Biochem. J. (1991) 279, 351–354 (Printed in Great Britain)

Pectin methylesterase, metal ions and plant cell-wall extension

The role of metal ions in plant cell-wall extension

Anne-Marie MOUSTACAS, Joannès NARI, Maguy BOREL, Georges NOAT and Jacques RICARD*
Centre de Biochimie et de Biologie Moléculaire du C.N.R.S., B.P. 71, 13402 Marseille Cédex 9, France

The study of pectin methylesterase and wall-loosening enzyme activities *in situ*, as well as the estimation of the electrostatic potential of the cell wall, suggest a coherent picture of the role played by metal ions and pH in cell-wall extension. Cell-wall growth brings about a decrease of local proton concentration because the electrostatic potential difference ($\Delta\psi$) of the wall decreases. This in turn activates pectin methylesterase, which restores the initial $\Delta\psi$ value. This process is amplified by the attraction of metal ions in the polyanionic cell-wall matrix. The amplification process is basically due to the release of enzyme molecules that were initially bound to 'blocks' of carboxy groups. This increase of metal-ion concentration also results in the activation of wall-loosening enzymes. Moreover, the apparent 'inhibition' of pectin methylesterase by high salt concentrations may be considered as a device which prevents the electrostatic potential from becoming too high.

INTRODUCTION

Clumps of soybean (*Glycine max*) cells in sterile culture represent a good system for studying cell-wall elongation and building up [1,2]. When transferred to a fresh culture medium, the cells first elongate and then divide in a more or less synchronous way. Pectin methylesterase demethylates cell-wall pectins [3,4] and is thus involved in the building up of the electrostatic potential difference, $\Delta\psi$, between the inside and the outside of the wall [1,2].

Previous studies have shown that the enzyme in free solution has a rather alkaline optimum pH and requires cations in order to be active [1]. At fixed pectin concentration, however, the free enzyme is inhibited by an excess of metal ions. We have shown in the preceding paper [5] that these complex effects are due to an interaction of metal ions not with the enzyme, but rather with pectin. As changes of $\Delta\psi$ result in changes of local cation concentration, which may in turn activate or inhibit the enzyme bound to pectin, one may speculate about the existence of feedback loops that control pectin methylesterase activity through pH changes and that involve the concentration of metal ions as well as the $\Delta\psi$ between the inside and the outside of the cell wall.

So far the regulation of the activity of pectin methylesterase in plant cell walls was mostly considered to result from the changes of local pH [1,2,6]. The aim of the present paper is to study how metal-ion concentration, together with cell-wall electrostatic potential, may control pectin methylesterase activity as well as cell-wall autolysis and extension.

MATERIAL AND METHODS

Soybean cell clumps were cultured in liquid medium as described by Gamborg *et al.* [7]. Cells were disrupted in a French press, as previously described, and the cell wall fragments were purified by centrifugation [8]. Pectin methylesterase was isolated from these fragments and purified [1]. Enzyme activity was monitored with either cell-wall fragments, or the soluble enzyme in the presence of apple pectin in a pH-stat. A solution of 0.01 M-NaOH was continuously introduced in the reaction vessel under continuous N_2 bubbling. The reaction proceeded in the presence of various NaCl concentrations (see the legends to the Figures). The pH was set at 7 and the temperature to 30 °C [1].

In some experiments (see Figs. 2 and 3) the temperature was maintained at 2 °C as to minimize the activity of pectin methylesterase, thus making it possible to monitor the pH- or salt-dependence of the building-up of the electrostatic potential of the wall. Proton efflux from cell-wall fragments was followed by titration, upon raising the ionic strength, in a reaction vessel containing the cell-wall fragments [9,10].

The internal concentration of protons was estimated from the knowledge of the hydration volume of the walls. This volume was obtained by subtracting the dried weight of the walls from their wet weight, which was measured after centrifugation (1000 *g* for 5 min) and careful draining off. The reproducibility of these determinations was about 95%. The 'swelling volume' thus estimated has been supposed, in the calculations, to be entirely accessible to protons. This assumption is probably not true, so that the values thus obtained are mean values, certainly underestimated with respect to the real ones. The internal concentration was calculated from the formula:

$$[H^+]_i = 10^{-pH} + n/v$$

in which pH is that of the bulk, n the number of protons (in mol) which appeared in the bulk phase and v the swelling volume (in litres). Then the $\Delta\psi$ between the wall and the outside was calculated (at 25 °C) using the Nernst formula:

$$\Delta\psi\ (mV) = 25.7 \ln([H^+]_o/[H^+]_i)$$

in which $[H^+]_o = 10^{-pH}$.

Autolysis experiments were conducted with cell-wall fragments, prepared as described above, and introduced in 0.05 M-succinate buffer, pH 5. At regular intervals, samples were removed and analysed for reducing sugars [2].

RESULTS AND INTERPRETATION

In the preceding paper [5] it was shown that soluble and purified pectin methylesterase displays an activity towards pectins that reaches a maximum value and then decreases as the ionic

Abbreviation used: $\Delta\psi$, electrostatic potential difference.

* To whom correspondence should be sent.

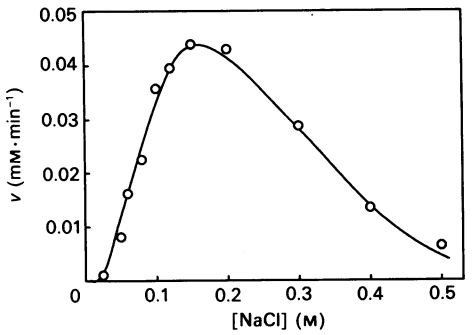


Fig. 1. Effect of Na^+ on bound pectin methylesterase activity

The initial steady-state rate of commercial pectin (concn. 0.025 mM) was determined with a pH-stat at pH 7 and 30 °C. The reaction rate was monitored in the presence of a suspension of soybean cell-wall fragments bearing pectin methylesterase. The titration vial (5 ml) contained 10 μl of cell walls.

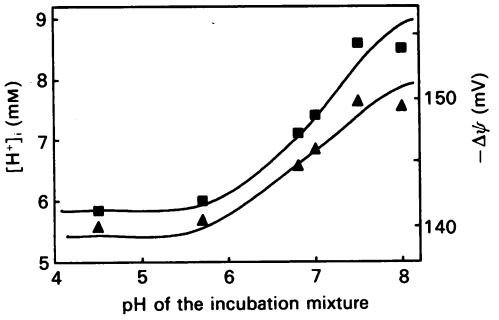


Fig. 2. Variation of $\Delta\psi$ (▲) and of the local proton concentration, $[H^+]_i$ (■), as a function of the pH of the preincubation mixture

The suspensions of cell-wall fragments were incubated 10 min at 2 °C in the presence of 0.2 M-NaCl at a pH whose value is shown on the abscissa. Then the fragments were acidified at pH 2 to block the enzymic activity and extensively rinsed. Afterwards the proton efflux was measured at pH 4.6. The values of $[H^+]_i$ and $\Delta\psi$ may be estimated if one knows the swelling volume of the cell-wall fragments. The value of $\Delta\psi$ (in mV) is defined by the expression:

$$\Delta\psi = 25.7 \ln([H^+]_o/[H^+]_i)$$

where $[H^+]_o$ is the bulk concentration equal to $10^{-4.6}$ M (see the Materials and methods section).

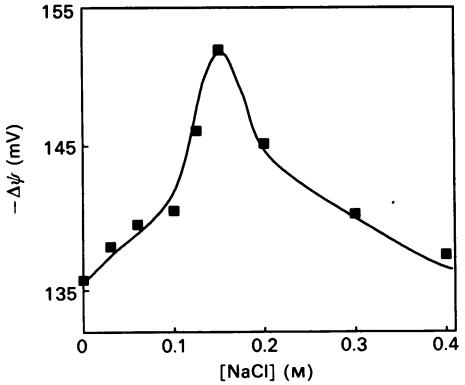


Fig. 3. Effect of Na^+ concentration on the building-up of the cell-wall $\Delta\psi$

The cell-wall suspension (1 ml of cell walls/3 ml of suspension) was incubated for 10 min at pH 7 and 2 °C with different concentrations of NaCl. After acidification at pH 2, the cell walls were rinsed several times and the proton efflux was measured at pH 4.6. $\Delta\psi$ was estimated by measuring the proton efflux from the cell walls (see the Materials and methods section).

concentration in the reaction medium is increased [5]. The same result is obtained with cell-wall-bound pectin methylesterase (Fig. 1).

If soybean cell-wall fragments are preincubated under alkaline pH conditions, then washed, transferred to pH 4.6 and submitted to ionic-strength jumps in the bulk phase, an efflux of protons is observed in this bulk phase [1]. This proton efflux results from the decrease of the $\Delta\psi$ value when the ionic strength in the bulk phase is raised. The extent of proton efflux increases as the pH of the preincubation medium is raised up to pH 7 or 8 [1]. From these data, one may estimate the variation of the $\Delta\psi$ between the wall and the bulk phase as a function of the pH of the preincubation medium (Fig. 2). It is clear that pH values of close to 8 activate pectin methylesterase *in situ* and therefore increase $\Delta\psi$. Since cell-wall pectin methylesterase is active only in the presence of cations, one may expect cation concentration to influence the building-up of $\Delta\psi$. This is exactly what is observed experimentally. If soybean cell-wall fragments are incubated at pH 7 with different salt concentrations, one observes, depending on that concentration, either an increase or a decrease of the $\Delta\psi$ of the cell wall (Fig. 3). It is worth noting that the maximum rate of cell-wall-bound pectin methylesterase and the maximum value of $\Delta\psi$ occur at the same salt concentration. This is a novel argument in favour of the view that pectin methylesterase is directly involved in the building-up of the electrostatic potential of the cell wall.

The process of wall-loosening accompanies cell extension and results in the release of reducing sugars from the wall [2]. Raising the ionic strength of a suspension of cell-wall fragments to 0.125 results in an increase in the activity of wall-loosening enzymes (Fig. 4). Since a preincubation of cell walls at pH 8 enhances pectin methylesterase activity and the cell-wall fixed charge density, one must expect that, under these conditions, the local concentration of cations be enhanced, thus stimulating wall-loosening-enzyme activity. This is precisely what is seen. Two samples of soybean cell-wall fragments were preincubated for 15 min, either at pH 5 or at pH 8, then washed and submitted to autolysis under the same experimental conditions at pH 5 in the presence of a succinate buffer. The rate of autolysis was found to be higher if the cell walls had been preincubated at pH 8 (Fig. 4).

The curve that describes the variation of pectin methylesterase activity in free solution as a function of metal-ion concentration displays a maximum [5]. This maximum, however, depends upon the pH of the reaction mixture [11–13]. For rather 'high' pH values (pH 8, for instance) the maximum pectin methylesterase activity was observed for 'low' salt concentration (Fig. 5). If, alternatively, the pH is lower (pH 5, for instance), the optimum enzyme activity requires much higher salt concentrations.

In the light of the above experiments, the role of $\Delta\psi$ in cell-wall extension and building-up is shown in Scheme 1. The basic idea of this model is that $\Delta\psi$ is the trigger of cell-wall extension, both through local pH changes and local changes of metal-ion concentrations. This model is thus an extension of a previous one that postulated that the control exerted by $\Delta\psi$ on cell elongation solely occurred through local changes of proton concentration.

When the cell wall extends, the fixed charge density, and therefore the $\Delta\psi$ value, decrease because pectins are incorporated in the wall as neutral, methylated, molecules [1,2]. Therefore the number of fixed charges remains constant as the volume of the wall increases. This indeed leads to the decrease of the $\Delta\psi$ value, as well as to a decrease of local concentrations of protons and metal ions. The local increase of pH stimulates pectin methylesterase activity, which then tends to restore the initial $\Delta\psi$ value (feedback process 2 in Scheme 1). This increase of the cell-wall $\Delta\psi$ brings about the attraction of cations in the negatively

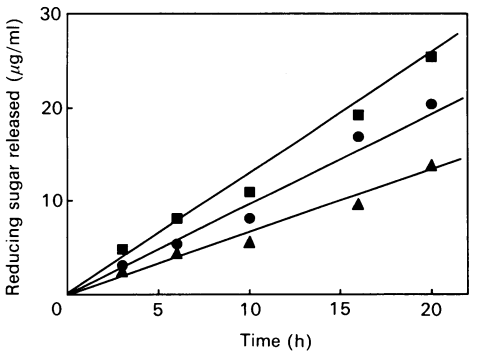


Fig. 4. Interplay between pectin methylesterase activity and cell-wall loosening and autolysis

Three samples of identical cell-wall fragments were prepared at pH 5. Wall fragments of the first sample (▲) were kept for 15 min at the same pH before the autolytic activity was measured as a function of time in succinate buffer, pH 5. The ionic strength of the assay mixture used for autolysis was 0.025. The cell-wall fragments of the second sample (■) were, as described above, incubated for 15 min at pH 5 before the autolytic activity was assayed as a function of time, in succinate buffer, pH 5. The ionic strength of the assay mixture used for autolysis was 0.125. The cell-wall fragments of the last sample (●) were adjusted, after 15 min, to pH 8, then washed, and adjusted with succinate buffer back to pH 5 and the autolytic activity was determined as a function of time. The ionic strength of the medium used for the autolysis was 0.025. For the three curves, the autolysis activity was estimated by the release of reducing sugars (glucose equivalents expressed in μg/ml) in the presence of 0.02% NaN_3. The autolytic activity was measured at 20 °C.

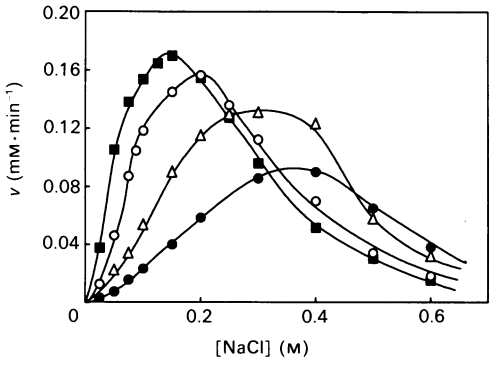
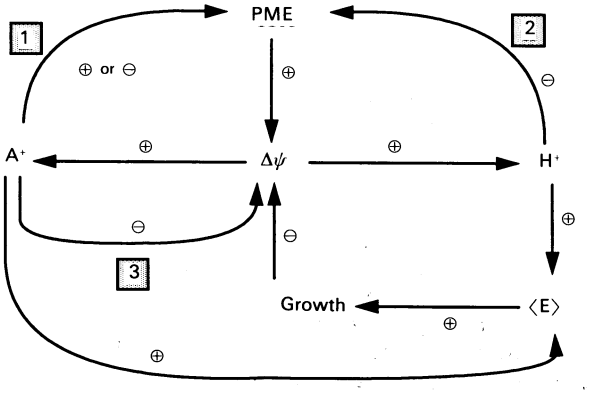


Fig. 5. Variation of the reaction rate of pectin hydrolysis as a function of metal-ion concentration and at different pH values

The pectin concentration was 0.025 mM and the enzyme concentration was equal to 3 nM. The rate is expressed in mM-methyl groups released. ■, pH 8; ○, pH 7; △, pH 6; ●, pH 5.

charged matrix and the amplification of the activity of pectin-bound methylesterase. This amplification process is made possible because the cation concentration required for optimum enzyme reaction depends upon the pH value (Fig. 5). The amplification of the enzyme response (feedback process 1 in Scheme 1) is due to the dissociation of pectin methylesterase from the 'blocks' of carboxy groups, as shown in the previous paper of this series [5]. The increase of concentration of both protons and metal ions in the wall stimulates wall-loosening enzymes and this results in turn to cell elongation. The growth



Scheme 1. Tentative model of the ionic control of pectin methylesterase (PME) activity and cell-wall extension

See the text for details. ⟨E⟩ represents the glucanases involved in plant cell-wall extension. The signs ' + ' and ' − ' refer to activation or increase, and inhibition or decrease, respectively.

process may thus be viewed as a concerted mechanism which involves both protons and metal ions.

If, owing to the activity of pectin methylesterase, the $\Delta\psi$ value becomes too high, the local pH may decrease to below the value required for optimum growth. Then feedback process 2 of Scheme 1 may result in the progressive blocking of pectin methylesterase and feedback loop 3 of the same Scheme tends to decrease the value of $\Delta\psi$. These devices should prevent the cell wall from accumulating a proton concentration which is too high for the optimum activity of wall-loosening enzymes.

DISCUSSION

The present paper offers some experimental data that either confirm previously published results [1,2] or offer some new insights about the mechanisms of cell-wall extension and building-up. So far, the idea that cell-wall $\Delta\psi$ was the trigger of growth has been put forward, and this implied that this control process was effected through the local changes of pH in the wall which, in turn, alters the intrinsic activity of pectin methylesterase. In the preceding paper [5], however, we showed that changes of metal-ion concentration may dramatically alter the activity of pectin-bound methylesterase. As the changes of $\Delta\psi$ should also alter the local concentration of metal ions, one should therefore expect that these ions should also be involved in the control of the cell growth. The present paper was devoted to these topics.

The part played by pectin methylesterase in the building-up of $\Delta\psi$ is supported by the results of Fig. 3. The metal-ion concentration that results in the maximum reaction rate of bound pectin methylesterase is also the one that brings about the largest $\Delta\psi$ value. Taking account of the previous results [5], this could hardly be mere coincidence.

If pectin methylesterase activity is stimulated by preincubation of cell-wall fragments under alkaline pH conditions, the attraction of protons is intense and the local pH may be as low as 2 (Fig. 2). It is very likely, however, that this value does not reflect the real pH conditions that prevail *in vivo*, because, in the experiments *in vitro* that have been reported here, the cell wall is not loaded with metal counterions.

Scheme 1 suggests that pH and metal ions play a dual role in the control of wall extension. Cell-wall growth brings about an increase in local pH, because the $\Delta\psi$ of the wall declines. This increase in pH stimulates the intrinsic activity of pectin methylesterase, which in turn tends to restore the initial $\Delta\psi$

value. This process is amplified by the attraction of metal ions in the polyanionic cell-wall matrix. Pectin methylesterase is not intrinsically activated by metal ions, but these ions tend to dissociate enzyme molecules from 'blocks' of carboxy groups of the cell wall [5,13,14]. Thus metal ions increase the number of enzyme molecules that are available to the process of demethylation of pectins. The release of pectin methylesterase molecules from cell-wall pectins, and the increase in their availability for the catalytic process, bring about increases in proton concentration and metal ions in the cell wall. Numerous studies have shown [8,15–17] that wall-loosening enzymes have an acidic optimum pH. Moreover, results in Fig. 4 of the present paper show that these enzymes are activated by high salt concentrations. As previously suggested, it may be concluded from these results that $\Delta\psi$ is the trigger of plant cell-wall growth.

It has been demonstrated by a number of authors [13,18–20] that some carboxy groups located in the vicinity of the ester bond to be cleaved are required for the enzyme reaction to occur. If these carboxy groups are blocked by metal ions, the enzyme reaction does not occur. Therefore pectin methylesterase must be inhibited by high salt concentrations. This inhibition may be viewed as a device which prevents the $\Delta\psi$ value from becoming too high, and therefore the local proton concentration to be larger than the one required for the fine tuning of wall-loosening enzymes.

All in all it appears that the electrostatic potential $\Delta\psi$ is the trigger of plant cell-wall extension and that pectin methylesterase, together with the proton and cation concentrations, play a major part in the cell growth process.

Received 11 December 1990/15 April 1991; accepted 22 April 1991

REFERENCES

1. Moustacas, A. M., Nari, J., Diamantidis, G., Noat, G., Crasnier, M., Borel, M. & Ricard, J. (1986) Eur. J. Biochem. **155**, 191–197
2. Nari, J., Noat, G., Diamantidis, G., Woudstra, M. & Ricard, J. (1986) Eur. J. Biochem. **155**, 199–202
3. Goldberg, R. & Prat, R. (1982) Plant Cell Physiol. **23**, 1145–1154
4. Goldberg, R. (1984) Physiol. Plant. **61**, 58–63
5. Nari, J., Noat, G. & Ricard, J. (1991) Biochem. J. **279**, 343–350
6. Ricard, J. & Noat, G. (1986) Eur. J. Biochem. **155**, 183–190
7. Gamborg, O. L., Miller, R. A. & Ojima, K. (1968) Exp. Cell Res. **50**, 151–158
8. Crasnier, M., Noat, G. & Ricard, J. (1980) Plant Cell Environ. **3**, 217–224
9. Crasnier, M., Ricard, J. & Noat, G. (1982) FEBS Lett. **144**, 309–312
10. Crasnier, M., Moustacas, A. M. & Ricard, J. (1985) Eur. J. Biochem. **151**, 187–190
11. MacDonnell, L. R., Jansen, E. F. & Lineweaver, H. (1945) Arch. Biochem. **6**, 389–401
12. Pithawala, H. R., Savur, G. R. & Sreenivasan, A. (1948) Arch. Biochem. **17**, 235–248
13. Lineweaver, H. & Jansen, E. F. (1951) Adv. Enzymol. Relat. Subj. Biochem. **11**, 267–295
14. Lineweaver, H. & Ballou, G. A. (1945) Arch. Biochem. **6**, 373–387
15. Nari, J., Noat, G., Ricard, J., Franchini, E. & Sauve, P. (1982/83) Plant Sci. Lett. **28**, 313–320
16. Nari, J., Noat, G. & Ricard, J. (1984) Eur. J. Biochem. **145**, 319–322
17. Taiz, L. (1984) Annu. Rev. Plant Physiol. **45**, 585–657
18. Deul, H. & Stutz, E. (1958) Adv. Enzymol. Relat. Subj. Biochem. **20**, 341–382
19. Lee, M. & MacMillan, J. D. (1970) Biochemistry **9**, 1930–1934
20. Rexova-Benkova, L. & Markovic, O. (1976) Adv. Carbohydr. Chem. Biochem. **33**, 323–385