CHAPTER III

RESULTS

3.1 Growth rate determination.

3.1.1 Growth of *A. halophytica* in various NaCl concentrations.

A. halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane which multiplies by binary fission as shown in Figure 3.1. The cells were grown photoautotrophically at 30 °C in BG_{11} plus 18 mM $NaNO_3$ and Turk Island Salt Solution plus modified BG_{11} medium at different NaCl concentrations, i.e., 0.5 M, 1.0 M, 1.5 M and 2.0 M NaCl. Bacterial growth was monitored spectrophotometrically by the increase of the optical density at 750 nm. Figure 3.2 shows that maxmal growth occurred in the medium containing 0.5 M NaCl. At 1.0 M, 1.5 M and 2.0 M NaCl, a reduction in growth was observed.

3.1.2 Choline functions as an osmoprotectant in *A. halophytica.*

We investigated the influence of high osmolarity media on the growth of the *A. halophytica*. When the osmolarity of the medium was increased by the addition of 0.5 M and 2.0 M NaCl. The addition of 1 mM glycine betaine largely relieved the detrimental effects of high osmolarity on the growth of cells. We tested the ability of exogenously provided choline to function as osmoprotectant. This compound served as precursor for glycine betaine synthesis in *Escherichia coli* (Lanfald and Strom,

1986). We found that choline functioned as efficient osmoprotectant when they were added at a concentration of 1 mM to the growth medium and strongly stimulated the growth of osmotically stressed cells (Figure 3.3). Choline was somewhat less efficient osmoprotectant than glycine betaine (Canovas et al., 1998).

3.1.3 Effect of choline concentration on growth at diferrent salinity of *A. halophytica.*

We tested how much choline was required to serve as an osmoprotectant. Cultures of *A. halophytica* were grown in high osmolarity medium containing various concentrations (0-50 mM) of choline. The addition of 1 mM choline to the growth medium was sufficient to cause partial osmoprotection, whereas lower choline concentrations had no stimulating effect. An increase in the concentration of exogenously provided choline to 1 mM further improved the growth of the osmotically stressed cells, but the presence of 50 mM choline did not result in a concomitant stimulation of growth. Thus, a relatively low concentration (1 mM) of choline suffices to protect *A. halophytica* from the deleterious effects of a high osmolarity environment (Figuer 3.4).

Figure 3.1 Microscopic picture of *A.halophytica* grown in Turk Island Salt Solution plus modified BG_{11} medium at day 14 (x2250).

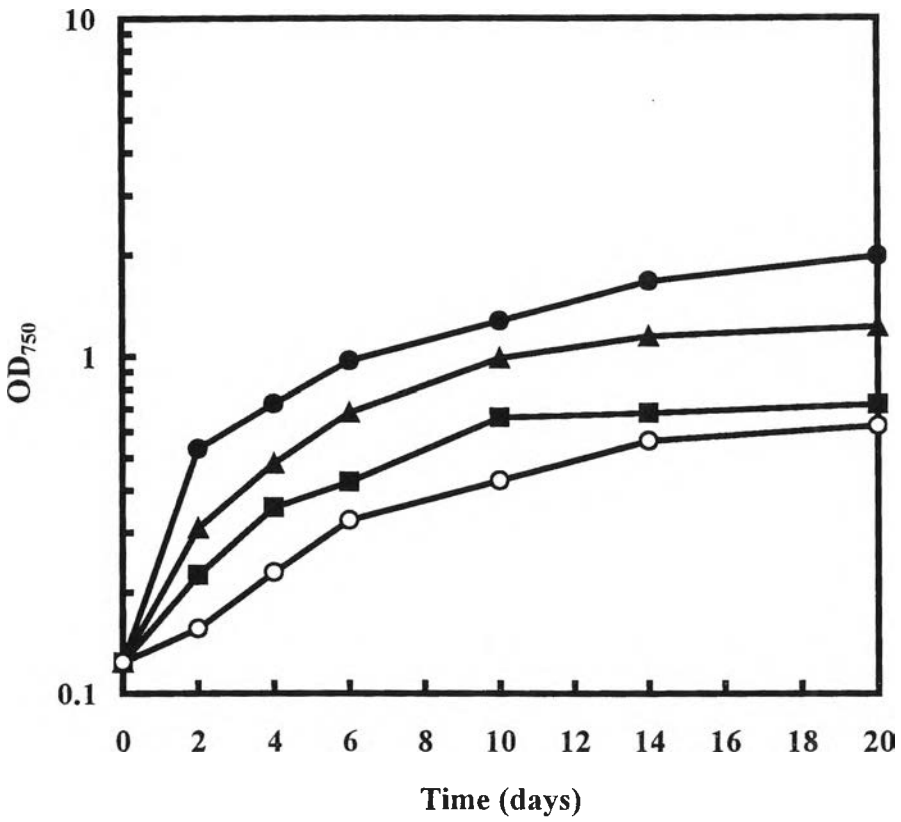

Figure 3.2 Growth of *A. halophytica* in Turk Island Salt Solution plus modified BG $_{11}$ medium containing various NaCl concentration. Symbol: ●, 0.5 M NaCl; ▲, 1.0 M NaCl; ■,1.5 M NaCl and ○, 2.0 M NaCl.

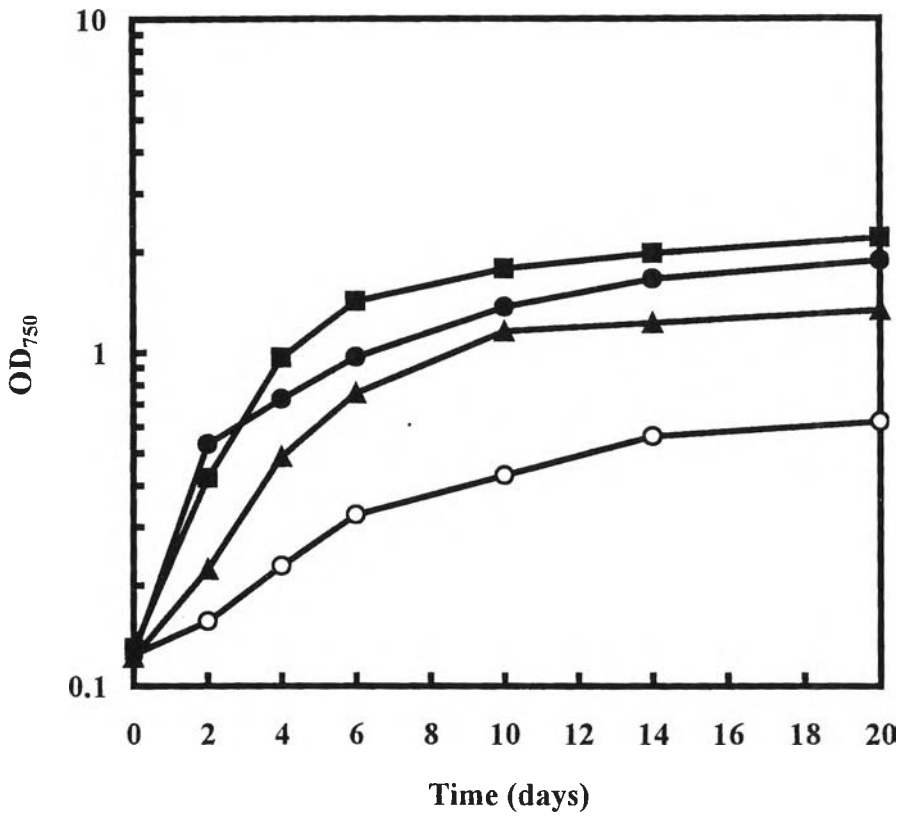

Figure 3.3 Choline functions as an osmoprotectant in *A. halophytica*. Symbol: ●, 0.5 M NaCl; ○, 2.0 M NaCl; ▲, 2.0 M NaCl in the presence of 1 mM choline and ■,2.0 M NaCl in the presence of 1 mM glycine betaine.

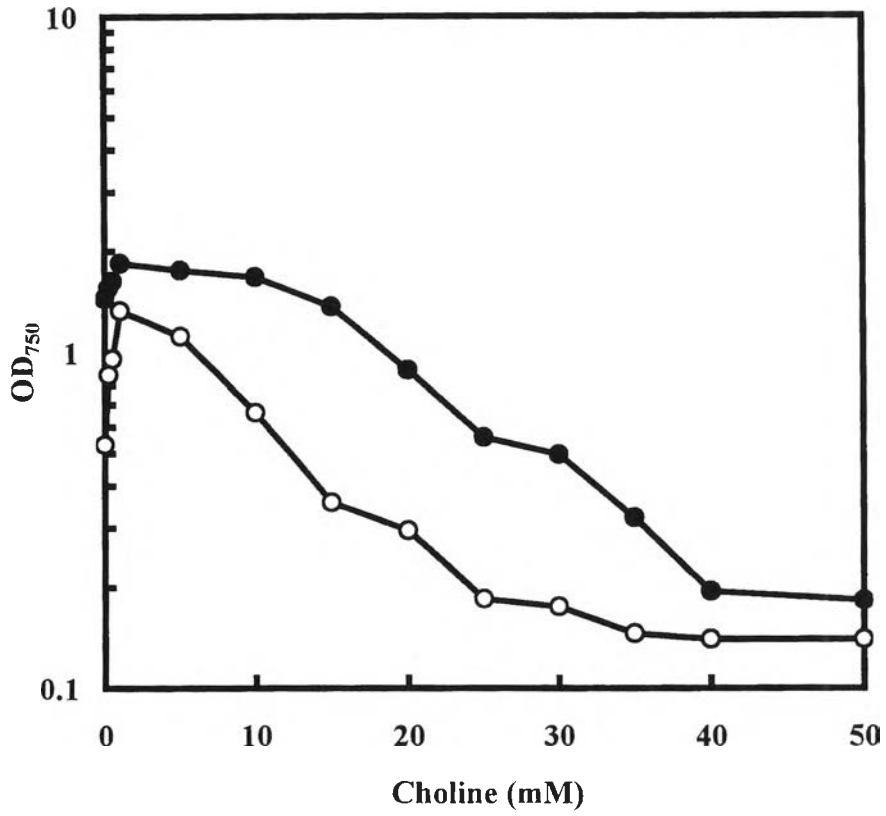

Figure 3.4 Effect of choline concentration on growth at different salinity of *A. halophytica* (10 days). Symbol: ●, 0.5 M NaCl and ○, 2.0 M NaCl.

3.1.4 Effect of choline on the salinity growth range of *A. halophytica.*

Choline stimulated the growth of *A. halophytica* in medium at salinities above 0.5 M NaCl, so that it extended the salinities range of growth at least 3.0 M NaCl. It is noteworthy that the growth of *A. halophytica* was also slightly stimulated by choline at 0.25 M NaCl (Figure 3.5). Thus, compared to a culture grown at 0.5 M NaCl, the growth rate of *A. halophytica* can be increased by two seemingly contradictory additives: increased NaCl concentration up to 2.0 M NaCl or an osmoprotectant such as glycine betaine or its precursor, choline. These observations present somewhat of a paradox, because these additives have contradictory effects: increasing the NaCl concentration presumably increases osmotic stress, while the osmoprotectants are believed to releive the inhibitory effects of high osmolarity.

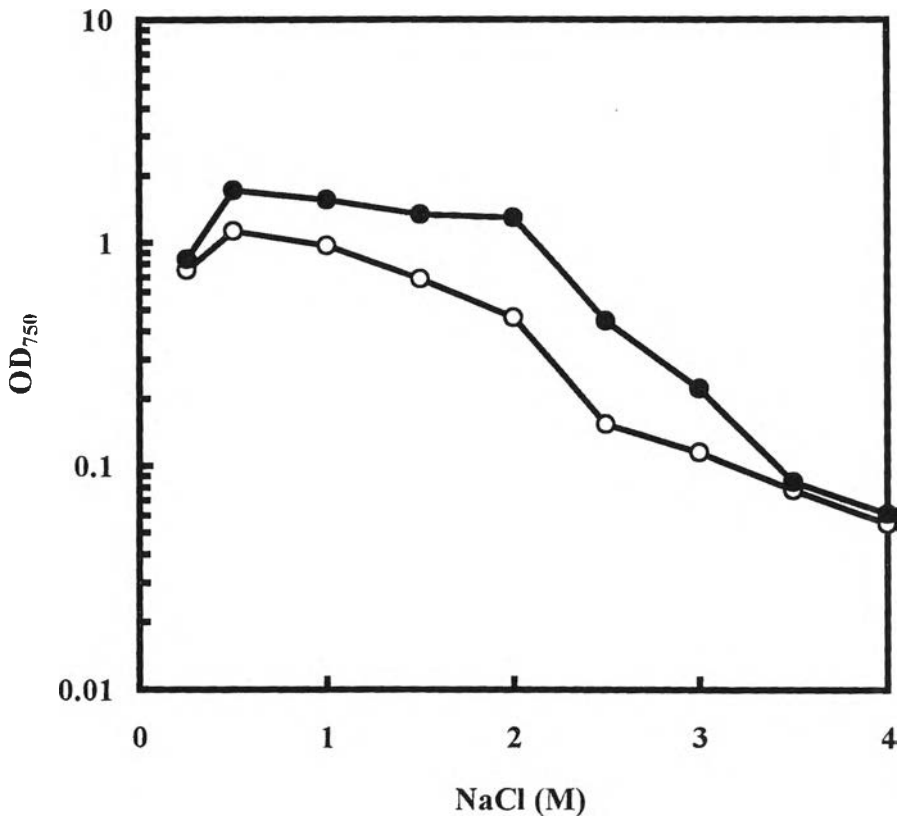

Figure 3.5 Effect of choline on the salinity growth range of *A. halophytica* (10 days).

Symbol: ○, minus 1 mM choline and ●, plus 1 mM choline.

3.2 Determination of [*methyl*-^{14}C]choline uptake system.

3.2.1 Uptake of [*methyl*-^{14}C]choline upon hyperosmotic stress.

Osmoprotection by including a compound into the system requires its intracellular accumulation. The uptake of choline was assayed in osmotic stress condition. We measured the initial rates of choline uptake in cells at control and stress conditions, using [*methyl*-^{14}C]choline as a substrate. There was substantial choline uptake of *A. halophytica* in osmotic stress condition, and this choline uptake activity was stimulated about three-fold when the osmolarity of the stress condition was raised by the increase of NaCl concentration from 0.5 M to 2.0 M (Figure 3.6).

3.2.2 Inhibition of choline uptake by chloramphenicol.

We investigated the inhibitors of chloramphenicol on choline uptake by blocking induction with chloramphenicol, a protein synthesis inhibitor. The uptake of [*methyl*-^{14}C]choline in response to osmotic stress with 2.0 M NaCl was much less than the uptake in uninhibited condition (Figure 3.7). Therefore, the choline-dependent stimulation of uptake that was observed in the absence of chloramphenicol appears to be dependent on the de novo protein synthesis of either a new transport protein or protein(s) regulating the activity of preexisting transport protein(s).

3.2.3 Effect of various osmoticums on choline uptake.

A high rate of choline uptake was dependent on energization of cells with osmoticum, such as mannitol, sorbitol and sucrose. Choline uptake was also stimulated by various osmoticums with the same osmolarity of stress condition. There was hardly any difference in stimulation whether the osmolarity was increased with mannitol, sorbitol and sucrose, indicating that the osmolarity, determined the uptake rate (Figure 3.8).

3.3 Characterization of choline uptake system.

3.3.1 Kinetics of choline uptake.

We also determined the concentration needed to saturate the uptake system of osmotic stress condition (0.5 M and 2.0 M NaCl) on the rate of choline uptake by *A. halophytica* (Figure 3.9). When cells were assayed with 50 μM [*methyl*-^{14}C]choline uptake was linear for 10 min (Figure 3.6). Initial uptake rates were determined over a wide rage of choline concentrations (100 to 700 μM). The choline uptake system was saturable and displayed typical Michaelis-Menten type kinetics (Figure 3.10). The Lineweaver-Burk Transformation of the data under these conditions, the line of best fit was performed by using a least squares linear regression, the apparent K_m values for control and stress condition were 278.6 and 256.4 μM respectively, the maximum velocity (V_{max}) was 17.6 and 35.7 nmol/min/mg protein respectively.

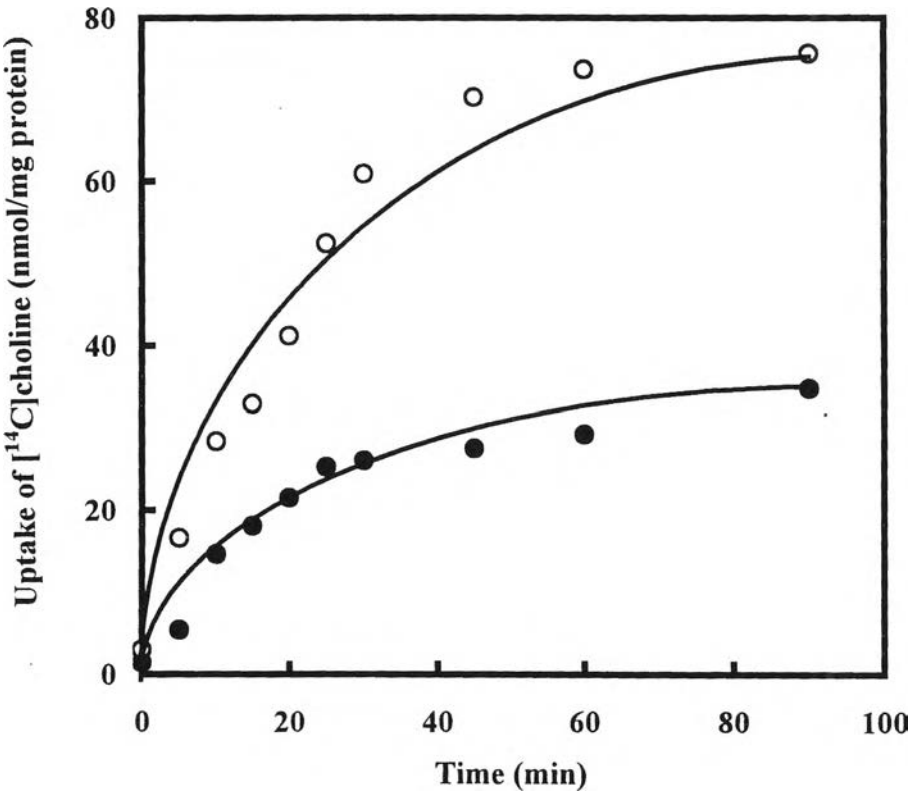

Figure 3.6 Time intervals of choline uptake by *A. halophytica*. Cells were incubated with 50 μM [*methyl*-^{14}C]choline under control (●, 0.5 M NaCl) and stress condition (○, 2.0 M NaCl).

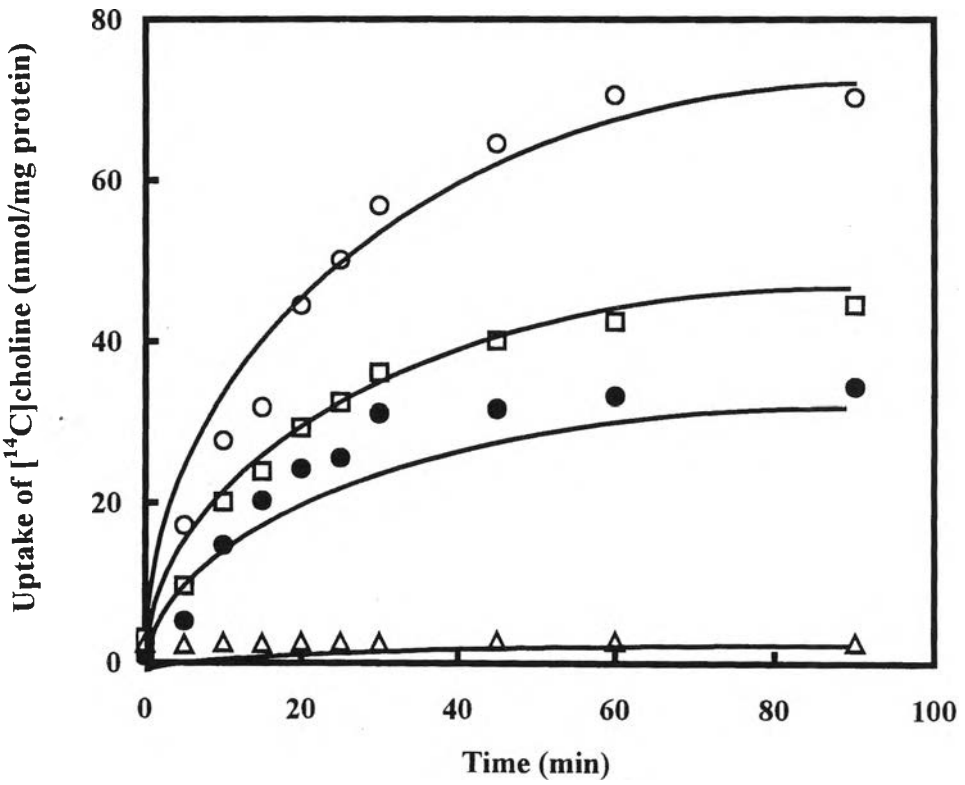

Figure 3.7 Inhibition by chloramphenicol of the uptake of [*methyl*-^{14}C]choline under control ●, 0.5 M NaCl and stress condition ○, 2.0 M NaCl. □, 2.0 M NaCl added with 100 μg/ml chloramphenicol and Δ, 2.0 M NaCl added with 500 μg/ml chloramphenicol.

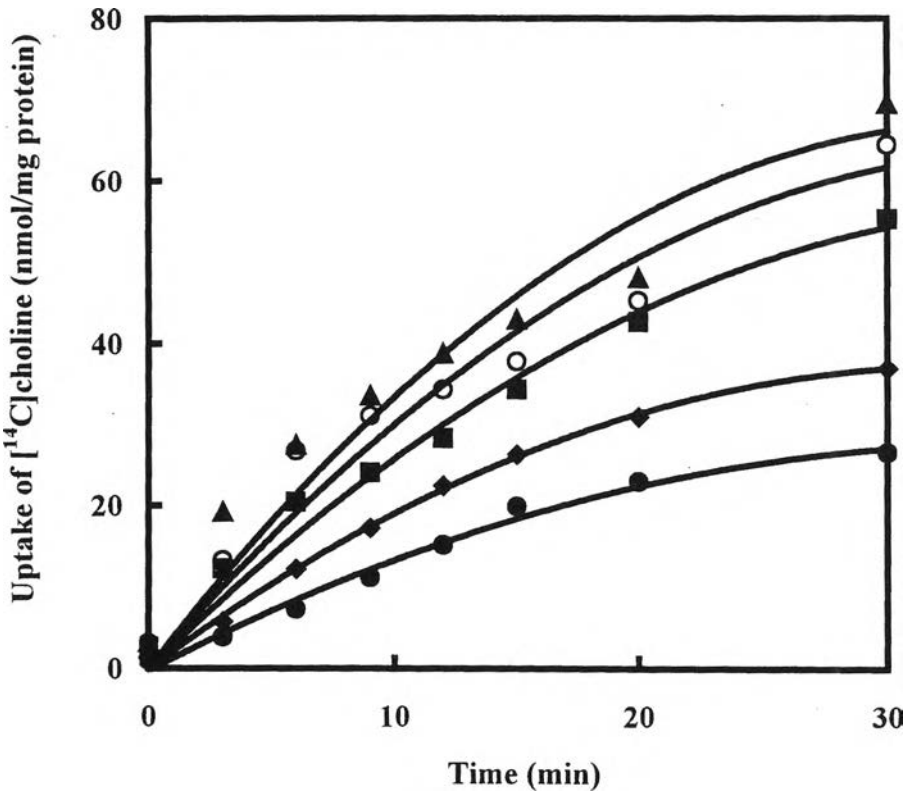

Figure 3.8 Effect of various osmoticums on [*methyl*-^{14}C]choline uptake under control (●, 0.5 M NaCl) and stress condition (○, 2.0 M NaCl). ▲, 0.5 M NaCl added 3.0 M Sorbitol; ■, 0.5 M NaCl added 3.0 M Mannitol and ♦, 0.5 M NaCl added 3.0 M Sucrose.

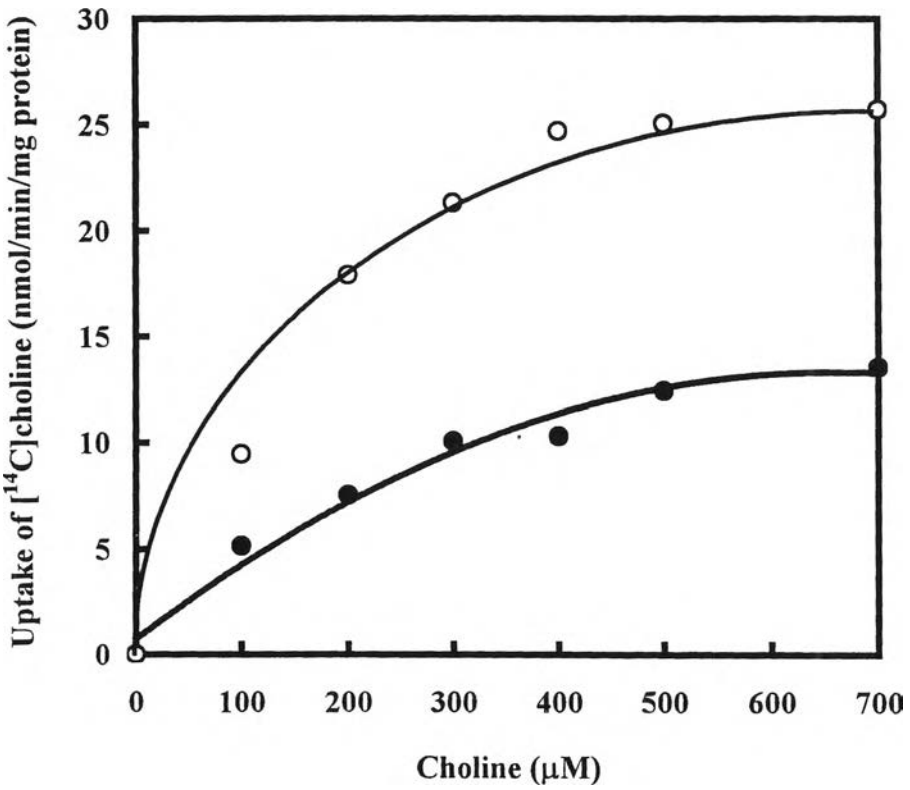

Figure 3.9 Kinetics of [*methyl*-^{14}C] choline uptake by *A. halophytica* under control (●, 0.5 M NaCl) and stress condition (○, 2.0 M NaCl).

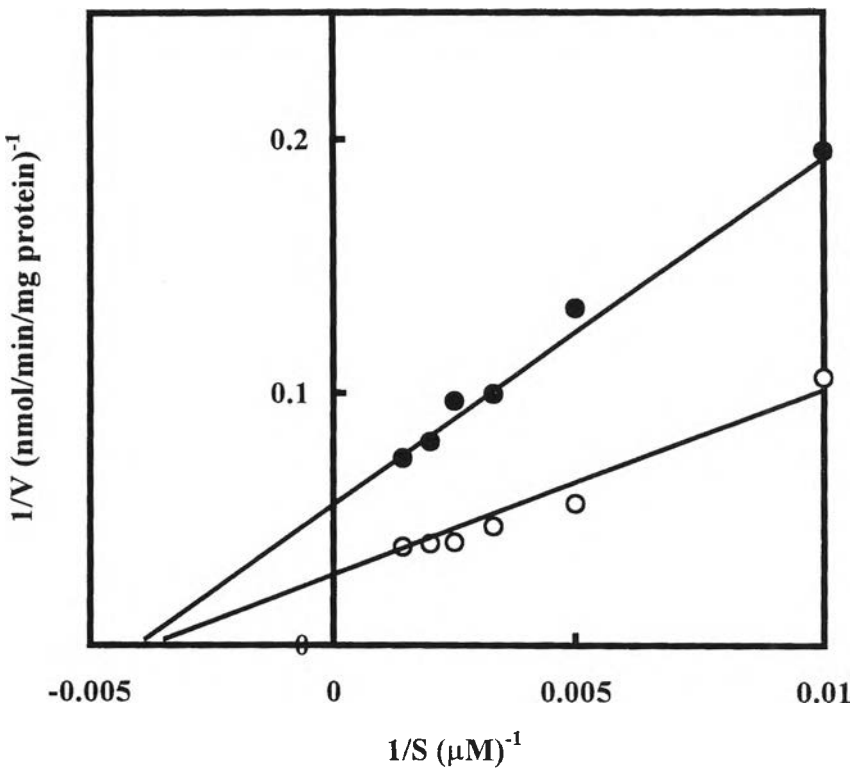

Figure 3.10 Lineweaver-Burk transformation of the data for control (●, 0.5 M NaCl) and stress condition (○, 2.0 M NaCl).

3.3.2 Na^+ requirement of the uptake system.

Choline uptake was dependent on Na^+ and 0.01 to 2.0 M NaCl supported maximum uptake rate. Of the concentrations of Na^+ tested, 2.0 M Na^+ supported the fastest rate of choline uptake (Figure 3.11). Markedly slower rates of uptake were obtained with 2.5 and 3.0 mM NaCl.

3.3.3 Substrate specificity of choline uptake.

The specificity of the system was studied by observing the initial rate of [*methyl*-^{14}C]choline uptake in the presence of 100 fold excess of unlabeled compounds (Table 2). The choline analog, acetylcholine, was effective competitor for choline uptake. Of the osmoregulatory molecules tested, L-proline did not compete with choline. Glycine betaine, which differs from choline by having a carboxyl group instead of an alcohol group, was not a very effective competitor. However, glycine betaine aldehyde, an intermediate in the oxidation of choline to glycine betaine, was a more effective competitor than glycine betaine. Glycine and ethanolamine did not compete for choline uptake but trimethylamine was also a good competitor. None of the amino acids tested, representing the 10 amino acid uptake system, appeared to be an effective competitor, although the basic amino acid arginine and lysine slightly reduced choline uptake.

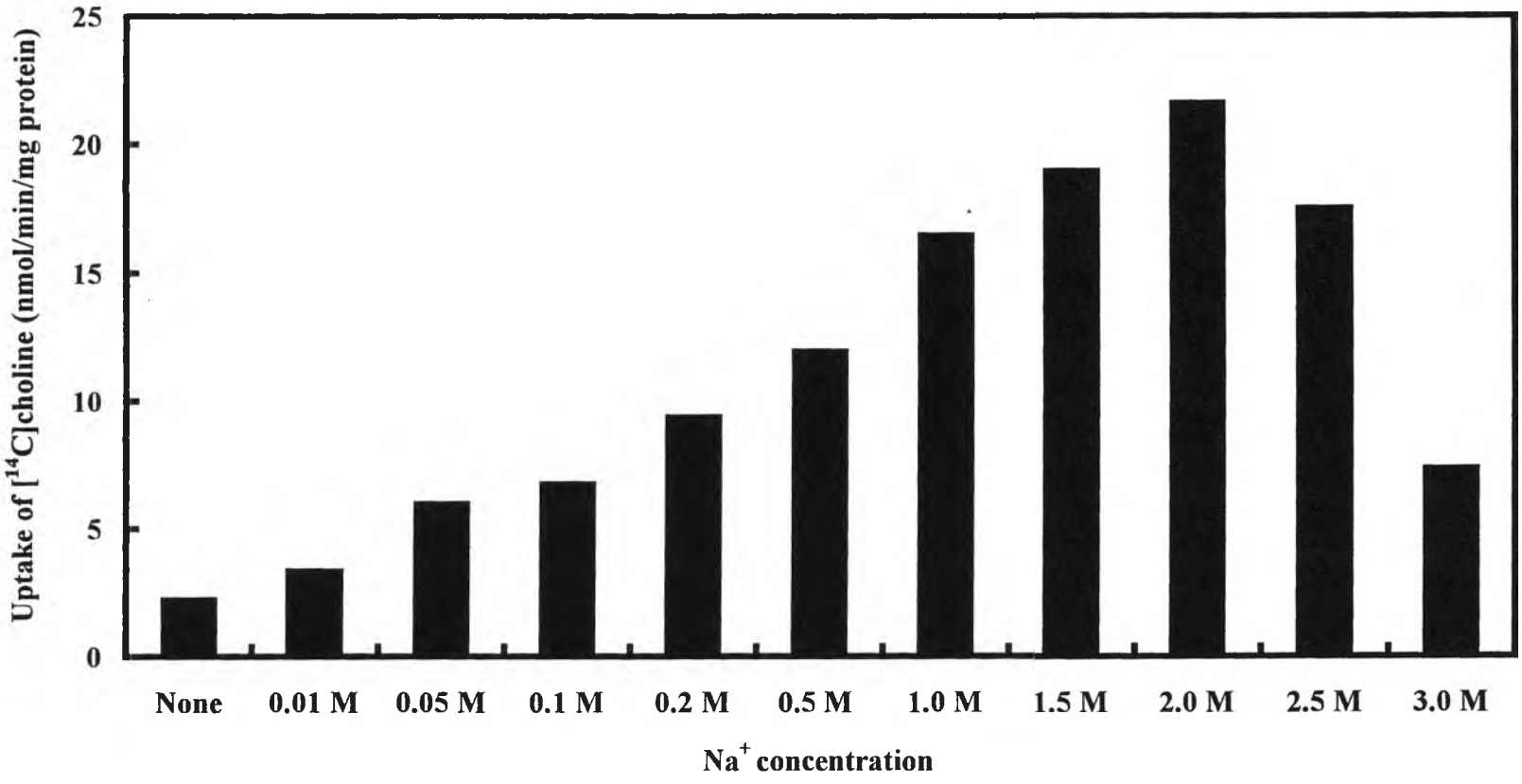

Figure 3.11 Effect of different Na^+ concentration on choline uptake.

Table 2 Effect of potential inhibitor on the initial rate of choline uptake.

Compound	% inhibition	
	Control condition	Stress condition
Acetylcholine	82	74
Phosphorylcholine	23	15
Ethanolamine	16	4
Trimethylamine	76	63
Glycine betaine aldehyde	93	87
Glycine betaine	18	5
Glycine	24	2
L-Carnitine	52	38
L-Alanine	6	2
L-Valine	3	0
L-Serine	8	0
L-Threonine	11	3
L-Phenylalanine	18	7
L-Cysteine	5	2
L-Glutamic acid	7	0
L-Lysine	17	12
L-Arginine	32	18
L-Proline	6	0

3.3.4 Effect of various inhibitors on choline uptake.

The effects of various agents that disrupt protein structure, ATPase activity, proton motive force and Na^+ electrochemical gradients on the initial rate of [*methyl*-^{14}C]choline uptake via osmotic stress condition were studied. The results were shown in Table 3. The inhibitors *N*-ethylmaleimide and sodium *p*-chloromercuribenzoate (affect protein structure), sodium arsenate and sodium fluoride (reaction involving high energy phosphate bond), dinitrophenol (electrochemcal gradient) and gramicidin D (Na^+ gradients), all inhibited choline uptake in both control and stress systems.

Table 3 Effect of various inhibitors on the initial rate of choline uptake.

Inhibitor	Concentration	% inhibition	
		Control condition	Stress condition
N-Ethylmaleimide	0.50 mM	97	86
Sodium *p*-chloromercuribenzoate	0.50 mM	94	81
Sodium arsenate	1.00 mM	95	73
Sodium fluoride	1.00 mM	63	52
Potassium cyanide	1.00 mM	83	77
Dinitrophenol	1.00 mM	79	67
Gramicidin D	1.00 μg/ml	74	65

3.4 Choline oxidation by choline dehydrogenase.

By radioisotopic assay for choline dehydrogenase, which catalyzes the oxidation of choline to glycine betaine, we measured its activity in *A. halophytica* grown under low and high salinity. High salinity of 2.0 M NaCl stimulated choline dehydrogenase in vivo, crude enzyme, and membrane and cytoplasmic fractions (Figure 3.12).

The crude enzyme fraction of stress culture condition was used to test effect of cation, metal ions and various reagents on choline dehydrogenase activity. Six substrate analogues (acetylcholine, phosphorylcholine, ethanolamine, glycine betaine, glycine betaine aldehyde or glycine) were tested. Table 4 show that phosphorylcholine, ethanolamine, glycine betaine and glycine were slightly inhibitory to the enzyme whereas acetylcholine and glycine betaine aldehyde greatly inhibited the enzyme activity. The enzyme activity was initially stimulated by increasing concentration of Na^+ and K^+ from 0 to 0.1 M above which the choline dehydrogenase activity was decreased (Figure 3.13). Effect of various metal ions and other compounds on the enzyme were examined (Table 5). $MnCl_2$, $CuSO_4$ and $AgNO_3$ caused more inhibition at the concentration of 1 mM, and some loss of enzyme activity was observed with SH reagents such as sodium *p*-chloromercuribenzoate. EDTA sodium arsenate,dinitrophenol and *N*-ethylmalemide were inhibitory to enzyme activity.

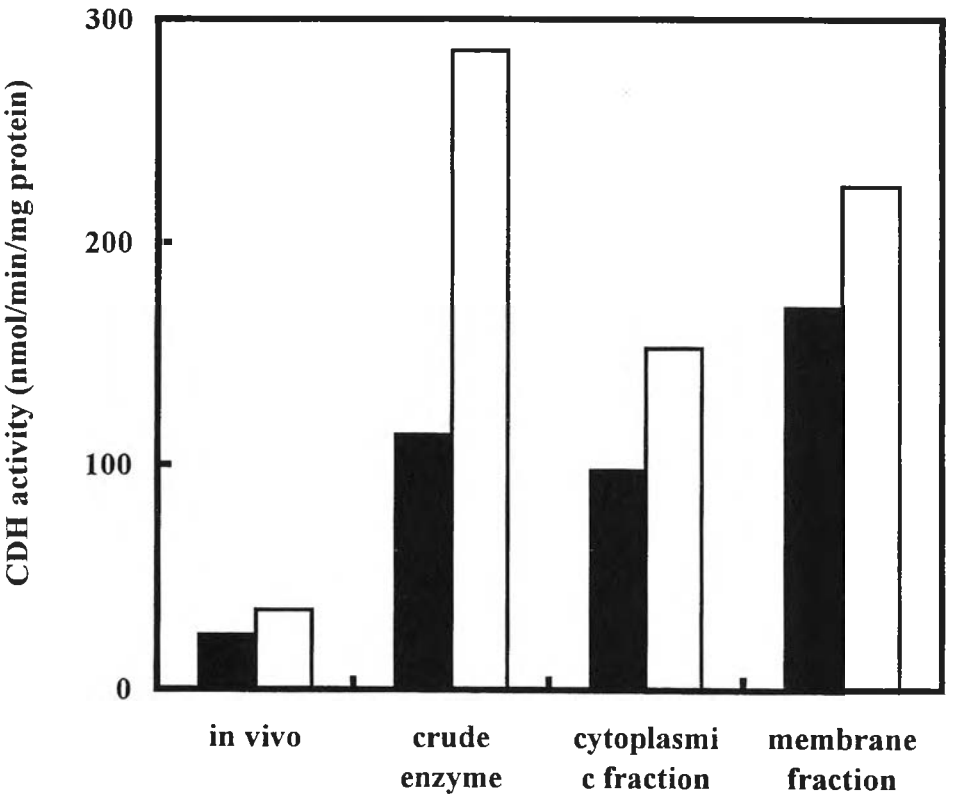

Figure 3.12 Choline dehydrogenase activity of *A. halophytica* in low (■, 0.5 M NaCl) and high salinties (□, 2.0 M NaCl).

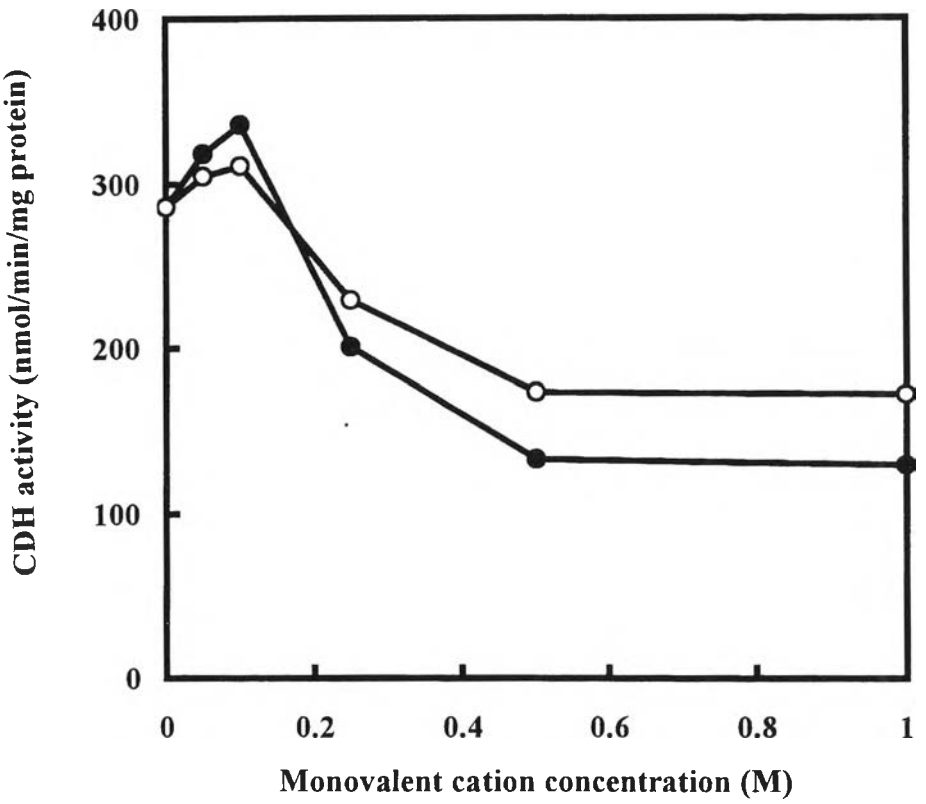

Figure 3.13 Effect of monovalent cations on choline dehydrogenase activity. Symbol: ●, NaCl and ○, KCl)

Table 4 Effect of choline analogues on the choline dehydrogenase activity.

Substrate analog	Concentration (mM)	% inhibition
Acetylcholine	1.0	79
Ethanolamine	1.0	18
Phosphorylcholine	1.0	26
Glycine betaine aldehyde	1.0	95
Glycine betaine	1.0	8
Glycine	1.0	17

Table 5 Effect of metal ions and various reagents on choline dehydrogenase activity.

Reagent	Concentration (mM)	% inhibition
$MnCl_2$	1.0	76
$CuSO_4$	1.0	80
$AgNO_3$	1.0	79
EDTA	1.0	11
N-Ethylmaleimide	1.0	38
Sodium *p*-chloromercuribenzoate	1.0	24
Sodium arsenate	1.0	22
Dinitrophenol	1.0	16

3.5 Molecular weight determination on SDS-PAGE

In the SDS-PAGE (Figure 3.14), lanes 1-4 showed the composition of periplasmic proteins obtained from cells adapted to 0.5 M and 2.0 M NaCl with clear differences in five proteins with apparent molecular masses of 19.9, 26.2, 34.6, 47.8 and 83.9 kDa (designated PP 1, PP 2, PP 3, PP 4 and PP 5). The molecular weight of these proteins was determined from calibration curve of log molecular weight of standard proteins and their R_f (Figure 3.15). The proteins with apparent molecular masses of 19.9, 26.2 and 47.8 (designated PP 1, PP 2 and PP 4) represented the most abundant proteins in all periplasmic preparations (lanes 1-4). Proteins with apparent molecular masses of 34.6 and 63.9 kDa (designated PP 3 and PP 5) represented the periplasmic fraction proteins which differed significantly between cells adapted to 0.5 M and 2.0 M NaCl (lanes 3 and 4).

3.6 Non-denaturing PAGE.

The native-PAGE pattern of periplasmic proteins obtained from cells adapted to 0.5 M and 2.0 M NaCl was compared (Figure 3.16) with comassie blue staining (lanes 1 and 2), choline dehydrogenase activity staining (lanes 5 and 6) and autoradiograph (lanes 8 and 9).

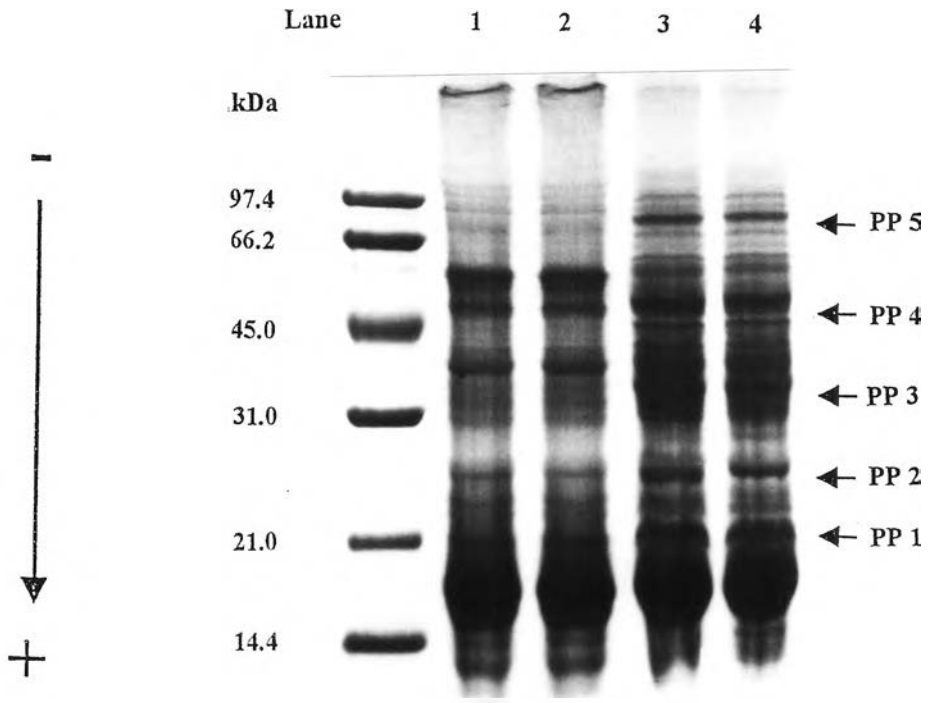

Figure 3.14 SDS-PAGE pattern of periplasmic proteins obtained from cells adapted to 0.5 M and 2.0 M NaCl.

Lane 1,2: periplasmic protein fraction in 0.5 M NaCl 50 μg

Lane 3,4: periplasmic protein fraction in 2.0 M NaCl 50 μg

Phosphorylase B	MW = 97.4 kDa
Bovine serum albumin	MW = 66.2 kDa
Ovalbulmin	MW = 45.0 kDa
Carbonic anhydrase	MW = 31.0 kDa
Soybean trypsin inhibitor	MW = 21.0 kDa
Lysozyme	MW = 14.4 kDa

PP 1, PP 2, PP 3, PP 4 and PP 5 indicated the position of the 19.9, 26.2, 34.6, 47.8 and 83.9 kDa proteins respectively.

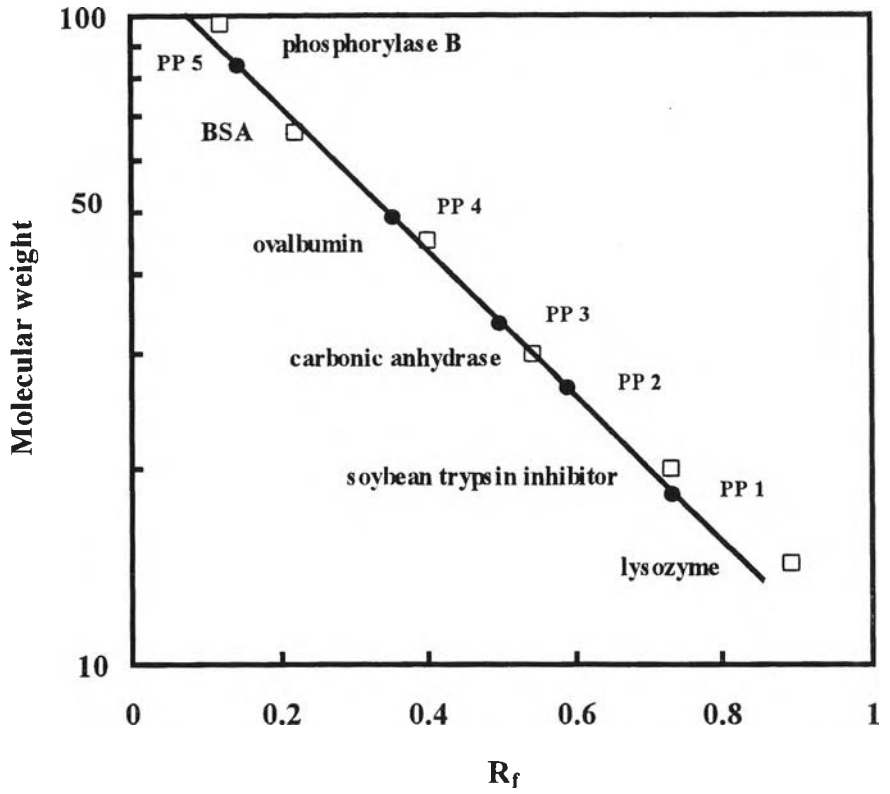

Figure 3.15 Molecular weight calibration curve of standard protein on 12% SDS-PAGE. Symbol: □, standard protein; ●, periplasmic protein fraction.

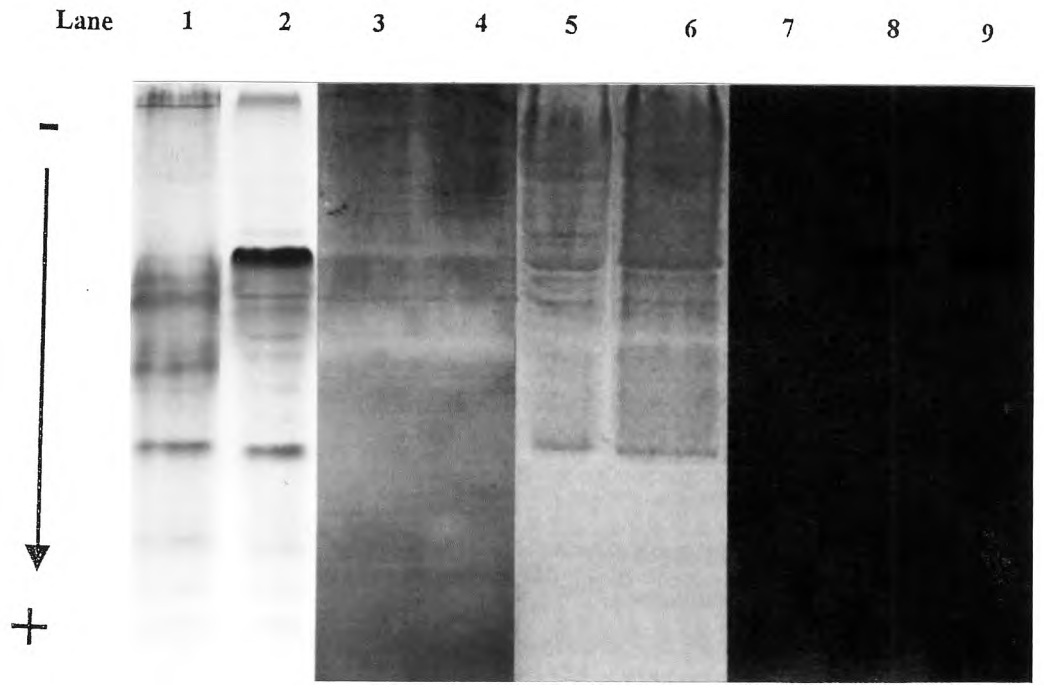

Figure 3.16 Non-denaturing PAGE pattern of periplasmic proteins obtained from cells adapted to 0.5 M and 2.0 M NaCl. Coomassie blue staining was used to detect protein (A). The native protein was stained for enzyme activity (B). The native protein was detected by autoradiograph (C).

Lane 1,2: periplasmic protein fraction in 0.5 M and 2.0 M NaCl 50 μg

Lane 3,4: periplasmic protein fraction in 0.5 M and 2.0 M NaCl
(activity staining with out choline) 50 μg

Lane 5,6: periplasmic protein fraction in 0.5 M and 2.0 M NaCl
(activity staining with choline) 50 μg

Lane 7: autoradiogram of [*methyl*-^{14}C]choline (control)

Lane 8,9: autoradiogram of periplasmic protein fraction
in 0.5 M 2.0 M NaCl 50 μg