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# Inhibition of plant vacuolar $H^+$-ATPase by diethylpyrocarbonate

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**Abstract**

Treatment of the tonoplast $H^+$-ATPase from mung bean seedlings (*Vigna radiata* L.) with histidine-specific modifier, diethyl pyrocarbonate (DEP), caused a marked loss of the ATP hydrolysis activity and the proton translocation in a concentration-dependent manner. The reaction order of inhibition was calculated to be 0.98, suggesting that at least one histidine residue of vacuolar $H^+$-ATPase was modified by DEP. The absorbance of the vacuolar $H^+$-ATPase at 240 nm was progressively increased after incubation with DEP, suggesting that *N*-carbethoxyhistidine had been formed. Hydroxylamine, which could break *N*-carbethoxyhistidine, reversed the absorbance change and partially restored the enzymic activity. The $pK_a$ of modified residues of vacuolar $H^+$-ATPase was kinetically determined to be 6.73, a value close to that of histidine. Thus, it is assuredly concluded that histidine residues of the vacuolar $H^+$-ATPase were modified by DEP. Kinetic analysis showed that $V_{max}$ but not $K_m$ of vacuolar $H^+$-ATPase was decreased by DEP. This result is interpreted as that the residual activity after DEP inhibition was primarily due to the unmodified enzyme molecules. Moreover, simultaneous presence of DEP and DCCD (*N*,*N*′-dicyclohexyl-carbodiimide), an inhibitor modified at proteolipid subunit of vacuolar $H^+$-ATPase, did not induce synergistic inhibition, indicating their independent effects. The stoichiometry studies further demonstrate that only one out of four histidine residues modified was involved in the inhibition of vacuolar $H^+$-ATPase by DEP. $Mg^{2+}$-ATP, the physiological substrate of vacuolar $H^+$-ATPase, but not its analogs, exerted preferentially partial protection against DEP, indicating that the histidine residue involved in the inhibition of enzymatic activity may locate at/or near the active site and directly participate in the binding of the substrate. 

*Keywords:* Tonoplast; Vacuolar $H^+$-ATPase; Diethylpyrocarbonate

## 1. Introduction

Vacuolar $H^+$-ATPase of higher plants plays an important role in the secondary active transport of solutes such as ions and metabolites [1,2]. Tonoplast ATPases are characteristically stimulated by $Cl^-$ and inhibited by $NO_3^-$, DIDS, NEM, NBD-Cl, and DCCD [1,3]. Molecular mass of tonoplast $H^+$-ATPase from higher plants was estimated to be 600–700 kDa [4,5]. Vacuolar $H^+$-ATPase contains seven to nine polypeptides as visualized on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis with two major bands of the molecular masses of

Abbreviations: BZ-ATP, 3-*O*-(4-benzoyl)benzoyladenosine 5′-triphosphate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DIDS, 4,4′diisothiocyanostilbene-2,2′-disulfonate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide

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65–80 kDa (A subunit) and 55–65 kDa (B subunit), and a 16 kDa protein (DCCD-binding proteolipid). Subunit A of vacuolar $H^+$-ATPase reacts with the substrate analogs, such as NBD-Cl and dialdehyde derivative of ATP [6–8], and is implicated as the catalytic site. The B subunit probably plays a regulatory role, since its binding by ATP analog Bz-ATP resulted in a complicated and uncompetitive inhibition [9]. The DCCD-binding proteolipid subunit c might be involved in the proton translocation and is preferentially labeled by radioactive DCCD [6,7].

The cDNA sequences and, as consequence, their deduced amino acid sequences of the A subunit from tonoplast $H^+$-ATPase of carrot roots, mung bean, and cotton were successfully obtained, respectively [10–12]. However, little is known on the essential amino acid residues involved in the active site, neither to say the structure and the function of plant vacuolar $H^+$-ATPase. Our previous work indicates, using substrate analog dialdehyde derivative of ATP, A subunit of vacuolar $H^+$-ATPase contains a lysine residue essential to the enzymic activity [8]. Other reports [13,14] revealed that the arginine residue is crucial for the tonoplast $H^+$-ATPase activity. The sensitivities of vacuolar $H^+$-ATPase to NEM, and NBD-Cl imply the presence of cysteine and/or tyrosine residues at active site [6,9]. Meanwhile, several lines of evidence showed that carboxylate modifier [$^{14}$C]DCCD could bind the 16 kDa proteolipid (subunit c) of vacuolar $H^+$-ATPase [6,7,9,15]. Nevertheless, no report addressed whether the catalytic subunit of vacuolar $H^+$-ATPase also possesses critical carboxylate residue. In spite of these results, further efforts are still required to identify other possible amino acid residues significant to the activity of vacuolar $H^+$-ATPase and to elucidate their roles in the enzymatic mechanism.

Histidine residues were shown to be involved in catalytic activities of F- and P-type $H^+$-ATPases [16–20]. In several reports, the protonation-deprotonation alternation of imidazole was believed to play critical roles in catalytic mechanism, substrate binding, and maintenance of enzyme conformation [21–23]. However, no evidence indicates whether histidine residues are also involved in the catalytic activity of plant vacuolar $H^+$-ATPase. Diethyl pyrocarbonate (ethoxyformic anhydride, DEP) has been widely used to identify histidine residues and determine their roles in enzymatic activity of various enzymes [22,23]. In this study, we demonstrated the presence of a histidine residue may be involved in the inhibition of enzymic reaction and $H^+$-translocation of plant vacuolar $H^+$-ATPase by diethyl pyrocarbonate. Kinetic and stoichiometry studies reveal that modification of one mol histidine/mol ATPase is sufficient to inhibit the enzymic activity of vacuolar $H^+$-ATPase.

## 2. Materials and methods

### 2.1. Plant materials

Seeds of *Vigna radiata* L. (mung bean) germinated at room temperature in the dark following soak in tap water for 24 h. Hypocotyls of 4-day-old etiolated seedlings were excised, chilled, and then used as starting materials.

### 2.2. Preparation of tonoplast and vacuolar $H^+$-ATPase

Tonoplast vesicles and purified vacuolar $H^+$-ATPase were prepared from etiolated hypocotyl according to methods modified from Matsuura-Endo et al. [5] as described previously [14,24].

### 2.3. Enzyme assay and protein determination

Activities of tonoplast $H^+$-ATPase were measured in a 1 ml reaction solution (30 mM Tris–HCl (pH 7.0), 3 mM $MgSO_4$, 50 mM KCl, 3 mM ATP, 0.5 mM sodium azide, 0.1 mM sodium vanadate, and 0.1 mM ammonium molybdate). After 30 min incubation at 37°C, the reaction was stopped by adding a solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) SDS, and 0.02% (w/v) ANSA (1-amino-2-naphthol-4-sulfonic acid) at room temperature. The released $P_i$ was measured spectrophotometrically [3].

Protein concentration was determined according to the modified Lowry method using bovine serum albumin as the standard [25].

### 2.4. Measurement of proton translocation

Proton translocation was measured as described previously using fluorescence quenching of Acridine orange [3]. The reaction mixture contained 5 mM Tris–HCl (pH 7.0), 250 mM sorbitol, 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 0.5 mM $NaN_3$, 0.1 mM Na-vanadate, 0.1 mM ammonium molybdate, 5 μM Acridine orange, and 10–20 μg/ml membrane protein. The fluorescence quenching was initiated by adding 3 mM $MgSO_4$. The ionophore gramicidin D (2 μg/ml) was added at the end of each assay.

### 2.5. Modification of $H^+$-ATPase with DEP

DEP was dissolved as stock with acetonitrile, in which it was stable for several weeks (data not shown). Vacuolar $H^+$-ATPase was modified with DEP in 50 mM Mops–KOH (pH 7.0) at room temperature. The concentration of acetonitrile in the incubation mixture was kept below 5% (v/v). At the end of incubation the mixture was diluted 25-fold with assay solution above and 50 mM imidazole. The modification was stopped and enzyme assay started immediately.

### 2.6. Kinetic analysis

Values of $K_m$ and $V_{max}$ were obtained from conventional Lineweaver-Burk plot. The plot of log$(A/A_0)$ versus time yields the apparent rate constant of inhibition, $k_{obs}$, at various concentrations of the modifier as previously described [14]. The reaction order, $n$, was calculated from the double logarithmic plot of $k_{obs}$ versus concentrations of DEP as follows [26–29]:

$$\log k_{obs} = n\log[\mathrm{DEP}] + \log k_1 \quad (1)$$

where $k_{obs}$ is the apparent reaction rate constant, $k_1$ is the second-order rate constant, and [DEP] is concentration of DEP.

The apparent rate constant of inhibition, $k_{obs}$, is pH-dependent according to the equation [27,30]:

$$1/k_{obs} = 1/k_{obs(max)} + [H^+]/(K_a k_{obs(max)}) \quad (2)$$

where $[H^+]$ is proton concentration, $k_{obs(max)}$ is maximum of $k_{obs}$, and $K_a$ is the apparent acidic dissociation constant of modified group. The value of p$K_a$ was then determined from the slope of the plot of $1/k_{obs}$ against $[H^+]$.

### 2.7. Stoichiometry studies

The stoichiometry is expressed as molar ratio of histidine residues modified by DEP to vacuolar $H^+$-ATPase. The number of *N*-carbethoxyhistidine residues formed was calculated by measuring the absorbance increase at 240 nm [23,23].

Furthermore, Tsou's plot was used to determine the number and type of histidine residues involved in the enzymic activity [26,27,31,32]:

$$(A/A_0)^{1/i} = (p-m)/p \quad (3)$$

where $A/A_0$ is the fraction of activity remaining when $m$ groups was modified, $i$ is the number of essential groups for activity, and $p$ is the total number of residues modified. The number of essential groups, $i$, is obtained if the plot of $A/A_0$ versus $m$ is linear.

However, if the plot above is not linear for all possible number of $i$, different types of modified residues are suggested. Another Tsou's equation is then employed [26,27,31,32]:

$$\log\{[nx/(A/A_0)^{1/i}]-p\} = \log(n-p) + [(\alpha-1)/i]\log(A/A_0) \quad (4)$$

where $n$ is the number of total modifiable groups, $x$ is the total fraction of enzymic activity remaining, $p$ is the number of residues with a reaction rate constant $k_1$, of which $i$ is essential, and finally $n-p$ is the number of residues reacted with reagent at the rate constant $\alpha k_1$. The real case is then determined from a best-fitted straight line in a plot of $\log\{[nx/(A/A_0)^{1/i}]-p\}$ against $\log(A/A_0)$ using suitable $p$ and $i$. The slope of the plot, $(\alpha-1)/i$, indicates the difference in the reaction rates for modified essential and nonessential residues [26,27,31,32].

### 2.8. Chemicals

DEP and hydroxylamine were obtained from Sigma. ATP and acetonitrile were purchased from Merck. All other chemicals were of the highest grade available from commercial sources.

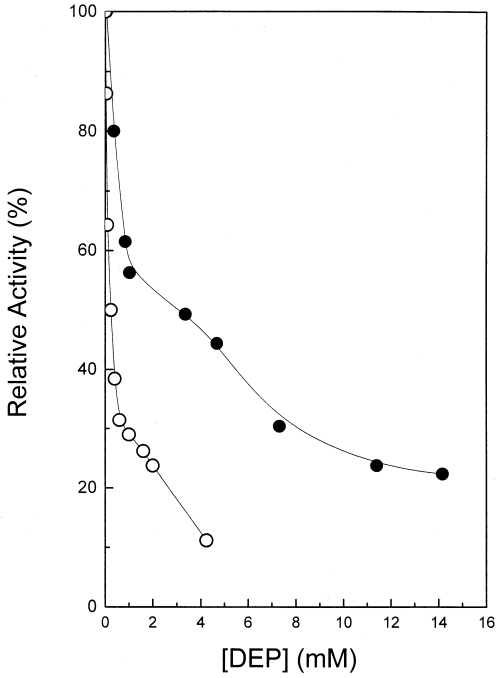


Fig. 1. Inhibition of vacuolar $H^+$-ATPase by DEP. Membrane protein (1.0 mg/ml) and solubilized $H^+$-ATPase (0.6 mg/ml) were incubated with various concentrations of DEP in 50 mM Mops–KOH (pH 7.0) at room temperature for 15 min. Enzymic activities were measured as described in Section 2. (●) ATP hydrolysis activity of membrane-bound $H^+$-ATPase; (○) ATP hydrolysis activity of solubilized $H^+$-ATPase. The control activities were 10.5 and 107.4 μmol $P_i$ released/mg protein per hour for membrane-bound and solubilized $H^+$-ATPases, respectively.

## 3. Results and discussion

### 3.1. Inhibition of vacuolar $H^+$-ATPase by DEP

Incubation of vacuolar $H^+$-ATPase with DEP resulted in a progressive loss of the enzymic activity in a concentration-dependent manner (Fig. 1). Half-maximal inhibition by DEP occurred at 3.2 and 0.12 mM for membrane-bound and solubilized $H^+$-ATPases, respectively. Carried-over acetonitrile (< 5%), in which DEP was dissolved as the stock, has negligible effect on $H^+$-ATPase activities under conditions used (data not shown). In parallel experiments, DEP inhibition of ATP-mediated $H^+$-translocation measured by fluorescence quenching of Ac-

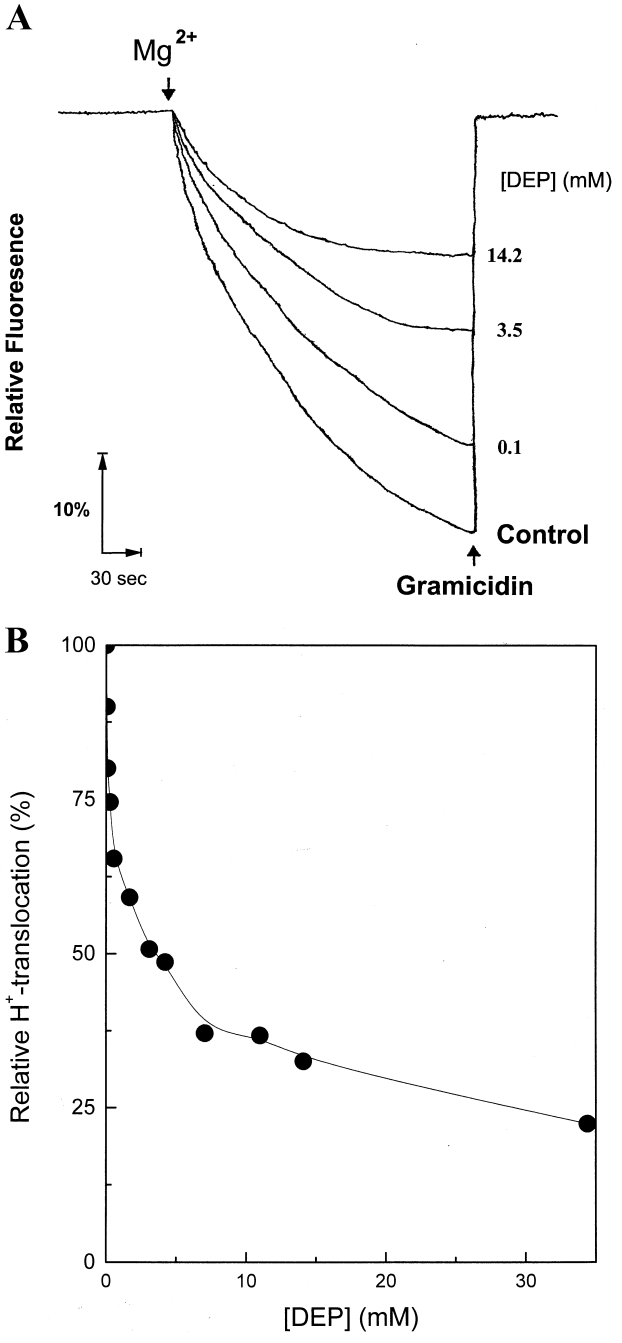


Fig. 2. Inhibition of $H^+$-translocation by DEP. Vesicles (1.0 mg/ml protein) were treated with various concentrations of DEP in 50 mM Mops–KOH at room temperature for 15 min. Proton translocation was measured as described in Section 2. (A) Reaction trace of fluorescence quenching of Acridine orange with various concentrations of DEP. The reaction was initiated by adding 3 mM $MgSO_4$. The ionophore gramicidin D (2 μg/ml) was added at the end of each assay. Excitation wavelength was 495 nm, while emission wavelength was 530 nm. (B) Concentration curve for the inhibition of proton translocation by DEP. Initial rate of fluorescence quenching was calculated from the changes of fluorescence in the first minute.

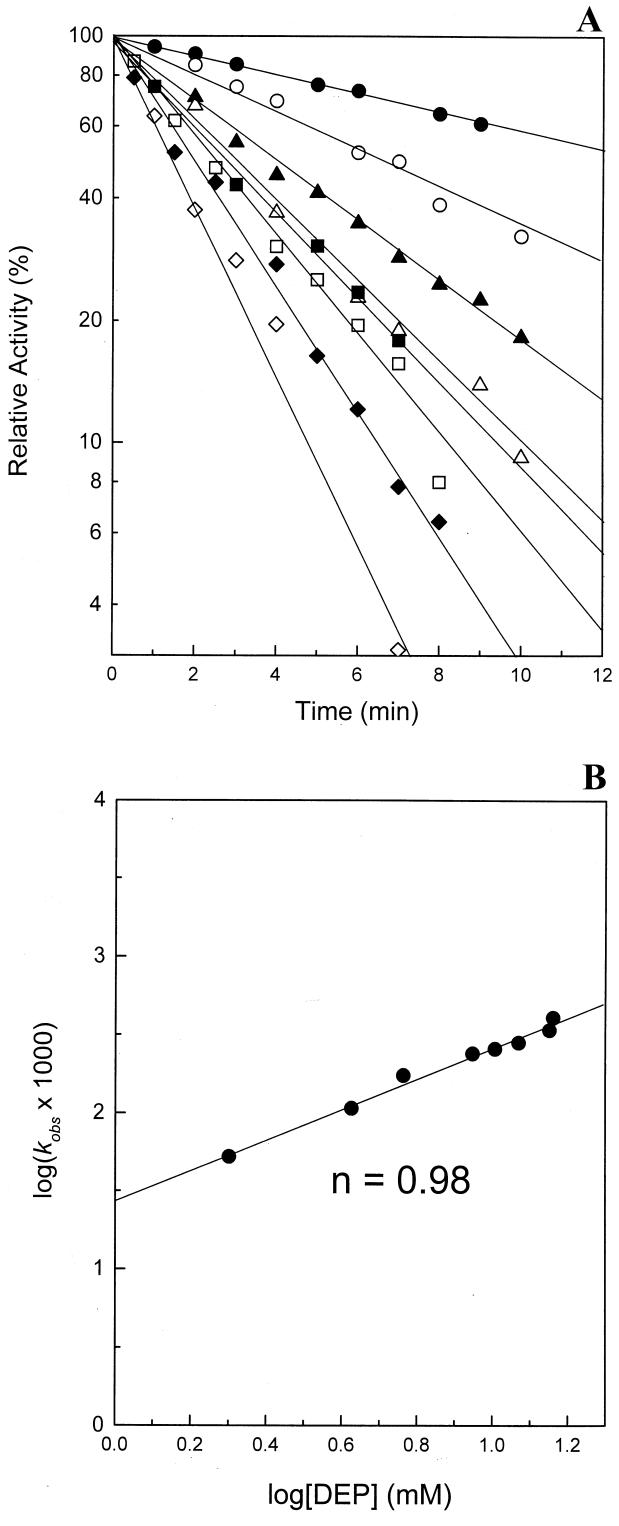


Fig. 3. DEP inhibition of purified vacuolar $H^+$-ATPase. (A) Time courses of inhibition of purified vacuolar $H^+$-ATPase by various concentrations of DEP. Inhibition treatment and enzyme assay of vacuolar $H^+$-ATPase were performed as described in Section 2. (●) 2 mM, (○) 4.2 mM, (▲) 5.8 mM, (△) 8.9 mM, (■) 10.2 mM, (□) 11.8 mM, (◆) 14.2 mM, and (◇) 14.6 mM DEP, respectively. The control activity was approximately 124.6 μmol $P_i$ released/mg protein per hour. (B) Double-logarithmic plot of the apparent rate constant of the inhibition ($k_{obs}$) against concentrations of DEP. A reaction order ($n$) of 0.98 was calculated.
←

ridine Orange was also observed (Fig. 2). The rate of proton translocation was markedly decreased following the treatment of tonoplast vesicles with DEP at various concentrations (Fig. 1, closed circles). The concentration for half-maximal inhibition of proton translocation is approximately at 4 mM DEP, similar to enzymic activity of membrane-bound $H^+$-ATPase. These results indicate that enzymic reaction and $H^+$-translocation of vacuolar $H^+$-ATPase share common mechanism, which could be inhibited by DEP. Moreover, solubilized $H^+$-ATPase is approximately 20-fold more sensitive to DEP than membrane-bound enzyme, suggesting that membrane might play a significant role against the accessibility of the inhibitor to the target amino acid residues in vacuolar $H^+$-ATPase.

Inhibition of vacuolar $H^+$-ATPase by DEP follows a simple and time-dependent kinetics. Fig. 3 depicts a pseudo-first order inhibition of vacuolar $H^+$-ATPase by DEP (Fig. 3A). The apparent rate constants of inhibition ($k_{obs}$) at given concentration of modifier were obtained from time course. Reaction order of vacuolar $H^+$-ATPase with DEP was then determined as 0.98 from double-logarithmic plot of $k_{obs}$ against DEP concentration (Fig. 3B), indicating that at least one histidine residue of the enzyme was involved in the inhibition by DEP. Lineweaver–Burk plot of native vacuolar $H^+$-ATPase yields $K_m$ and $V_{max}$ values of 0.37 mM and 137 μmol $P_i$ released/mg protein per hour, respectively (Fig. 4). However, after incubating with DEP, $K_m$ of vacuolar $H^+$-ATPase remains unchanged, while $V_{max}$ is apparently decreased with increasing concentration of the modifier. This inhibition mode is interpreted as that residual activities after the DEP inhibition might come directly from the unmodified enzyme molecules.

### 3.2. Characteristics of DEP-modified $H^+$-ATPase

According to earlier reports, DEP could react with histidine, tyrosine, lysine, cysteine, and serine residues [23]. The characterization of DEP-derivatized residues in vacuolar $H^+$-ATPase was therefore investigated to determine which residues were modified. First of all, the UV absorption spectra of modified and native $H^+$-ATPases were scrutinized. The difference spectra of DEP-labeled against native $H^+$-ATPases at various time intervals are shown in Fig. 5. A maximum near 240 nm, a spectral characteristic of *N*-carbethoxyhistidine, appeared progressively as incubation time increased. These spectral properties indicate that histidine residues of vacuolar $H^+$-ATPase were primarily modified by DEP [23]. Furthermore, no change of absorbance at 278 nm was found (data not shown), excluding possible involvement of tyrosine residues in the DEP modification [23].

Hydroxylamine could deacylate DEP-derivatized histidine moieties of the enzyme and consequently restores its activity [23]. When vacuolar $H^+$-ATPase was treated with DEP, the absorption at 240 nm was increased as previously mentioned (Fig. 6). However, as hydroxylamine was subsequently added, the absorption change was partially reversed, indicating that modified histidine moieties were deacylated. In parallel experiments, the activity of vacuolar $H^+$-ATPase was also measured following the treatment of DEP and hydroxylamine. Hydroxylamine alone exerts no significant effect on the enzymic activity of control $H^+$-ATPase (data not shown). DEP inhibition of vacuolar $H^+$-ATPase could be substantially removed by hydroxylamine and enzymic activity restored to approximately 50–75% of control. The time course for recovery of enzymic activity coincides perfectly with that of the absorption change along the treatment of DEP and hydroxylamine, a distinct feature for *N*-carbethoxyhistidine (data not shown).

The pH dependence of DEP inhibition of vacuolar $H^+$-ATPase was further investigated (Fig. 7A). The degree of DEP inhibition of vacuolar $H^+$-ATPase increased concomitantly with a rise in pH. The $1/k_{obs}$ of DEP inhibition at various pH values versus proton concentration was plotted according to Eq. 2

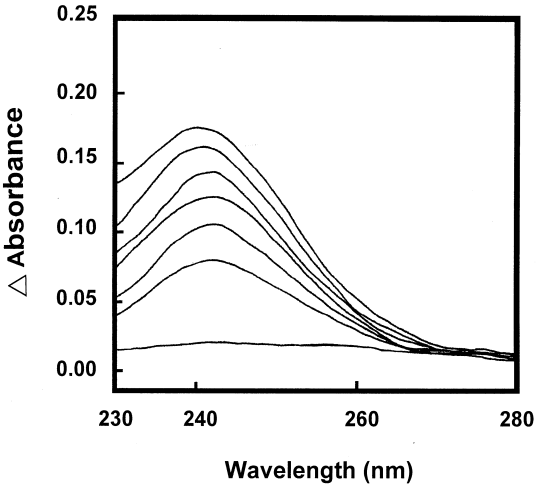


Fig. 5. Difference absorption spectra for the modification of vacuolar $H^+$-ATPase by DEP. Purified vacuolar $H^+$-ATPase (0.33 mg/ml) was incubated with 1.6 mM DEP in 50 mM Mops–KOH (pH 7.0). The difference absorption spectra of DEP-ATPase were taken against the control enzyme solution. After the addition of the modifier, the difference absorption spectra were scanned from 230 to 300 nm for 0, 3, 6, 9, 15, 24, and 30 min, respectively. The difference absorbance was increased as incubation time prolonged (curves from bottom up).

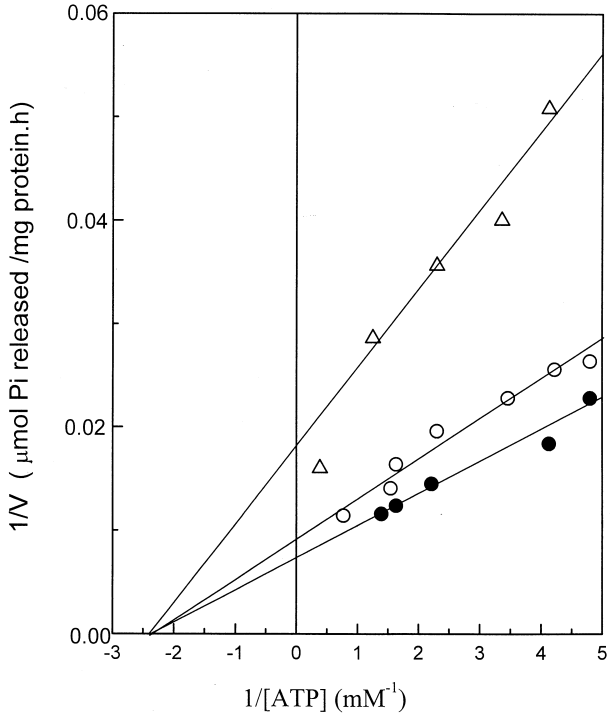


Fig. 4. Lineweaver–Burk plots of DEP inhibition of vacuolar $H^+$-ATPase. Purified vacuolar $H^+$-ATPase (0.6 mg/ml) was treated with DEP at the concentrations as indicated in 50 mM Mops–KOH (pH 7.0) at room temperature. Enzymic activities were determined in an assay medium with various concentrations of ATP as described in Fig. 1. (●) 0 mM, (○) 3.5 mM, and (△) 14.2 mM DEP, respectively.

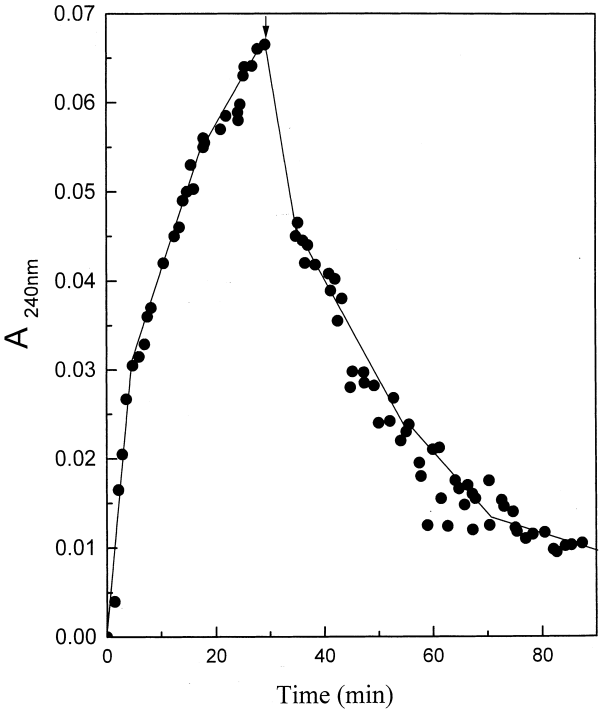


Fig. 6. Restoration of absorbance of DEP-ATPase by hydroxylamine. Purified vacuolar $H^+$-ATPase (0.67 mg/ml) was treated with 1.6 mM DEP in 50 mM Mops–KOH (pH 7.0). The absorption changes at 240 nm were monitored during incubation periods. After 20 min, 0.2 M hydroxylamine (pH 7.0) was added to the mixture (as arrows indicate).

(Fig. 7B). From the slope, the p$K_a$ for the modified amino acid residues was calculated to be 6.73, a value similar to that of histidine [22,30]. Previous reports demonstrated that DEP reacted only with unprotonated imidazoles and histidine residues [22,23,30]. We were thus convinced assuredly by evidence above, that histidine residues were the most probable candidates for DEP inhibition of vacuolar $H^+$-ATPase under our conditions. Since DEP also inhibited ATP-mediated proton transport, it is possible that protonation-deprotonation of histidine residue plays an important role in reaction mechanism and regulation of vacuolar $H^+$-ATPase. A similar conclusion for significance of histidine residue in proton translocation was also obtained from the studies in $H^+$/organic cation antiport system of rat renal brush-border membrane [33].

Moreover, it is well demonstrated that DCCD could bind the 16 kDa proteolipid (subunit c) of vacuolar $H^+$-ATPase, resulting in inhibition of its enzymatic reaction and proton translocation [6,7,9,15]. The additivity of inhibition of vacuolar $H^+$-ATPase by DEP and DCCD was then examined (Table 1). Simultaneous presence of DEP and DCCD did not induce any synergistic inhibition of enzymatic reaction or proton translocation, indicating that both modifications are independent. This result is conceivable, since these two inhibitors primarily modify distinct amino acid residues in different subunits. Modification of one subunit did not exert any significant long-distance effect on the other.

### 3.3. Protection against DEP inhibition

Protection studies offer a proper means to determine whether the histidine residue modified is present in the active domain. When vacuolar $H^+$-ATPase was incubated with 3.5 mM DEP, the enzy-

Table 1
Inhibition of vacuolar $H^+$-ATPase by DCCD and DEP[a]

| Addition | Membrane-bound $H^+$-ATPase | | | | Purified $H^+$-ATPase | |
|---|---|---|---|---|---|---|
| | Specific activity | | $H^+$-translocation | | | |
| | μmol $mg^{-1}$ $h^{-1}$ | Inhibition (%) | ΔF/F | Inhibition (%) | μmol $mg^{-1}$ $h^{-1}$ | Inhibition (%) |
| None | 14.0 ± 0.2 | 100 | 116.9 ± 0.7 | 100 | 124.7 ± 7.6 | 100 |
| DEPC[b] | 7.3 ± 0.1 | 52.2 | 77.4 ± 2.3 | 66.0 | 62.7 ± 10.1 | 50.3 |
| DCCD[c] | 7.8 ± 0.1 | 55.7 | 66.9 ± 8.2 | 59.8 | 74.8 ± 2.9 | 60.0 |
| DEPC+DCCD[d] | 4.2 ± 0.5 | 31.2 | 56.4 ± 6.5 | 48.2 | 33.7 ± 2.3 | 27.0 |

[a]Reaction conditions for inhibition and measurements of specific activities and proton translocation were as indicated in Figs. 1 and 2.
[b]Concentrations of DEP for membrane-bound and purified vacuolar $H^+$-ATPase were 1.0 and 0.2 mM, respectively.
[c]Concentrations of DCCD for membrane-bound and purified vacuolar $H^+$-ATPase were 0.25 mM and 5 μM, respectively.
[d]Both inhibitors were present in reaction mixture simultaneously.

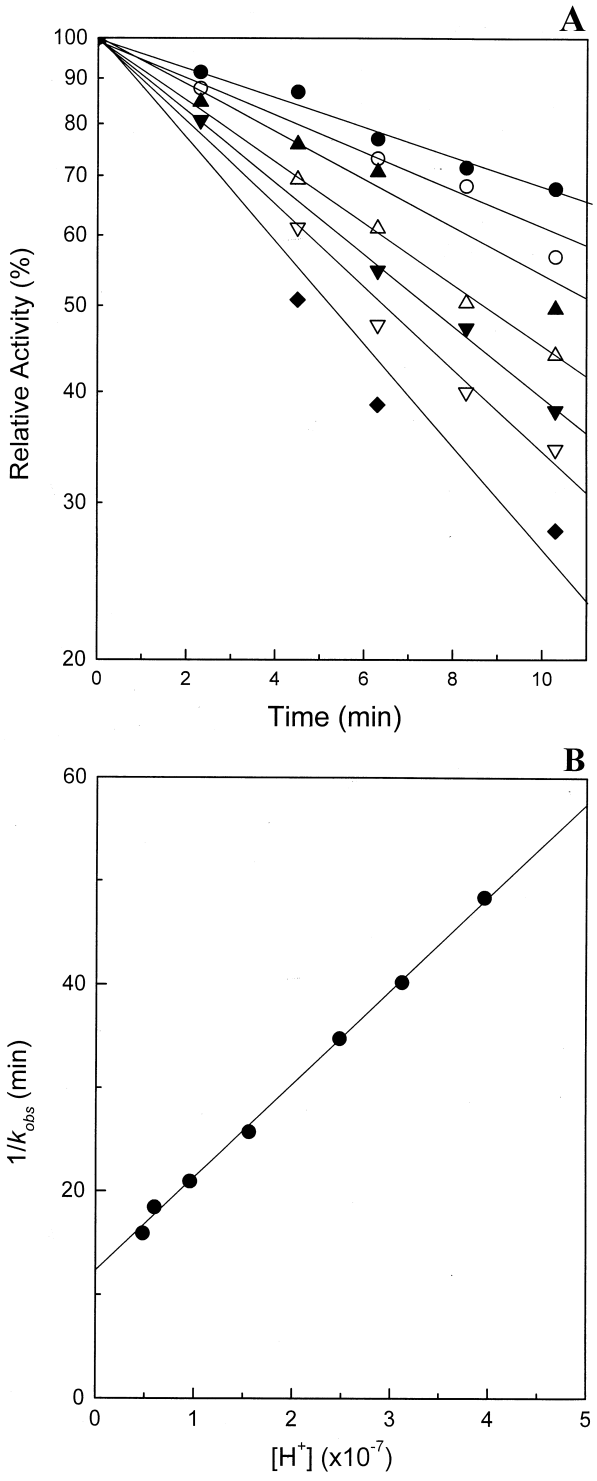


Fig. 7. The pH dependence of DEP inhibition of vacuolar $H^+$-ATPase. Purified vacuolar $H^+$-ATPase (0.6 mg/ml) was incubated with 1.6 mM DEP in 50 mM Mops–KOH at various pH values. At time intervals, residual activities of the enzyme were assayed as described in Fig. 1. The pH values were (●) 6.4, (○) 6.5, (▲) 6.6, (△) 6.8, (▼) 7.0, (▽) 7.2, and (◆) 7.3, respectively. (A) Time courses of DEP inhibition of vacuolar $H^+$-ATPase at different pH. (B) Plot of $1/k_{obs}$ at various pH against proton concentration. A $pK_a$ of 6.73 for modified amino acid residues was determined.

mic activity was completely inhibited. However, in the presence of physiological substrate $Mg^{2+}$-ATP, the enzymic activity was elevated accordingly (data not shown). For instance, 10 mM ATP provided approximately 50% protection of vacuolar $H^+$-ATPase against DEP inhibition (Table 2). The presence of the $Mg^{2+}$ is crucial for the efficacy of the protection by ATP. In contrast, substrate analogues, such as ADP, AMP, GTP, CTP, and UTP, exert less protection effects against the DEP inhibition of vacuolar

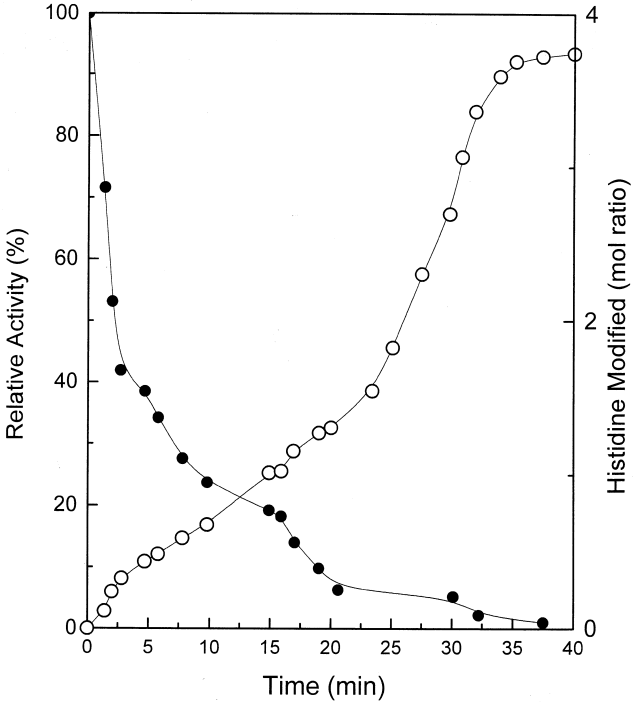


Fig. 8. Relationship between the residual enzyme activity and the number of histidine residues modified by DEP. Purified $H^+$-ATPase (1.0 mg/ml) was incubated with 3.0 mM DEP in 50 mM Mops–KOH (pH 7.0) at room temperature. At time intervals, activity (●) and the amount of labeling (○) of vacuolar $H^+$-ATPase were determined as described in Section 2. The number of histidine residues modified was calculated on the basis of absorption change at 240 nm using extinction coefficient of $\varepsilon_{240\,nm} = 3200\ M^{-1}\ cm^{-1}$. The control ATPase activity was 127 μmol $P_i$ released/mg protein per hour.

$H^+$-ATPase. We therefore believe that the histidine residue investigated might not directly interact with nucleoside moiety of the substrate. Moreover, neither did inorganic phosphate nor its analogs, such as pyrophosphate, *p*-nitrophenylphosphate, imidodiphosphate, phosphoserine, and phosphothreonine, provide substantial protection of vacuolar $H^+$-ATPase against DEP inhibition, suggesting that the histidine residue involved does not probably reside at a site for phosphate-binding. However, the preferential protection by $Mg^{2+}$-ATP indicates that the physiological substrate might compete specifically with DEP. Taking all this into account, we speculate that the histidine observed might locate at the door-front to the active site of vacuolar $H^+$-ATPase. Its modification by large moiety of DEP prevents the entrance of substrate to the catalytic domain of the enzyme. Nevertheless, we cannot exclude the possibility that DEP modifies the histidine residue in a domain other than the active site, inducing a conformational change and consequently the loss of enzymic activity.

### 3.4. Stoichiometry of the labeling

Fig. 8 depicts the relationship between the degree of DEP inhibition of vacuolar $H^+$-ATPase and the extent of histidine labeling which was determined spectrophotometrically using an extinction coefficient of $\varepsilon_{240\,nm} = 3200\ M^{-1}\ cm^{-1}$ (Fig. 8) [22,23]. The correlation between DEP inhibition and histidine labeling of vacuolar $H^+$-ATPase is not linear. Extrapolation of the relationship yields approximately four histidine residues to be labeled when enzymic activity of vacuolar $H^+$-ATPase was completely inhibited. This complicated relationship and the reaction order of 0.98 obtained kinetically prompted us to further employed Tsou's method to analyze how these labeled histidine residues are participating in DEP inhibition of the ATP hydrolysis. At first, we used the plot of $A/A_0$ versus $m$ (Eq. 3) to calculate the number of the crucial histidine residues. However, no suitable straight line was yielded for all possible numbers of essential residues assumed (data not shown). This negative result suggests that all modifiable residues may have different susceptibility to DEP attack [26]. Therefore, we subsequently used the plot of $\log\{[nx/(A/A_0)^{1/i}]-p\}$ against $\log(A/A_0)$ (Eq. 4) to verify this speculation (Fig. 9). Alternatively, a best-fitted straight line was obtained assuming $n=4$ and $p=i=1$. Furthermore, the value of $\alpha$, proportionality of reaction rate for modified residue other than

Table 2
Protection of the tonoplast ATPase activity against inactivation by DEP[a]

| Protectors (10 mM) | Activity as substrate (μmol $P_i$ released/mg protein/h) (%) | Protection against DEP | |
|---|---|---|---|
| | | +$MgSO_4$ (%) | −$MgSO_4$ (%) |
| ATP | 108.3 (100.0%) | 43.0 | 19.7 |
| ADP | 12.6 (11.6%) | 11.8 | 6.6 |
| AMP | 6.9 (6.5%) | 17.0 | 8.9 |
| GTP | 16.8 (15.5%) | 12.1 | 5.2 |
| CTP | 17.4 (16.1%) | 17.9 | 7.4 |
| UTP | 2.4 (2.2%) | 17.3 | 11.3 |
| $PP_i$ | 30.9 (28.5%) | 24.6 | 5.5 |
| $P_i$ | – | 19.6 | 10.4 |
| *p*-Nitrophenyl phosphate | 1.5 (1.5%) | 10.0 | 10.5 |
| Imidodiphosphate | 14.1 (13.0%) | 15.7 | 8.7 |
| Phosphoserine | 7.5 (6.8%) | 13.1 | 3.5 |
| Phosphothreonine | 9.3 (8.5%) | 11.1 | 3.6 |

[a]Vacuolar $H^+$-ATPase (0.6 mg/ml) was preincubated with 10 mM protectors at room temperature in the presence or absence of 10 mM $MgCl_2$. After addition of 3.5 mM DEP for 30 min, aliquots (20 μl) were removed and assayed for ATPase activity as described in Section 2. The percentage of protection was calculated according to the equation:

$$\text{Percentage protection} = \frac{[SA_{(protected)} - SA_{(unprotected)}]}{[SA_{(control)} - SA_{(unprotected)}]} \times 100$$

SA, specific activity.

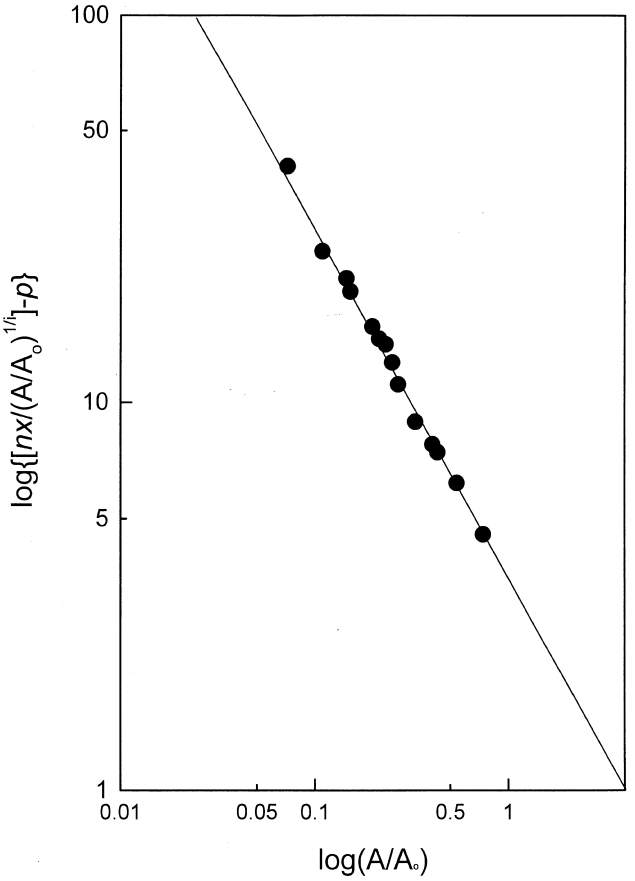


Fig. 9. Tsuo's analysis of the modification of vacuolar $H^+$-ATPase by DEP. The experimental conditions were the same as those shown in Fig. 8. A best-fitted straight line according to Eq. 4 was obtained, assuming $n=4$ and $p=i=1$. The $\alpha$ value of 0.1 was calculated from the slope of the plot.

most essential one, was calculated to be 0.1. Taking all Tsuo's parameters into account, it is possible that one out of four histidine residues modified is essentially involved in the inhibition of ATPase activity and the reaction rate of this important residue with DEP is approximately 10-fold (1/0.1) faster than those not participating in the inhibition.

Recently, cDNA sequence and deduced amino-acid sequence of catalytic subunit (A) of higher plants, eubacteria, red alga, yeast, and bovine have been reported [10–12,34–38]. Sequences of A subunit from wide variety of sources show strikingly high homology. In addition, six regions within these sequences, designated A–F, are highly conserved as compared to the β (catalytic) subunit of F-type $H^+$-ATPases [10]. Subunit A of mung bean vacuolar $H^+$-ATPase contains seven histidine residues, of which only one is located in the homologous region A, residues 73–101 [11]. There are two adjacent histidine residues in the non-homologous region that accommodates a stretch of a palindromic DNA sequence. The region between residues 231–260 of A subunit of mung bean vacuolar $H^+$-ATPase is homologous to residues 129–158 of the β subunit of *Escherichia coli* $F_1$-ATPase, which is believed to include the universal nucleotide-binding site [39]. However, this fragment in A subunit of vacuolar $H^+$-ATPase did not contain any histidine residue [39]. Thus, we speculate that the histidine in the region of residues 73–101 is the most possible candidate involved in the catalytic reaction of the vacuolar $H^+$-ATPase and soundly in the inhibition of the enzyme by DEP in this study. If this is the case, the conserved fragment of residues 73–101 may fold into the active pocket of vacuolar $H^+$-ATPase and participate in the enzymic activity as well as proton translocation. However, we cannot exclude the possibility that this stretch is not in the active domain, but its modification, at least by DEP, may cause a long-range effect on the enzymatic activity. The verification of our suggestion requires further investigations.

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## References

[1] H. Sze, J. Ward, S. Lai, J. Bioenerg. Biomembr. 24 (1992) 371–381.
[2] B.J. Barkla, O. Pantoja, Annu. Rev. Plant Physiol. Mol. Biol. 47 (1996) 159–184.
[3] M.Y. Wang, Y.H. Lin, W.M. Chow, T.P. Chung, R.L. Pan, Plant Physiol. 90 (1989) 475–481.
[4] R.V. Parry, J.C. Tunner, P.A. Rea, J. Biol. Chem. 264 (1989) 20025–20032.
[5] C. Matsuura-Endo, M. Maeshima, S. Yoshida, Eur. J. Biochem. 187 (1980) 745–751.
[6] S. Mandala, L. Taiz, J. Biol. Chem. 261 (1986) 12850–12855.

[7] S.K. Randall, H. Sze, J. Biol. Chem. 262 (1987) 7135–7141.
[8] W.M. Chow, C.M. Tzeng, C.S. Chen, S.Y. Kuo, M.Y. Wang, R.L. Pan, Plant Physiol. 98 (1992) 44–52.
[9] M.H. Manolson, P.A. Rea, R.J. Poole, J. Biol. Chem. 260 (1985) 12273–12279.
[10] L. Zimniak, P. Dittrich, J.P. Gogarten, H. Kibak, L. Taiz, J. Biol. Chem. 263 (1988) 9102–9112.
[11] S.J. Chiu, S.H. Hung, L.Y. Lin, R.L. Pan, Plant Physiol. 109 (1995) 1125–1127.
[12] T.A. Wilkins, Plant Physiol. 102 (1993) 679–680.
[13] A.B. Bennett, K. Borcherts, Biochim. Biophys. Acta 1023 (1990) 119–123.
[14] S.Y. Kuo, C.M. Tzeng, W.J. Lin, S.S. Jiang, S.S. Hung, R.L. Pan, Bot. Bull. Acad. Sin. 40 (1999) 127–134.
[15] S.K. Randall, H. Sze, J. Biol. Chem. 261 (1986) 1364–1371.
[16] C. Godinot, F. Penin, D.C. Gautheron, Arch. Biochem. Biophys. 192 (1979) 225–234.
[17] D. Khananshvili, Z. Gromet-Elhanan, FEBS Lett. 159 (1983) 271–274.
[18] F. Guerrieri, F. Zanotti, Y.W. Che, R. Scarfo, S. Papa, Biochim. Biophys. Acta 892 (1987) 284–293.
[19] N. Morjana, G.A. Scarborough, Biochim. Biophys. Acta 985 (1989) 19–25.
[20] L.H. Gildensoph, D.P. Briskin, Plant Physiol. 94 (1990) 319–323.
[21] M. Grouselle, A.A. Thiam, J. Pudles, Eur. J. Biol. 39 (1973) 431–441.
[22] E.W. Miles, Methods Enzymol. 47 (1977) 431–442.
[23] R.L. Lundblad, C.M. Noyes, Chemical Reagents for Protein Modification, Vol. I, CRC Press, Boca Raton, FL, 1985, pp. 105–125.
[24] Y.R. Tsai, S.J. Yang, S.S. Jiang, S.J. Ko, S.S. Hung, S.Y. Kuo, R.L. Pan, J. Protein Chem. 17 (2) (1998) 161–172.
[25] E. Larson, B. Howlett, A.T. Jagendorf, Anal. Biochem. 155 (1986) 243–248.
[26] T. Gomi, M. Fujioka, Biochemistry 22 (1983) 137–143.
[27] E.T. Rakitzis, Biochem. J. 217 (1984) 341–351.
[28] C.M. Topham, Biochem. J. 227 (1985) 1025–1026.
[29] H.M. Levy, P.D. Leber, E.M. Ryan, J. Biol. Chem. 238 (1963) 3654–3659.
[30] J. Cousineau, E. Meighen, Biochemistry 15 (1976) 4992–5000.
[31] E.T. Rakitzis, J. Theor. Biol. 70 (1978) 461–465.
[32] K. Horiike, D.B. McCormick, J. Theor. Biol. 79 (1979) 403–414.
[33] R. Hori, H. Maegawa, M. Kato, T. Katsura, K. Inui, J. Biol. Chem. 264 (1989) 12232–12237.
[34] S. Tsutsumi, K. Denda, K. Yokoyama, T. Oshima, T. Daid, M. Yoshida, Biochim. Biophys. Acta 1098 (1991) 13–20.
[35] K. Ziegler, G. Hauska, N. Nelson, Biochim. Biophys. Acta 1230 (1995) 202–206.
[36] K. Puopolo, C. Kumamoto, I. Adach, M. Forgac, J. Biol. Chem. 266 (1991) 24564–24572.
[37] E.J. Bowman, K. Tenney, B.J. Bowman, J. Biol. Chem. 260 (1988) 12273–12279.
[38] B.J. Bowman, R. Allen, M.A. Wechser, E.J. Bowman, J. Biol. Chem. 263 (1988) 14002–14007.
[39] N. Nelson, L. Taiz, Trends Biochem. Sci. 14 (1989) 113–116.