การแลกเปลี่ยนโซเดียม/โปรตอนในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*

นางสาวอาภรณ์ บัวหลวง

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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SODIUM/PROTON EXCHANGE IN HALOTOLERANT CYANOBACTERIUM

Aphanothece halophytica

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สถาบันวิทยบริการ
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A Thesis Submitted in Partial Fulfillment of the Requirements

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อาภรณ์ บัวหลวง : การแลกเปลี่ยนโซเดียม/โปรตอนในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*. (SODIUM/PROTON EXCHANGE IN HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*) อ. ที่ปรึกษา: รศ.ดร. อรัญ อินเจริญศักดิ์, 72 หน้า.

ไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* สามารถเจริญเติบโตได้ภายใต้สภาวะแวดล้อมที่มีความเข้มข้นของเกลือโซเดียมคลอไรด์ 0.5 โมลาร์ (สภาวะปกติ) และ 2.0 โมลาร์ (สภาวะเครียด) มีกลไกในการขับโซเดียมออกจากเซลล์เป็นการรักษาระดับโซเดียมภายในเซลล์ให้เหมาะสมเพื่อไม่ให้เป็นอันตราย ต่อกระบวนการเมแทบอลิซึม การแลกเปลี่ยนโซเดียม/โปรตอนเป็นกลไกหนึ่งในการควบคุมปริมาณโซเดียม ภายในเซลล์งานวิจัยนี้ ทำการศึกษาการแลกเปลี่ยนโซเดียม/โปรตอนในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* พบว่าเซลล์ที่เติบโตเหมาะสมเมื่อความขุ่นของเซลล์ที่ค่าการดูดกลืนแสง 730 นาโนเมตร อยู่ในช่วง 0.5-0.9 หรือเซลล์ในช่วงระยะแบ่งเซลล์ พบการแลกเปลี่ยนโซเดียม/โปรตอนสูงที่สุด จึงนำเซลล์ในช่วงนี้มาทำการติดตามแอกทิวิตีต่อไป โดยทำการศึกษาผลกระทบของตัวยับยั้งพลังงาน และไอโอโนฟอร์ชนิดต่างๆ ต่อการแลกเปลี่ยนโซเดียม/โปรตอน พบว่า valinomycin, CCCP, vanadate และ DCCD ซึ่งตัวยับยั้งดังกล่าวทำลายเกรเดียนท์ของโปรตอนและเกี่ยวข้องกับความต่างศักย์ของไอออน มีผลในการยับยั้งการแลกเปลี่ยน อีกทั้ง monensin เกี่ยวข้องกับการแลกเปลี่ยนโซเดียม ให้ผลยับยั้งเช่นกัน amiloride ซึ่งเป็นตัวยับยั้งที่จำเพาะกับโซเดียม/โปรตอนแอนติพอร์เตอร์ต่อแบคทีเรีย ตอบสนองต่อแอคทิวิตีของแอนติพอร์ตเตอร์เช่นกัน นอกจากนี้การแลกเปลี่ยนโซเดียม/โปรตอนยังถูกยับยั้งด้วย sodium azide อีกด้วย ซึ่งจากผลการลองแสดงว่า การแลกเปลี่ยนโซเดียม/โปรตอน เกี่ยวข้องกับเกรเดียนท์ของโปรตอนและเกี่ยวข้องกับความต่างศักย์ของไอออน ต่อมาศึกษาบทบาทของความเป็นกรด-ด่างต่อการแลกเปลี่ยนโซเดียม/โปรตอนพบว่า สามารถทำงานได้ในช่วงกว้าง pH 5.0-10.0 นอกจากนี้ ไอออนอื่น อาทิ โปแทสเซียม ลิเทียม แคลเซียม และแมกนีเซียมมีการแลกเปลี่ยนกับโปรตอนได้ ต่อมาศึกษาผลทางจลนพลศาสตร์ต่อการแลกเปลี่ยนโซเดียม/โปรตอนพบว่า เซลล์มีค่าคงที่ของ Michaelis-menten ต่อ โซเดียม แคลเซียม และลิเทียม ไอออน เท่ากับ 23.7, 4.4 และ 36.0 มิลลิโมลาร์ ตามลำดับ และมีค่าอัตราเร็วสูงสุดเท่ากับ 15.0, 14.5 และ 16.0% fluorescence ตามลำดับ

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ภาควิชา........................ชีวเคมี.............ลายมือชื่อนิสิต...
สาขาวิชา......................ชีวเคมี............ลายมือชื่ออาจารย์ที่ปรึกษา...
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APORN BUALUANG: SODIUM/PROTON EXCHANGE IN HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*. THESIS ADVISOR: ASSOC.PROF. ARAN INCHAROENSAKDI, Ph. D., 72 pp.

The halotolerant cyanobacterium *Aphanothece halophytica* grown under non-stress (0.5 M NaCl) and stress condition (2.0 M NaCl) provided a maximal Na^+/H^+ exchange when the optical density of culture reached 0.5-0.9 (7th day). The Na^+/H^+ exchange of *Aphanothece* was shown to be dissipated by protonophores, namely valinomycin and CCCP. Na^+/H^+ exchange was strongly inhibited by monensin. Orthovanadate and DCCD also inhibited Na^+/H^+ exchange. Amiloride, a Na^+/H^+ antiporterer inhibitor, showed inhibition of Na^+/H^+ exchange. The Na^+/H^+ exchange was sensitive to sodium azide, an ATP formation inhibitor. These results strongly favour the concept that respiratory energy is used for proton efflux and that the resulting proton motive force may be involved in Na^+/H^+ exchange. A role for antiport activity in pH regulation can also explain the activity of Na^+/H^+ exchange over a broad pH range extending to the alkaline condition (pH 5.0-10.0). The cation/H^+ exchange activity was observed when either mono-valent cations (Na^+, K^+, Li^+) or di-valent cations (Ca^{2+} and Mg^{2+}) served as a cation. The K_m values for Na^+, Ca^{2+}, and Li^+ were 23.7, 4.4 and 36.0 mM, respectively, whereas the V_{max} values were 15.0, 14.5 and 16.0 % fluorescence respectively.

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Department.....Biochemistry............Student's signature......Aporn Bualuang......

Field of study......Biochemistry..... Advisor's signature..........................

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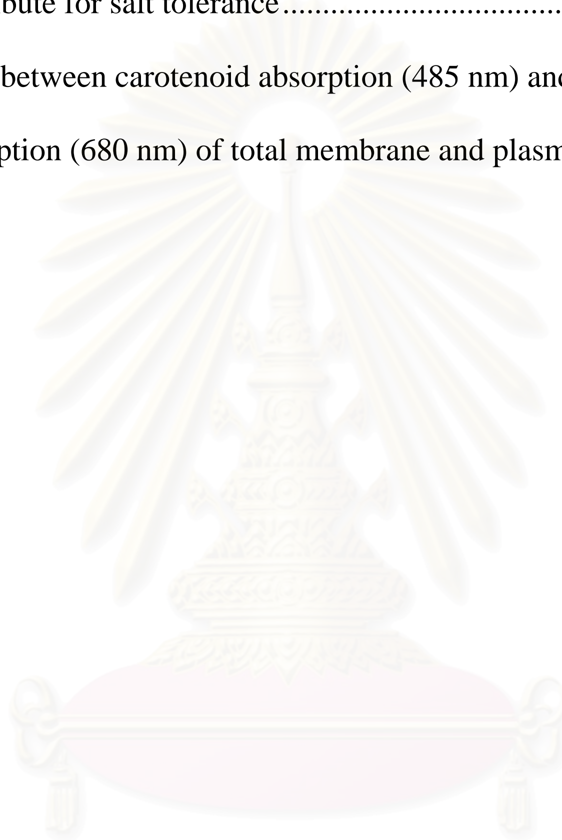

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LIST OF ABBREVIATIONS

A Absorbance

ATP Adenosine triphosphate

BSA Bovine serum albumin

$CaCl_2$ Calcium chloride

CCCP Carbonyl cyanidetrichloromethoxyphenylhydrazone

Chl Chlorophyll

cm Centimeter

CO_2 Carbondioxide

^{o}C Degree celsius

DCCD *N, N*-dicyclohexylcarbodiimide

DTT Dithiothreitol

ΔpH pH gradient or proton gradient

$\Delta\psi$ Membrane potential

ΔNa Sodium gradient

EDTA Ethylenediamine tetraacetic acid

g Gram

Hepes N-2-hydroxyethylpiperazine-N-ethanesulfonic

HCl Hydrochloric acid

KCN Potassium cyanide

KCl Potassium chloride

l Litre

LiCl Lithium cholride

lux Photometric (light density)

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min	Minute
ul	Microliter
MES	2-(N-morpholine)-ethane sulphonic acid
ml	Milliliter
mg	Milligram
Mg_2SO_4	Magnesium sulfate
mM	Millimolar
MW	Molecular weight marker
μM	Micromolar
M	Molar
NaN_3	Sodium azide
NaCl	Sodium chloride
nm	Nanometer
OD	Optical density
PEG	Polyethylene glycol
PMF	Proton motive force
rpm	Revolution per minute

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CHAPTER I

Introduction

Twenty percentages of the world's cultivated land and nearly half of all irrigated lands are affected by high salinity (Flower and Yeo, 1995). High salinity causes ion imbalance and hyperosmotic stress in plants. Organisms that thrive in hypersaline environments possess specific mechanisms for the adjustment of their internal osmotic status. One such mechanism is the ability to accumulate low-molecular-weight organic-compatible solutes such as sugars, some amino acids, and quaternary ammonium compounds (Rhodes and Hanson, 1993; Hanson *et al.*, 1994; Bettina and Bremer, 1998).

Salinity is one of many limiting factors in crop productivity and much effort has been made to understand salt tolerance. Although the ability to adapt to fluctuations in the external salinity is fundamental to survival of organisms, the mechanisms responsible for osmotic adaptation have been largely unknown. Upon the changing salinity of environment, all microorganisms need to balance their cytoplasm with the osmotic pressure exerted by the external medium. To adjust the internal osmotic status that enables organisms to survive in hypersaline environment, several strategies have been reported, for example regulation of ions, the accumulation of organic molecules, quenching of active oxygen, chaperone for protein folding (stress protein), molecular evolution of the salt tolerance enzyme, and the modification of membrane lipid composition.

The mechanisms responsible for osmotic adaptation have been elucidated only relatively recently. There are remarkable similarities between bacteria and plants because organisms from both kingdoms accumulate the same set of cytoplasm solutes

upon exposure to conditions of hyperosmolarity. Thus, it is likely that there will be close parallels in the mechanisms that theses organisms employ to regulate response to osmotic stress (Csonka and Hanson, 1991).

Living cells adapt to water stress by two basic schemes. The first strategy is accumulation of organic solutes to control their internal water activity, maintain the appropriate cell volume and turgor pressure, and protect intracellular macromolecules (Le Rudulier *et al.*, 1984; Galinski and Truper, 1994). The compatible solutes, also called osmolytes, include sugars, amino acids and their derivatives, polyols and their derivatives, betaine, and ectoines (Wood *et al.*, 2001)

The compatible solute

The compatible solutes in general, are low molecular weight organic compounds that accumulate to high intracellular levels under osmotic stress and are compatible with the metabolism of the cell. For example, glycine betaine is compatible solute in halotolerant bacteria, archaea, and cyanobacteria. It is not synthesized by most microorganisms but is taken up from the medium and used for osmoadaptation (Wood, 1999) The compatible solutes are shown in Fig. 1.1.

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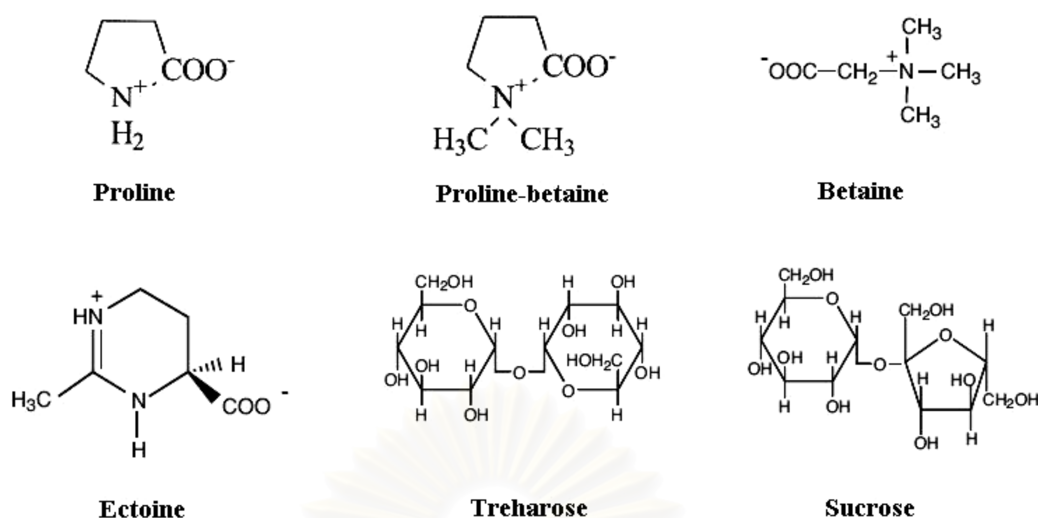

Figure 1.1 Organic compatible solutes found in living organisms.

A very important quaternary ammonium compound commonly used in cellular osmoregulation is glycine betaine (*N, N, N*-trimethylglycine), a derivative of amino acid glycine, is evidence in a number of diverse microbial systems, including enteric bacteria (Andresen *et al.*, 1988), soil bacteria (Smith *et al.*, 1988), *a* halophilic bacteria (Galinski *et al.*, 1994), cyanobacteria (Mackey, 1984) and metanogenic archaea bacteria (Robertson *et al.*, 1990). In higher plant, some plants in families Chenopodaceae, Amaranthaceae and Gramineae, for example, accumulate glycine betaine in response to salt stress or drought (Hanson *et al.*, 1985; Brouquisse *et al.*, 1989; Ishitani *et al.*, 1993) reported that glycine betaine synthesis occurs in chloroplasts of spinach leaves. Subsequently (Robinson, 1986) reported the accumulation of glycine betaine up to 0.3 M in spinach chloroplast provide osmotic adjustment during salt stress.

Glycine-betaine (betaine) is a trimethylamine produced by two different general pathways for synthesizing betaine. The oxidative pathway can utilize one enzyme to change choline to betaine (choline oxidase in Gram-positive soil bacteria;

Actinopolyspora halophila) or require two soluble enzymes to oxidize choline to betaine with betaine-aldehyde as intermediate (choline monooxygenase and betaine-aldehyde dehydrogenase in higher plants and choline dehydrogenase and betaine-aldehyde dehydrogenase in marine invertebrates and bacteria including *E. coli*). Several microorganisms can also generate betaine by successively methylating glycine. GSMT (glycine sarcosine methyltransferase) and SDMT (sarcosine dimethylglycine methyltransferase) in *Halorhodospira halochloris* and *Aphanothece halophytica* transfer the methyl group of S-adenosylmethionine to two different types of amines. Betaine synthesis from glycine in a halotolerant photosynthetic organism *A. halophila* was also carried out by GSMT and DGMT activities. Only one methanogen, *Methanohalophilus portucalensis*, has been shown to synthesize betaine de novo. The last result suggests that betaine accumulation is likely regulated by the internal K^+ concentration in these cells. In most cells where it is accumulated as an osmolyte, the betaine is actively transported from complex medium. The pathway for glycine betaine synthesis is shown in Fig. 1.2.

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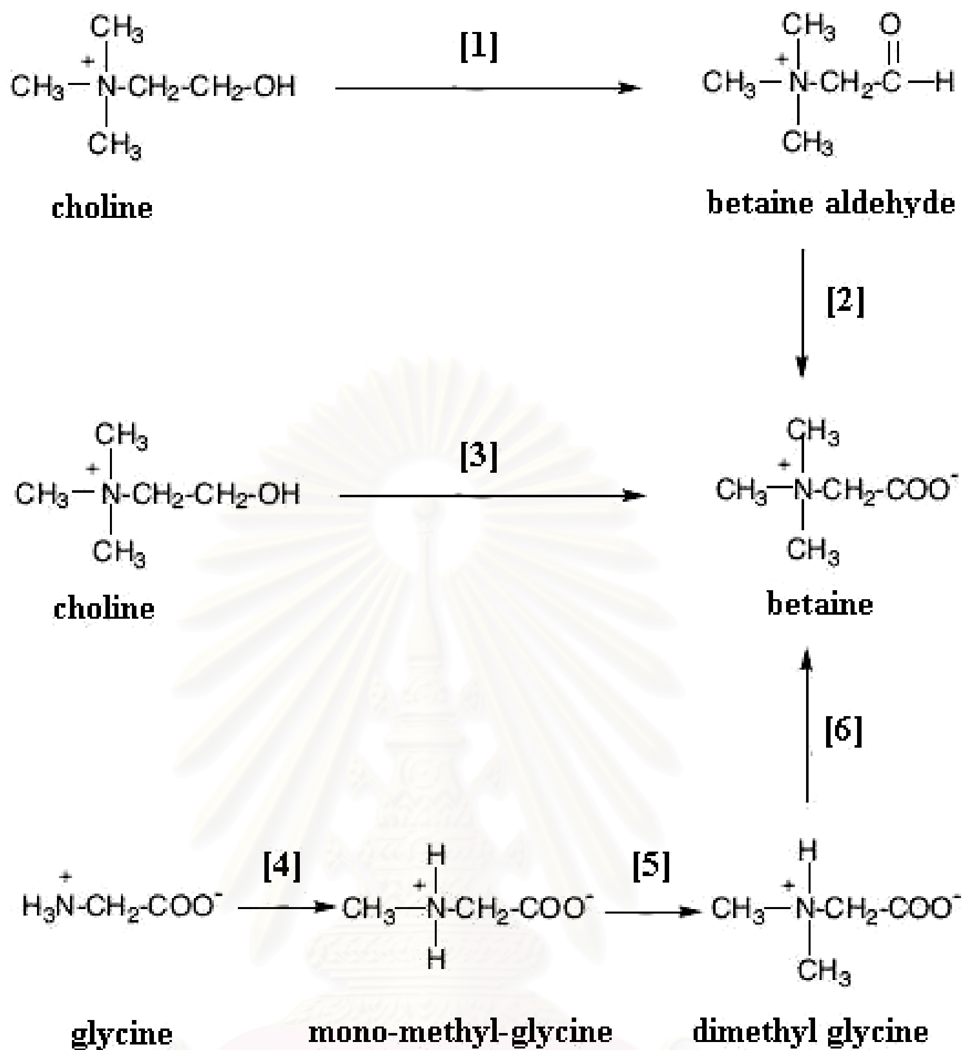

Figure 1.2 glycine betaine pathways (1 = choline dehydrogenase (bacteria) or choline-monooxygenase (plants), 2 = glycine-betaine aldehyde dehydrogenase,3 = choline oxidase, 4 = glycine-sarcosine methyl transferase, 5, 6 = dimethyl glycine methyl transferase)

The second strategy for adaptation to water stress is accumulation of very high intracellular concentration of ions such as Na^+ and K^+ (Ventosa *et al.*, 1998). Since biological membranes are permeable to water, cells cannot maintain the water activity of their cytoplasm higher than that of the surrounding brine, because this would lead to a rapid loss of water to the environment. Therefore, any microorganism living at high salt concentrations may be expected to keep its cytoplasm at least isoosmotic with the extracellular environment. Build up of a turgor pressure requires a hyperosmotic cytoplasm (Oren, 1999)

Osmotic balance in microbes can be maintained by accumulation of high cytoplasmic concentrations of KCl. As a consequence, the intracellular enzymes and macromolecules must be adapted to high ionic strength. In fact, intracellular enzymes of the microbes using the KCl-strategy are not only fully active at high KCl concentrations, but also require monovalent cations (preferably K^+) for stability. It has been shown that bacteria using this strategy have intracellular proteins with higher proportion of acidic amino acids and lower proportion of nonpolar residues than proteins usually (Lanyi, 1974). The KCl-strategy is typical for halophilic, anaerobic, heterotrophic eubacteria (*Haloanaerobiales*) (Oren, 1986; Rengpipat *et al.*, 1988; Oren, 1997) and extremely halophilic archaebacteria (*Halobacteriales*) (Lanyi, 1974) with the exception of extremely halophilic methanogens. The anaerobic heterotrophic halophiles also accumulate significant amounts of NaCl. Extremely large amounts of KCl can be accumulated by halophilic archaebacteria. Intracellular concentrations of several metal K+ have been reported for some *Halobacterium* strains (Lanyi, 1974)

When organisms are subjected to a sudden increase in hyperosmotic condition, they usually respond with an adaptation reaction. Upon the exposure to high salinity, extrusion of sodium ion is needed to balance and maintain sodium level. Hence all

cells have sodium extrusion via the Na^+/H^+ antiporters which catalyze the exchange of Na^+ for H^+ across membranes. The primary energy source for this system in most organisms is the proton electrochemical gradient ($\mu_{H}{}^{+}$) across the cytoplasmic membrane. This proton electrochemical gradient is derived either from respiratory electron transport or at the expense of ATP formed during substrate-level phosphorylation by activity of the membrane ATPase. The Na^+/H^+ antiporters are membrane proteins that are essential for maintenance of the balance between Na^+ and K^+ in plant, fungal bacterial cells, in particular when the organism lacks primary Na^+ pumps or when the Na^+ pumps are not operative.

Na^+/H^+ antiporters

The Na^+/H^+ antiporter (exchanger) is a ubiquitous membrane protein localized in cytoplasmic and organellar membranes and is present in virtually all cell types, including bacteria and the cells of plants and mammals. This integral membrane protein transports Na^+ and H^+ in opposite directions across cell membranes. The direction of exchange is dependent solely upon the ions' electrochemical gradient, requiring no additional metabolic energy. In higher eukaryotic cells, Na^+/H^+ antiporters function to remove excess protons from the cytosol by taking up Na^+ from the external environment, a process that is driven by the sodium gradient generated by Na^+/K^+ATPase (Figure 1.3A). In bacteria and yeast, Na^+/H^+ antiporters is in the opposite direction, and intracellular Na^+ is removed utilizing the H^+ gradient generated by the plasma-membrane H^+-ATPase (Figure 1.3B) . In mammalian and fungal cells, Na^+/H^+ antiporters is electroneutral, with a stoichiometry of 1:1. Conversely, bacterial Na^+/H^+ antiporters is electrogenic. In *Escherichia coli*, NhaA

exchanges 2 H^+ per 1 Na^+ and NhaB exchanges 3 H^+ per 2 Na^+. In addition, all Na^+/H^+ antiporters are able to transport Li^+ in exchange for H^+. (Wiebe *et al.*, 2001)

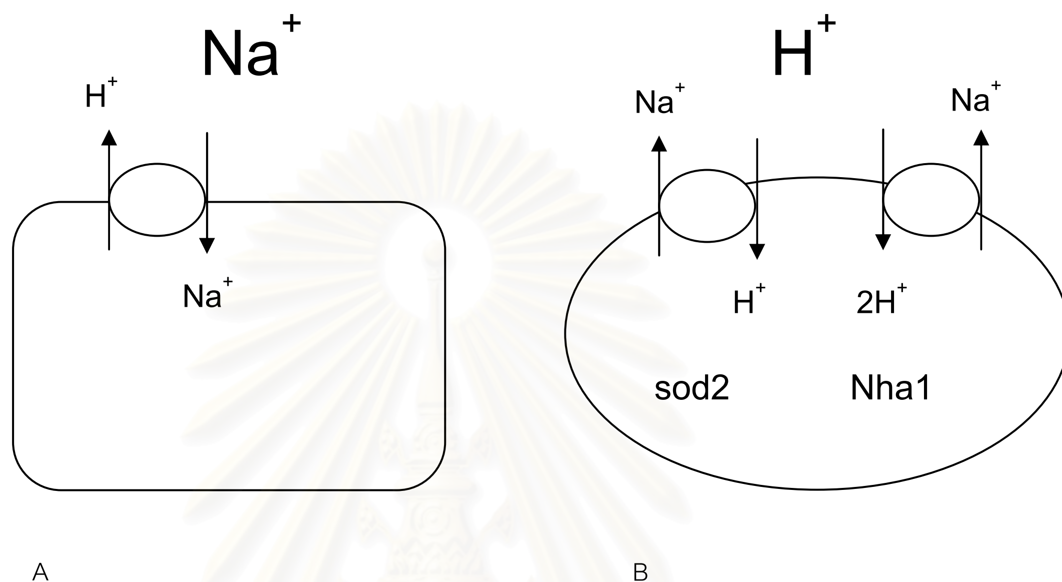

Figure 1.3 Schematic diagram illustrating typical activity of Na^+/H^+ exchanger isoforms in mammalian cells, yeast and *E. coli* (A) Typical activity of the mammalian Na^+/H^+ exchanger, NHE1 isoform. The large `$Na^+$' indicates the higher sodium concentration outside the cell driving activity of the protein. (B) Activity of $Na^+/H^+$ exchangers involved in salt tolerance, the yeast $Na^+/H^+$ exchanger sod2 of *Schizosaccharomyces pombe* and NhaA of *E. coli.* The large `H^+' indicates the higher H^+ concentration driving the activity of these forms of the protein

The importance of Na^+/H^+ antiporters has been shown to be involved in diverse physiological processes, e.g., extrusion of sodium which is toxic if it accumulates too high concentration in cells (Dover and Padan, 2001), play a role for internal pH regulation and cell volume, involve in morphogenesis, extruding the H^+ generated during metabolism, and also crucial for salt tolerance in particular when the organism lacks primary Na^+ pumps or when the Na^+ pumps are not operative such as in plants or cyanobacteria (Karpel *et al.*, 1988; Blumwald *et al.*, 2000; Soong *et al.*, 2000; Inaba *et al.*, 2001)

The genomic sequence projects of several species from bacteria to higher plants and humans have been emerging. They reveal the Na^+/H^+ antiporters cluster in several families, at least eight families of Na^+/H^+ antiporters have been reported, i.e. 1) NhaA, 2) NhaB, 3) NhaC 4) ChaA (CAX), 5) NhaP (NHE, NHX), 6) NapA, 7) NhaD, and 8) Sod2. Among them, the physiological and molecular genetic studies of the Na^+/H^+ antiporters system in *E.coli* have been well studied and contribute extensively to the understanding of the physiology of Na^+/H^+ antiporters. Three antiporters in *E. coli*, NhaA, NhaB and ChaA, are known and their functional characteristics have been well described (Padan and Schuldiner 1996. Bacterail Na^+/H^+ antiporters: molecular biology, biochemistry, and physiology. In Handbook of Biological Physics (Konings *et al*., 1986; Rosen, 1986). In human, the existence of a total of nine NHE paralogs (NHE1-9) in the entire genome sequence, which differ in subcellular location, has been reported (Brett *et al.*, 2005). The Na^+/H^+ exchanger isoform1 (NHE1) is first discovered and it is universally present in the plasma membrane of mammalian cells. It plays a key role in the regulation of internal pH, but not for Na^+ extrusion. All isoforms of NHE have a similar structure. Generally it consists of an N-terminal integral membrane domain, which catalyzes the Na^+/H^+ exchange, and a long C-

terminal cytoplasmic domain, which functions as a regulatory membrane (Orlowski and Grinstein, 1997). In plant, *Arabidopsis thaliana*, the exploration of Na^+/H^+ antiporters has been reported from the recent cloning and screening mutants in which defective to salt tolerance, i. e., the AtNHX1 gene encodes a tonoplast Na^+/H^+ antiporter has been cloned and identified by the similarity to the yeast vacuolar antiporter NHX1 (Gaxiola *et al.*, 1999). The SOS1 gene encoding a plasma membrane Na^+/H^+ antiporters has been identified by screening of the mutants defective in salt tolerance. It is the first isolation of the Na^+ efflux transport across plasma membrane in Arabidopsis. This putative gene plays a major role for Na^+ extrusion across plasma membrane and controls long distance Na^+ transport from root to shoot (Shi *et al.*, 2000; Shi *et al.*, 2003). The progress on the recent cloning of these Na^+/H^+ antiporters in Arabidopsis provides the basis for the understanding on physiology and molecular biology of salt tolerance.

The completion of genomic sequencing of *Arabidopsis thaliana* in the past few years provides an information for assigning members to gene families Its genome reveals many genes encoding membrane transport proteins homolog of Na^+/H^+ antiporters, i.e, the families CPA1 (The monovalent Cation:Proton antiporter-1), CPA2 (The monovalent Cation:Proton antiporter-2) and NhaD (Maser *et al.*, 2001). The CPA1 family, arose from ancestral NhaP genes in prokaryotes, consists of a member of 8 genes (AtNHX1-8), such as the AtNHX1 and AtNHX7 (SOS1). These two genes are crucial and contribute for salt tolerance in Arabidopsis (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001) The CPA2 family shares origin with prokaryotic Na^+/H^+ antiporter, consists of 28 genes (AtCHX1-28) in Arabidopsis genome. However, only limited information on the significance of these genes is available. The probable functions of the AtCHX genes have been reported

very recently by the expression pattern in transgenic plants and suppressing gene functions (Song *et al.*, 2004; Sze *et al.*, 2004). It reveals that several AtCHX genes specifically expressed in the male gametophyte and sporophytic tissues and developmentally regulated during gametophyte (Sze *et al.*, 2004) The AtCHX17, which expressed in the epidermal and cortical cells of mature root zone, is believed to be involve in K^+ transport since the knock-out mutant plants accumulate less K^+ in the roots when stress with salt or K^+ starvation (Cellier *et al.*, 2004). T he AtCHX23, which shows a homology with SOS1, was functionally analyzed. This putative gene encodes a $Na^+(K^+)/H^+$ antiporter on the chloroplast envelope and functions in pH homeostasis and chloroplast development (Song *et al*., 2004). These experimental results suggest the multiplicity of homolog putative Na^+, K^+/H^+ antiporters in Arabidopsis which have various transport modes with different targets. The multiplicity of AtCHX genes is conserved in higher plants but not found in animals (Cellier *et al*., 2004; Sze *et al*., 2004).

Based on their primary structure, a similar membrane topology can be predicted for all isoforms, with 10–12 membrane-spanning (M) regions at the N terminus and a large cytoplasmic region at the C terminus (Fig. 1.4) (Wiebe *et al*., 2001)

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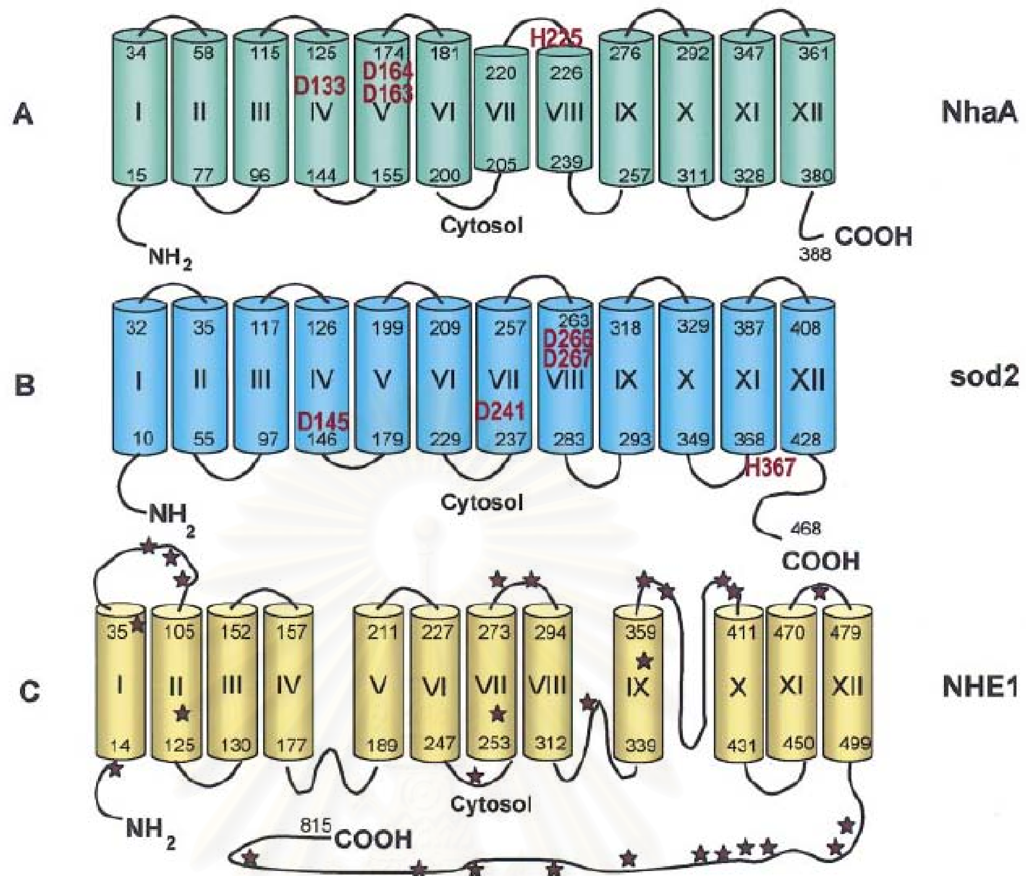

Figure 1.4 Models of the secondary structure of Na^+/H^+ exchangers (A) Model of NhaA based on the findings by Rothman *et al.* (B) Model of sod2 based on predictions using the program TopPred II (C) Model of mammalian, human NHE1 isoform of the Na^+/H^+ exchanger based on the findings by (Wakabayashi *et al*, 2000).

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To understand salt-stress tolerance in relation to oxygenic photosynthesis, a cyanobacteria is one of a useful model since they conduct plant-like oxygenic photosynthesis. Phylogenetic analysis using multiple criteria strongly suggested the cyanobacteria are an earlier evolution species and can serve as an ancestor of higher plant. In the recent year, the entire genome sequences of several cyanobacteria, namely *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC7120, *Thermosynechococcus* sp. BP-1, are available and will be invaluable for assigning members to gene families. Among them, *Synechocystis* sp. PCC6803, which its genome size 3.6 MB, represents an ideal model for study stress responses. It is a simple autotroph-photosynthesis, ROS scavenging, respiration and chloroplast functions. The organism can be transformed with very high efficiency relying on homologous recombination.

In cyanobacteria, the existence of a concentration gradient of Na^+ directed inward, which is dependent on the photosynthetic or respiratory metabolism has been demonstrated, indicates the existence of an active transport system extruding Na^+ from cyanobacterial cells. This active transport system is well known by the function of Na^+/H^+ antiporters. The activities of the circulation of H^+ and Na^+ by the Na^+/H^+ antiporter appeared to be involved in the intracellular Na^+ homeostasis of a unicellular *Synechococcus* sp. PCC6311. The Na^+/H^+ antiporter activities have been shown in cyanobacterial strains grown at different sodium concentration. Interestingly, a variety of Na^+/H^+ antiporter seems to cooperate in cyanobacteria, the whole genome sequencing of *Synechocystis* sp. PCC6803 appears to have five putative Na^+/H^+ antiporters, slr1727 (NhaS1); sll0273 (NhaS2); sll0683 (NhaS3); slr1595 (NhaS4) and slr0415 (NhaS5). These putative genes show very strong homologies to those found in higher plants. Phylogenetic relationship of these antiporters suggested the NhaS1 and NhaS2 closely resemble with the eukaryotic type while the NhaS3, NhaS4 and NhaS5

closely resemble with the prokaryotic ones (Blumwald *et al.*, 1984; Padan *et al.*, 1988; Nitschmann and Packer, 1992; Kaneko *et al.*, 1996)

To understand the physiological functions of the Na^+/H^+ antiporters in cyanobacteria, gene disruption leads to new insight into the physiological roles. In addition to gene disruption, the functional expression of these Na^+/H^+ antiporters in the mutant lacking antiporter genes also provide a powerful tool to identify and study Na^+/H^+ antiporters.

To date, functional characterization of Na^+/H^+ antiporters in cyanabacteria has been analyzed from only two species, the unicellular cyanobacterium *Synechocystis* sp. PCC6803 and the halotolerant cyanobacterium *A. halophytica*. The putative genes encoding the Na^+/H^+ antiporter from *Synechocystis* sp. PCC6803 have been functionally characterized and expressed in the salt-sensitive *E.coli* mutant in which three putative Na^+/H^+ antiporters are disrupted. Among them, only the NhaS1 (eukaryotic type), and NhaS3 (prokaryotic type) complemented the salt-sensitive mutant *E.coli* and could exhibit the exchange activity of $Na^+(Li^+)$ for H^+ (Ohyama *et al.*, 1994; Hamada *et al.*, 2001; Inaba *et al.*, 2001; Waditee *et al.*, 2001).

NhaS1 (SynNhaP1) could exchange activity of $Na^+(Li^+)$ for H^+ over a wide range of pH from 5 to 9 and their activities were insensitive to amiloride. Kinetic analysis revealed that NhaS1 (SynNhaP1) encodes a low affinity Na^+/H^+ antiporter. The homologous gene of NhaS1(SynNhaP1), ApNhaP1, was isolated from a halotolerant cyanobacterium, *A. halophytica*. The gene product of ApNhaP1 exhibited the Na^+/H^+ antiporter activity over a wide pH range between 5 and 9 but had virtually no activity for Li^+/H^+ antiporter activity. Interestingly, ApNhaP1 could exhibit the exchange activity of Ca^{2+} for H^+ at alkaline pH.

Topological models of NhaS1(SynNhaP1)and ApNhaP1 predicted the presence of 11 segments of trans-membrane with a long hydrophilic cytoplasmic tail in the C-terminal part (123 amino acid residues in case of SynNhaP1 and 124 amino acid residues in case of ApNhaP1). Functional studies of the chimera proteins by exchanging the C-terminus between NhaS1(SynNhaP1) and ApNhaP1 and vice versa could serve the plausible role and providing the importance of the C-terminal tail for ion specificity. The putative NhaS1(SynNhaP1) exhibited the exchange of Na^+(Li^+) for H^+ whereas ApNhaP1 exhibited the exchange of Na^+, Ca^{2+} for H^+. Although both of them contained a similar length of a C-terminal tail but their charges were different. ApNhaP1 had more positive charge [22(+), 14(-)] while SynNhaP1 had more negative charge [15(+), 24(-)]. The chimera ASNhaP, in which a long C-terminus of ApNhaP1 replaced with SynNhaP1, could exhibit the exchange of Li^+ for H^+ which never been observed in the wild type ApNhaP1. The chimera between ApNhaP1 and NhaS1 (SynNhaP1) demonstrated the importance of the charge on C-terminal tail and affected to their ion specificity. The truncated versions of NhaS1 (SynNhaP1) by deletion of C-terminus is not only diminished the exchange of antiporter activity but also increased the affinity for Na^+ and Li^+ ion (unpublished data). From these results suggested that the C-terminal tail of NhaP played a role for ion specificity and affecting to the Km of the exchange activity (Ohyama *et al.*, 1994; Hamada *et al.*, 2001; Inaba *et al.*, 2001; Waditee *et al.*, 2001).

A long tail region residing in the cytoplasmic part also apparent in the SOS1 (a plasma membrane type Na^+/H^+ antiporter in Arabidopsis), AtNHX1 (a tonoplast Na^+/H^+ antiporter), NHE isoform in mammalian, and Nha1p in yeast *Saccharomyces cerevisiae.* The homology of these long tails region is very low. In NHE, and a long hydrophilic cytoplasmic region are structural diverse and accumulating evidence

suggested this region mediates interactions with other proteins, therefore functions as a regulatory part. It had the evidence that the Nha1p C-terminus involves in the cell response to sudden changes in environmental osmolarity, and very recently it has been shown that a conserved domain in the tail region of Nha1p play an important roles in localization and salinity resistant cell-growth. In the plant plasma membrane type Na^+/H^+ antiporter SOS1, the long C-terminal portion has been shown to reside in the cytoplasmic space and necessary for salt tolerance. The single amino acid substitution mutants in the hydrophilic tail of SOS1, i.e. sos1-8 (G777E) and sos1-9 (G784D), were inactive for SOS1 function. In a vacuolar type Na^+/H^+ antiporter, AtNHX1, deletion of the hydrophilic C-terminal tail caused a dramatic increase of Na^+/H^+ antiport activity. The C-terminal of AtNHX1 appears to be involved in the determination of the ion selectivity of the transporter. Thus, the functions of C-terminal tail in eukayotic Na^+/H^+ antiporters seem to have various mode (Denker *et al.*, 2000; Lehoux *et al.*, 2001; Pang *et al.*, 2001; Kinclova *et al.*, 2002; Mitsui *et al.*, 2004).

Another type of Na^+/H^+ antiporter in *Synechocystis* sp. PCC 6803, which is classified as the prokaryotic type, consists of NhaS3, NhaS4 and NhaS5. It should be noted that only the NhaS3 could exhibit the exchange activity of Na^+/Li^+ for H^+ when it was expressed in the salt-sensitive mutant *E.coli*. NhaS3 shares homology with NapA from *Enterococcus hirae*, GerN from *Bacillus cereus*, and CPA2 of higher plants. Topological model of NhaS3 predicted 11 segments of transmembrane and a long C-terminal tail is absent. NhaS3 could exchange activity of Na^+, Li^+ for H^+ in a pH dependent fashion. The exchange activities of Na^+ or Li^+ for H^+ are restricted at alkaline pH. The homologous gene of NhaS3 was isolated from a halotolerant cyanobacterium, *A. halophytica*. It was found that *A. halophytica* contains at least two

homologous genes of NhaS3. Interestingly, one of gene product has the exchange activities of Na^+ for H^+/K^+. It should be note that the extreme halophile and thermophile harbour the Nap type antiporter in their genome. Comparisons with the apparent orthologues in *Noctoc punctiforme*, *Anabaena* sp. PCC 7120 and Marine cyanobacteria show that NhaS3 is the most highly conserve of Na^+/H^+ antiporter found among cyanobacteria (Strausak *et al.*, 1993; Southworth *et al.*, 2001)

The presence of the multiple Na^+/H^+ antiporters in cyanobacteria suggested they might play overlapping functions, or might have various transport modes. From the gene knockout analysis of the five putative Na^+/H^+ antiporters in *Synechocystis* sp. PCC 6803 indicated the role of these Na^+/H^+ antiporters for the growth under high-salinity condition as well as high alkaline pH (Table1.1). Among five putative genes, only NhaS3 seems to be crucial for NaCl tolerance. The attempt to knockout of NhaS3 from different group, this gene failed to make a complete segregate. Although the partial segregate of NhaS3 was constructed, it revealed that the mutant, in which NhaS3 was disrupted, became less tolerance for salt than that could observe in the wild type. Therefore, this antiporter might be the most crucial and necessary for salt tolerance in cyanobacteria (Elanskaya *et al.*, 2002; Wang *et al.*, 2002).

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Table 1.1 Molecular basis of the gene knock-out of five putative Na^+/H^+ antiporters in *Synechocystis* sp. PCC 6803

Putative Na^+/H^+ antiporter	Phenotype/affect	References
-slr1727 (NhaS1)	-No NaCl sensitivity	-Wang *et al*., 1999
-sll0273 (NhaS2)	-low salt sensitivity, shift the pH preference to acidic region	-Wang *et al*., 1999; Mikkat *et al*., 2000
-sll0683 (NhaS3)	-High salt sensitivity (using partial segregation of mutant)	-Inaba *et al*., 2001; Wang *et al*., 1999
-slr1595 (NhaS4)	-low salt sensitivity (similar to NhaS2)	-Inaba *et al*., 2001; Wang *et al*., 1999
-slr0415 (NhaS5)	-No NaCl sensitivity	-Inaba *et al*., 2001
-NhaS1/NhaS4	-No NaCl sensitivity	- Elanskaya *,et al.,* 2002
-NhaS1/NhaS5	-No NaCl sensitivity	- Elanskaya *,et al.,* 2002
-NhaS1/NhaS4/NhaS5	-No NaCl sensitivity	- Elanskaya *,et al.,* 2002

The other complete segregation of four putative Na^+/H^+ antiporters revealed no salt-sensitive phenotype, but somehow reflects physiological roles of these putative genes. For the disruption of NhaS1 in *Synechocystis,* the mutant NhaS1 cells had no salt-sensitive phenotype, suggesting this putative Na^+/H^+ antiporter does not crucial for salt tolerance in *Synechocystis*. However, the contribution for salt tolerance by the homolog NhaS1 had been studied by overexpression of ApNhaP1 from a halotolerant cyanobacterium, *A. halophytica*. The overexpressing of ApNhaP1 in a fresh water

cyanobacterium, *Synechococcus* sp. PCC 7942, conferred salt tolerance, and capable of growth in seawater (Waditee *et al.*, 2001). The comparable situation supporting the crucial role of NhaS1 was demonstrated in Arabidopsis. This has been extensively studied for the role of NhaS1 homologue, SOS1 in Arabidopsis for salt tolerance. The overexpression of this plasma membrane Na^+/H^+ antiporter gene, SOS1, in *Arabidopsis thaliana* enhanced salt tolerance.

The disruption of NhaS2 caused the mutant cells unable to grow at low sodium concentration and exhibited an increased sensitivity towards potassium ion, even at low concentrations. From this result, it has been proposed that the NhaS2 is required for Na^+ influx (Mikkat *et al.*, 2000). In addition, the knockout of NhaS2 dramatically shifts the pH preference of *Synechocystis* to the acidic region, suggesting this antiporter implicated in determining the pH optimum in the *Synechocystis*. The mutant cells of NhaS4 were observed quite similar phenotype as the disruption of NhaS2, but it was sensitive to acidic pH (low pH). The expressing of NhaS4 in an *E.coli* mutant is tolerant to K^+depleted medium, suggesting NhaS4 facilitates K^+ uptake.

From the gene knockout analysis of the putative genes of Na^+/H^+ antiporter could provide the plausible physiological functions. But the presence of multiple Na^+/H^+ antiporters with potentially overlapping function, which is not completely characterized, needs to be investigated in many points. The progress in genome sequence of higher plants suggested the complexity of Na^+/H^+ antiporters in their genomes. For example, in Arabidopsis genome presents the number of genes involves in Na^+ transport and some of them were identified to play a crucial role during salt stress. **Table 1.2** shows physiological roles of Arabidopsis genes, AtNHX1, AtNHX7(SOS1), AtCHX17, AtCHX23, and AtHKT, in which contribute for salt

tolerance. Numerous investigations have led to the conclusion that these genes play important role to expel Na^+ across plasma membrane, sequestrate Na^+ into a tonoplast and or function to re-circulate or remove large amount of Na^+ from shoot. These results provide the number and nature of some genes that are implicated in stress tolerance. However, due to the complexity of salinity stresses responses, the function of the novel antiporters need to be investigated further.

Table 1.2 Physiological roles of the Arabidopsis genes in which contribute for salt tolerance

Putative Na^+/H^+ antiporter	Functions	References
-AtNHX1	-Compartmentation of Na^+ into a tonoplast	-Gaxiola *et al*., 1999
-AtNHX7 (SOS1)	-Na^+ efflux across plasma membrane and controls long distance Na^+ transport from root to shoot	-Shi *et al*., 2000; 2003
-AtCHX17	-K^+ transport	-Sze *et al*., 2004; Cellier *et al*.,2004
-AtCHX23	-pH homeostasis and chloroplast development	-Song *et al*., 2004
-AtHKT1	- Na^+ re-circulation from shoot to root	- Berthomieu *et al.,* 2003 Apse *et al*., 2003

In other plants, the genes encoding Na^+/H^+ antiporters have been cloned, such as barley, cotton, maize, rice. The recent cloning of the Na^+/H^+ antiporters from rice

reveals the expression level was induced under salt stress condition. Overexpression of the rice Na^+/H^+ antiporter, OsNHX1, enhanced the salt tolerance of transgenic rice cells and plants (Apse *et al.*, 2003; Berthomieu *et al.*, 2003; Fukuda *et al.*, 2004; Wu *et al.*, 2004; Vasekina *et al.*, 2005; Zorb *et al.*, 2005).

4. Objectives

The organism used in this study is the unicellular halotolerant cyanobacterium, *A. halophytica* (This alga is classified into Chroococcales order, Chroococcacean cyanobacteria subgroup) (Geitler, 1932; Stanier *et al.*, 1971). This organism is an extremely halotolerant organism that can grow in a wide range of salinity conditions from 0.25-3.0 M NaCl (Takabe, 1988). In general, there are a number of protective mechanisms for cells thriving in changing salinity environment. Previously, *A. halophytica* was shown to accumulate a compatible solute, glycinebetaine, upon the increase in the external salinity (Takabe, 1988). Recently, it has been reported that Na^+/H^+ antiporters from *A. halophytica* can make *E. coli* and a freshwater cyanobacterium *Synechococcus* PCC 7942 tolerant to high salinity (Waditee *et al.*, 2002).

The main work of this thesis is to study Na^+/H^+ exchange under normal and high salinity condition in *A. halophytica*.

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CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Instruments

Autoclave HV-110	: Hirayama, Japan
Autopipette	: Pipetman, Gilson, France
Centrifuge, refrigerated centrifuge	: Model J-21C, Beckman Instrument Inc, USA
Digital lux meter FT710	: Taiwan
French pressure cell	: SIM-Aminco Spectronic Instrument, USA
Illuminated/Refrigerated orbital	: Sanyo, England
Laminar flow BVT-124	: International Scientific Supply, Thailand
Luminescence spectrometer LS55	: Perkin Elmer, USA
Microcentrifuge 5417C	: Eppendoff, German
pH meter pH900	: Precisa, Switzerland
Spectrophotometer UV-240	: Shimadzu, Japan and Du series 650: Beckman, USA
Ultracentrifuge Optima L-100 XP	: Beckman coulter, USA
Vortex	: Model K-550-GE: Scientific Industries, USA

2.2 Chemicals

Amiloride	: Sigma, USA
Ammonium chloride	: Katayama Chem, Japan

Acridine orange : BDH Chemicals Ltd, England

Calcium chloride : Merck Ag Darmstadt, Germany

Carbonyl cyanide 3-chlorophynyl hydrazone (CCCP): Sigma, USA

Choline chloride : Sigma, USA

Coomasie brilliant blue G-250 : Sigma, USA

Coomasie brilliant blue R-251 : Sigma, USA

Dextran T 500 :Amercharm bioscience, Sweden

Dithiothreitol (DTT) : Sigma, USA

Ethylenediaminetetraacetic acid (EDTA) : Sigma, USA

Ethanol : Katayama Chem, Japan

Ferric sulfate : Mallinckrodt Chemical, USA

HEPES : Sigma, USA

L-lactate : Sigma, USA

Lithium chloride : Sigma, USA

Magnesium chloride : Merck Ag Darmstadt, Germany

Magnesium sulfate : Merck Ag Darmstadt, Germany

MES : Sigma, USA

Monensin : Sigma, USA

N, N'-diclyclohexylcarbodiimide (DCCD) : Sigma, USA

Polyethylene glycol : Sigma, USA

Potassium chloride : Merck Ag Darmatadt, Germany

Sodium azide : Sigma, USA

Sodium nitrate : Sigma, USA

Sodium chloride : Sigma, USA

Sodium cyanide : Sigma, USA

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Sodium ionophore	: Fluka, German
Sorbitol	: BDH, England
Sucrose	: Fluka, German
Tris-hydrochloride	: USB, USA
Triton X-100	: Packard, USA
Valinomycin	: Sigma, USA

2.3 Bacterial strains

Aphanothece halophytica was initially isolated from Solar Lake in Israel. The organism was kindly provided by Dr. T. Takabe of Nagoya University, Japan.

Methods

2.4 Culture conditions

A. halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane which multiplies by binary fission as shown in Figure 3.1. Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described in Appendix A except that the NaCl concentration of the culture medium was adjusted to a 0.5 M for normal condition and 2.0 M for salt-stress condition. Cotton-plugged 250 ml conical flasks containing 100 ml of medium were used and shaken with 160 rpm on a reciprocal shaker without supplementation of condensed CO_2 gas. The culture flasks were incubated at 30 °C under continuous fluorescent white light (30 $\mu Em^{-2}s^{-1}$).

2.5 Measurements of Na^+/H^+ exchange by intact cell

Na^+/H^+ exchange was measured by the acridine orange fluorescence quenching technique using Perkin-Elmer fluorimeter (Luminescence Spectrometer, LS-55) exciting light was set at 493 nm and emission light was at 525 nm. The assay mixture (total volume 1 ml) was composed of 0.5 M sorbitol, 10 mM HEPES/Tris buffer(pH 7.6), and 1 µM acridine orange. *A. halophytica* cells (25 µg protein) were added to the assay mixture which caused the accumulation of dye and fluorescence guenching (Q). To assess Na^+/H^+ exchange, NaCl solution was added to the mixture. Fluorescence increase (ΔQ) due to the excretion of H^+ by antiporters was monitored. (Elanskaya *et al.,* 2001)

2.5.1 Effect of NaCl on Na^+/H^+ exchange

A. halophytica cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described Appendix A except that the NaCl concentration of the culture medium was adjusted to a 0.5 M. After seven days, cell culture of 100 ml was harvested at late exponential growth phase. The cells were concentrated by centrifugation at 8,000 rpm for 20 min, washed twice with the media that same salt concentration. *A. halophytica* cells were added to the assay mixture (25 µg). Where indicated, NaCl solution was added to the mixture to adjust the final concentration from 0 to 200 mM.

2.5.2 Growth-independent Na^+/H^+ exchange

Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described Appendix A except that the NaCl concentration of the culture medium was adjusted to a 0.5 M for normal condition and 2.0 M for salt-stress condition. At various time intervals for 2-15 days cells were

harvested and measured Na^+/H^+ exchange as described above. For salt conditions, NaCl solution was added to adjust the final concentration to 100 mM.

2.5.3 Effect of pH on Na^+/H^+ exchange

Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described in Appendix A except that the NaCl concentration of the culture medium was adjusted to 0.5 M. Dependence of pH on Na^+/H^+ exchange was tested by varying the pH, 10 mM MES/Tris buffer (pH 5-6); 10 mM Hepes/Tris (pH 7-8) and 10 mM Glycine-NaOH buffer (pH 9-10) was used as the buffer and measuring Na^+/H^+ exchange as described in section 2.5. For salt condition, NaCl solution was added to the mixture to adjust the final concentration to 100 mM.

2.5.4 Effect of cations on H^+ exchange in *A. halophytica*

Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described in Appendix A except that the NaCl concentration of the culture medium was adjusted to a 0.5 M. The cations/H^+ exchange was monitored by adding 100 mM of either KCl, LiCl, $CaCl_2$ or $MgCl_2$ to the assay mixture. In case where the effect of the combination of two or three cations were tested, an equal concentration of each cation was added so that the final concentration was 100 mM.

2.5.5 Effect of metabolic inhibitors on Na^+/H^+ exchange

Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described in Appendix A except that the NaCl concentration of the culture medium was adjusted to 0.5 M. Inhibitors including ionophores, ATPase inhibitors, energy dissipators were added to the reaction mixture

incubated for 30 min before initiation of the reaction. For salt conditon, NaCl solution was added to the mixture to adjust the final concentration to 100 mM.

2.6 Preparation of plasma membrane in *A. halophytica* cells

A. halophytica was grown in BG_{11} medium containing 0.5 M NaCl. After seven days, cells attained late exponential growth phase. The cells were harvested by centrifugation at 8,000 rpm for 20 min and washed twice with 10 mM Tris-HCl buffer, pH 7.6, containing 0.25 M sucrose to protect the cells from lysis, 5 mM DTT and 0.14 M Chlorine chloride (TCDS buffer). The cells of were collected and the plasma membranes were prepared according to the method by Murata and Omata (1988). The cells were re-suspended in TCDS buffer (see Appendix B) and passed through a pre-cooled French press cell operating at 4000 psi followed by centrifugation at 8,000 rpm for 20 min to remove unbroken cells and cell debris. The resulting dark blue green supernatant contained a crude membrane mixture and was used as starting material for sucrose gradient and two phase partitioning.

2.6.1 Sucrose gradient separation

The procedure was carried out according to Omata *et al*. (1985) The dark blue green supernatant was centrifuged at 100,000 g for 1 hr and pellet made up to a sucrose concentration of 50% (w/v) by adding 90% sucrose solution. A 3 ml aliquot was placed at the bottom of a 12 ml centrifuge tube, overlaid with 2 ml of 39%, 3 ml of 30%, and 2 ml of 10% sucrose solutions (w/v), and centrifuged at 130,000g for 16 h at 4°C in a swinging bucket rotor (Beckman SW 41). Plasma membrane formed a band in the 30% sucrose layer and thylakoid membranes at the interface between the 39 and 50% sucrose layers. Cell walls were pelleted at the

bottom. The plasma membrane was withdrawn from the gradient and collected by centrifugation at 300,000g for1 h after 3-fold dilution with Tris-HCl buffer (pH 7.6) containing 0.25 M sucrose .

2.6.2 Aqueous polymer two-phase partitioning

The procedure was carried out according to Norling *et al.* (1994). Crude membranes prepared as described in section 2.6 were centrifuged at 100,000 g for 1 hr. This pellet was suspended to a total volume of 5 ml in 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.6, and 5 g of the suspension was added to the 10 g phase mixture (see Appendix C) to give a 15 g phase system with a final concentration of 5.6 % (w/w) Dextran T-500, 5.6 % (w/w) polyethylene glycol, 0.25 M sucrose and 5 mM Tris-HCl buffer, pH 7.6. The partitioning was performed by turning the tube gently upside-down 20-30 times at 4 ^{o}C. After mixing, phase setting was facilitated by centrifugation for 5 min at 3,000 g and the upper and lower phases were collected separately. Plasma membranes were in lower phase. The lower phase was diluted 2-fold with Tris-HCl buffer (pH 7.6) containing 0.25 M sucrose and plasma membranes were collected by ultra centrifugation at 100,000 g for 1 hr.

2.6.3 Absorption spectra

As a purity criterion for plasma membrane, the ratio between the absorption at 485 nm (carotenoid) and at 680 nm (chlorophyll) was used, since plasma membranes contain carotenoids absorbing at 485 nm but do not contain chlorophyll which absorbs at 680 nm (Murata and Omata, 1988). Absorption spectra of the different membrane fractions were measured at room temperature with spectrophotometer. The samples were measured for the absorption ratio between

carotenoid absorption (485 nm) and chlorophyll absorption (680 nm) after increasing number of partitioning steps. For comparison, the 485/680 absorption ratio for the total mixture of cyanobacterial membranes (crude membrane) before partitioning was also determined (Norling *et al*., 1994).

2.6.4 Electron micrographs of plasma membrane vesicles

Plasma membrane vesicles were analyzed by electron microscopy. Membrane vesicles were stored in -80 ^{o}C before used. Optical microscopy was performed with a microscope (Axioskop FL, Carl Zeiss, Gottingen, Germany) that was equipped with a high-definition image-capture camera (model HC-1000, Fujix, Tokyo). For electron microscopy, cells were pelleted by centrifugation at 3,000*g* for 5 min and then immediately fixed for 1 h with 2% (v/v) glutaraldehyde in 100 mM sodium phosphate (pH 7.2). After rinsing overnight in sodium phosphate buffer, samples were post-fixed in 1% (v/v) osmium tetraoxide for 1 h before dehydration by passage through a graded ethanol series (50%–100%, v/v). Then samples were infiltrated with and embedded in resin (Araldite CY-212, Ouken, Tokyo). Thin sections were mounted on copper grids, stained with uranyl acetate, and examined under an electron microscope (model 1200EX, JEOL, Tokyo).

2.6.5 Measurements of Na^+/H^+ exchange by plasma membrane vesicles.

The exchange activity was estimated from the changes of ΔpH (transmembrane pH gradient) by the addition of salt to the reaction mixture, which contained 10 mM Tris-HCl (titrated with HCl to the indicated pH), 5 mM $MgCl_2$, 0.14 M choline chloride, 1 μM acridine orange, and membrane vesicles (25 μg of protein) in a volume of 1 ml. The ΔpH was monitored with acridine orange fluorescence,

which was obtained by excitation at 493 nm and emission at 525 nm. Before the addition of salt, Tris-DL-lactate (2 mM) was added to initiate fluorescence quenching (Q) due to respiration. Lactate energizes the vesicle and accumulates H^+ intravesiculary, which causes the accumulation of dye and fluorescence quenching (Q). Upon the addition of salt (5 mM), fluorescence increases because of the excretion of H^+ by antiporters and dequenched fluorescence (ΔQ) was monitored. Then, NH_4Cl (25 mM) was added to dissipate ΔpH.

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CHAPTER III

RESULTS

3.1 Na^+/H^+ exchange by intact cell

A. halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane (Fig. 3.1). The cells multiply by binary fission. The cells were grown photoautotrophically in a BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution, pH of medium was adjusted to 7.6 at different NaCl concentrations**.** To examine the exchange activity, cells were monitored by measuring the accumulation of dye induced fluorescence quenching (Q) and salt induced fluorescence dequenching (ΔQ) as shown in Fig. 3.2. The data were shown as % Fluorescence [(ΔQ /Q) x 100].

3.1.1 Effect of NaCl on Na^+/H^+ exchange

We tested the effect of NaCl on Na^+/H^+ exchange by intact cells and found that increasing the concentration of NaCl up to 200 mM resulted in a saturable fluorescence rate above 100 mM NaCl (Fig. 3.3). The results indicated that the higher amount of H^+ or Na^+/H^+ exchange was enhanced by NaCl. Michaelis-Menten kinetics were revealed, and calculated apparent K_m values of approximately 23.7 mM and 22.1 mM , whereas V_{max} value were 15.0 and 20.0 % fluorescence for NaCl in *A. halophytica* grown in 0.5 and 2.0 mM NaCl, respectively.

3.1.2 Growth-independent Na^+/H^+ exchange

Growth of *A. halophytica* cells in the medium containing 2 M NaCl showed the similar growth pattern to cells grown in 0.5 M NaCl up to 20 days (Fig. 3.4). However, growth was not as rapid as that of normal cells (0.5 M NaCl) indicating salt stress affected the growth rate of *A. halophytica.* In addition, cells grown both under 2.0 M and 0.5 M NaCl also showed the highest fluorescence values of 20% at around 7-days suggesting that higher amount of H^+ was extruded from cells (Fig. 3.5). Although salt has caused the reduction of the growth rate, it has no effect on the activity of Na^+/H^+ exchange.

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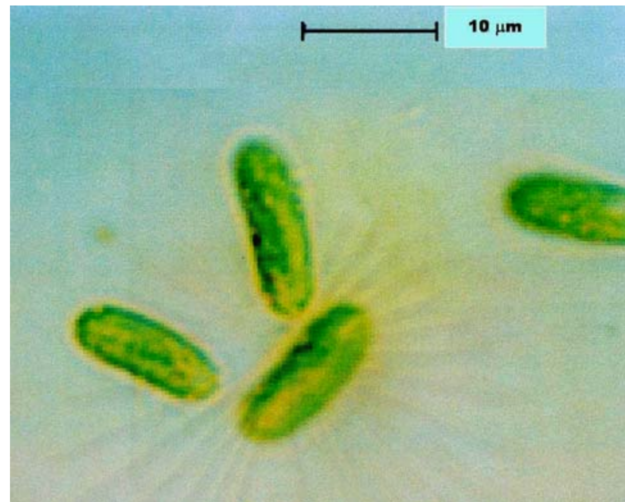

Figure 3.1 Microscopic picture of *Aphanothece halophytica* grown in Turk Island Salt Solution plus BG_{11} at 14 days (x 2250).

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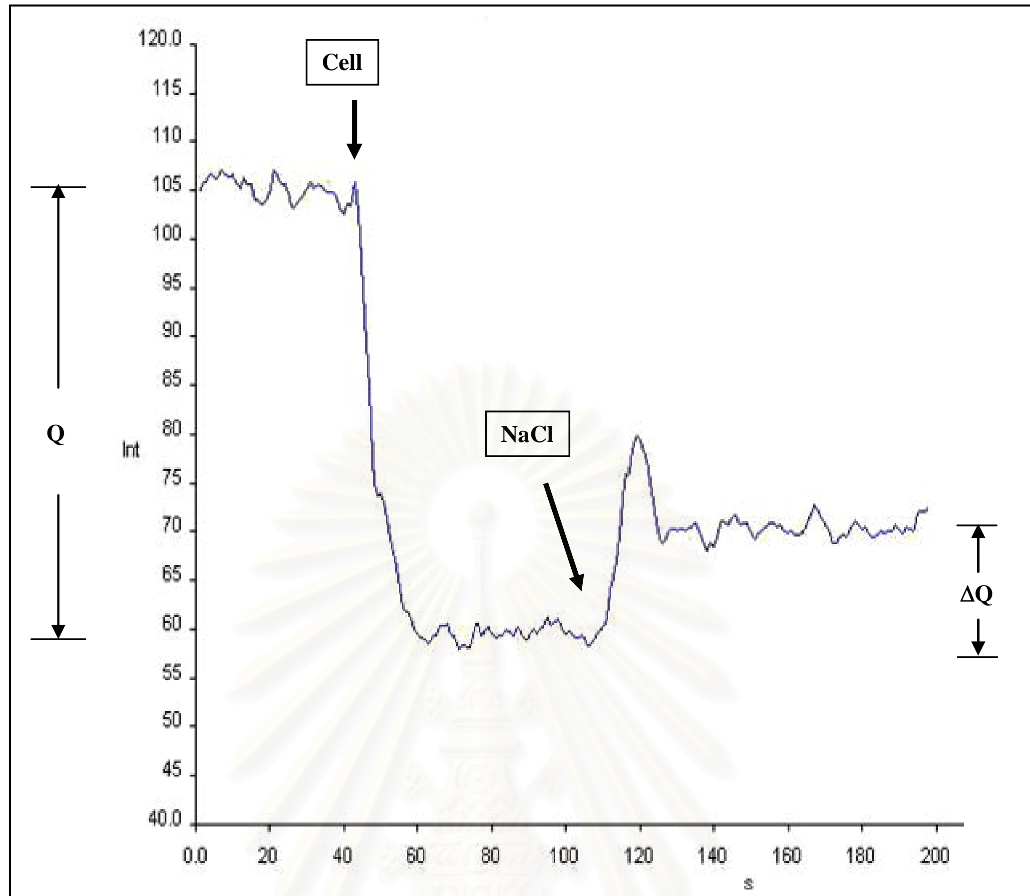

Figure 3.2 The diagram of Na^+/H^+ exchange as measured by the acridine orange fluorescence quenching method as described in Materials and Methods.

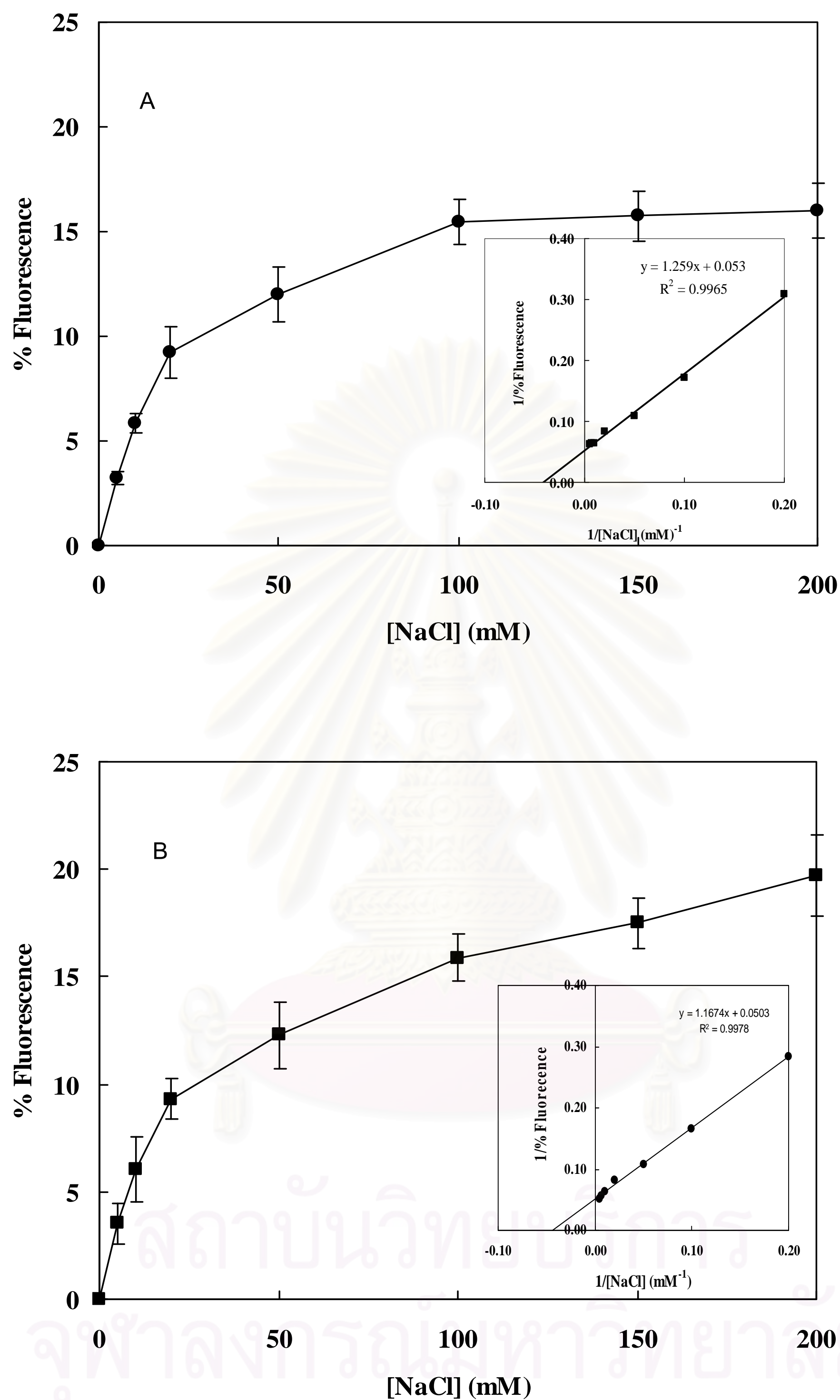

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Figure 3.3 Effect of NaCl on Na^+/H^+ exchange. A) cells grown in 0.5 M NaCl B).cells grown in 2.0 M NaCl. The data are the average of 3 replicates $\pm$ S.E. (n=3)

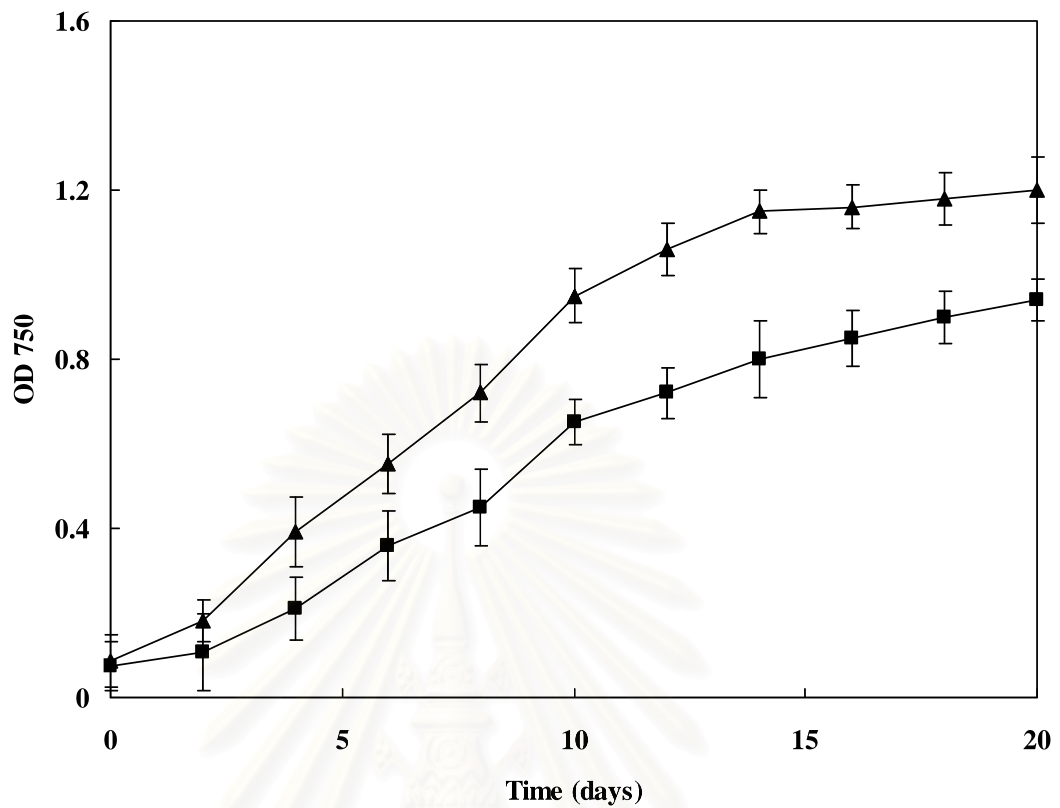

Figure 3.4 Growth of *A. halophytica* in Turk Island Salt Solution plus modified BG11 medium. Symbols represent cells grown in 0.5 M NaCl (▲) and in 2.0 M NaCl (■).The data are the average of 3 replicates ± S.E. (n=3).

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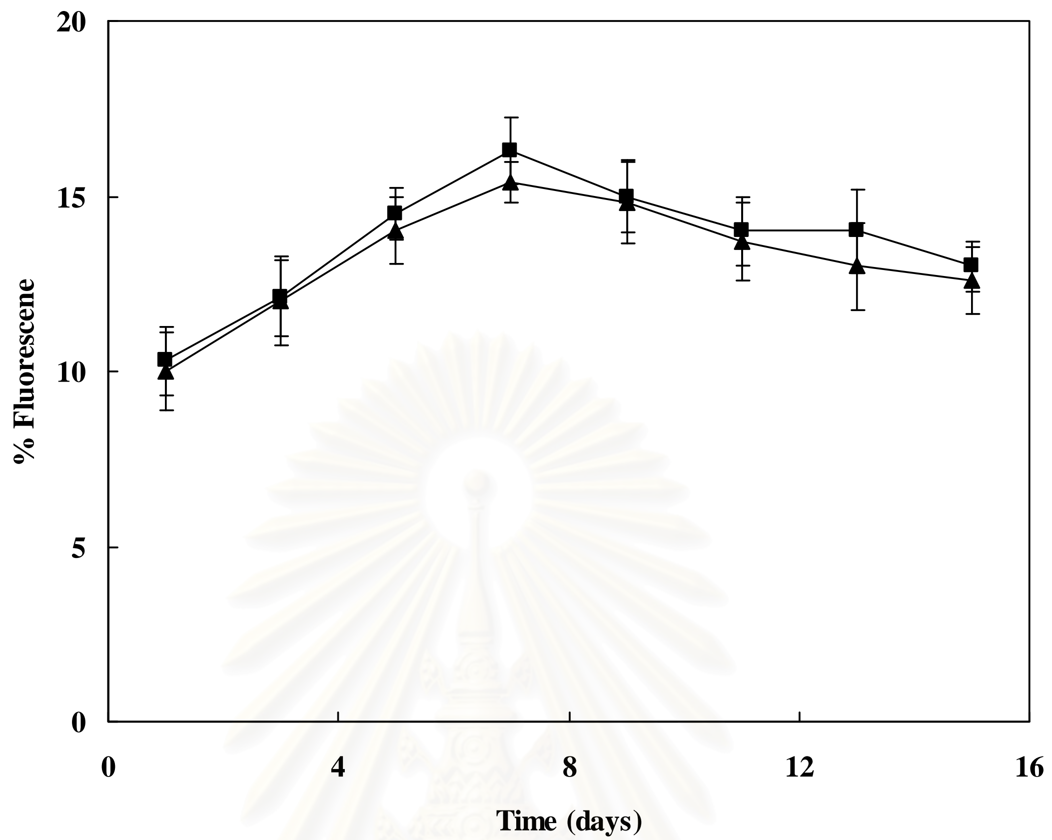

Figure 3.5 Na^+/H^+ exchange of cell grown in 0.5 M NaCl (▲) and cells grown in 2.0 M NaCl (■) at various growth stage. The data are the average of 3 replicates ± S.E. (n=3)

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3.1.3 Effect of external pH on Na^+/H^+ exchange

Since Na^+/H^+ exchange is involved in H^+ circulation, we therefore further tested whether the changes in external pH could affect its activity. Among the pH values tested, highest fluorescence value of 25 % occurred at neutral pH of 7.0 (Fig. 3.6). However, alkaline conditions gave slightly higher activity than acidic condition. The results indicated that the optimal pH for Na^+/H^+ exchange was 7.0

3.1.4 Effect of cations on H^+ exchange in *A. halophytica*

To determine cation on H^+ exchange under various types of cation such as Na^+, Li^+, K^+, Ca^{2+} and Mg^{2+}. As shown in Fig. 3.7, the cation/H^+ exchange provided the maximal activity with 20 % fluorescence when addition 0f 100 mM NaCl. While, other monovalent cations, namely, Li^+ and K^+ were able to provide 19 and 14 % fluorescence, respectively. Furthermor, divalent cation, such as Mg^{2+} showed the lowest activity with 11 % fluorescence. In this study, indicating that these cation/H^+ exchange were able to work under various types of cations. In addition, the combination f cations such as Na^+ plus K^+, Na^+ plus Li^+, and Na^+ plus Ca^{2+} *etc*.(Fig. 3.8) were used to observe the activity of cation/H^+ exchange. The activity was roughly in range of 13-18 % fluorescence, suggesting that they did not highly change of activity by combination of cations. The kinetics of cation/H^+ exchange by *A. halophytica* werw studied by various LiCl and $CaCl_2$ concentrations ranging from 0-200 Mm (Fig. 3.9). The exchange system was saturable and displayed typical Michaelis-Menten kinetics. Lineweaver-Berke transformation of this data gave the following kinetic parameter: K_m was 36.0 mM, V_{max} was 16.0 % fluorescence for LiCl and: K_m was 4.4 mM, V_{max} was 14.5 % fluorescence for $CaCl_2$.

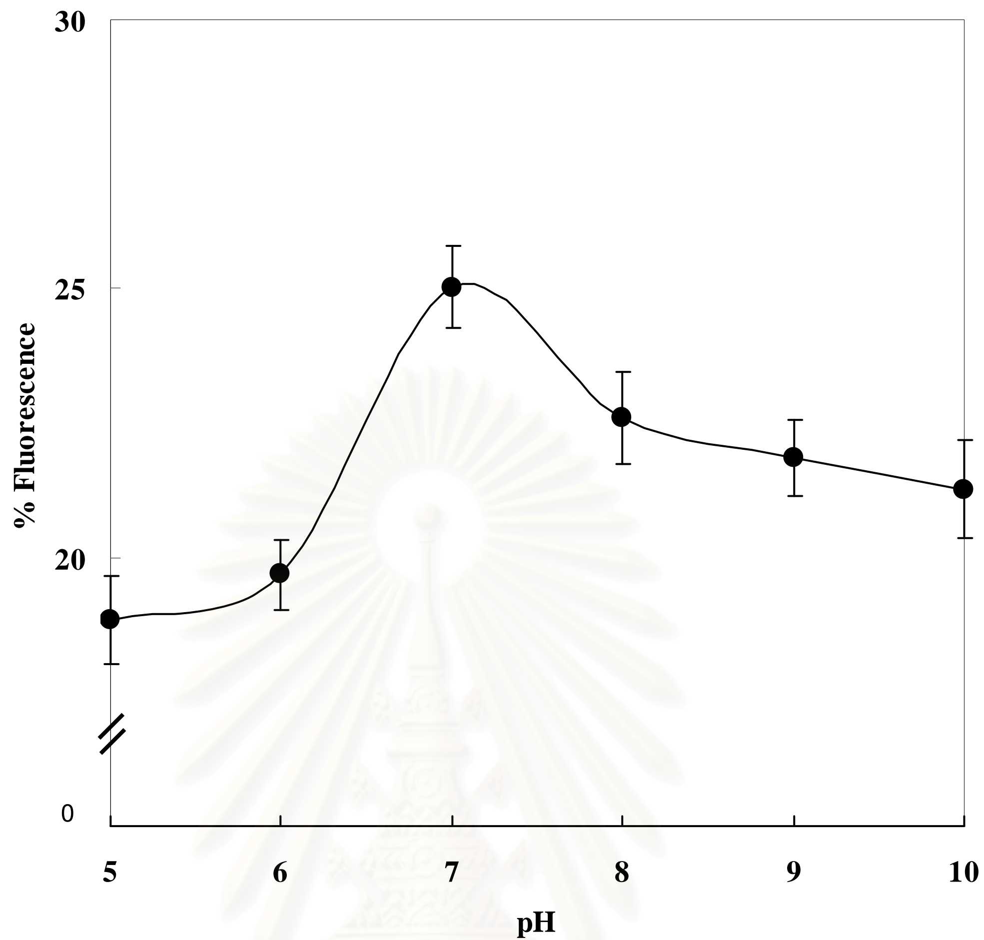

Figure 3.6 Effect of pH on Na^+/H^+ exchange of cells grown in 0.5 M NaCl. The data are the average of 3 replicates ± S.E. (n=3).

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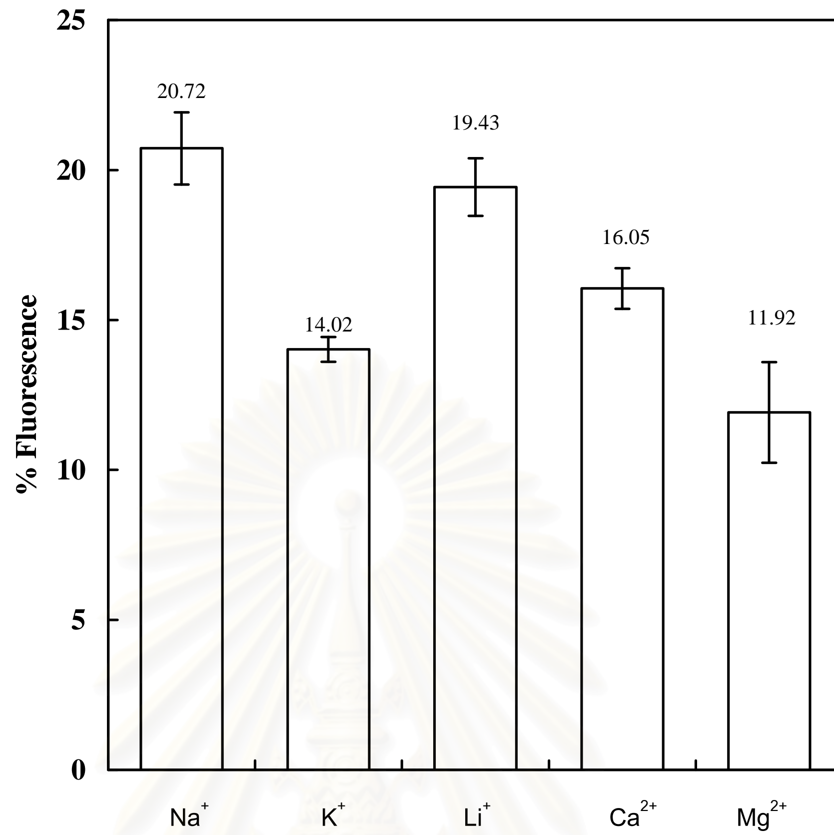

Figure 3.7 Effects of mono cation, divalent cation on cation/H^+ exchange of cells grown in 0.5 M NaCl The data are the average of 3 replicates ± S.E. (n=3).

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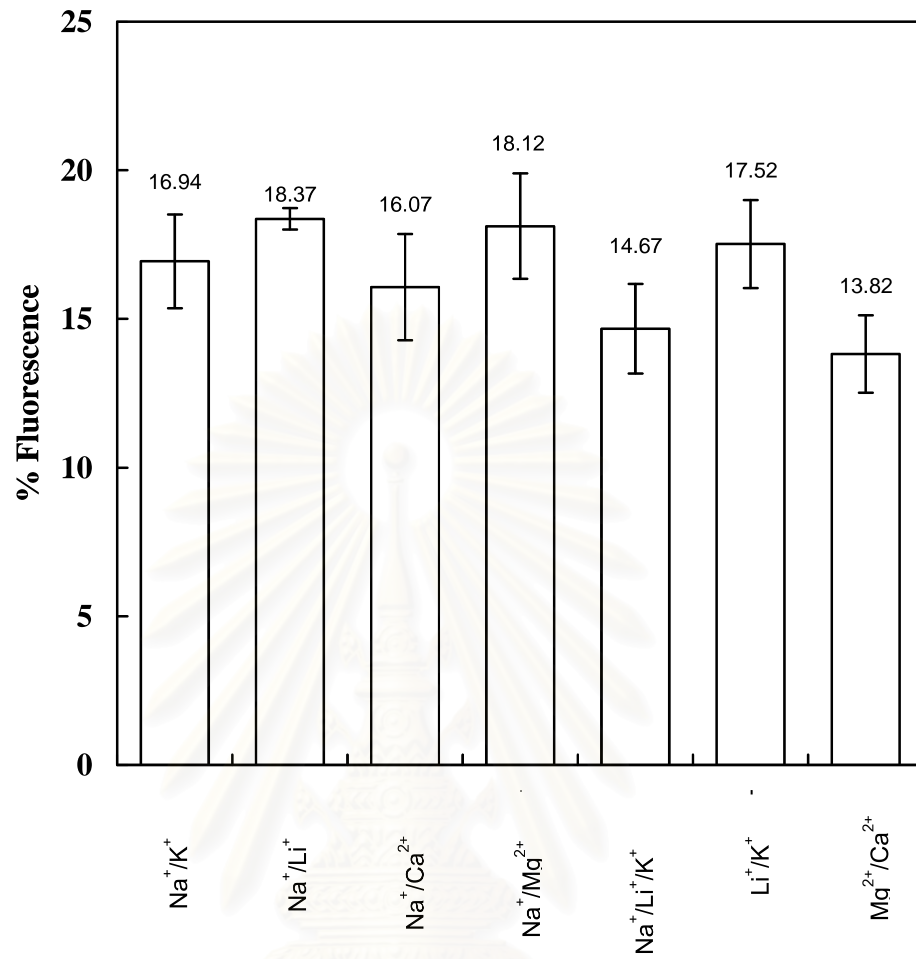

Figure 3.8 Effect of combination of cation on cation/H^+ exchange of cells grown in 0.5 M NaCl. The data are the average of 3 replicates ± S.E. (n=3).

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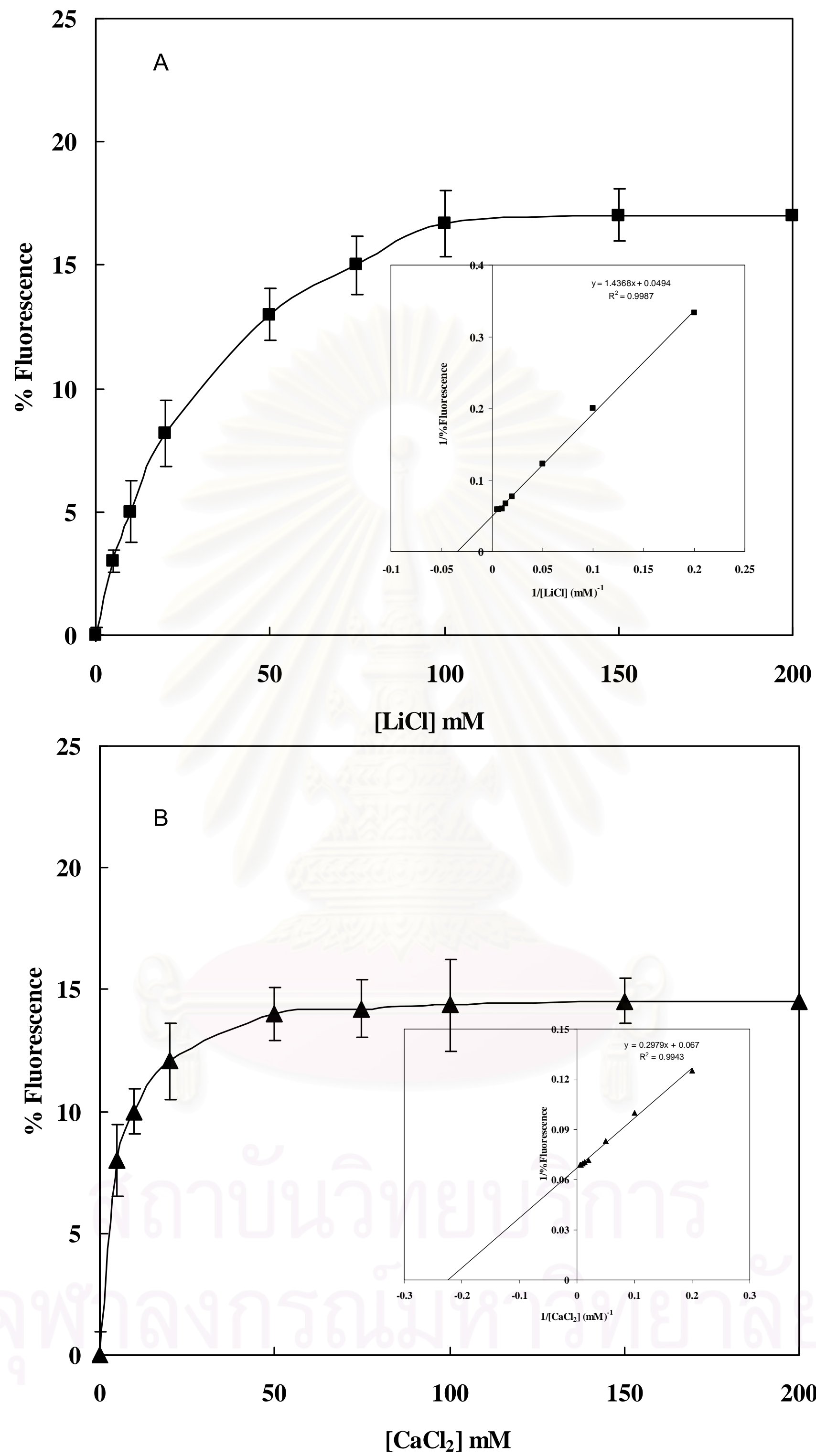

Figure 3.9 Effect of LiCl (A) and $CaCl_2$(B) on Na^+/H^+ exchange of cells grown in 0.5 M NaCl. The data are the average of 3 replicates ± S.E. (n=3).

3.1.5 Inhibition of Na^+/H^+ exchange by metabolic inhibitors

To determine the requirement of energy for Na^+/H^+ exchange, the effects of some inhibitors on the activity were studied. The results in Fig. 3.10 show that sodium azide, which is involved in ATP formation, was effective inhibitor of Na^+/H^+ exchange. *N, N'* dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor causing a reduction in pH gradient, also effectively inhibited Na^+/H^+ exchange. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton conductor, inhibited Na^+/H^+ exchange. Valinomycin, an inhibitor collapsing the electrical potential with a reduction in $\Delta\psi$, inhibited Na^+/H^+ exchange. Similarly inhibition of Na^+/H^+ exchange was also observed with amiloride, an inhibitor of Na^+/H^+ exchanger. Na^+-gradient dissipators, monensin and Na^+-ionophore, caused strong inhibition of Na^+/H^+ exchange. All these results indicate that the proton motive force plays an important role in activity of Na^+/H^+ exchange.

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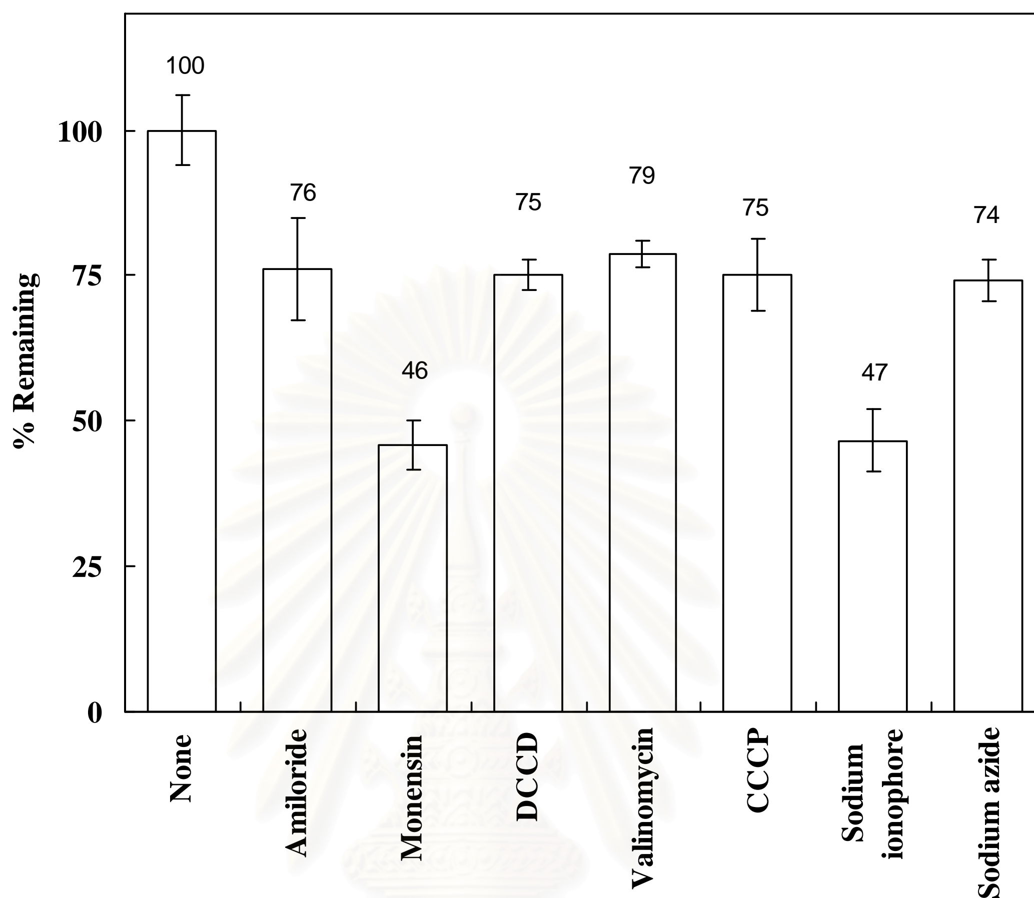

Figure 3.10 Inhibition of Na^+/H^+ exchange by metabolic inhibitors of cells grown in 0.5 M NaCl. The data are the average of 3 replicates $\pm$ S.E. (n=3).

3.2 Preparation of plasma membrane in *A. halophytica* cells

3.2.1 Sucrose gradient separation

Plasma membrane formed a band in the 30% sucrose layer and thylakoid membranes at the interface between the 39 and 50% sucrose layers. Cell walls were pelleted at the bottom (Fig. 3.11)

3.2.2 Aqueous polymer two-phase partitioning

The procedure was carried out according to Norling, et al. (1994). Crude membranes prepared as described in section 2.6 were centrifuged at 100,000 g for 1 hr. This pellet was suspended to a total volume of 5 ml in 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.6, and 5 g of the suspension was added to the 10 g phase mixture (see Appendix D) to give a 15 g phase system with a final concentration of 5.6 % (w/w) Dextran T-500, 5.6 % (w/w) polyethylene glycol, 0.25 M sucrose and 5 mM Tris-HCl buffer, pH 7.6. The partitioning was performed by turning the tube gently upside-down 20-30 times at 4 ^{o}C. After mixing, phase setting was facilitated by centrifugation for 5 min at 3,000 g and the upper and lower phases were collected separately. Plasma membranes were in lower phase. (Fig. 3.12)

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3.2.3 Electron micrograph of isolated plasma membranes

Figure 3.13 shows an electronmicrograph obtained from the plasma membranes preparation. The membranes shows vesicle diameter in the range of 0.1-0.22 m. The electrodense, amorphous structures seen in the electron micrograph are probably aggregates of lipophilic compounds often seen in membrane preparations

3.2.4 Absorption spectra

Table 3.1 shows absorption ratio between carotenoid absorption (485 nm) and chlorophyll absorption (680 nm). The absorption ratio at 485/680 nm was increase from 1.70 in total membrane to 6.5 in plasma membrane (sucrose gradient separation) and 1.75 in two-phase system, indicating that purification and pronounced enrichment of the plasma membrane.

3.3 Na^+/H^+ exchange by plasma membrane vesicle

According to the material and method plasma membrane vesicle was measured for Na^+/H^+ exchange. However, we could not detect activity using that method.

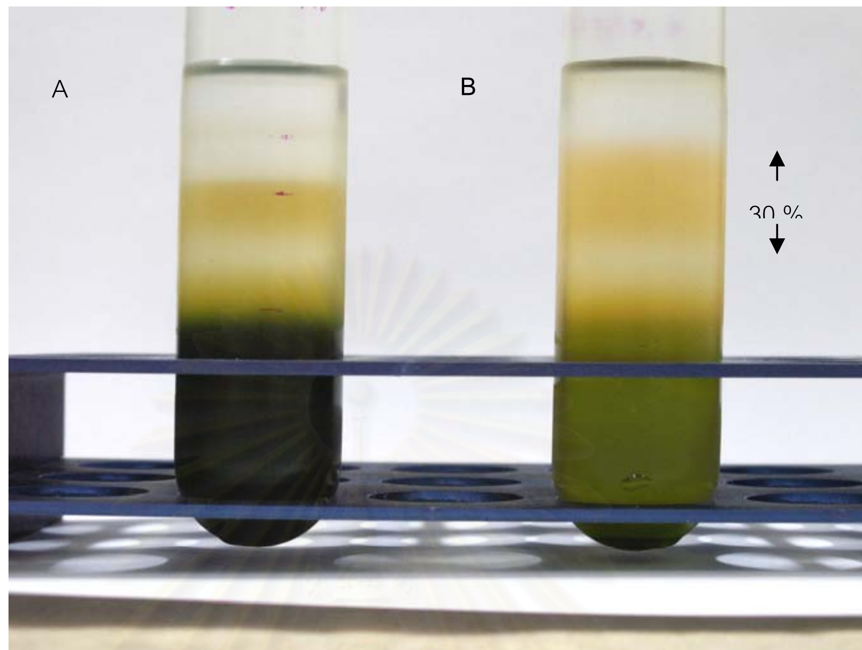

Figure 3.11 Appearance of plasma membrane as separated by sucrose gradient centrifugation**.** A) cell grown in 0.5 M NaCl and B) cell grown in 2.0 M NaCl

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Figure 3.12 Partition of total cyanobacteria membrane in two-phase system (5.6 % dextran/5.6 % PEG).

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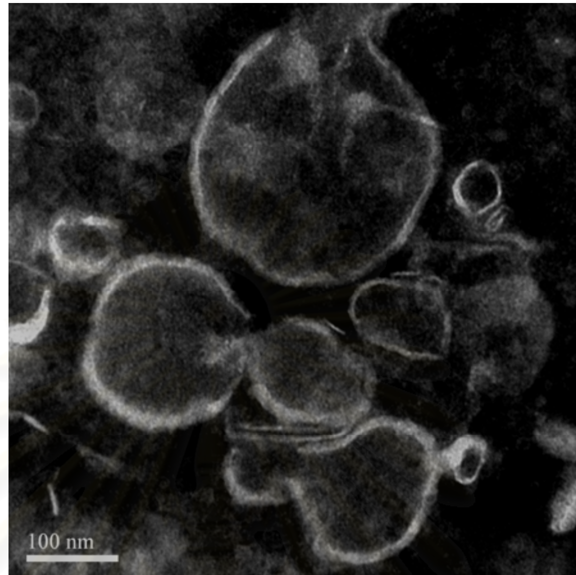

Figure 3.13 Electron micrograph of the plasma membrane preparation. Scale bar = 0.1 m.

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Table 3.1 Ratio between carotenoid absorption (485 nm) and chlorophyll absorption (680 nm) of total membrane and plasma membrane.

Fraction	A_{485}/A_{680}
Total membrane	1.7
Sucrose gradient	6.5
Two phase phase partitoning	1.75

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CHAPTER IV

DISCUSSION

The results presented in this study demonstrated the existence of Na^+/H^+ exchange in halotolerant cyanobacterium *Aphanothece halophytica*. Indeed, *A. halophytica* grown under normal condition (0.5 M NaCl) and stress condition (2 M NaCl) show no different pattern of growth rate, suggesting that cells have mechanisms that allow them to adapt to changes in the concentration of salt in the environment. Among these mechanisms, one of the most clearly understood systems is the extrusion of sodium ion needed to balance and maintain sodium level. Previously, the response of *A. halophytica* to an increase in salinity showed the increased expression of Na^+/H^+ exchange under salt stress conditions (Waditee *et al*, 2001 and Wutipraditkul *et al*, 2005).

It is worthwhile noting that there is an apparent disparity of the data between an exchange and the growth rate of cells. *A. halophytica* cells grown from one day until stationary stage were used to monitor of Na^+/ H^+ exchange. The results provide the data that exchange of intact cells at log phase (seven days) was much greater than the others, suggesting that the optimal growth condition used for further assay exchange should be the cells grown in range of log stage. Results in Fig. 3.3 exhibited the significant exchange of Na^+/H^+ exchange measured by using the whole cells following the concentration gradient of Na^+. Increasing of NaCl concentration resulted in the increasing percent of fluorescence. Michaelis-Menten kinetics were revealed, and calculated apparent K_m values of approximately 23.7 mM and 22.1 mM for NaCl in *A. halophytica* grown in 0.5 and 2.0 M NaCl, respectively. Previously, the

everted membrane vesicles of *E.coli* contained Ap-NhaP were used to observe the Na^+/H^+ antiporter activity, the apparent K_m and V_{max} values of ApNhaP1 for Na^+ was 1.75 mM and 49 (% dequenching) at pH 7.0 and did not change so much upon the change of pH (pH 8.0 and 9.0) (Waditee *et al.*, 2006), whilst everted membrane vesicles of *E.coli* containing NhaD-G327S of *Alkalimonas amylolytica* showed the apparent K_m value of 3 mM for NaCl (Liu *et al.*, 2005). Moreover, the low affinity and high affinity Na^+/H^+ antiporter of nhaS1 and nhaS3 *of Synechocystis* sp. PCC6803 *in E. coli* transformant exhibited the apparent K_m values of 7.7 mM and 0.7 mM, respectively (Inaba *et al*, 2001)

One of the properties of Na^+/H^+ exchange is their pH dependence and roles in salt tolerance at alkaline pH. The results in Fig. 3.6 showed that the activity was observed over a wide range, which was maximal at pH value of 7.0 whereas alkaline condition gave higher exchange than acidic condition. This is similar to the previous results for Ap-NapA and Ap-NhaP type Na^+/H^+ antiporters, which exhibited high exchange activities over a wide range (pH 5.0-9.0) (Waditee *et al*, 2001 and Wutipraditkul *et al*, 2005).

Monovalent cation/proton antipoters play dominant roles in cells that have several antiporters catalyzing similar reactions (Padan *et al.*, 2005). The Na^+/H^+ antiporter could use K^+, Li^+ and Ca^+ as a coupling ion; for example, *E. coli* ChaA has the proton/cation exchange activity with Na^+ or Ca^{2+}, but not Li^+ or K^+ (Ivey *et al.*, 1993; Ohyama *et al.*, 1994). Correspondingly, Ap-NhaP could use Na^+ and Ca^{2+} (Waditee *et al*, 2001), whereas Ap-NapA could use Na^+ and Li^+ (Wutipraditkul *et al*, 2005). According to the present results in Fig. 3.7, *A. halophytica* cells could use K^+, Li^+, $Mg^{2+}$and Ca^{2+} exchange for H^+. Increasing the concentration of mono-cations gradient resulted in an increase of exchanger activity in Fig. 3.9. Michaelis-Menten

kinetics were revealed, and calculated apparent K_m values were approximately 36.0 mM and 4.4 mM for LiCl and $CaCl_2$, respectively.

Hence all cells have sodium extrusion via the Na^+/H^+ antiporters which catalyze the exchange of Na^+ for H^+ across membranes. The primary energy source for this system in most organisms is the proton electrochemical gradient across the cytoplasmic membrane. This proton electrochemical gradient is derived either from respiratory electron transport or at the expense of ATP formed during substrate-level phosphorylation by activity of the membrane ATPase (Wiebe *et al*, 2001). The energy of the proton motive force may be converted to a sodium motive force through Na^+/H^+ antiport activity as found in *Alteromonas haloplanktis* (Niven and MacLeod, 1978), *Halobacterium halobium* (Lanyi and MacDonald, 1976), *alkalophilic bacilli* (Mandel, 1980) *Mycoplasma mycoides* (Benyoucef *et al.*, 1982). The present results shown in Fig. 3.10 provide evidence that Na^+/H^+ exchange required energy. The exchange was inhibited when ATP formation inhibitor, namely sodium azide, was added. The Na^+/H^+ exchange was inhibited by *N, N*′ dicyclohexylcarbodiimide (DCCD), pH gradient dissipator and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton conductor. Furthermore, valinomycin, an inhibitor collapsing the electrical potential with a reduction in $\Delta\psi$, inhibited Na^+/H^+ exchange. These results suggested that the exchange is dependent on proton gradient. Besides, the abolition of Na^+ transport observed in the presence of either the Na^+ ionophore, monensin suggested that the transport is dependent on Na^+ gradient. Similarly inhibition of Na^+/H^+ exchange was also observed with amiloride, an inhibitor of Na^+/H^+ exchanger. All these results indicate that the proton motive force plays an important role in activity of Na^+/H^+ exchange in *A. halophytica*.

Membrane vesicles prepared from *A. halophytica* cells grown under non-stress (0.5 M NaCl) and stress condition (2.0 M NaCl) showed no Na^+/H^+ exchanger activity compared to whole cells. It is possible that the preparations consisted entirely of damaged membrane vesicle. However, detailed studies to elucidate the role of Na^+/H^+ antiporter are needed for *A. halophytica*.

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CHAPTER V

CONCLUSIONS

The results of the present investigation can be summarized as follows:

1. Sodium extrusion within cells depends on Na^+ gradient and plays an important role in exchange of Na^+/H^+ exchange in *A. halophytica*.
2. Michaelis-Menten kinetics were revealed, and calculated apparent K_m values were approximately 23.7 mM and 22.1 mM for NaCl in *A. halophytica* grown in 0.5 and 2.0 M NaCl respectively.
3. The metabolic inhibitor, namely, sodium azide and ionophores, namely CCCP, DCCD, valinomycin, and monensin and ATPase inhibitor, namely, vanadate inhibited Na^+/H^+ exchange.
4. Na^+/H^+ exchange of *A. halophytica* depends on pH, it showed maximal activity at pH 7.0. Besides, alkaline condition gave slightly higher activity than acidic condition.
5. The cation/H^+ exchange could also occur with ions such as K^+, Li^+ and Ca^{2+}.

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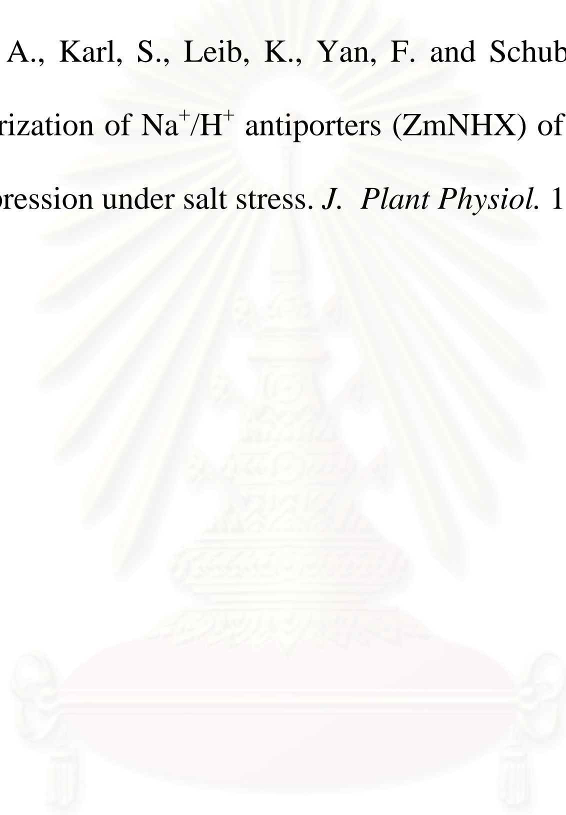

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APPENDICES

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APPENDIX A

Turk Island Salt Solution + modified BG11 medium contained the following components:

1. Preparation of Turk Island Salt Solution

Stock solution A:	KCl	33.3	g
	$MgCl_2.6H_20$	275.0	g
	$CaCl_2.2H_20$	73.3	g
	and made up to 5 litres with distilled water .		
Stock solution B:	$MgSO_4.7\ H_20$	347.0	g
	and then made up to 5 litres with distilled water .		

To make Turk Island Salt Solution, 500 ml of Stock solution A was added to 500 ml of Stock solution B. To this mixture 140.8 g NaCl was added and then final volume was made to 5 litres with distilled water .

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2. Composition of modified BG_{11} medium (BG_{11} medium + $NaNO_3$ Solution)

$NaNO_3$	(75g / 500 ml)	50	ml
KH_2PO_4	(8g / 200 ml)	5	ml
$MgSO_4.7H_20$	(15g / 200 ml)	5	ml
$CaCl_2.2H_20$	(7.2g/ 200 ml)	5	ml

Na_2CO_3 (4g/ 200 ml) 5 ml

Citric acid (1.2g / 200 ml) 5 ml

$EDTA.Na_2$ (0.2g / 200 ml) 5 ml

$FeSO_4.7H_20$ (1.2g / 200 ml) 5 ml

*Trace element A_5 solution + Co 5 ml

*Trace element A_5 solution + Co contained the following component in gram per litres H_3PO_4 : 2.86 ; $ZnSO_4.7H_2O$: 0.2 ; $CuSO_4.5H_2O$: 0.08 ; $MnCl_2.4H_2O$: 1.81 ; $Na_2MnO_4.2H_2O$: 0.39 ; $Co(NO_3)_2.6H_2O$: 0.049.

Culture medium of *Aphanothece halophytica* was prepared by adding all solution of item 2 at indicated volume to 5 litres of Turk Island Salt Solution and the pH was adjusted to 7.6 by slowly adding 2.0 M NaOH. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minutes.

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APPENDIX B

TCDS buffer

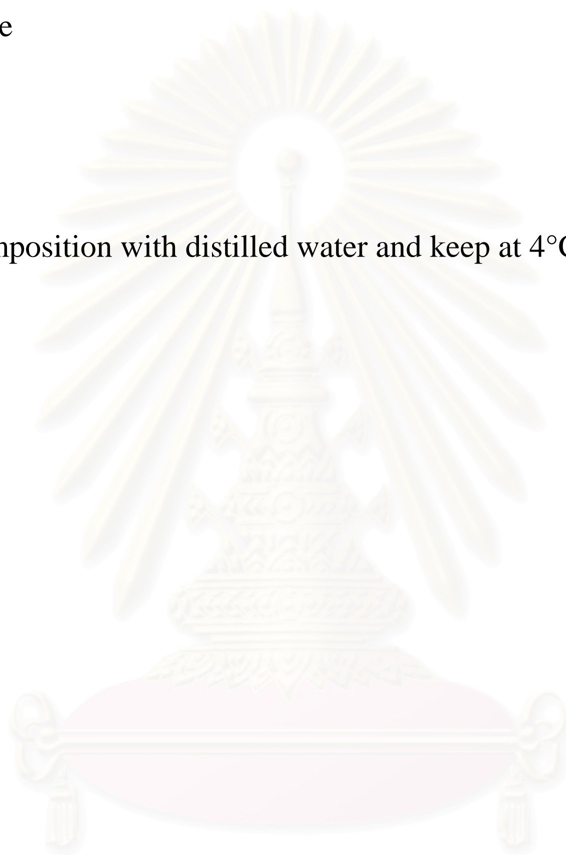
10 mM Tris-HCl pH 7.6

140 mM Choline chloride

250 mM Sucrose

5 mM DTT

Dissolve all composition with distilled water and keep at 4°C



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APPENDIX C

Preparation of dextran and polyethylene glycol

Phase mixture and phase system used in plasma membrane purification (Larsson, et al., 1987)

Step	Phase mixture	Phase system
20% (w/w) Dextran T-500	4.20 g	14.00 g
40% (w/w) Polyethylene glycol 3350	2.10 g	7.00 g
Sucrose (solid)	1.28 g	4.28 g
0.2 M Tris-HCl, pH 7.6	0.38 ml	1.25 ml
Add water to a final weight of:	10.00 g	50.00 g

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BIOGRAPHY

Miss. Aporn Bualuang was born on January 16, 1979 in Nakornpathom, Thailand. She graduated with a Bachelor of Science degree in Biology from Facculty of Science, Silapakorn University, Nakornpathom, Thailand in 2001. She has further studied for the Master degree in Biochemistry Program, Chulalongkorn University since 2003.

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