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Pectin methylesterase, metal ions and plant cell-wall extension

Hydrolysis of pectin by plant cell-wall pectin methylesterase

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The hydrolysis of *p*-nitrophenyl acetate catalysed by pectin methylesterase is competitively inhibited by pectin and does not require metal ions to occur. The results suggest that the activation by metal ions may be explained by assuming that they interact with the substrate rather than with the enzyme. With pectin used as substrate, metal ions are required in order to allow the hydrolysis to occur in the presence of pectin methylesterase. This is explained by the existence of 'blocks' of carboxy groups on pectin that may trap enzyme molecules and thus prevent the enzyme reaction occurring. Metal ions may interact with these negatively charged groups, thus allowing the enzyme to interact with the ester bonds to be cleaved. At high concentrations, however, metal ions inhibit the enzyme reaction. This is again understandable on the basis of the view that some carboxy groups must be adjacent to the ester bond to be cleaved in order to allow the reaction to proceed. Indeed, if these groups are blocked by metal ions, the enzyme reaction cannot occur, and this is the reason for the apparent inhibition of the reaction by high concentrations of metal ions. Methylene Blue, which may be bound to pectin, may replace metal ions in the 'activation' and 'inhibition' of the enzyme reaction. A kinetic model based on these results has been proposed and fits the kinetic data very well. All the available results favour the view that metal ions do not affect the reaction through a direct interaction with enzyme, but rather with pectin.

INTRODUCTION

Owing to the presence of polygalacturonic acids in the plant cell wall, this organelle is negatively charged and behaves, under most pH conditions, as an insoluble polyanion [1–4]. The existence of the fixed negative charges in the primary cell wall generates a difference of electrostatic potential, $\Delta\psi$, between the inside and the outside of this cell wall [5,6]. Theoretical considerations, as well as a number of experimental results, suggest that this electrostatic potential difference ($\Delta\psi$) may serve as a trigger for plant cell growth [6–8]. As the pectins are incorporated in the cell wall in a methylated state, one should expect the fixed charge density to decline as the cell extends. This decline is not observed experimentally [6,7]. It is therefore mandatory to assume that a subtle molecular mechanism exists that may adjust the difference of electrostatic potential between the inside and the outside of the wall. The central element of this multimolecular device is the enzyme pectin methylesterase, which may demethylate pectins that have been incorporated in the cell wall. The optimum pH of the enzyme activity is about 7.5. Therefore, as the cell wall extends, the fixed charge density declines and the local pH tends to increase, thus activating pectin methylesterase, which restores the initial charge density [6–8].

Although pectin methylesterase most likely plays a major role in cell elongation, its mode of action is far from understood. Probably the simplest chemical reaction that may be catalysed by pectin methylesterase is the hydrolysis of *p*-nitrophenyl acetate [6], and it is therefore of interest to use the information that has been obtained with this artificial substrate in order to understand how pectin methylesterase works with its real substrate in the cell wall. Of particular interest in this respect is the role played by metal ions in the enzyme reaction [9–14]. It is well known that Ca^{2+}, for instance, may be taken up, or alternatively released, by the plant cell wall, and that this process certainly plays a major role in cell-wall extension. Moreover, metal ions are apparently strictly required for pectin methylesterase activity on natural pectins. As this enzyme is most probably involved in the building up of the electrostatic potential of the cell wall, understanding its mechanism of action is certainly of interest [6–8].

The aim of the present paper is precisely to answer these unsolved questions.

MATERIALS AND METHODS

Pectin methylesterase was isolated and purified from cell wall fragments of soybean (*Glycine max*) cells, as previously described [6]. Soybean cell clusters were cultured *in vitro* as described by Gamborg *et al.* [15]. The cells were disrupted in a French press, and the cell-wall fragments were prepared as described previously [16]. The pectin methylesterase was isolated and purified from these fragments.

The hydrolysis of *p*-nitrophenyl acetate was monitored, under steady-state conditions, by measuring *p*-nitrophenol production at pH 7 and at 30 °C [17]. The incubation mixture (3 ml) contained 1 mM-phosphate buffer, pH 7, and a fixed concentration of NaCl, KCl, $MgCl_2$ or $CaCl_2$, as well as the substrate *p*-nitrophenyl acetate [6]. Before the reaction was initiated by the enzyme, the substrate *p*-nitrophenyl acetate was dissolved in a small volume of ethanol; the final concentration of this solvent in the reaction mixture was 2%. The product formed, *p*-nitrophenol, was continuously monitored at 400 nm with a DU 8630 Philips spectrophotometer connected to an Apple microcomputer. The reaction was also monitored by titrimetry with a pH-stat (Radiometer VIT 90) by measuring the appearance of acetate during the reaction. A solution of 0.01 M-NaOH was used to maintain the pH of the reaction mixture at 7.

Pectin hydrolysis was mainly followed on a commercial pectin preparation extracted from apples (Fluka) or citrus fruit (Serva). Before use, pectin solutions were chromatographed through Sephadex G-100 (Pharmacia), and the main peak of mean molecular mass 40 kDa was collected and utilized in the

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

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experiments. The degree of methylation of pectins was estimated by the method of Wood & Siddiqui [18], and their content in uronic acid by the method of Blumenkrantz & Asboe-Hansen [19]. The mean degree of methylation of the pectin utilized was 70%. Some experiments were done with polygalacturonic acid methylated in the laboratory [20]. In that case the methylation degree varied from 30 to 70%. In all the cases the reaction was monitored in the pH-stat.

Absorbance spectra of Methylene Blue bound to pectin were recorded with a Kontron Uvicord 860 spectrophotometer. Intrinsic fluorescence spectra of pectin methylesterase or spectra in the presence of terbium [21] or of 2-*p*-toluidinylnaphthalene-6-sulphonate were carried out with a Kontron SVM 25 spectrofluorimeter.

Protein concentration was determined by using the Bradford technique [22]. The binding constant of K^+ on carboxylate groups of pectin was determined at pH 7 by using a potassium electrode (Radiometer F2312K), which permits one to determine free K^+ in a mixture of pectin and KCl.

In order to determine whether pectin methylesterase activity was inhibited by typical serine- or thiol-group-reacting reagents, the enzyme (0.1 μM) was incubated with either methanesulphonyl fluoride (2 mM), iodoacetamide (2 mM), 5,5′-dithiobis-(2-nitrobenzoate) (100 μM) or diethyl pyrocarbonate (100 μM). The enzyme activity was monitored, after two hours, in the presence of *p*-nitrophenyl acetate or pectin.

Fitting of kinetic equations to the rate data was effected through a standard non-linear-regression analysis procedure [23].

RESULTS

Soybean cell-wall pectin methylesterase catalyses the hydrolysis of *p*-nitrophenyl acetate, even in the absence of a metal ion. The overall process follows Michaelis–Menten kinetics. Univalent or bivalent cations (K^+, Na^+, Mg^{2+}, Ca^{2+}), however, stimulate the hydrolytic activity of the enzyme without affecting to any significant extent the $V_{max.}$ of the reaction (Figs. 1*a* and 1*b*). The intercepts of the primary plots are thus, within the error limits of the experiments, independent of the nature of the metal and of its concentration.

Moreover, the hydrolysis of *p*-nitrophenyl acetate is competitively inhibited by pectin (Fig. 2), and no inhibition of the reaction was observed with any of the active site-directed reagents tested (see the Materials and methods section).

All the results that are presented below were obtained with a monodisperse preparation of pectin of average molecular mass equal to 40 kDa. Its mean degree of methylation was 70%. However, using pectin preparations of lower degree of methylation (down to 30%) does not qualitatively affect the kinetic results obtained. That is, the individual reaction rate measurements are different, but the overall kinetic scheme remains unchanged.

In the absence of metal ions the hydrolysis of pectin catalysed by pectin methylesterase is negligible. This activity is strongly enhanced by metal ions. The reaction rate of hydrolysis, however, is inhibited by an excess of metal [9–14] (Fig. 3). The maximum reaction rate is obtained for quite different values of salt concentration, but the value of this maximum rate is the same regardless the nature of the cation. The effect observed is certainly not exclusively related to the difference of ionic strength of these different salts [9,11,24,25]. For instance, the concentration of NaCl that gives the maximum rate is 0.15 M, whereas that of $CaCl_2$ is 0.015 M, that is, a ratio of 10 between the two, whereas the ratio of the corresponding ionic strength is equal to 3. Moreover, the lower the pectin concentration the lower is the salt concentration that gives the maximum activation of the reaction (Fig. 4).

With respect to pectin concentration, the primary Lineweaver–Burk plots are linear and parallel in the range of salt concentrations that result in an activation of the enzyme (Fig. 5). For higher metal-ion concentrations, the reciprocal plots are no

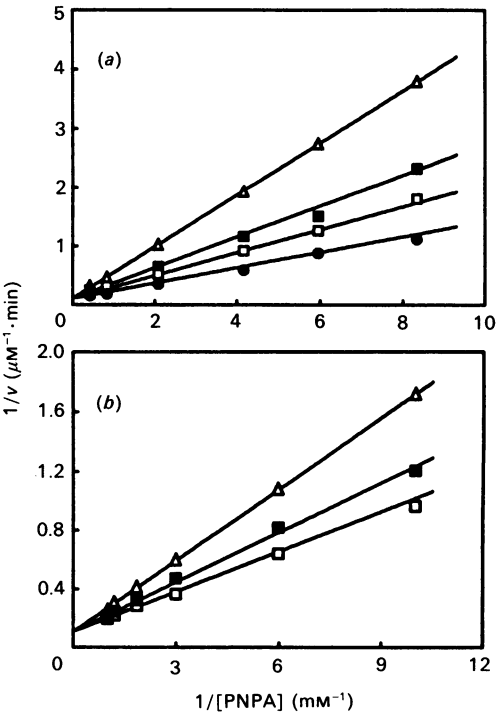


Fig. 1. Lineweaver–Burk plots of *p*-nitrophenyl acetate (PNPA) hydrolysis by pectin methylesterase in the presence of Na^+ and Ca^{2+}

(*a*) The enzyme reaction was monitored in the presence of NaCl. The salt concentrations used were: 0.015 M (△), 0.05 M (■), 0.2 M (□) and 0.4 M (●). Other results obtained for different metal-ion concentrations are not shown here for the sake of clarity. (*b*) The enzyme reaction was monitored in the presence of $CaCl_2$. The salt concentrations used were: 0.016 M (△), 0.1 M (■) and 0.166 M (□). For clarity, some results that have been obtained for different metal ion concentrations are not presented here. For both *a* and *b*, the pH was 7 (1 mM-phosphate buffer). The enzyme concentration was 24 nM.

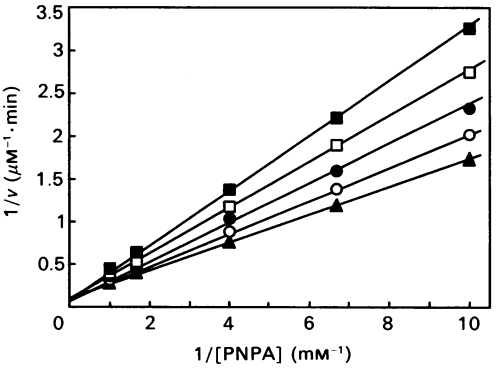


Fig. 2. Inhibition by pectin of the hydrolysis of *p*-nitrophenyl acetate (PNPA) by pectin methylesterase

The pH was 7.0 (1 mM-phosphate buffer) and *I* 0.28 (NaCl). The enzyme concentration was 24 nM. Pectin concentrations were: 0 (▲), 0.025 mM (○), 0.05 mM (●), 0.095 mM (□) and 0.15 mM (■).

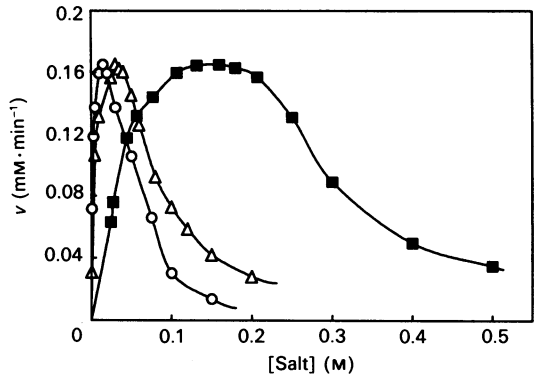


Fig. 3. Rate of pectin hydrolysis as a function of salt concentration

The pH of the reaction mixture was 7.8. The concentrations of enzyme and pectin were 3 nM and 0.025 mM respectively. The rate is expressed as mM-methyl groups released/min. Symbols: ■, NaCl; △, $MgCl_2$; ○, $CaCl_2$.

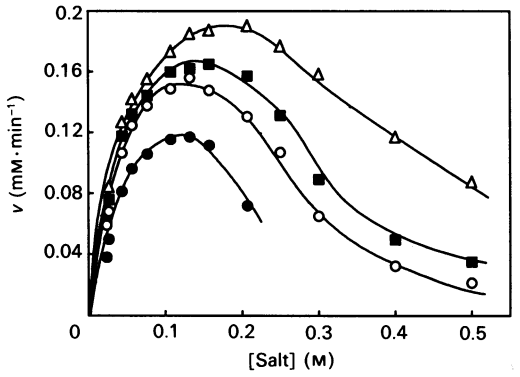


Fig. 4. Rate of pectin hydrolysis as a function of NaCl concentration for different concentrations of pectin

The pH of the reaction mixture was 7.8. The enzyme concentration was 3 nM. The rate is expressed as mM-methyl groups released/min during the reaction. The following symbols are used for different pectin concentrations: △, 0.1 mM (0.4%); ■, 0.025 mM (0.1%); ○, 0.015 mM (0.06%); ●, 0.005 mM (0.02%).

longer parallel and converge at the same point on the ordinate axis. Then the metal apparently behaves as a competitive inhibitor. The primary reciprocal plots, obtained with respect to the metal-ion concentration, are more complex. They present a minimum which tends to be shifted towards low salt concentrations as the concentration of pectin is increased (Fig. 6). These results have been obtained with Na^+ and apple pectin, but qualitatively similar ones were obtained with other metal ions (for instance K^+, Ca^{2+} or Mg^{2+}) or with pectins of different origin and different degrees of methylation.

The secondary plots of the intercepts and the slopes of the data of Fig. 5 are thus non-linear functions of metal-ion concentration (results not shown).

It has convincingly been shown by a number of authors [14,25,26] that polygalacturonic acid behaves as a competitive inhibitor of the reaction. The extent of this inhibition, however, decreases, upon increasing the salt concentration (Fig. 7). This implies that metal ions, once bound to polygalacturonate, relieve the inhibition exerted by this polyanion.

Several dyes, such as Methylene Blue and Acridine Orange, may be bound to polycarboxylic polyelectrolytes [27,28]. If several molecules of Methylene Blue bind to adjacent carboxylate groups (stacking), one may observe a change in the spectrum of

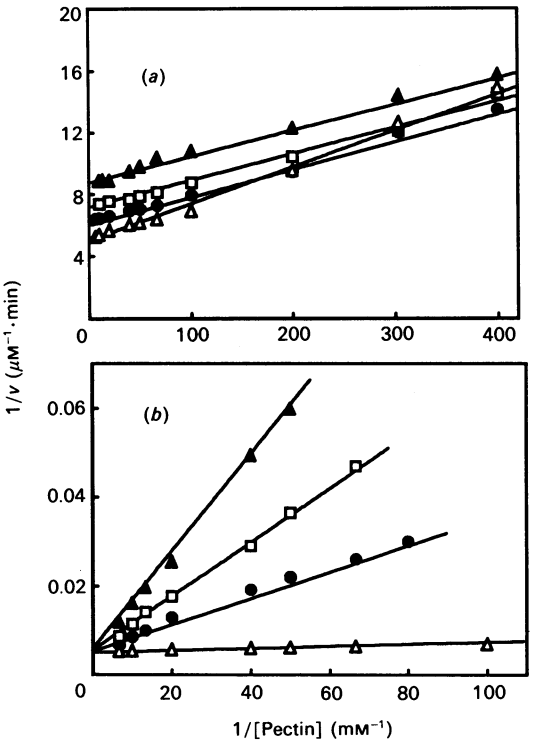


Fig. 5. Lineweaver–Burk plots of pectin hydrolysis obtained under different conditions of metal-ion concentration

The pH of the reaction mixture was 7.8 and the concentration of pectin methylesterase was 3 nM. The various symbols (points, squares etc.) correspond to means of experimental data and the plots are theoretical lines fitted to these data by using eqn. (10). (*a*) NaCl concentrations: ▲, 0.0443 M; □, 0.057 M; ●, 0.077 M; △, 0.132 M; (*b*) salt concentrations: △, 0.132 M; ●, 0.4 M; □, 0.5 M; ▲, 0.6 M.

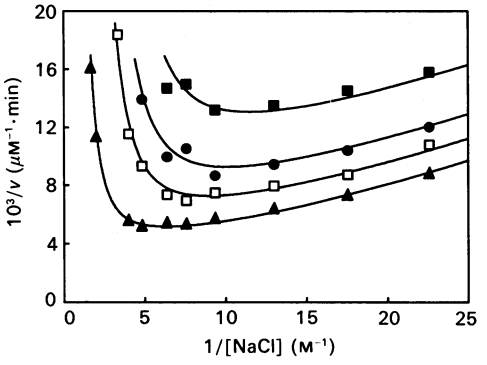


Fig. 6. Lineweaver–Burk plots of pectin hydrolysis as a function of the reciprocal of NaCl concentration

The same experimental conditions as for Fig. 5 were used. The various symbols (points, squares, etc.) are means of experimental data; the curves are theoretical (eqn. 10). The symbols used for the different pectin concentrations are: ■, 0.0025 mM; ●, 0.005 mM; □, 0.01 mM; ▲, 0.1 mM. For clarity, only some data among those available have been presented.

Methylene Blue (Fig. 8). The spectrum of Methylene Blue shows a peak at 665 nm and a shoulder at 610 nm. Addition of polygalacturonic acid, or of pectin, to a solution of Methylene Blue results in a decrease in A_{570} and an increase in A_{665} (Fig. 8).

The variation of the absorbance, ΔA, at 665 nm, of Methylene Blue is different depending on the pectin/Methylene Blue ratio.

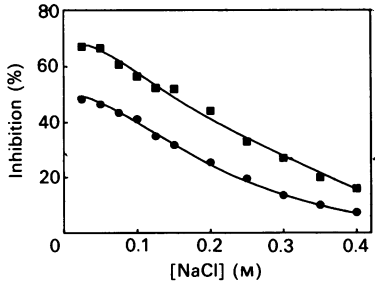


Fig. 7. Inhibition of pectin methylesterase reaction by polygalacturonic acid for different metal-ion concentrations

The pH of the reaction mixture was 7.8. The concentrations of enzyme and pectin were 3 nM and 0.025 mM respectively. v_0 is the reaction rate obtained in the absence of polygalacturonic acid and v_I the one obtained in the presence of polygalacturonic acid. The percentage inhibition obtained is expressed as 100 $(v_0-v_I)/v_0$. This index is plotted as a function of the NaCl concentration for two different concentrations of polygalacturonic acid, namely 2.5 mM (■) and 5 mM (●).

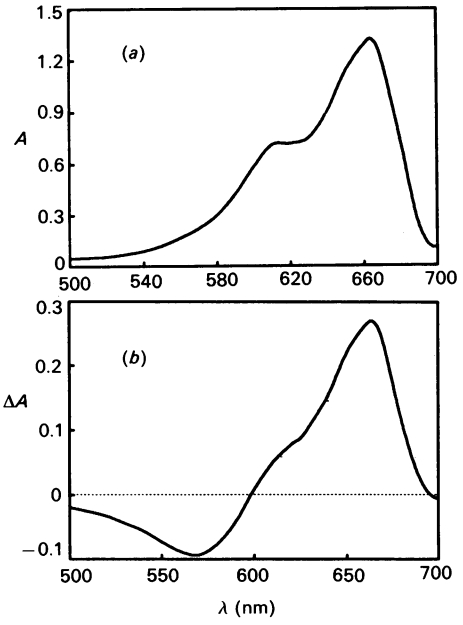


Fig. 8. Binding of Methylene Blue to pectin

(*a*) Spectrum of Methylene Blue in free solution (concn. 16 μM). (*b*) Difference spectrum of pectin-bound Methylene Blue against free Methylene Blue. In both cuvettes the concentration of Methylene Blue was 14.9 μM, and the concentration of pectin in one of the two cuvettes was 104 μM

The maximum ΔA value is obtained for a ratio of about 15 and decreases above this value (Fig. 9). The variation of ΔA as a function of the pectin/Methylene Blue ratio correlates well with the variation of stacking of Methylene Blue on the polymer. As shown also in Fig. 9, this stacking phenomenon tends to fade away in the presence of metal ions that interact with carboxylate groups.

The occurrence of stacking in the binding of Methylene Blue to pectin molecules shows that there exist, in this polymer, blocks of carboxylate groups that may bind either the dye molecules or the metal ions [29–31].

In the range of salt concentrations that result in enzyme activation, Methylene Blue enhances the rate of hydrolysis of

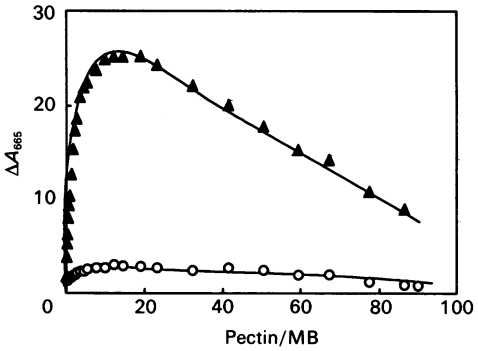


Fig. 9. Variation of the absorbance of pectin-bound Methylene Blue as a function of the pectin/Methylene Blue (MB) ratio

The concentration of Methylene Blue in the reaction mixture was 20 μM. The concentration of pectin is expressed in mM unmethylated uronic acid. The ΔA_{665} value, determined from the difference spectra of the mixture pectin plus Methylene Blue against Methylene Blue, is plotted as a function of the pectin/Methylene Blue ratio, either in the absence (▲) or in the presence (○) of 5 mM-NaCl.

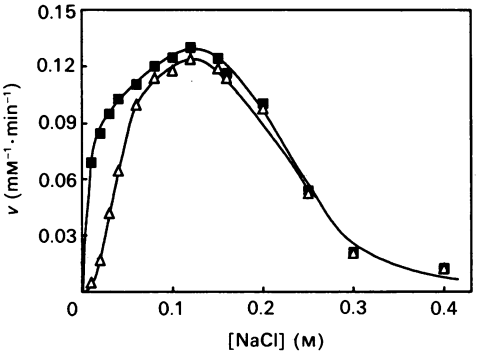


Fig. 10. Activation of pectin methylesterase reaction by Methylene Blue

The pH of the reaction mixture was 7.8. The enzyme concentration was 3 nM, and that of pectin was 0.005 mM. The reaction rate is expressed as mM-methyl groups released/min. Symbols: ■, 1 mM-Methylene Blue; △, no Methylene Blue.

pectin in the presence of pectin methylesterase (Fig. 10). However, over the range of metal-ion concentration where inhibition of the reaction is observed, Methylene Blue has no effect on the enzyme reaction rate (Fig. 10).

Pectin may be hydrolysed at a high rate in the presence of pectin methylesterase and Methylene Blue, but at very low concentrations of metal ions. The reaction rate varies as a function of the concentration of Methylene Blue, and displays a maximum for a given concentration of Methylene Blue. Above that value the reaction is inhibited by an excess of Methylene Blue. The larger the pectin concentration, the greater is the Methylene Blue concentration that results in the maximum reaction rate (Fig. 11). In other words, the situation is qualitatively similar to the one already described with metal ions.

The binding of metal ions to pectin, and in particular to the blocks of carboxylate groups, was directly detected by using a potassium electrode. This binding process does not display any significant co-operativity (results not shown).

As in the case of *p*-nitrophenyl acetate, which has already been mentioned, no inhibition of the reaction rate was observed with any of the thiol- and serine-group-reacting reagents tested (see the Materials and methods section).

Neither the fluorescence spectra of the enzyme (1.5 μM) nor

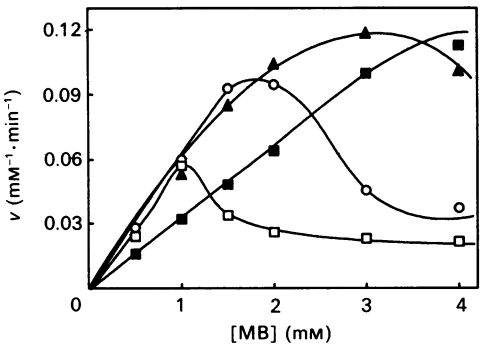


Fig. 11. Variation of pectin methylesterase reaction rate as a function of Methylene Blue concentration

The pH of the reaction mixtures was 7.8 and the ionic strength of the various solutions of pectin have been adjusted to 0.005. The pectin concentrations were: 0.1 mM (■), 0.02 mM (▲), 0.01 mM (○) and 0.005 mM (□).

those of fluorescent probes (150 μM of 2-*p*-toluidinylnaphthalene-6-sulphonate or 1 mM-terbium) were perturbed by the addition of salts (not shown).

INTERPRETATION AND DISCUSSION

The mechanism of pectin hydrolysis catalysed by pectin methylesterase should take into account the following experimental results.

(1) Pectin methylesterase is able to hydrolyse *p*-nitrophenyl acetate in the absence of metal ions. The lack of dependence of the $V_{max.}$ of the reaction on the metal-ion concentration implies that the metal is not involved in the release of the two products, nitrophenol and acetate. Thus the observed effect is not a simple ionic one on the rate constants of the product release. Moreover, the fact that no change in fluorescence spectra of the enzyme, or of the fluorescent probes, was observed in the presence of salts suggests that the metal probably increases the catalytic rate by interacting with the substrate rather than with the enzyme. As pectin inhibits competitively the hydrolysis of *p*-nitrophenyl acetate, this notion must also hold for pectin as substrate.

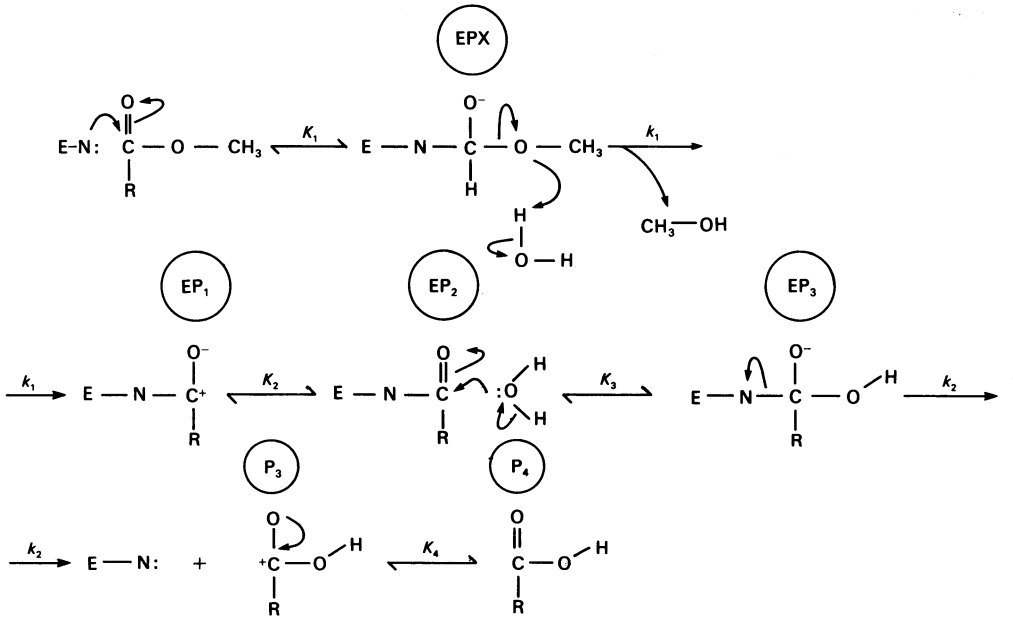
(2) Blocks of carboxylate groups may trap enzyme molecules, thus preventing them from reacting with the $-COO-CH_3$ groups to be hydrolysed. Metal ions may relieve this inhibition by binding to carboxylate groups of the pectin. This process may be mimicked by Methylene Blue.

(3) Binding of metal ions to the carboxylate groups of the blocks of pectin molecules is a non-co-operative process [32–34].

(4) Carboxylate groups located in the vicinity of the $-COO-CH_3$ groups that are subjected to hydrolysis are required in order to allow this hydrolysis to occur [10,11,35–37]. It thus appears that these carboxylate groups should interact with the active site of the enzyme [11]. Binding of metal ions to these groups should therefore inhibit the enzyme activity. The inhibition of the enzyme activity at high salt concentration should therefore be ascribed to these effects.

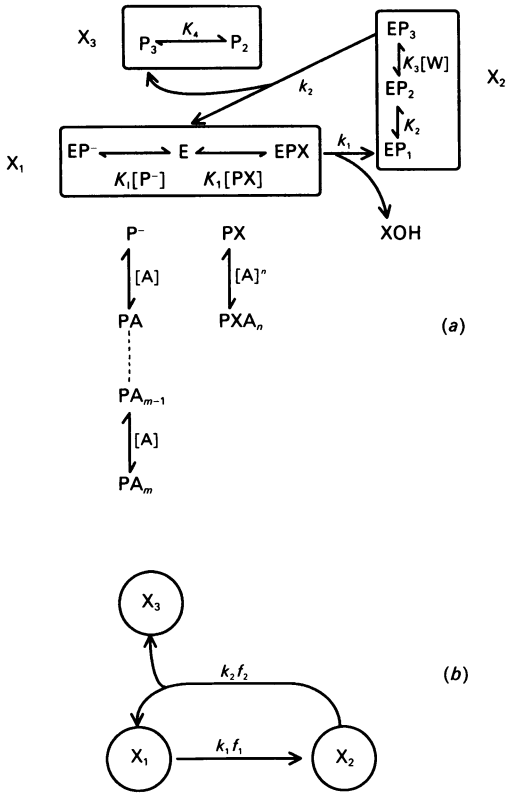
The mechanism of the hydrolysis of pectin by pectin methylesterase should be basically similar to the one already described for the hydrolysis of *p*-nitrophenyl acetate and other carboxylic esters [38,39]. This mechanism is shown in Scheme 1, and involves a nucleophilic attack of a basic group of the enzyme on the ester, followed by the release of methanol and another nucleophilic attack of a water molecule on the acyl-enzyme intermediate, thus resulting in the regeneration of the free enzyme and the appearance of a carboxylic acid.

The simplest kinetic scheme which takes into account the mechanism shown in Scheme 1 and accommodates the experimental findings is shown in Scheme 2. This model is formulated as if a protein macromolecule were reacting with a small ligand molecule, which is, indeed, not the case, for both the enzyme and pectin are macromolecules. The model of Scheme 2, however, is still meaningful, for even if the enzyme slides along the pectin molecule during the reaction, as shown in Scheme 3,



Scheme 1. Tentative mechanism of pectin hydrolysis in the presence of pectin methylesterase

E represents the enzyme and –N a nucleophile group of this enzyme. PX is the ester. EP_1, EP_2 and EP_3 are covalent intermediates. The two products are shown as XOH (methanol) and P_3/P_4 (carboxylic acid). K_1, K_2, K_3 and K_4 are affinity constants (units M^{-1} or dimensionless), where k_1 and k_2 are rate constants (units s^{-1}).



Scheme 2. A simple kinetic scheme that may account for the kinetic data

The symbolism is the same as in Scheme 1. P^- represents polygalacturonic acid blocks. (*a*) This kinetic scheme is in agreement with the reaction mechanism of Scheme 1. The reaction steps that are located within the 'boxes', X_1, X_2 and X_3, are assumed to be 'fast' equilibrium steps. X is the methyl group and W a water molecule. $P_1 \ldots P_4$ represent the reaction intermediates of the reaction scheme of Scheme 1. P^- represents a 'block' of negatively charged groups of pectin. PX is a region of pectin that is able to undergo hydrolysis. A is a cation that may be bound non-co-operatively to the 'blocks' and co-operatively to the carboxy groups that are adjacent to a region of pectin molecule that may undergo hydrolysis. (*b*) 'Contraction' of the above kinetic scheme. X_1, X_2 and X_3 are the 'boxes' pertaining to the fast equilibrium steps. f_1 and f_2 are the fractionation factors defined in the text.

the whole process may still be represented by the classical Michaelis–Menten formalism.

In agreement with the results described above, the model of Scheme 2 postulates that some enzyme molecules may be trapped by the blocks of carboxylate groups. This is represented by EP^- in the model. Metal ions, A, may bind to the carboxylate groups of these blocks to form the complexes $PA \ldots PA_m$, thus preventing the enzyme from being bound to the blocks. Moreover, the metal ions may also be bound to the carboxylate groups located in the vicinity of the $-COO-CH_3$ group which is going to be hydrolysed. For reasons that will be discussed below, this process is assumed to be highly co-operative and is represented by the step

$$PX \overset{[A]^n}{\rightleftharpoons} PXA_n$$

in the kinetic scheme of Scheme 2.

If a number of steps, namely:

$$EP^- \rightleftharpoons E \rightleftharpoons EPX$$
$$EP_1 \rightleftharpoons EP_2 \rightleftharpoons EP_3$$
$$P_3 \rightleftharpoons P_4 \qquad (1)$$

remain in fast equilibrium, whereas the overall process is in steady state, the kinetic scheme of Scheme 2(*a*) may be 'contracted', as shown in Scheme 2(*b*). In this Scheme one has:

$$X_1 = [E]+[EP^-]+[EPX]$$
$$X_2 = [EP_1]+[EP_2]+[EP_3]$$
$$X_3 = [P_3]+[P_4] \qquad (2)$$

and f_1 and f_2 represent the fractionation factors [40,41], namely:

$$f_1 = \frac{[EPX]}{[E]+[EP^-]+[EPX]} = \frac{K_1[PX]}{1+K_1[P^-]+K_1[PX]}$$
$$f_2 = \frac{[EP_3]}{[EP_1]+[EP_2]+[EP_3]} = \frac{K_2K_3[W]}{1+K_2+K_2K_3[W]} \qquad (3)$$

where [W] is the 'concentration' of water. As:

one must have: $$K_2K_3[W] \gg 1+K_2 \qquad (4)$$
$$f_2 = 1 \qquad (5)$$

Then, under these conditions, the steady-state rate assumes the simple form:

$$\frac{v}{[E]_0} = \frac{k_1K_1[PX]}{1+K_1[P^-]+\frac{k_1+k_2}{k_2}K_1[PX]} \qquad (6)$$

where $[E]_0$ is the total enzyme concentration. Moreover, one must have, according to Scheme 2:

$$[P^-] = \frac{[P]_0}{(1+K[A])^m}$$
$$[PX] = \frac{[PX]_0}{1+K'[A]^n} \qquad (7)$$

In these expressions $[P]_0$ is the total concentration of carboxylate groups in the blocks, $[PX]_0$ the total concentration of ester groups that are adjacent to carboxylate groups that have bound, or that have not bound, a metal, and K and K' are the relevant constants of binding of the metal ion to these two types of carboxylate groups. As there is always a large excess of the metal ion over the pectin, the free concentration of A is nearly identical with the corresponding total concentration.

Inserting eqns. (7) into eqn. (6) yields:

$$\frac{v}{[E]_0} = \frac{k_1K_1[PX]_0}{1+K'[A]^n+\frac{k_1+k_2}{k_2}K_1[PX]_0+\frac{1+K'[A]^n}{(1+K[A])^m}K_1[P]_0} \qquad (8)$$

Varying the concentration of pectin in solution results in varying both the concentrations of $[PX]_0$ and $[P]_0$ to the same extent. In fact, one must have:

$$\alpha = \frac{[P]_0}{[PX]_0} \qquad (9)$$

where α is a constant whose value depends upon the degree of

$$E+S_1S_2\text{---}S_n \rightleftharpoons E\,S_1S_2\text{---}S_n \rightleftharpoons P_1ES_2\text{---}S_n \rightleftharpoons \cdots \rightleftharpoons P_1P_2\text{---}ES_n \rightarrow P_1P_2\text{---}P_n+E$$

Scheme 3. Kinetic scheme showing that an enzyme sliding along a linear polymer may display Michaelis–Menten kinetics

$S_1 \ldots S_n$ represent the units of the polymer that are going to undergo the reaction. Similarly, $P_1 \ldots P_n$ represent the units that have been transformed into the corresponding product.

Table 1. Parameter values for the hydrolysis of pectin in the presence of pectin methylesterase

In order to decrease the number of independent parameters in the Table, it has been arbitrarily postulated that $m = n$. If this assumption were not made, some standard-error values would have been much larger. These parameter values should, therefore, be taken more as an illustration of the quantitative predictions of the model, than as real estimations of the parameters.

Parameter	Value
$1/k_1K_1$	$(16.72 \pm 1.09) \times 10^{-3}$ min
K'	370.80 ± 21.56 M^{-1}
$n = m$	3.37 ± 0.07
$\frac{1}{k_1} + \frac{1}{k_2}$	4774 ± 315 M^{-1}·min
$\alpha K_I/k_1K_1$	$(70.66 \pm 28.11) \times 10^3$ M^{-1}·min
K	30.99 ± 8.30 M^{-1}

methylation of pectin. Therefore eqn. (8) may be rewritten in reciprocal form as:

$$\frac{[E]_0}{v} = \frac{1}{k_1K_1}(1 + K'[A]^n)\frac{1}{[PX]_0} + \left(\frac{1}{k_1} + \frac{1}{k_2}\right) + \frac{K_I\alpha}{k_1K_1}\frac{1 + K'[A]^n}{(1 + K[A])^m} \quad (10)$$

In this form it becomes obvious that the reciprocal plots must be linear relative to pectin and may be parallel or convergent depending upon the metal-ion concentration. The primary rate data have been fitted to this equation, and the fits obtained are satisfactory (see Figs. 5 and 6). The parameter values that may be derived by non-linear least-squares fitting of the data are given in Table 1.

The fact that metal ions appear to be required in order to allow the enzymic hydrolysis of pectin to occur does not imply that the enzyme mechanism of pectin hydrolysis is basically different from that of *p*-nitrophenyl acetate hydrolysis. The enhancement of the reaction rate of pectin hydrolysis by metal ions appears mostly due to a relief of the inhibition of the enzyme bound to the blocks of the carboxylate groups of the pectin molecule. The experimental support of this idea mostly originates from the observation that metal ions suppress the inhibition exerted by polygalacturonic acid on the enzyme reaction. Since the molecules of polygalacturonic acid precisely bear these blocks, the activation of the enzyme reaction by metals is due to the relief of an inhibition. In this work, direct binding studies have shown that cations indeed bind to carboxylate groups of pectin. This is by no means a novel result, for different authors have reached the same conclusion by using different techniques, namely c.d. [42] and ^{13}C n.m.r. The results of Ravanat & Rinaudot [43] are of particular interest. By using ^{13}C and ^1H n.m.r. of polygalacturonic and galacturonic acids, they showed that Na^+ ions are efficiently bound to polygalacturonic acid and that there is a great difference between the conformation of the acidic and the neutral forms of this polymer.

A number of authors have proposed the view that the carboxylate groups adjacent to the ester bond to be cleaved are required to allow a correct interaction between the enzyme and the pectin [11,36,37]. Masking these groups with metal ions should therefore result in an inhibition of the enzyme reaction by high concentrations of the metal ion. This inhibition may thus be ascribed to an interaction between these carboxylate groups and the metal ions.

The existence of the blocks of carboxylate groups in natural pectins can be confirmed by the stacking of Methylene Blue on the polyelectrolyte. The observation that the stacking may be antagonized by metal ions is quite consistent with the view that Methylene Blue interacts with pectin, exactly as metal ions do. This view is directly confirmed by the finding that Methylene Blue can replace metal ions and can activate, or inhibit, pectin methylesterase, depending on its concentration. The behaviour of Methylene Blue in the kinetics of pectin hydrolysis is, in all respects, similar to the one observed with metal ions. Although the possibility that the metal ion interacts with the enzyme cannot be excluded, there is no experimental result that supports this view.

The very strong inhibition of the enzyme reaction by an excess of metal ions can be understood only if there is a strong positive co-operativity in the binding of the metal ion to the carboxylate groups adjacent to the ester bond to be cleaved. Kinetic evidence, however, favours the view that metal ions bind to pectin and that the binding process follows two binding modes. The first mode is a non-co-operative one and pertains to the binding of metal ions to the blocks of carboxylate groups. The corresponding binding constant, K, does not vary significantly with the ionic strength. The second mode is a co-operative one and pertains to the binding of metal in the vicinity of ester bonds. In the model it has been assumed for simplicity that the corresponding binding constant, K', does not depend upon the ionic strength. Moreover, it has been postulated that $m = n$. This assumption is made for simplicity and because eqn. (10) contains too many parameters to allow their precise estimation. This is indeed a rough assumption which deprives the parameters m and n of any physical significance. Moreover, one may recall that the pectin preparation used was not a monodisperse one from the point of view of the methyl-group distribution. Obviously this has no qualitative importance in the context of the proposed model, but different quantitative results probably could be obtained with other pectins because of a different proportion between carboxylate blocks and sites of hydrolysis.

All the results presented in the present work favour the view that metal ions do not affect the reaction catalysed by pectin methylesterase through a direct interaction with the enzyme but rather with the substrate. There is little doubt that they have some relevance to the way the enzyme behaves *in situ*. One may speculate, for instance, that moderate concentrations of metal ions bring about the dissociation of some enzyme molecules from the blocks of carboxylate groups in the cell walls, thus making this enzyme available to the process of hydrolysis of ester bonds. As the enzyme reactions proceed in the cell wall, more and more negative charges are produced, thus resulting in an enhancement of the attraction of metal ions in this organelle. The inhibition of pectin methylesterase activity may thus be viewed as a device that controls the ionic atmosphere of the cell wall. This important matter is considered in the following paper [44].

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