A novel enzyme activity involving the demethylation of specific partially methylated oligogalacturonides

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Studies of the enzymic digestion of pectic substrates using different polygalacturonase (PG) preparations have revealed evidence for a previously unreported enzyme activity carried out by a contaminating enzyme in one of the preparations. This observed activity involves the demethylation of specific oligogalacturonides, namely 2-methyltrigalacturonic acid and 2,3-dimethyltetragalacturonic acid. However, no large-scale demethylation of highly methylated polymeric substrates is found, demonstrating that the enzyme responsible is not a conventional pectin methylesterase (PME). Furthermore, it has been shown that a commercial sample of fungal PME from *Aspergillus niger* demethylates all of the oligogalacturonides present as primary

products of endo-PG digestion, in contrast with the activity observed here. On the basis of the known methyl ester distribution of the endo-PG-generated fragments and knowledge of which of these oligogalacturonides are demethylated, it is concluded that the observed activity can be explained by the existence of an *exo*-acting methylesterase that attacks the non-reducing end of the oligogalacturonide molecules.

Key words: capillary electrophoresis, pectin digests, pectin methylesterase, polygalacturonase, polysaccharide-degrading enzymes.

INTRODUCTION

The ubiquitous occurrence of the polysaccharide pectin in the cell walls of land plants, taken together with the large number of pectin-modifying enzymes encoded in the genomes of plants [1] and their pathogens [2], clearly points to the importance of pectin, and pectin-derived compounds, in diverse aspects of plant physiology. Plant-directed modification of the cell wall in response to a myriad of environmentally triggered stimuli, for example during fruit ripening, and the degradation of the cell wall by invading pathogens, graphically illustrate the macroscopic consequences of pectin modification. Indeed, the products of pectin digestion, the oligogalacturonides, play a key role in triggering plant defence [3]. Hence, it is important for many areas of plant science that the enzymic processing of both pectin and the oligogalacturonides can be understood in detail.

Much progress in the determination of pectin fine structure has been made in recent years, largely on the basis of chain-fragmentation methodologies and the development of methods for the detection, separation and sequencing of the resultant oligogalacturonide fragments using anion-exchange chromatography and MS [4–6]. Recently, one of us (M.A.K.W.) and others [7] have developed a method using capillary electrophoresis (CE) as an additional tool for the separation and quantification of oligogalacturonide fragments obtained from the enzymic digestion of pectic substrates [7]. The work reported herein originates from the use of this technique in the simple comparison of digest patterns obtained upon the incubation of polygalacturonic acid (PGA) and 31 % methyl-esterified pectin, with a commercially available endo-polygalacturonase (endo-PG) preparation and a pure endo-PG II isoform.

EXPERIMENTAL

Substrates

The two pectin samples used were derived from lemon peel and were supplied by CP Kelco ApS, DK 4623 Lille Skensved, Denmark. Initial characterization of the degrees of methyl-esterification (DM) was carried out by the manufacturer using a titration method and these were found to be 31.1 and 77.8 %. The samples were supplied as powder and (5 mg/ml) solutions were prepared by heating in deionized water at 60 °C for 20 min. PGA was purchased from Sigma–Aldrich Corp., St. Louis, MO, U.S.A. and prepared similarly.

Enzymes

A commercially available preparation of endo-PG from *Aspergillus niger* was obtained from Megazyme International Ireland Limited, Bray, County Wicklow, Republic of Ireland. Previous work on *A. niger* preparations suggest that this sample is likely to consist predominantly of the endo-PG II [8]. The pure endo-PG II (EC 3.2.1.15) isoform was prepared as described previously [9]. In all digests reported here, 380 ng of protein (in 0.2 ml of acetate buffer, pH 5) was added to 0.5 ml of 0.5 % (w/w) substrate in 50 mM acetate buffer, pH 4.2 (equivalent to 1.5 units for the pure endo-PG II and 0.34 unit for the Megazyme preparation). The fungal pectin methylesterase (PME) used was a commercially available sample from *A. niger* and was obtained from Novo. Exo-polygalacturonase (exo-PG) from *A. tubingenensis*, was obtained as described previously [10].

Abbreviations used: BGE, background electrolyte; CE, capillary electrophoresis; DM, degree of methyl-esterification; endo-PG, endo-polygalacturonase; exo-PG, exo-polygalacturonase; HPAEC, high-performance anion-exchange chromatography; PGA, polygalacturonic acid; PME, pectin methylesterase.

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Buffers

Phosphate buffer, pH 7.0, was used as a CE background electrolyte (BGE) and was prepared by titrating aq. 50 mM NaH_2PO_4 with 1 M NaOH. Sodium acetate, pH 5.0, was used as a high-performance anion-exchange chromatography (HPAEC) eluent and was prepared by titrating 1 M sodium acetate with 1 M acetic acid. All buffers were filtered through 0.2- μm -pore-size filters (Whatman Biosystems Ltd., Maidstone, Kent, U.K.).

HPAEC

HPAEC was carried out using a Dionex-300 system (Dionex Corporation, Camberley, Surrey, U.K.) equipped with a Carbowac PA100 guard column and a PA1 column. The separation was carried out at pH 5.0, and post-column addition of NaOH allowed for the use of pulsed electrochemical detection, as reported previously. Samples were eluted with a 65 min linear gradient of 0.05–0.7 M sodium acetate using a flow rate of 0.5 ml $\cdot$ min $^{-1}$, which was typically made up to 0.8 ml $\cdot$ min $^{-1}$ with the NaOH.

Fraction collection

For use in CE, fractions from HPAEC were collected and subsequently diluted post collection in order to achieve a sample ionic strength of 50 mM prior to injection. In contrast with higher ionic strengths, which caused significant peak broadening, this was found to give negligible perturbation to the CE electropherograms, and dilution was favoured over desalting procedures. In order to collect sufficient quantities of the relevant methyl-esterified oligomers so that they could be clearly observed, even after dilution, a partial digest of a 5% pectin sample was carried out and injected from a 500 μl injection loop. While this lead to significant peak broadening compared with the experiment carried out at lower column loadings, the chromatogram was still clearly recognizable and individual peaks of interest sufficiently resolved to allow fraction collection. In all 100 fractions (0.25 ml each) were collected.

CE

Experiments were carried out using an automated CE system (HP 3D) equipped with a diode-array detector. Electrophoresis was carried out in a fused silica capillary of internal diameter 50 μm and a total length of 46.5 cm (40 cm from inlet to detector). The capillary incorporated an extended light-path detection window (150 μm) and was thermostatically controlled at 25 $^{\circ}\text{C}$. All new capillaries were conditioned by rinsing for 30 min with 1 M NaOH, 30 min with a 0.1 M NaOH solution, 15 min with water and 30 min with BGE. Between runs the capillary was washed for 2 min with 1 M NaOH, 2 min with 0.1 M NaOH, 1 min with water and 2 min with BGE. Detection was carried out using UV absorbance at 191 nm with a bandwidth of 2 nm. Samples were loaded hydrodynamically (various injection times at 5 kPa, typically giving injection volumes of the order of 10 nl), and typically electrophoresed across a potential difference of 20 kV. All experiments were carried out at normal polarity (inlet anodic) unless otherwise stated.

RESULTS AND DISCUSSION

Figure 1 shows the comparison of enzymic digests of PGA and a low-DM (31%) pectin, obtained after 48 h incubation at room temperature with the commercially available PG preparation and the pure endo-PG II. The PGA digests, (a) and (b), can be

seen to be identical within experimental uncertainties, strongly indicating that the commercial enzyme sample predominantly consists of endo-PG II. The digest pattern of the 31%-DM pectin produced by the commercial enzyme preparation, Figure 1(c), is comparable with that found in previous work [7], and the peaks are labelled according to the assignments made therein. (The notation n^m is used throughout in order to indicate an n -mer of galacturonic acid with m groups methyl-esterified.) However, it can be seen that, whereas the general appearance of the two digests using the two different enzyme sources, (c) and (d), is similar, there are some striking differences between them. Perhaps most obvious is the fact that there are clearly two extra peaks observed in the endo-PG II digest migrating at about 5.6 and 6.4 min. These were identified using a previously described methodology [7] in which a digest was separated using HPAEC and collected fractions of known oligogalacturonides, identified in previous studies [5,11], were 'spiked' back into the CE runs. Figure 2 shows the results from the application of this methodology and confirms that the extra peaks are in fact 2-methyltrigalacturonic acid (3^1) and 2,3-dimethyltetragalacturonic acid (4^2). The position of the methyl groups within the digest fragments has been identified previously by tandem MS [11], although in this case the assignment is trivial as it is well known that endo-PG subsites -1 and $+1$ must be un-esterified in order for the chain to be cleaved [12], and consequently (neglecting end effects) fragments cannot be terminated at either end by methyl-esterified residues. In fact, these 'extra' oligomeric fragments are expected products of endo-PG II digestion [11], so that the pertinent question is not why they appear in the digest performed with the pure isoform, but rather why they have not been observed, here or in previous work [7], in digests performed with the commercial enzyme preparation.

On closer examination of Figure 1, with the aid of peak integration algorithms, it is clear that the total amounts of the species 3^0 and 4^1 are also different in runs (c) and (d), while other peaks have unmodified normalized peak areas (these were assessed from the average results for three independent experiments). Moreover, the total normalized peak areas for both digests were identical within experimental uncertainty. The fact that the total number of galacturonic acid residues was found to

Figure 1 Electropherograms of enzyme digests

Digests of PGA (top trace, combined traces a and b) and pectin (DM = 31%) (traces c and d) obtained after 48 h incubation at room temperature with the commercially available PG preparation (traces a and c) and the pure endo-PG II (traces b and d) are shown.

Figure 2 Electropherograms of 'spiked' enzyme digests

The traces show an endo-PG II pectin (DM = 31%) digest 'spiked' with individual oligogalacturonides 4² (trace a) and 3¹ (trace b).

Figure 3 Electropherograms of oligogalacturonide incubations

Substrates were incubated for 24 h at room temperature with the commercial endo-PG preparation (continuous thick lines) and with a control consisting of the commercial PG preparation heated to 95 °C for 10 min (○).

be the same in both enzyme digests implies that all species have been detected in both cases and that differences between the patterns simply represents a redistribution of galacturonic acid residues between individual species. On the basis of the observed differences and peak areas this could be explained if in the digest generated by the commercial enzyme the 3¹ species generated by endo-PG II digestion are further modified to produce 3⁰, and similar post-endo-PG II processing of 4² to produce 4¹ takes place. A clear candidate for such specific processing of partially methylated oligogalacturonic acid species would be an enzyme present in the commercial PG sample as an impurity. This was initially supported by the fact that addition of the commercial enzyme preparation to samples pre-digested with the pure endo-PG isoform did produce further processing of the 3¹ and 4² species as described.

Figure 4 Electropherograms obtained from high-DM polymeric substrates

Trace (a), a highly methylated pectin substrate (DM = 78%); traces (b) and (c), the same sample after incubation with the commercially available PG preparation (trace b) and the pure endo-PG II (trace c).

This hypothesis has been investigated further by incubating single oligogalacturonide species with the commercial endo-PG preparation and with a control consisting of the commercial preparation heated to 95 °C for 10 min in order to denature any enzymes present. Figure 3 shows the results from this experiment and clearly demonstrates that 3¹ is indeed degraded to 3⁰ in the presence of the commercial enzyme preparation, but not with the heated sample. Similarly, 4² is de-methylated to 4¹ in the presence of the commercial enzyme preparation but not with the heated sample. [The product identities are inferred from the migration times (cf. Figure 1 for example).] In contrast, the oligogalacturonides 4¹, 5¹ and 5² are not de-esterified. This is clearly consistent with the results obtained from the digests and indeed supports the hypothesis that there is an additional enzymic processing of specific oligogalacturonides in the sample incubated with the commercial PG preparation. It is noteworthy that this experiment was carried out with two different batches of the enzyme preparation and the same results were found in both cases. In addition, the samples were rerun after 1 month and though all the 3¹ and 4² species had been completely processed to give 3⁰ and 4¹, the same results were found with regard to the 4¹, 5¹, and 5² species.

In order to assess the possibility that the observed specificity reflects the nature of the subsite architecture of a conventional PME, the activity of the commercial PG preparation towards highly methylated polymeric substrates was examined. Figure 4 shows a comparison of the electropherograms obtained from (a) a highly methyl-esterified (78%) pectic substrate and the sample after extensive incubation with (b) the commercial PG preparation and (c) the pure endo-PG II. It is well established that CE can be used for the analysis of intact pectic polymers as well as digest fragments [13,14], and the large peak migrating at about 4.5 min is the polymer itself. It can clearly be seen that the results of the enzyme incubations are comparable in two important respects. First, there is no significant change in the migration-time distribution of the polymeric material, indicating an unchanged charge density and thus degree of esterification. Secondly, the very small amount of oligogalacturonide peaks migrating at later times is indication that minimal digestion has taken place. For such high-DM substrates this is entirely expected

Figure 5 Electropherogram of a fungal-PME-treated digest

Trace (a), the endo-PG II pectin (DM = 31%) digest; trace (b), the same digest after incubation with a fungal PME.

for endo-PG II. However, any conventional PME activity in the commercial enzyme preparation would have been expected to introduce a large degree of endo-PG II susceptibility into the polymeric structure via de-methylation, and this is not seen. A selection of other high-DM pectins were used as a substrate, and the same equivalence was found in all cases. Furthermore, the same experiment was carried out at pH 4 and pH 7 (broadly speaking the optima for fungal and plant PMEs respectively [15]), and the same results were found, even over extended incubation periods of weeks. While plant PMEs are known that are not able to completely de-esterify highly methylated substrates [16], have different action patterns as a function of pH [17] and exhibit quite specific activities towards different oligogalacturonides, particularly as a function of degree of polymerization [15], enzymes of this type would be expected to be rampantly active towards highly methyl-esterified substrates such as those used here. Although the results of this experiment do not preclude the possibility of an enzymic de-methylation occurring purely at the chain ends, they do strongly suggest that the enzyme impurity in the commercial sample is not a conventionally known PME.

In order to further demonstrate the uniqueness of the specific activity described here, the digest obtained from the 31%-DM pectin substrate by endo-PG II, as shown in Figure 1(d), was incubated with a sample of commercially available fungal PME from *A. niger*. Figure 5 shows the endo-PG II digest compared with that obtained after incubation with the commercial fungal PME. It is clear that this enzyme de-esterifies all the present digest products and does not exhibit the same oligogalacturonide specificity found after incubation with the commercial PG preparation. This result is consistent with those obtained in recent MS studies of the mode of action of *A. niger* PME on fully methyl-esterified oligogalacturonides, namely that only methyl ester groups at the non-reducing end were resistant to de-esterification [18].

In order to understand the possible origin of the exquisite specificity in the action of the proposed enzyme, the distribution of the methyl groups within the oligogalacturonides has been considered. (This is already known because the individual fragments have been obtained by fraction-collecting from an

Figure 6 Effect of the intramolecular distribution of methyl-esterification

The Figure shows the major partially methylated products of the endo-PG II digest of a pectin substrate (DM = 31%), displayed in a manner similar to that used in [11], together with the result of incubation of these oligogalacturonides with the commercially available PG preparation. The reducing end is on the right hand side. Those oligogalacturonides that are modified are highlighted by the grey horizontal box. The small black ovals represent the methyl groups.

endo-PG II digest and sequenced [11].) Figure 6 shows a detailed picture of the major products of the endo-PG II digest of the pectic substrate (after [11]) together with the result of incubation of these oligogalacturonides with the commercially available PG preparation. It should be noted that although the results described so far show that the 4² is modified to 4¹, they do not show whether the product is the 2-methyl or 3-methyl species. This was determined unequivocally by using another pectin-degrading enzyme, an exo-PG, that is known to cleave single galacturonic acid units from the non-reducing end, providing that both the first and the second residue from the non-reducing end are free from methyl-esterification [6]. A 100 µl portion of the 4¹ sample produced by the de-methylating action of the newly proposed enzyme were incubated with around 0.005 unit of exo-PG, at room temperature, for 48 h. Figure 7 shows the electropherograms obtained from (a) the original pectin digest (reproduced from Figure 1), (b) the 4¹ fragment produced by enzymic de-methylation from 4² and (c) the incubation of (b) with exo-PG. It can clearly be seen that the 4¹ fragment is indeed processed by exo-PG to produce 1⁰ and 3¹, thus confirming the position of the methyl group to be as shown in Figure 6. It appears, then, that only methyl groups next to the non-reducing end are removed as a result of the newly discovered enzymic activity. From our data we conclude that this activity represents a novel exo-acting methylesterase that attacks the non-reducing end. To our knowledge this is the first time that such an enzyme activity has been identified.

It is tempting to speculate that such an exo-acting PME has evolved as a degradation partner to the exo-PGs. As described above, it is well known that these enzymes cleave single galacturonic acid units from the non-reducing end and that cleavage requires both the first and the second residue from the non-reducing end to be free from methyl-esterification [6]. From the methyl-esterification patterns of the major endo-PG II fragments (Figure 6) it is clear that only 3¹ and 4² are inert to exo-PG action [11] and it is these oligomers in particular that are found to be further modified in the work described here. Furthermore, the ensuing processing removes the methyl groups from the second

Figure 7 Electropherograms of enzyme digests

Trace (a), pectin (DM = 31%) digest obtained after 48 h incubation at room temperature with endo-PG II; trace (b), the 4^1 fragment arising from the proposed enzymic modification of 4^2 ; trace (c), the result of incubating the 4^1 fragment with exo-PG at room temperature for 48 h.

residue from the non-reducing end, the very ones that would thwart the advance of an exo-PG.

We hope that, ultimately, the exo-PME can be obtained in isolation from the endo-PG preparation in which it has currently been found. This would open up a number of exciting possibilities. In particular, the sequencing of such a protein would lead to an increased structure–function understanding and the identification of its encoding gene. Eventually, it may also be possible to utilize the complementarity of exo-PME and exo-PG in order to sequence single chains of partially methylated galacturonic acid by performing stepwise degradation from the non-reducing end.

CONCLUSION

There is clear evidence from the comparison of pectic digests that a commercially available endo-PG preparation does not simply contain endo-PG II. Further studies using this enzyme preparation with single oligogalacturonides support the view that whereas 3^1 and 4^2 are de-methylated to produce 3^0 and 4^1 respectively, the species 4^1 , 5^1 , 5^2 and 6^2 are stable. A control experiment in which the enzyme preparation was heated to 95 °C before addition to the oligogalacturonides showed no such activity, confirming the enzymic nature of the de-methylation process. Furthermore, the preparation in question shows no significant de-methylating activity towards highly methylated polymeric substrates (over and above what might be expected from non-reducing ends), and it has been shown that conventionally known fungal PME's would be expected to de-esterify all the oligogalacturonide species present as endo-PG II digest products. Considering the known distribution of the methyl groups on the oligogalacturonide fragments from endo-PG II digests, it is concluded that the commercially available endo-PG preparation contains a hitherto unknown exo-acting methyl-esterase.

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