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DIFFERENT FORMS OF TOMATO PECTINESTERASE HAVE DIFFERENT KINETIC PROPERTIES

ANDREW G. S. WARRILOW and M. GEORGE JONES*

Department of Biochemistry, University of Wales Aberystwyth, Penglais, Aberystwyth, Dyfed SY23 3DD, U.K.

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; pectinesterase; isoenzymes; kinetic properties.

Abstract—The kinetic properties of the three main forms (A–C) of pectinesterase (PE) found in pericarp of the tomato variety Ailsa Craig were investigated. Differences in the effects of salt on the three forms were correlated with the degree of charge carried by each form, as reflected in their isoelectric points (A: 8.9, B: 9.7 and C: 9.9). All three forms showed Michaelis–Menten kinetics, with K_m varying with salt concentration and degree of esterification (DE) of the substrate. PE-C generally exhibited lower values for K_m than PE-A, and was more effective at de-esterifying pectin with low DE. A large difference was seen in the K_i for inhibition by polygalacturonate (A: 11.0 mM, B: 1.2 mM, C: 0.7 mM). Calcium was particularly effective in activating PE-C. PE-C was completely inactive once the pH was reduced to 5.0, while PE-A maintained more than 60% of maximum activity at this pH. These results suggest that the different kinetic properties of the three forms of PE reflect different physiological functions. PE-A should be able to continue working during the processes of disruption and degradation that typify fruit ripening, while the other forms (particularly PE-C) have their activity under continuous fine control by local ionic and pH conditions in the wall, and are more likely candidates for a role in the modulation of cell wall growth.

INTRODUCTION

The pectic polymers of plant cell walls contain a high proportion of galacturonic acid residues. When the pectins are first synthesized, most of these galacturonate residues are methyl esterified. Pectinesterase (PE) (EC 3.1.1.11) is the cell wall enzyme which hydrolyses these methoxyl groups during subsequent cell development. The consequences of PE action are varied and potentially important in several areas of plant development, as well as in plant–microbe interactions. De-esterified pectins are able to interact with calcium ions to form gels, with consequent effects on the mechanical properties of the cell wall [1]. De-esterified pectins are susceptible to the subsequent action of polygalacturonases [2], which may be important in fruit ripening and abscission, as well as in the degradation of plant cell walls by saprophytic and pathogenic microbes. The de-methylation of pectins also leads to changes in the pH within the wall, with associated electrostatic changes. This effect has been made the basis of a hypothetical model for the regulation of plant cell growth which gives a pivotal role to the action of PE [3]. To increase our understanding of the function of this enzyme in plant growth and development, more information is needed about the factors which influence the activity of PE.

PE occurs in tomato fruits in multiple forms [4, 5], the most abundant of which has been studied extensively [6, 7]. We recently described the occurrence of three forms (A–C) of PE in pericarp of the cultivar Ailsa Craig [8]. Several of the molecular characteristics of PE-B and PE-C were found to be distinct from those of the main form, PE-A. In particular, the second most abundant form, PE-C, has a completely different *N*-terminal sequence to that of PE-A. Moreover, PE-A only appears to occur in fruits, while forms with the chromatographic characteristics of PE-B and C occur in hypocotyls, and roots only appear to contain PE-C [8]. De-esterification of pectins in different parts of the plant or at different times in development may therefore involve different forms of PE. In order to assess the functional significance of the production of more than one form of PE by the tomato plant, more data are needed about the similarities and differences between these forms. This paper reports studies on the kinetic properties of the three different forms of PE described earlier [8] and on the effects of factors such as ionic environment and pH, which may influence PE activity within the cell wall.

*Author to whom correspondence should be addressed. Current address: Institute of Biological Sciences, Stapledon Building, UWA, Aberystwyth, Dyfed SY23 3DD, U.K.

RESULTS AND DISCUSSION

Effects of ionic strength on PE activity

Study and interpretation of the kinetics of PE action is complicated by the effect of cations on PE activity. It has been known for many years that the activity of PE preparations from a variety of species may be increased by the addition of cations to the incubation mixture [9]. Pressey and Avants [4] found that the different forms of PE in tomato showed differing responses to salt concentration, but did not present any detailed data.

Our initial studies on the activity of the different isoforms of PE from tomato pericarp found that the ionic strength of the incubation mixture had a much greater effect on the activities of PE-B and PE-C than on that of PE-A. The relationship between NaCl concentration and PE activity at pH 7.5 and with a methoxyl concentration of 15 mM is shown in Fig. 1. In the absence of added cations, PE-A showed three-quarters of its maximum activity, while PE-B showed only a quarter of maximum activity and PE-C was completely inactive. The optimum salt concentration for PE-A activity was 50 mM, similar to that observed by Lee and Macmillan [6] and contrasting with the optimum of 200 to 250 mM for PE-B and C. In all three cases, higher salt concentrations caused progressive inhibition of PE activity.

The activation of PE by cations was explained by Lineweaver and Ballou [10] as the indirect effect of an interaction between the cations and blocks of negative charges on the pectin substrate, rather than a direct interaction of cations with the enzyme itself. In the process of de-esterification, pectin must obviously bind specifically to the active site of PE, to form a catalytically-active complex. However, at pHs at which PE carries an overall positive charge, the enzyme may also interact electrostatically with the negative charges on blocks of galacturonate residues on the pectin, forming non-specific, catalytically-inactive complexes. Lineweaver and Ballou [10] suggested that the activating effect of cations is to shield the negative charges on the pectin, reducing the formation of inactive enzyme–pectin complexes and so increasing PE activity indirectly.

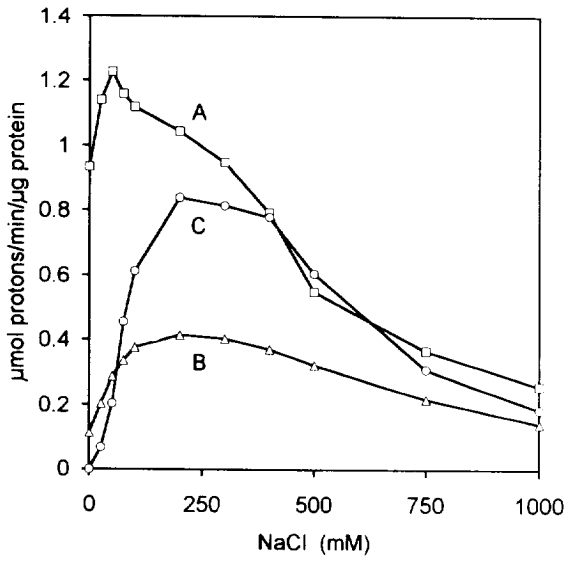

Fig. 1. Effect of NaCl concentration on activity of PE-A (squares), PE-B (triangles) and PE-C (circles). Assays were performed in 0.6% (w/v) Fluka apple pectin (equivalent to 15 mM methoxyl groups) at pH 7.5.

If this hypothesis is correct, one would expect that the concentration of salt needed to achieve maximum reaction rate would be lower in less-concentrated solutions of pectin. To test this, the three tomato PEs were assayed in various concentrations of NaCl, with a pectin concentration equivalent to 1.5 mM methoxyl groups. The optimum NaCl concentrations were indeed reduced to 30, 150 and 150 mM for A, B and C, respectively.

A further inference from the Lineweaver explanation of cation activation is that the effect of salt on the activity of the different PEs must reflect differences in the overall positive charge carried by the different PEs at a particular pH. The three PEs were subjected to isoelectric focusing and their isoelectric points estimated to be 8.9 (A), 9.7 (B) and 9.9 (C). These results indicate that at pH 7.5, PE-C carries a much greater positive charge than PE-A, with PE-B in between. Since PE-C shows no activity at this pH in the absence of added cations, we can conclude that all of this form of the enzyme is bound to the pectin as inactive complexes under these conditions, while the bulk of form A is free to hydrolyse methoxyl groups.

If the effect of salt on PE activity is a consequence of the extent to which a given isoform carries an overall positive charge, one would expect that the optimum salt concentration would vary with pH. At lower pHs the enzyme will carry a greater overall positive charge and so bind more tightly to the blocks of negative charges on the pectin, requiring a higher salt concentration to dissociate these inactive complexes and allow maximum enzyme activity. This was indeed found to be the case for each of the three forms of PE. A reduction in pH from 7.5 to 5.5 increased the value of the optimum salt concentration to 200 mM for PE-A activity, 400 mM for B and 450 mM for C.

Since the overall positive charge on a PE molecule increases as the pH falls, one would also expect an effect of salt concentration on the pH–activity curve for the enzyme. All three forms of PE have pH–activity profiles with an optimum at around pH 8, with activity falling off progressively into the acidic region. For PE-A, increasing the NaCl concentration from 0 to 200 mM shifted the pH–activity profile by 2.5 units into the acidic region, while a similar effect was seen for PE-B and PE-C when the concentration was increased from 50 to 600 mM.

Effect of substrate concentration on reaction rate

Since there was such a clear difference between PE-A and the other two forms in the effect of salt concentration on activity, we investigated the effect of substrate concentration (Fluka apple pectin, from 0.25 mM to 10 mM

methoxyl) on reaction rate for the three forms of PE in various concentrations of salt. The results were analysed by non-linear regression and the behaviour of all three forms of the enzyme generally showed good fit to the Michaelis–Menten equation. Values for the Michaelis constant varied for all three PEs with variation in salt concentration (Fig. 2). Again, there was a noticeable difference between the behaviour of PE-B and C on the one hand, and that of PE-A on the other, with the K_m values for PE-A increasing more quickly with increase in ionic strength than those for the other two forms. Interestingly, the lowest values for K_m (0.14, 0.16 and 0.06 mM for A, B and C, respectively) were obtained in all three cases at salt concentrations below the optimum for maximum activity for each isoform. This presumably reflects the complexity of the interactions occurring between PE, pectin and any cations present.

Over the range of salt concentrations that result in activation of each form of the enzyme, Lineweaver–Burk plots with respect to pectin concentration were linear for all three forms of PE, but were neither convergent nor parallel. This lack of an obvious relationship between salt concentration and kinetic parameters in tomato contrasts with the situation described for soybean PE [11], where the Lineweaver–Burk plots are described as parallel, and again reflects the complexity of the ionic interactions occurring in this system. The observed optimum NaCl concentration must represent the balance point between the two opposing processes of cation activation and cation inhibition.

The data in Fig. 1 show that at higher salt concentrations, all three forms of PE are progressively inhibited. Kinetic analysis of the inhibitory effect of higher concentrations of NaCl on PE activity (from 50 to 500 mM for A and 200 to 600 mM for B and C) indicated that the cation apparently behaves as a competitive inhibitor for all three PEs (i.e. variation in K_m but not in V_{max}). Explanations of the inhibitory effect of excess cations on PE activity are based on the work of Solms and Deuel [12] who proposed that, in order to form an active enzyme–substrate complex, the PE binds to an initiation site on the pectin at which a galacturonate residue is in the close vicinity of the methyl galacturonate residue which is to be hydrolysed. The inhibitory effect of high cation concentrations is then ascribed [11] to competition between the enzyme and cations for binding to these initiation site galacturonate residues. The observation that the effects of high salt concentration on the three forms of tomato PE all involve competitive inhibition indicates that all three act through a similar process of binding to the pectin.

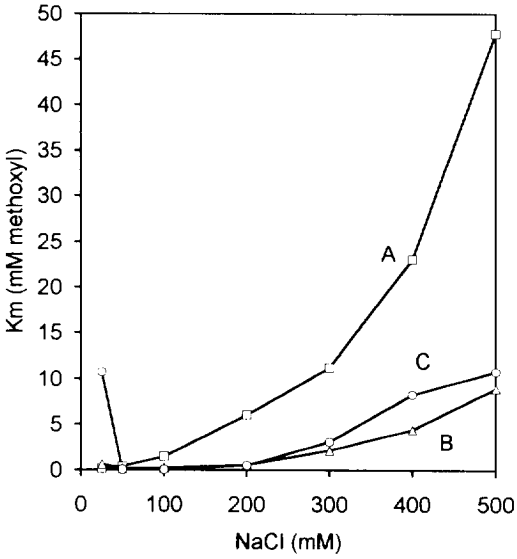

Fig. 2. Variation of Michaelis constant with NaCl concentration for PE-A (squares), PE-B (triangles) and PE-C (circles). Rates of reaction were determined with apple pectin over a range from 0.25 to 10 mM methoxyl.

Effect of polygalacturonate on PE activity

The result of PE action on pectin is, in effect, the generation of polygalacturonic acid. For each form of PE, we determined the effect of substrate concentration on reaction rate in the presence and absence of polygalacturonate (10 mM with respect to anhydrogalacturonan residues) at the optimum salt concentration for that form. Polygalacturonate was a competitive inhibitor of the activity of all three forms of tomato PE and again a large difference was seen between PE-A and the other two forms, with calculated K_i values of 11.0, 1.2 and 0.7 mM for PEs A, B and C, respectively. These results, with the PE form with the highest pI value being inhibited the most, support the idea that the inhibitory effect of polygalacturonate is due to electrostatic interactions between positively-charged PE molecules and blocks of negatively-charged residues on the polygalacturonate, resulting in the formation of inactive complexes. Electrostatic interactions between the enzyme and blocks of polygalacturonate in the wall account for the need for solutions of high ionic strength to solubilize these proteins from cell wall preparations and may have physiological significance during development.

Effect of degree of esterification on PE kinetics

The behaviour of the three forms of PE with substrates of different degrees of esterification (DE) was studied using a set of commercial citrus pectins with DEs from 78% down to 32%. Each form of PE was assayed at its optimum salt concentration and the kinetic constants determined by non-linear regression. Values of V_{max} for each form of PE showed little variation with DE of the substrate, whereas values for K_m varied considerably with DE (Fig. 3). PE-B and PE-C had similar values for K_m for each of the pectins used in this experiment, with the values of K_m falling as the DE of the pectin fell to 30%. The Michaelis constants for PE-A also declined to DE 39%, but then increased at lower DE. In commercial pectins such as these, in which lower values of DE are produced by increased alkaline saponification of the pectin, the lower the DE the greater the frequency of initiation sites

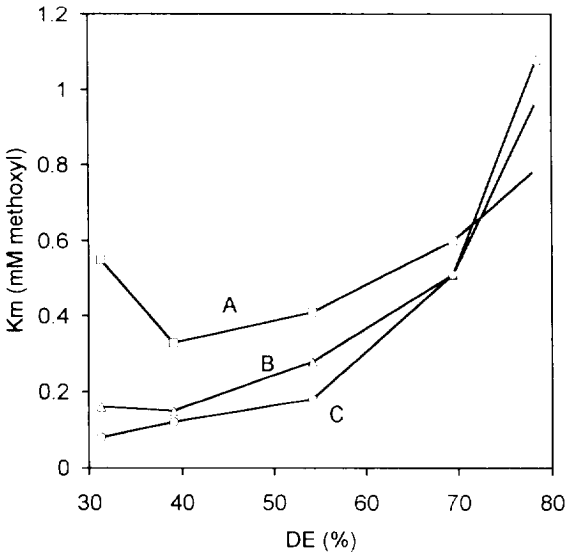

Fig. 3. Variation of Michaelis constant with degree of esterification of citrus pectin for PE-A (squares), PE-B (triangles) and PE-C (circles). Assays were performed in 50 mM NaCl (PE-A) or 200 mM NaCl (PE-B and C).

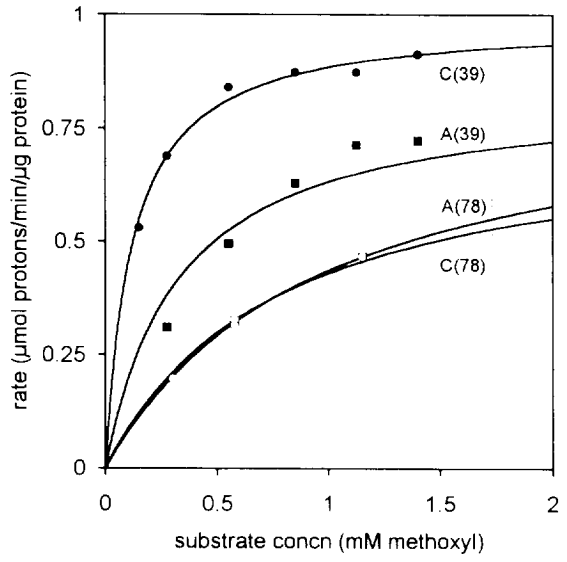

Fig. 4. Rates of de-esterification of two citrus pectins (open symbols = DE 78%, closed symbols = DE 39%) by PE-A (squares) and PE-C (circles). Curves were fitted to experimental data points by non-linear regression.

for PE action (i.e. galacturonate residues adjacent to methyl galacturonate residues). Since an increase in the number of initiation sites effectively increases the initial substrate concentration, there would be expected to be increased rate of reaction as the DE of the pectin falls. However, even allowing for this, there is still a noticeable difference in the behaviour of PE-A from that of the other two forms with low DE pectin. Figure 4 shows the rates of de-esterification by PE-A and PE-C on two pectins of different DE. With a pectin of high (78%) DE there is little difference in the action of these two forms. In contrast, with a substrate of DE 39%, PE-C is significantly more effective at de-esterifying the pectin than PE-A. PE-C may therefore be able to act in a wider range of situations *in vivo* than can PE-A.

PE activity in the presence of calcium

The data described above show that the activity of the three forms of PE can be regulated *in vitro* by the cationic environment in which the enzyme finds itself, and cations and pH are good candidates for factors regulating PE activity *in vivo* also. However, the range of NaCl concentration used for these experiments was much higher than would normally be experienced *in vivo* by tomato PE. A more likely candidate for the cationic regulation of PE activity *in vivo* is calcium, since divalent cations are known to be more effective at activating PE than monovalent cations [10] and give maximum activation at lower concentrations. Figure 5 shows the effect of low concentrations of calcium on relative PE activity (the actual maximum rates of activity seen with calcium were slightly lower in all three cases than those seen with sodium in Fig. 1). The most striking feature of these data is that the activity of PE-C can be modulated across virtually its complete range by a variation in calcium concentration from zero to just 8 mM.

To obtain an idea of the effect of pH on the behaviour of the three PEs under more natural ionic conditions, pH–activity profiles for the three PEs were determined in the presence of 6 mM $CaCl_2$ (Fig. 6). All three forms are most active at alkaline and neutral pH, with activity falling off into the acidic region. However, the effect of fall

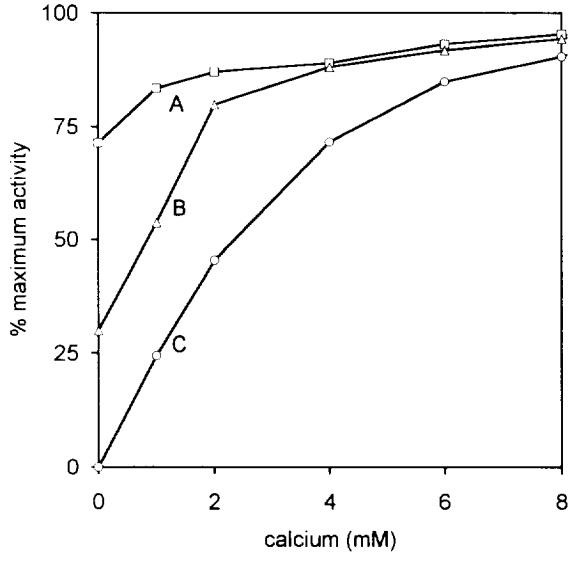

Fig. 5. Effect of calcium concentration on activity of PE-A (squares), PE-B (triangles) and PE-C (circles). Assays were performed with apple pectin (15 mM methoxyl) at pH 7.5.

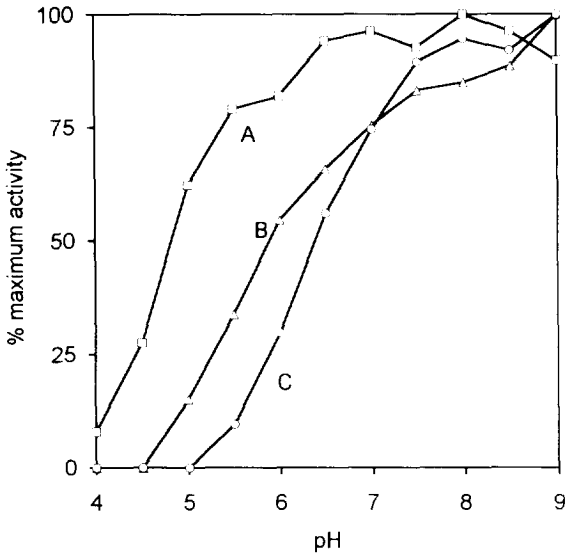

Fig. 6. Effect of pH on activity of PE-A (squares), PE-B (triangles) and PE-C (circles), assayed in 6 mM $CaCl_2$ with 15 mM methoxyl apple pectin.

in pH on activity was noticeably less marked with PE-A than with the other two forms. At pH 5.5 PE-A maintains 80% of its maximum activity, while PE-C only shows a third of its maximum rate. A further fall in pH to pH 5 renders PE-C completely inactive, while PE-A continues to work at more than 60% maximum activity.

Possible functions of the different forms of pectinesterase

The results of our previous paper [8] demonstrated that the different forms of PE found in tomato fruits have significant differences in molecular composition. The data described in this paper show that one of the consequences of these differences in molecular composition is that the different forms of PE have different isoelectric points. As a result of the complex electrostatic interactions between enzyme, substrate and cations, this in turn results in them having very different kinetic properties, which may reflect different functional roles for the different forms.

PE-A only appears to be expressed in fruits [8, 13], suggesting that it has a specific role in fruit development, perhaps particularly in the ripening process. However, it is clear from the results obtained using transgenic plants antisense for PE-A [13, 14] that increases in the level of this form of the enzyme do not act as the trigger to turn on fruit ripening. The data presented here show that the activity of PE-A is affected relatively little by the range of cation concentration or pH likely to be encountered in the wall. Its properties suggest that this form of PE is able to continue working during the processes of disruption and degradation that typify fruit ripening. We therefore envisage the role of PE-A in the fruit as being able to continue the demethylation of pectins (necessary for subsequent depolymerization by polygalacturonases) when the degeneration of fruit tissues creates conditions of pH and ionic environment in which the other forms of PE would be unable to operate. PE-A is also less likely to be inhibited by polygalacturonate produced during fruit ripening.

Although PE-C catalyses the same reaction as PE-A, it has a completely different *N*-terminal sequence [8], suggesting different evolutionary origins and perhaps a different developmental function. The *N*-terminal sequence of PE-C bears a close homology to that of the putative non-specific lipid transfer protein of tomato [15]. The reaction catalysed by these much smaller proteins *in vivo* is still quite uncertain, although interestingly a similar protein from *Arabidopsis* has recently been shown to be located primarily in the cell wall [16]. The data presented here show that PE-B and PE-C have kinetic properties indicating that their activity will be under much closer fine control by local ionic and pH conditions than is that of PE-A. This, together with its widespread distribution in the growing tissues of the plant, makes PE-C a likely candidate for a role in the modulation of cell wall growth, as postulated by Ricard [3].

EXPERIMENTAL

Prepn of extracts. PE was extracted from green fruits of tomato (variety Ailsa Craig) as described previously [8]. The three main forms were sepd by cation exchange chromatography and each purified further by gel filtration [8].

Determination of PE activity. A pHstat was used to monitor PE activity. The standard reaction mixt. consisted of 15 ml pectin soln (Fluka apple pectin or Bulmer citrus pectin) containing the appropriate concn of salt and adjusted to the relevant pH. The NaCl concn and pectin concn were varied for different kinetic experiments. The reaction was started by addition of 0.1 ml enzyme sample. The reaction temp. was 22° (± 1°) and the pH was kept constant by titration with 50 mM NaOH. Background rate assays were performed to confirm that there was no significant uptake of CO_2 by the reaction mixt. over the time of the assay. PE activity is expressed as μmol H^+ formed min^{-1} μg^{-1} protein (protein measured by Biorad dye-binding assay). Fitting of kinetic equations to the rate data was effected through a standard non-linear regression analysis procedure, using the Enzfitter programme written by R. J. Leatherbarrow (Biosoft, Cambridge).

Isoelectric focusing of PEs. This was done using an LKB Multiphor flat bed apparatus. The gel was 5% acrylamide, containing 2.5% Pharmalytes (Sigma, pH 3–10). Samples were focused at a constant 200 V for 4 hr. Pharmalytes were removed by soaking the gel in two changes of 0.64 M trichloroacetic acid containing 3.5% 5-sulphosalicylic acid. The gel was fixed in 40% (v/v) MeOH in 10% (w/v) TCA and stained with Coomassie Blue R-250. Standard proteins used as markers were lactate dehydrogenase, trypsinogen, equine cytochrome c and lysozyme.

Characterization of Bulmer pectins. The ester content of each of the five Bulmer pectins was determined by saponification and titration. The method of ref. [17] was used to determine the anhydrogalacturonan content of each pectin.

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