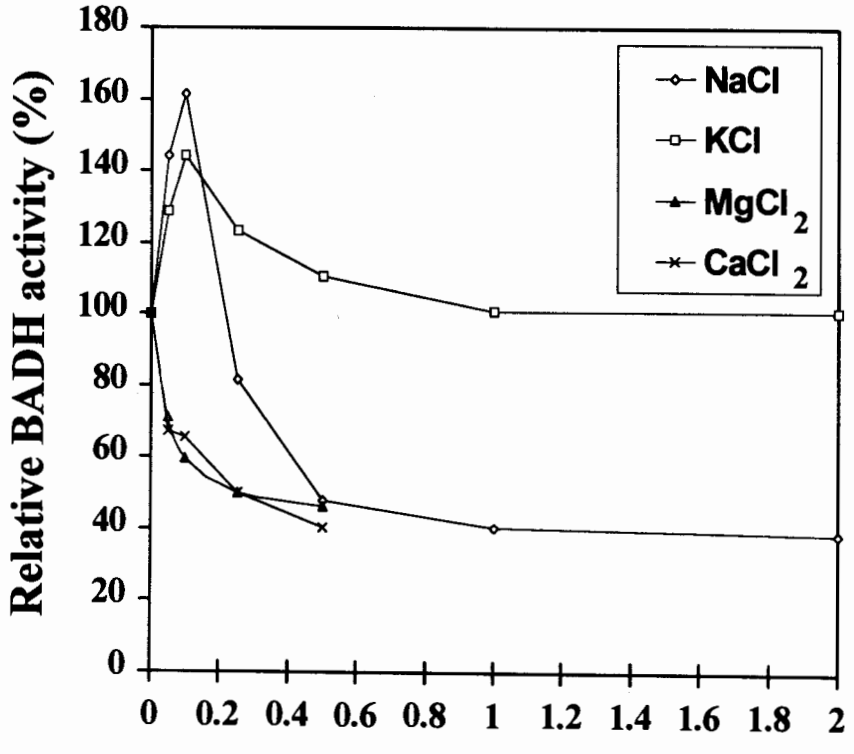

Fig.4 Effect of salts on the relative activity of betaine aldehyde dehydrogenase from *Aphanothece halophytica*.

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 specific 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, *i.e.*, 4.43 versus 1.03 μmol min^{-1} mg^{-1}. The result appeared to be in line with the previous finding that showed an 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.[21]

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 step of hydroxyapatite chromatography only slightly increased the specific activity of the enzyme. This was in sharp contrast with the results of the purification of betaine aldehyde dehydrogenase from spinach leaves showing a nearly 3-fold increase in the specific activity after hydroxyapatite chromatography.[22] It should be noted that the present study used hydroxyapatite (Type I) from Sigma Chemical whereas a Bio-Gel HTP from Biorad was used for the study in spinach leaves. The specific activity of BADH in the present study was somewhat similar to that reported for BADH of horseshoe crab[16] and about one and two orders of magnitude higher than BADH from spinach and *E. coli* respectively.[18,23]

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[24] and 63[22] kDa. The tetramers of subunits of 55[23] and 58[25] 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.[16,18,22,23] So far only BADH from *P. aeruginosa* A-16 has been reported to prefer $NADP^+$ to NAD^+ as a coenzyme.[26] *A. halophytica* BADH showed a relatively narrow pH optimum of 7.5 similar to that of BADH from horseshoe crab.[16] For spinach and *E. coli* the BADH had broader pH optima spanning 2 pH units.[22,23] 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[18,22] and about 3-times higher than the V_{max} for *E. coli* BADH.[23] 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.[16,18,22,23,26] The analogs of betaine aldehyde could inhibit BADH activity with varying degree of inhibition (Table 2). Aldehyde compounds 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.[21]

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. 4). 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.[2] 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+}.[16]

The observed salt-induced rise in *A. halophytica* BADH specific activity 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.[21] Previous report by Ishitani *et al.*[27] 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.

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บทคัดย่อ

การทำบริสุทธิ์เอนไซม์บีเทนอัลดีฮายด์ดีไฮโดรจีเนสที่แยกได้จากไซยาโนแบคทีเรียชนิดทนความเค็ม *Aphanothece halophytica* โดยใช้แอมโมเนียมซัลเฟตตกตะกอนโปรตีนออกจากสารละลายเซลล์ที่ถูกทำให้แตกด้วยไลโซไซม์ ผ่านคอลัมน์ดีอีเออี เซลลูโลส และคอลัมน์ไฮดรอกซีอะพาไทท์ พบว่าผลสุดท้ายสามารถทำเอนไซม์ให้บริสุทธิ์ได้ 18 เท่า มีแอคติวิตีจำเพาะ 298.6 ไมโครโมล นาที$^{-1}$ มิลลิกรัม$^{-1}$ เอนไซม์ประกอบไปด้วย 4 หน่วยย่อยเหมือนกันที่มีขนาดโมเลกุลหน่วยย่อยละ 30 กิโลดาลตัน พีเอชและอุณหภูมิที่เหมาะสมสำหรับการทำงานของเอนไซม์มีค่า 7.5 และ 25°ซ. ตามลำดับ ทั้งเอนเอดี และเอนเอดีพีสามารถทำหน้าที่เป็นโคเอนไซม์ได้โดยที่มีค่า Km เป็น 71.4 และ 100 ไมโครโมลาร์ตามลำดับ อะเซทาลดีฮายด์ยับยั้งการทำงานของเอนไซม์ได้อย่างรุนแรง เอน-เมธิลเลทเทดสับสเตรทอะนาลอกก็สามารถยับยั้งการทำงานของเอนไซม์ได้โดยที่ไกลซีนบีเทนมีผลยับยั้งเพียงเล็กน้อย ไดไธโอเธรทอลสามารถเพิ่มประสิทธิภาพการทำงานของเอนไซม์แต่พาราคลอโรเมอคิวริฟินิลซัลโฟเนตยับยั้งการทำงานโดยสิ้นเชิง โปแตสเซียมคลอไรด์และโซเดียมคลอไรด์ที่ความเข้มข้นต่ำๆ ไล่ขึ้นมาจนถึง 0.1 โมลาร์ กระตุ้นการทำงานของเอนไซม์ แต่ถ้าความเข้มข้นสูงกว่า 0.1 โมลาร์ โปแตสเซียมคลอไรด์มีผลกระตุ้นลดน้อยลงและโซเดียมคลอไรด์มีผลยับยั้ง การเพิ่มความเค็มภายนอกเซลล์สามารถทำให้แอคติวิตีจำเพาะของเอนไซม์เพิ่มขึ้น