

Effects of Multivalent Cations on Cell Wall-Associated Acid Phosphatase Activity

Received for publication December 21, 1987 and in revised form March 2, 1988

SHU-I TU*, JANINE N. BROUILLETTE, GERALD NAGAHASHI, AND THOMAS F. KUMOSINSKI
 United States Department of Agriculture, Agricultural Research Service, North Atlantic Area, Eastern
 Regional Research Center, 600 E. Mermaid Lane, Philadelphia, Pennsylvania 19118

ABSTRACT

Primary cell walls, free from cytoplasmic contamination were prepared from corn (*Zea mays* L.) roots and potato (*Solanum tuberosum*) tubers. After EDTA treatment, the bound acid phosphatase activities were measured in the presence of various multivalent cations. Under the conditions of minimized Donnan effect and at pH 4.2, the bound enzyme activity of potato tuber cell walls (PCW) was stimulated by Cu^{2+} , Mg^{2+} , Zn^{2+} , and Mn^{2+} ; unaffected by Ba^{2+} , Cd^{2+} , and Pb^{2+} ; and inhibited by Al^{3+} . The bound acid phosphatase of PCW was stimulated by a low concentration but inhibited by a higher concentration of Hg^{2+} . On the other hand, in the case of corn root cell walls (CCW), only inhibition of the bound acid phosphatase by Al^{3+} and Hg^{2+} was observed. Kinetic analyses revealed that PCW acid phosphatase exhibited a negative cooperativity under all employed experimental conditions except in the presence of Mg^{2+} . In contrast, CCW acid phosphatase showed no cooperative behavior. The presence of Ca^{2+} significantly reduced the effects of Hg^{2+} or Al^{3+} , but not Mg^{2+} , to the bound cell wall acid phosphatases. The salt solubilized (free) acid phosphatases from both PCW and CCW were not affected by the presence of tested cations except for Hg^{2+} or Al^{3+} which caused a Ca^{2+} -insensitive inhibition of the enzymes. The induced stimulation or inhibition of bound acid phosphatases was quantitatively related to cation binding in the cell wall structure.

Most plant cell walls form an extracellular network that is composed of cellulose fibers interconnected by the pectic-type polysaccharides (13). In addition to the carbohydrate components, the primary cell walls also contain several different types of proteins. One type of structural protein is the hydroxy proline-rich glycoproteins, which may be important for cell recognition and disease resistance (12). Functionally identifiable enzymes are also found in the primary cell walls (11) including peroxidases (9), glycosidases (11), and phosphatases (6). Presumably, these enzymes are important in cell wall metabolism, nutrient transport, recognition, and disease resistance.

Plant cell walls contain many ionizable groups. Thus, they may be regarded as immobilized polyelectrolytes. The ionic behavior of cell walls has been described by the well-known theoretical models of Donnan (17) or Gouy-Chapman (19). More recently (7), the classical Donnan theory and activity coefficient concept were used to describe ion-ion and ion-water interactions in cell walls. On the other hand, the relative adsorption of cations on isolated cell walls was shown to be accountable by mass-action expressions of ion exchange (2). A model, simultaneously taking electrostatic interactions and specific binding into consideration, was also developed for predicting ionic equilibrium concentrations in cell walls (18). These ionic properties may

significantly modify the activity of certain cell wall-bound enzymes.

It was reported that cell wall-bound acid phosphatase, but not the solubilized enzyme, is activated by increasing the ionic strength of the reaction mixture (15). The apparent activation may be attributed to a decrease of the Donnan potential, which can inhibit the movement of negatively charge substrate to the structure of cell walls. This explanation was later supported by the Ca^{2+} binding experiment at low ionic strength (5). The cell wall acid phosphatase, like other phosphatases, may be important in hydrolyzing and solubilizing organic soil phosphate-containing macromolecules independent of soil microbial activity (3). Wall bound acid phosphatases of different plant sources have been isolated (6, 20). The minimum mol wt of cell wall acid phosphatase is $\sim 100,000$ (6). However, detailed characterization of these isolated enzymes remains to be established.

A recent report from our laboratory (14) described a new and rapid procedure for the isolation of primary cell walls from potato tubers and corn roots. The isolated cell walls were free from the contamination of other subcellular organelles. It was reported that the pI values of extracted bound enzymes were all greater than 7.0 while the similar enzymes of cytosolic origin had pI values lower than 7.0. This result suggests the positive charges of cell wall enzymes may result in their binding to negatively charged wall structures. This suggestion is consistent with the finding that the majority of acid phosphatase is located in the pectin-rich region of sycamore cell walls (4). Since little information is available on the effects of metal ion binding on the cell wall-bound acid phosphatase activity under the condition of a minimum Donnan potential, we investigated the influence of different multivalent cations on both bound and salt-solubilized acid phosphatase activity associated with the primary cell walls of potato tubers and corn roots. The results were analyzed in terms of classical simple Michaelis-Menten kinetics and ion binding equilibria.

MATERIALS AND METHODS

Isolation of Plant Cell Walls. Corn seeds (*Zea mays* FBR 73, Illinois Foundation Seeds)¹ were germinated on filter paper saturated with 0.1 mM CaCl_2 in the dark at 28°C for 3 d as previously described (21). About 16 to 20 g fresh weight of cortex tissue stripped from stele of the primary roots were frozen with liquid nitrogen and pulverized with a mortar and pestle. The pulverized cortex was suspended in 500 mL of isolation medium containing 0.1 M Hepes-Mes (pH 7.8), 0.3 M sucrose, 5 mM 2-mercaptoethanol, 2 mM $\text{Na}_2\text{S}_2\text{O}_5$, and 5 mM sodium EDTA. The suspension

¹ Reference to brand or firm does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

was placed in a Parr Bomb under nitrogen pressure (1500 psi) for 10 min at 4°C before extrusion to atmospheric pressure. The cell wall material was twice washed with cold deionized water and trapped on a cheesecloth sieve. The cell walls were then suspended in 200 mL of fresh homogenization solution and sonicated with a Heat System 225 sonicator at 4°C with 50% pulsed cycle at power setting 7 for 7 min. After this treatment, the cell walls were washed extensively with a cold, aqueous solution of 1 mM 2-mercaptoethanol. For potato (*Solanum tuberosum*) tuber cell wall preparation, tubers were peeled and parenchyma tissue was processed as described previously (14). The homogenized tissue was then subjected to Parr bomb treatment and other further purification procedures identical to those applied for corn roots. Based on electron microscopic observation and marker enzyme analysis, the cell walls obtained were free from cytoplasmic contamination (14).

Salt Extracted of Cell Wall Acid Phosphatase Activity. The acid phosphatase activity of both CCW² and PCW was also partially solubilized by salt extraction method as described by Sugawara *et al.* (20) for potato tuber cell walls and Crasiner and Giordani (4) for sycamore cell walls. PCW and CCW obtained from 20 g tissues were incubated in 100 mL 1.5 M NaCl solution containing 1 mM 2-mercaptoethanol for 16 h at 4°C. The volumes of the protein solutions were reduced to ~8 mL by the use of an Amicon ultrafiltration cell (cut-off mol wt ~10,000). The concentrated protein solutions were then dialyzed against ice-cold H₂O (36 h) to remove residual 2-mercaptoethanol.

EDTA Treatment of Isolated Cell Walls. Cell walls isolated from corn roots and potato tubers were suspended in 100 mL of a solution containing 50 mM EDTA, pH 7.5 and were constantly stirred for 1.5 h at 4°C. After this treatment, the cell walls were repeatedly washed with cold deionized water (resistivity >10 megohm) by suspension and filtration. The treated cell walls were finally resuspended in 50 mM Na-acetate, pH 4.2. This procedure effectively removed most of the divalent cations bound to corn root cell walls. For example, the calcium contents of corn root cell walls used in this study were typically 0.134% (33.5 nmol Ca²⁺/mg cell walls) and 0.019% (4.75 nmol Ca²⁺/mg cell walls) by weight before and after the EDTA treatment, respectively. The potato cell walls contained only small amounts of Ca²⁺ (0.020% by weight, 5.0 nmol/mg cell walls) and further EDTA treatment did not significantly alter this Ca²⁺ content. For calcium content determination, cell walls (~20 mg) were first dried in a crucible and then completely oxidized by flame. After dissolving the residue in the crucible with 5 mL of deionized water, the calcium concentration was determined with a radiometer model F2002 calcium-ion electrode. We observed that the cell wall samples developed a deep yellow color in alkaline medium. To prevent possible permanent changes of the samples, pH 7.5 was chosen as a compromise for EDTA treatment.

Measurement of Acid Phosphatase Activity. The acid phosphatase activity of cell walls was measured by hydrolysis of PNP-P. An appropriate amount (~0.1 mg) of cell walls was suspended in 1 mL of a solution containing 50 mM Na-acetate (pH 4.2) and various concentrations of sulfates (Cu²⁺, Hg²⁺, Mg²⁺, Al³⁺) or chlorides (Zn²⁺, Mn²⁺, Ba²⁺, Cd²⁺, Ca²⁺, Pb²⁺) of different di- or trivalent cations. After an incubation for 10 min at 22°C, PNP-P (4 mM, unless indicated otherwise) was added to start the reaction. After 10 min, the reaction was stopped by the addition of 1 mL of 1.0 M Na₂CO₃. This addition changed the pH of the solution from 4.2 to higher than 10. The amount of PNP produced was determined by its absorbance at 405 nm

(extinction coefficient ϵ at 405 nm = 8.71 mM⁻¹cm⁻¹). The hydrolysis rate was linear within 10 min under the employed conditions. Furthermore, the nonenzymic hydrolysis and the enzymic hydrolysis in basic medium (adding Na₂CO₃ prior to PNP-P) over the period of 10 min was negligible. The solubilized acid phosphatase activity was determined in a similar manner.

Calculation of the Binding Related to Activity Change. The theoretical details of the analysis of cation binding that affects the enzyme activity are discussed in "Results." For example, in the calculation of the parameters in the equation:

$$A = \frac{A_1}{1 + K_a [M^n]} + \frac{CA_1 K_a [M^n]^n}{1 + K_a [M^n]^n}$$

n was first assigned an integral of 1. From input data of A , A_1 , C , and $[M]$, the value of K_a was estimated by a Gauss-Newton nonlinear regression program developed in our research center. In the regression process, the K_a value was varied until a minimum of an overall root-mean-square deviation between observed and calculated activity was reached. The value of n was then increased by 1, and the nonlinear regression was repeated. The n value chosen to describe the function-linked binding was the one which gave the lowest root-mean-square deviation for the fit and minimum error in K_a .

RESULTS

Sensitivity of Bound Acid Phosphatase to Multivalent Cations.

The activity of plant cell wall acid phosphatase has been shown to be modulated by the binding of Ca²⁺ and the ionic strength of the bulk solution (5, 15). Since cell walls are known to contain a high percentage of negatively charged residues, the observed modulation is attributable to an electrostatic interaction between cell walls and the negatively charged enzyme substrate (PNP-P). Whether the effect of Ca²⁺ is unique or not has not been systematically investigated. In order to gain certain insights into this question, the effects of multivalent cations, such as Ca²⁺, that may show comparable interaction with the immobilized negative charges of cell walls were determined on PCW-bound and CCW-bound acid phosphatases.

As shown in Table I, under the condition of nearly constant ionic strength, the addition of various cations produced different effects on bound acid phosphatase activity. In the case of CCW-bound acid phosphatase, with the exception of Al³⁺ or Hg²⁺, all tested cations including Ca²⁺ appear to have had negligible effect. In contrast, the PCW-bound acid phosphatase was strongly stimulated (>100% increase in activity) by the presence of 5 mM of Cu²⁺, Mg²⁺, Zn²⁺ and Mn²⁺. The rest of tested cations (Ba²⁺, Cd²⁺, Ca²⁺, Pb²⁺) showed weak or no stimulatory effects to PCW acid phosphatase. For both PCW and CCW, the bound enzyme was inhibited by Al³⁺ and Hg²⁺.

Concentration Dependence Multivalent Cation Effects. The results shown in Table I clearly demonstrate that depending on the source of cell walls, the tested cations may be either stimulatory, inhibitory, or have no effect on acid phosphatase activity. The electrostatic interaction model, which emphasizes the effect of increasing the concentration of PNP-P near the catalytic site of the enzyme by neutralization of cell wall negative charges with cation binding, has been suggested as the origin of Ca²⁺ modulation of acid phosphatase in sycamore (15) and cultured soy bean cells (5). It appears that this model may not be adequate to account for the observed effects mentioned in Table I.

In order to gain further insight, a more detailed study of the cation concentration dependence of the enzyme activity was performed. Because of their physiological or toxicological importance, we chose Mg²⁺, Hg²⁺, and Al³⁺ for the study. By

² Abbreviations: PCW, cell walls of potato tubers; CCW, cell walls of corn root cells; C-APase, solubilized acid phosphatase from CCW; P-APase, solubilized acid phosphatase from PCW; PNP-P, p-nitrophenyl phosphate; pI, isoelectric point.

CATION BINDING AFFECTS ACID PHOSPHATASE IN CELL WALLS

Table I. *Effect of Multivalent Cations on Cell Wall-Bound Acid Phosphatase Activity*

The acid phosphatase activity of EDTA-Treated CCW and PCW was determined as mentioned in "Material and Methods" and expressed as $\mu\text{mol Pi}$ released per mg of dry cell walls per h. The values shown represent an average of three determinations with an error as $\pm 10\%$.

Cation ^a (5 mM concn)	Corn Root Cell Wall		Potato Tuber Cell Wall	
	Activity	Relative activity	Activity	Relative activity
	$\mu\text{mol Pi/mg}\cdot\text{h}$		$\mu\text{mole Pi/mg}\cdot\text{h}$	
None	0.217	100.0	0.054	100.0
Cu ²⁺	0.225	103.3	0.256	475.1
Mg ²⁺	0.206	94.7	0.222	411.0
Zn ²⁺	0.220	101.3	0.190	351.6
Mn ²⁺	0.209	96.0	0.148	274.4
Ba ²⁺	0.216	99.3	0.075	139.7
Cd ²⁺	0.215	98.7	0.065	119.9
Ca ²⁺	0.213	98.0	0.062	114.8
Pb ²⁺	0.222	102.0	0.055	102.0
Al ³⁺	0.099	45.4	0.025	46.9
Hg ²⁺	0.023	10.7	0.000	0.0

^a In addition to 50 mM Na-acetate, salts of indicated cations were also added.

keeping the substrate concentration at a constant level (4 mM), the effect of increasing cation concentration on the bound acid phosphatases was determined. The results are summarized in Figures 1 and 2. It should be mentioned that the results shown are not due to the variation of ionic strength (I) of the solution. The addition of salts of multivalent cations at most changed the ionic strength of the solution from 0.03 (50 mM Na-acetate at pH 4.2) to 0.045 (50 mM Na-acetate + 2 mM Al₂(SO₄)₃). The bound acid phosphatase activity of both PCW and CCW observed in 50 mM Na-acetate (I = 0.03) was essentially the same as that measured in 50 mM Na-acetate + 25 mM KCl (I = 0.055). It appears that the effects of Donnan potential generated by the polyanionic PCW and CCW may have been sufficiently minimized by 50 mM Na-acetate at pH 4.2.

The data in Figure 1 indicate that the bound acid phosphatase of PCW reached a 50% maximum stimulation in the presence of 0.3 mM Mg²⁺. The PCW-bound acid phosphatase activity was first stimulated and then inhibited by Hg²⁺ or Al³⁺. However, the stimulation caused by Hg²⁺ or Al³⁺ at low concentration levels was not observed for CCW-bound acid phosphatase (Fig. 2). These results suggest that there are two, rather than one, types of function-linked binding processes for Hg²⁺ and Al³⁺ in PCW.

Effect of Multivalent Cations on Enzyme Kinetics. Free acid phosphatase isolated from the cell walls of sycamore cells obeys simple Michaelis-Menten kinetics (5). But, in bound form, the enzyme exhibits a negative cooperativity which can be eliminated by increasing the ionic strength (≥ 0.1) of the solution.

The kinetic patterns of PCW-bound and CCW-bound acid phosphatases observed under our experimental conditions are shown in Figures 3 and 4. For PCW-bound acid phosphatase (Fig. 3), in the absence of added multivalent cations, the 1/V versus 1/S plot reveals a negative cooperativity. The addition of Ca²⁺ did not change this kinetic response. An increase of Hg²⁺ concentration, not only substantially inhibited the enzyme activity but also enhanced the apparent negative cooperativity (data not shown). On the other hand, the presence of Mg²⁺ caused PCW-bound acid phosphatase to obey the simple Michaelis-Menten scheme. For CCW-bound acid phosphatase (Fig. 4), it appears that the enzymic reaction followed the Michaelis-Menten kinetics under all the conditions tested. The molecular basis for the difference between PCW acid phosphatase and CCW acid phosphatase remains to be determined. The determined K_m and V_{max} for the enzymes are listed in Table II.

Combined Effects of Multivalent Cations. When the bound

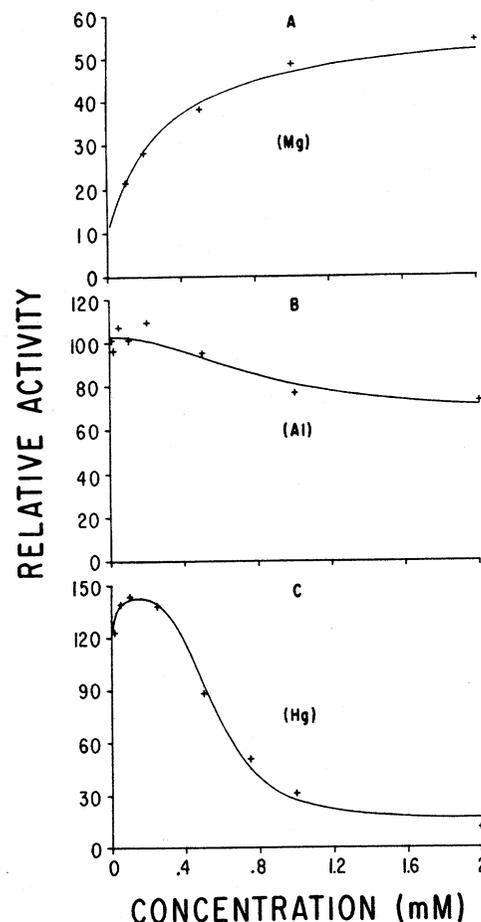


FIG. 1. Effects of multivalent cations on the acid phosphatase activity of PCW. The enzyme activity was measured in the solution mentioned in "Material and Methods" with the addition of various concentrations of indicated cations. The acid phosphatase activity obtained, in the absence of added cations, was assigned as 10 in (A) and as 100 in (B) and (C). The solid lines represent the best fit obtained by function-linked binding analysis.

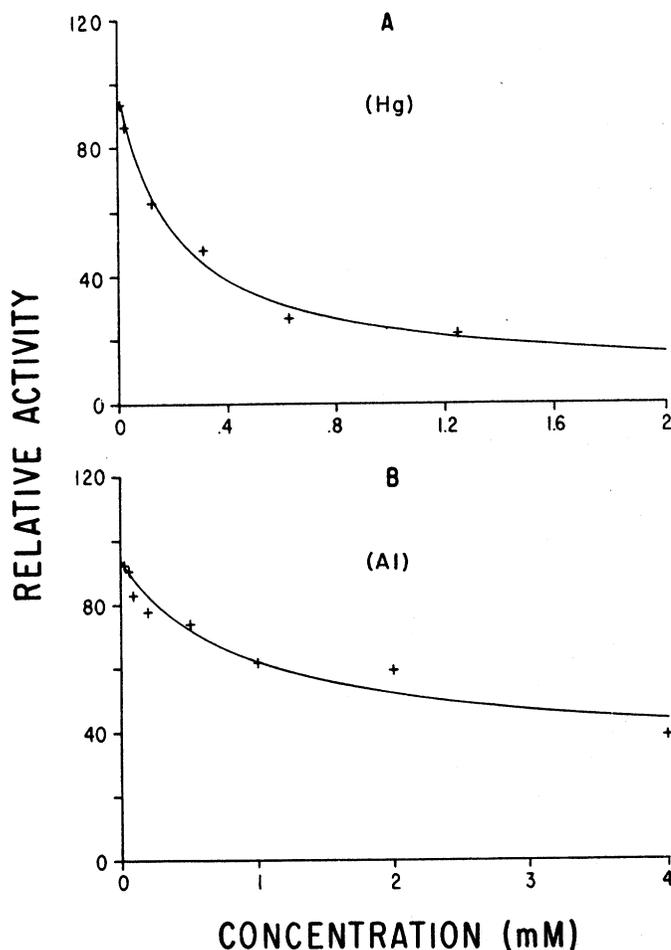


FIG. 2. Effects of multivalent cations on the acid phosphatase activity of CCW. The enzyme activity, in the presence of various concentration of indicated cations, was measured as described in Figure 1. The acid phosphatase activity (basal) obtained in the absence of added cations, was assigned as 100. The addition of MgSO_4 up to 5 mM did not cause any significant change of the basal activity. The solid lines represent the best fits obtained by function-linked binding analysis.

acid phosphatase activity was determined in the presence of two different multivalent cations, the combined effects could be determined. As shown in Table III, we observed that the inhibitory efficiency of Hg^{2+} or Al^{3+} to the bound acid phosphatase was not significantly affected by the presence of Mg^{2+} . On the other hand, the presence of Ca^{2+} substantially decreased the inhibitory power of Hg^{2+} and Al^{3+} . Figure 5 clearly shows that the inhibition efficiency of Hg^{2+} and Al^{3+} decreased as the concentration of Ca^{2+} increased. In terms of enzyme kinetics, the presence of the two different cations ($\text{Mg}^{2+} + \text{Ca}^{2+}$, $\text{Mg}^{2+} + \text{Hg}^{2+}$, $\text{Hg}^{2+} + \text{Ca}^{2+}$) did not alter the linear response ($1/V$ versus $1/S$) of CCW-bound acid phosphatase. However, for PCW-bound acid phosphatase, the linear response obtained in the presence of Mg^{2+} (Fig. 3B) was changed to a negative-cooperativity pattern by the addition of Hg^{2+} but not Ca^{2+} .

These results seem to suggest several possibilities: (a) Mg^{2+} and Ca^{2+} probably do not share the same binding sites; (b) Ca^{2+} binding may affect the interaction of Hg^{2+} and/or Al^{3+} with the enzymes; and (c) only some Hg^{2+} and/or Al^{3+} binding site(s) may be common for Mg^{2+} or alternatively, Mg^{2+} does not share any site(s) with Hg^{2+} but the effect of Hg^{2+} binding overrides Mg^{2+} stimulation. The exact origin of observed combined effects remains to be established.

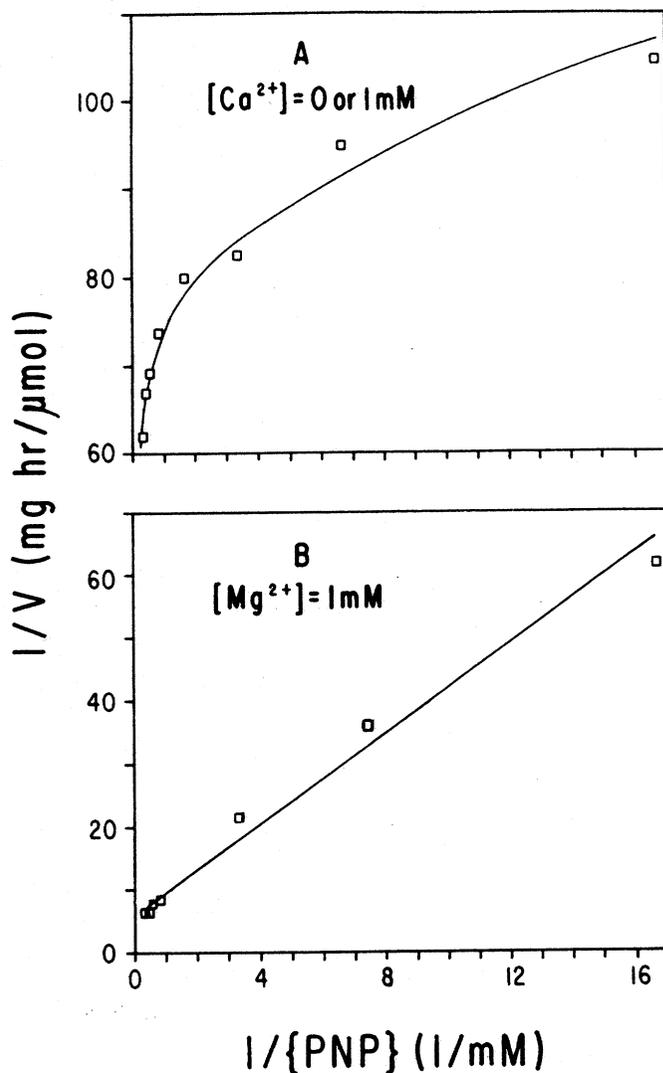


FIG. 3. $1/V$ versus $1/S$ plots of PCW acid phosphatase activity. The acid phosphatase activity of PCW was measured in the presence of various concentrations of PNP. (A) In the absence of any added cations or 1 mM Ca^{2+} , negative cooperativity was observed. The presence of Hg^{2+} (>0.5 mM) significantly inhibited the enzyme activity and also enhanced the apparent negative cooperativity. (B) In the presence of 1.0 mM Mg^{2+} , linear relationship between $1/V$ and $1/S$ was observed.

Effects of Multivalent Cations on Solubilized Acid Phosphatase Activity. The acid phosphatase activity associated with plant cell walls may be partially solubilized by salt treatment (4, 14, 20). When CCW was treated with 1.5 M NaCl, approximately 15% of the total acid phosphatase activity was released to the solution. On the other hand, similar treatment to PCW released ~50% of acid phosphatase activity. The sensitivity of solubilized acid phosphatase activity to the presence of multivalent cations was tested, and the results are summarized in Table IV. For convenience, we defined the solubilized activity obtained from CCW and PCW as C-APase and P-APase, respectively. For C-APase, the presence of cations yielded effects similar to those observed for bound acid phosphatase of CCW (see Tables I and III). However, unlike the bound acid phosphatase of CCW, Ca^{2+} failed to relieve the inhibitory effect of either Hg^{2+} or Al^{3+} on the solubilized enzyme activity. The effects of the cations on P-APase activity, as shown in Table IV, were quite different from those of bound acid phosphatase of PCW. A comparison with the data described in Tables I and III, indicated that none of the

CATON BINDING AFFECTS ACID PHOSPHATASE IN CELL WALLS

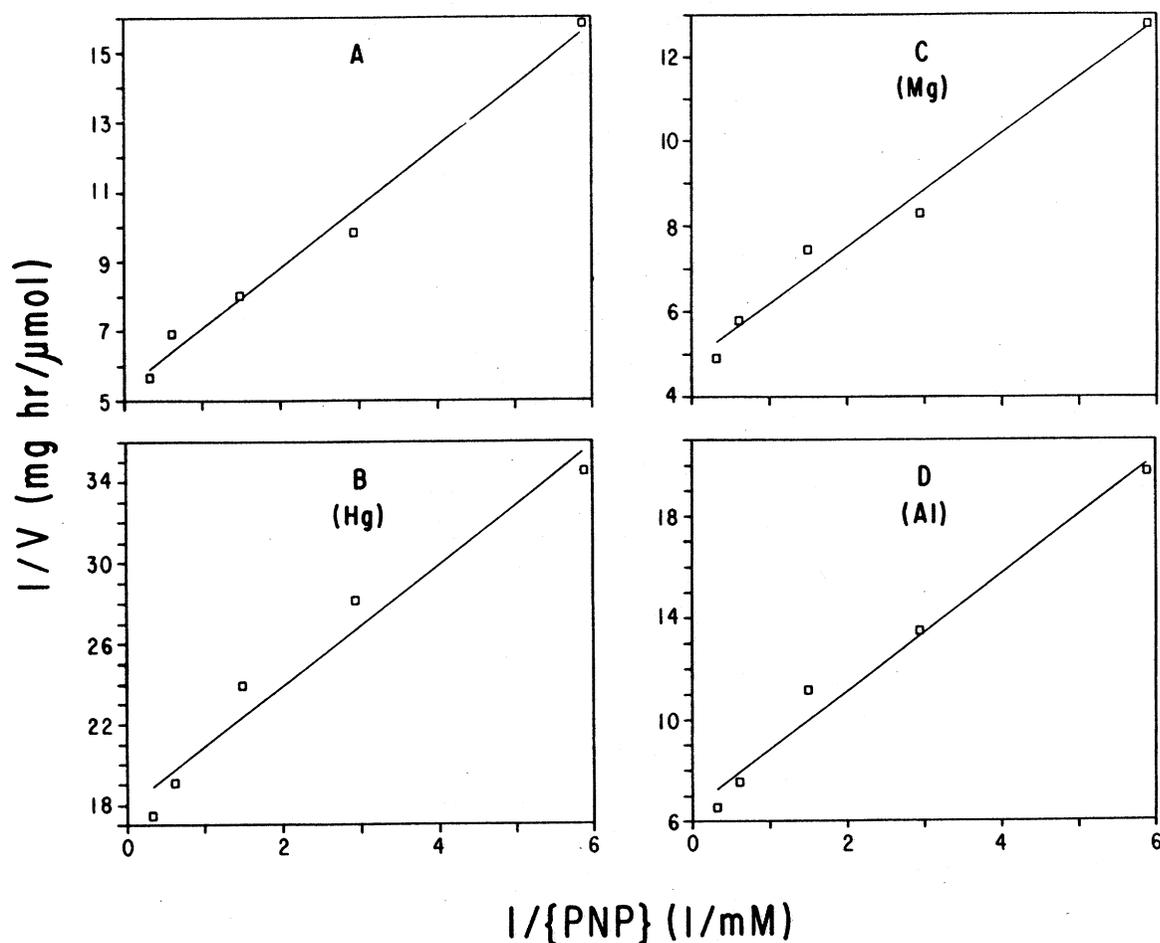


FIG. 4. The $1/V$ versus $1/S$ plots of CCW acid phosphatase activity. The CCW acid phosphatase activity, as a function of the substrate concentration, was determined in the presence (A) of zero or (B, C, D) 1 mM indicated cations.

Table II. Kinetic Parameters of Cell Wall-Bound Acid Phosphatase

Data shown in Figure 3 and 4 were analyzed according to simple Michaelis-Menten kinetic scheme.

Sample	K_m	V_{max}
	mM	μ mol/mg·h
CCW	0.33	0.190
CCW and Mg ²⁺ ^a	0.27	0.210
CCW and Ca ²⁺	0.18	0.160
CCW and Al ³⁺	0.35	0.150
CCW and Hg ²⁺	0.17	0.056
PCW	Negative cooperativity	
PCW and Mg ²⁺	0.45	0.123

^a All cations added at the rate of 1 mM.

tested cations could stimulate the activity of P-APase. Furthermore, the presence of Ca²⁺ provided no protection against the inhibition caused by Hg²⁺ and Al³⁺. These results strongly suggest that the structures of CCW and PCW can regulate the responses of bound acid phosphatases to the presence of multivalent cations. The specific activities of the bound acid phosphatases of PCW and CCW when expressed in terms of protein content, as listed in Tables I and III, would be in the same general ranges of solubilized enzymes since the protein accounts for about 10% of the dry weight of plant cell walls (16).

Possible Origin of Hg²⁺ Effects. The fact that both the bound and the free acid phosphatases were inhibited by Hg²⁺ indicated a direct interaction between the cation and the enzymes. It is

Table III. Combined Effects of Metal Ions on Bound Acid Phosphatase Activity

Experiment	Acid Phosphatase Activity
	%
<i>Corn Root Cell Wall</i>	
Control (no cations) ^a	100
Hg ²⁺ and Mg ²⁺ ^b	1-4 ^c
Hg ²⁺ and Ca ²⁺	35
Hg ²⁺	11
Al ³⁺ and Mg ²⁺	37
Al ³⁺ and Ca ²⁺	48
Al ³⁺	37
<i>Potato Tuber Cell Wall</i>	
Control (no cations)	100
2 mM Hg ²⁺ and 2 mM Mg ²⁺	8
1.25 mM Hg ²⁺ and 1.25 mM Ca ²⁺	95

^a No di- or trivalent cations were added to the basic assaying solutions. ^b In CCW, all cations added at a rate of 5 mM. ^c The values represent an average of three determinations with an error as $\pm 5\%$.

well known that Hg²⁺ reacts readily with protein —SH groups to form complexes. The observation that Ca²⁺ protects only the bound acid phosphatases suggests the accessibility of the —SH group is controlled by binding of Ca in cell wall structure. The uniqueness of this —SH group was also supported by the inability of *N*-ethylmaleimide, a powerful alkylation reagent for —SH

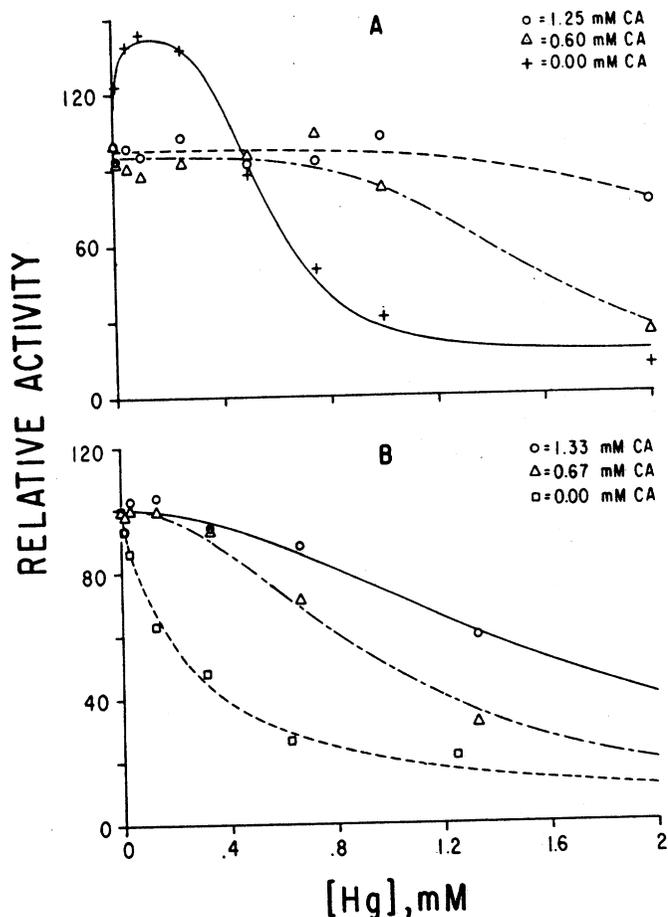


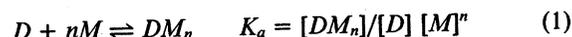
FIG. 5. Effects of Ca^{2+} on Hg^{2+} -inhibition. The acid phosphatase activity was measured in the presence of various amounts of Hg^{2+} but keeping Ca^{2+} concentration at indicated levels. The activity obtained in the absence of Hg^{2+} that was not affected by the concentration of Ca^{2+} was assigned as 100. The relationships obtained in PCW and CCW are shown in (A) and (B), respectively. The best fit lines, based on function-linked analysis, are also shown.

group, to inhibit the bound acid phosphatase of PCW and CCW. An incubation of the cell walls with 0.8 mM *N*-ethylmaleimide for 2 h at 22°C and pH 4.2, failed to induce any observable activity change of the bound enzymes. Under similar conditions, the presence of 0.8 mM Hg^{2+} substantially decreased the enzyme activity (Figs. 1 and 2).

Cation Binding Induced Activity Change. To quantitatively

express the effects of metal ion binding to bound acid phosphatase, the simple Michaelis-Menten kinetic analysis employed in Figure 2 and Table II would be of limited value. For example, in order to obtain the inhibition constant, K_i of Hg^{2+} for CCW-bound acid phosphatase, the linear transformation of the conventional enzyme kinetic method requires substantially more experimental information for a reasonable estimation. However, this information may be obtained by the use of the thermodynamic linkage concept (22) in combination with nonlinear Gauss-Newton data analysis of the experimental results. We assumed that the observed functional change of the acid phosphatase activity was due to a certain rather unique binding that may constitute only part of the total binding of tested cations to the components of cell walls. In order to simplify the analysis we further assumed that the molecules of acid phosphatase were uniformly distributed among many equivalent subdomains in the structure of cell walls. Certain binding of n , and only n , metal ions to the subdomain (either to the enzyme directly or to its immediate environment, or both) would cause a concentration dependent change in the activity of the enzyme.

For the simplest case, consider the following binding equilibrium between the subdomain (D) containing the enzyme and metal ions (M):



in which DM_n and D represent the domains with and without functionally effective (stimulation or inhibition) metal ion binding, respectively. K_a is the association equilibrium constant. By keeping the substrate concentration (4 mM PNP-P) in large excess over the K_M values (see Table II), the observed apparent enzyme activity, A , in the presence of tested cations, as determined by the hydrolysis of PNP-P is then:

$$A = (A_1 [D] + A_2 [DM_n])/([D] + [DM_n]) \quad (2)$$

in which A_1 and A_2 are activity of D and DM_n , respectively. A_2 may be either greater or smaller than A_1 . Or:

$$A_2 = CA_1, \quad (3)$$

C is greater than or smaller than 1 for stimulation or inhibition of the activity, respectively. The values of A_1 and C can be easily estimated from the data shown in Figures 1 and 2. Equation 2 may be reduced to:

$$A = \frac{A_1}{1 + K_a [M]^n} + \frac{CA_1 \cdot K_a [M]^n}{1 + K_a [M]^n} \quad (4)$$

Plant cell walls contain relatively small amount of protein. It has been shown that proteins constitute about 10% of the total dry weight of cell walls (16). The majority of cell wall protein is in the form of hydroxyproline-rich glycoproteins, which are

Table IV. Effects of Multivalent Cations on Solubilized Acid Phosphatase

The activity of solubilized acid phosphatase was determined by the same procedure as mentioned in Table I and is expressed as $\mu\text{mol Pi}$ released per mg of total solubilized protein per h. The values represent an average of two determinations with an error as $\pm 5\%$.

Cations	P-APase	Relative Activity	C-APase	Relative Activity
	$\mu\text{mol Pi}/\text{mg}\cdot\text{h}$	%	$\mu\text{mol Pi}/\text{mg}\cdot\text{h}$	%
None	4.64	100	6.88	100
Ca^{2+a}	3.71	80	6.67	97
Mg^{2+}	3.94	85	6.40	93
Hg^{2+}	0.28	6	1.51	22
Mn^{2+}	4.96	107		
$\text{Mg}^{2+} + \text{Hg}^{2+}$	0.32	7	1.72	25
$\text{Ca}^{2+} + \text{Hg}^{2+}$	0.28	6	1.31	19

^a All cations added at the rate of 2 mM.

CATION BINDING AFFECTS ACID PHOSPHATASE IN CELL WALLS

characteristic structural components of cell walls of higher plants (2). The acid phosphatase isolated from various cell wall preparations (6, 20) has a mol wt of approximately 100,000. Thus, even if we assume all the protein in PCW and CCW is in the form of acid phosphatase, the maximum enzyme content would be 1.0 nmol/mg cell wall (dry weight). Since the maximum activity related binding of tested cations as shown by Equation 1 would be in the same order of magnitude as the enzyme content, the concentration term $[M]$ in Equation 4 may be approximated by the total concentration of M added. With the quantities A , A_1 , C , and $[M]$ as known, the application of a nonlinear Gauss-Newton regression of the data shown in Figure 1 is possible. The results of this analysis are summarized in Table V. In most cases, the best fittings were obtained by the use of Equation 4. However, for the activity related Hg^{2+} -binding to PCW, this equation was obviously insufficient. The activity vs Hg^{2+} concentration dependence shown in Figure 1 indicates that the binding of Hg^{2+} at low concentration levels stimulated the activity, and at higher concentration of Hg^{2+} , inhibition became predominant. A simple model to account for this observation is to assume that there are two independent, activity-related binding sites of Hg^{2+} that may occur in the subdomains of PCW. The binding involved may be represented as:



The observed activity, A , can then be expressed as

$$A = \frac{A_1}{Z} + \frac{CA_1 K_n [M]^n}{Z} + \frac{C'A_1 (K_n [M]^n + K_n K_m [M]^{m+n})}{Z}, \quad (5)$$

where

$$Z = 1 + K_n [M]^n + K_m [M]^m + K_n K_m [M]^{m+n},$$

in which C and C' are greater and smaller than 1.0, respectively. While Equation 5 appears to be complex, the actual data fitting turns out to be rather simple because the term of $[M]^{m+n}$ has a relatively negligible contribution. Using this model, the data were best fitted with $n = 1$ and $m = 4$. On the other hand, a sequential binding model which specifies the binding of n occurring before m did not provide a satisfactory fitting of the obtained results. It should be mentioned that other possible models could not be

excluded by this analysis. However, it is quite possible that the presence of calcium not only abolishes the stimulation of low level Hg^{2+} but also substantially decreases the inhibitory binding of Hg^{2+} . Presumably, Ca^{2+} can bind to the functional domain more easily than Hg^{2+} , or the acid phosphatase is more sensitive to Ca^{2+} binding.

DISCUSSION

Although the physiological roles of plant cell wall-associated phosphatase activity remains to be firmly established, in root tissue the enzyme may function in hydrolyzing and mobilizing phosphorus from organic compounds in the soil for plant nutrition (3). This view is supported by the observation of an elevated root cell wall phosphatase activity under phosphate deficiency conditions (1). In the structure of cell walls, the acid phosphatase activity has been found to be concentrated on the negatively charged exterior surface of the cell wall (4). Thus, it is expected that the acid phosphatase activity may be modified by the electrical status of cell walls (3). In the present study, we demonstrated that the bound acid phosphatase activity, but not the solubilized forms, can be further affected by the binding of multivalent cations to the cell wall structure. While it is not possible to pinpoint the exact activity-related binding site(s) from the experimental results, our study of the salt-extracted protein fraction of PCW and CCW indicated that at least Hg^{2+} and Al^{3+} may interact directly to the acid phosphatase. Furthermore, the protective Ca-binding is most likely associated with the environment of the enzyme in the cell wall structure.

It is interesting to note that the responses of PCW-bound acid phosphatase to Mg^{2+} and Hg^{2+} are different from those of CCW-bound acid phosphatase. Although monocot (*e.g.* corn) and dicot (*e.g.* potato) cell walls differ in composition, *e.g.* pectic content (8), the exact molecular origin of this observed difference remains to be established. However, this observation suggests that the structure of either the acid phosphatase, or the enzyme-containing subdomain, or both of PCW is different from that of CCW.

Because of the relatively high content of negative charges, plant cell walls can function as efficient cation exchangers (2, 7). Based on electron spin resonance measurement of Mn^{2+} binding, it was estimated that cell walls of cortical tissues of apple fruit contain ~500 nmol Mn^{2+} binding sites per mg of dry weight of cell walls (10). Although the exact total binding capacity of divalent cations was not determined for PCW and CCW in this study, it is reasonable to assume that the cell walls contain considerably more multivalent cation binding sites than acid phosphatase. The activity-related binding may only represent a

Table V. Cation Binding Parameters Determined from Activity Change

Activity related metal ion binding was analyzed as described in text using data shown in Figures 1, 2, and 5.

Experiment	K (Association) ^a	n^a	RMS (\pm) ^b	Effect
			%	
PCW and Mg	$3.78 \text{ mM}^{-1} \pm 18\%$	1	15	Stimulation
PCW and Al	$1.40 \text{ mM}^{-1} \pm 41\%$	2	4	Inhibition
PCW Hg^c	$108.4 \text{ mM}^{-1} \pm 58\%$	1	8	Stimulation
	$1.38 \times 10^4 \text{ mM}^{-4} \pm 10\%$	4	3	Inhibition
PCW and Hg (0.6 mM Ca)	$6.55 \times 10^{-4} \text{ mM}^{-4} \pm 8\%$	4	6	Inhibition
PCW and Hg (1.2 mM Ca)	$1.60 \times 10^{-7} \text{ mM}^{-4} \pm 7\%$	4	4	Inhibition
CCW and Hg	$4.82 \text{ mM}^{-1} \pm 18\%$	1	2	Inhibition
CCW and Hg (0.7 mM Ca)	$1.06 \text{ mM}^{-2} \pm 5\%$	2	2	Inhibition
CCW and Hg (1.3 mM Ca)	$0.13 \text{ mM}^{-2} \pm 8\%$	2	3	Inhibition
CCW and Al (5.0 mM Mg)	$1.08 \text{ mM}^{-1} \pm 51\%$	1	4	Inhibition

^a In the case of only set of K and n , best fitting was obtained with Equation 1. ^b RMS refers to the overall deviation between observed and calculated values of A (activity). ^c For the effect of Hg on PCW acid phosphatase activity, Equation 5 was used.

small fraction of the total binding. Thus, the binding constants listed in Table V should not be confused with the binding determined by either equilibrium dialysis or electrochemical activity analysis. Nevertheless, the employed analysis provides a convenient quantitative measurement on the effect of metal ion binding to the function of an enzyme in a complex physicochemical environment, such as the cell wall of plants.

LITERATURE CITED

1. BIELESKI RL 1974 Development of an externally-located alkaline phosphatase as a response to phosphorus deficiency. In AR Ferguson, MM Gresswell, eds, Mechanism of Regulation of Plant Growth. R Soc NZ Bull, pp 165-170
2. BUSH DS, JG McCOLL 1987 Mass-action expressions of ion exchange applied to Ca^{2+} , H^+ , K^+ , and Mg^{2+} sorption on isolated cell walls of leaves from brassica oleracea. Plant Physiol 85: 247-260
3. CHANG CW, RS BANDURSKI 1964 Exocellular enzymes of corn roots. Plant Physiol 39: 60-64
4. CRASINER M, R GIORDANI 1985 Elution of acid phosphatase from sycamore cell walls. Plant Sci 40: 35-41
5. CRASINER M, A-M MOUSTACAS, J RICARD 1985 Electrostatic effects and calcium ion concentration as modulators of acid phosphatase bound to plant cell walls. Eur J Biochem 151: 187-190
6. CRASINER M, G NOAT, J RICARD 1980 Purification and molecular properties of acid phosphatase from sycamore cell walls. Plant Cell Environ 3: 217-224
7. DEMARTY M, C MORVAN, M THELLIER 1978 Exchange properties of isolated cell walls of *Lemna minor* L. Plant Physiol 62: 477-481
8. DEY PM, K BRINSON 1984 Plant cell-walls. Adv Carbohydr Chem Biochem 42: 265-382
9. FRY SC 1979 Phenolic components of primary cell wall and their possible role in the hormonal regulation of growth. Planta 146: 343-351
10. IRWIN PL, MD SEVILLA, CL STOUTD 1985 ESR spectroscopic evidence for hydration- and temperature-dependent spatial perturbations of a higher plant cell wall paramagnetic ion lattice. Biochem Biophys Acta 842: 76-83
11. LAMPORT DTA 1970 Cell wall metabolism. Annu Rev Plant Physiol 21: 235-270
12. LAMPORT DTA, JW CATT 1981 Glycoproteins and enzymes of cell wall. In W Tanner, F Loewus, eds, Encyclopedia of Plant Physiology, New Series, Vol 13B. Springer-Verlag, Berlin, pp 133-165
13. MCNEIL M, AG DARVILL, P ALBERSHEIM 1979 The structural polymers of the primary cell walls of dicots. Prog Chem Org Nat Prod 37: 191-249
14. NAGAHASHI G, TS SEIBLES 1986 Purification of plant cell walls: isoelectric focusing of CaCl_2 extracted enzymes. Protoplasma 134: 102-110
15. NOAT G, M CRASINER, J RICARD 1980 Ionic control of acid phosphatase activity in plant cell wall. Plant Cell Environ 3:225-229
16. O'NEILL MA, K ROBERTS 1981 Methylation analysis of cell wall glycoproteins and glycopeptides from *Chlamydomonas reinhardtii*. Phytochemistry 20: 25-28
17. PITMAN M, U LÜTTGE, D KRAMER, E BALL 1974 Free space characteristics of barley leaf slices. Aust J Plant Physiol 1: 65-75
18. SENTENAC H, C GRIGNON 1981 A model predicting ionic equilibrium concentrations in cell walls. Plant Physiol 68: 415-419
19. SHONE MGT 1966 The initial uptake of ions by barley roots. J Exp Bot 17: 89-95
20. SUGAWARA S, Y INAMOTO, M USHIJIMA 1981 Resolution and some properties of acid phosphatase isoenzymes bound to the cell wall of potato tubers. Agric Biol Chem 45: 1767-1773
21. TU S-I, JN BROUILLETTE 1987 Metal ion inhibition of corn root plasma membrane ATPase. Phytochemistry 26: 65-69
22. WYMAN J 1964 Linked functions and reciprocal effects in hemoglobin: a second look. Adv Protein Chem 19: 223-286