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PII:S0144-8617(96)00005-7

Carbohydrate Polymers 29 (1996) 129–136

0144-8617/96 $15.00+.00

Nature of sites hydrolyzable by endopolygalacturonase in partially-esterified homogalacturonans

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(Received 30 November 1995; accepted 3 December 1995)

Endopolygalacturonases (EPGs) hydrolyze glycosidic linkages between galacturonic acid (GalA) residues in polygalacturonans, a major fraction of plant pectins. However, most polygalacturonans occur naturally with some of the GalA residues methylesterified. Methylesterification is known to inhibit the activity of EPG. A commercial apple pectin sample was partially saponified to give three pectin samples of degree of methylesterification (DM) 52, 32, and 16. Each was digested to completion by a cloned EPG of *Erwinia carotovora* subsp. *carotovora* and the sizes of the resulting fragments were determined by HPLC. A mathematical model was devised to predict the distribution of fragment lengths expected from the three different DM pectins depending on how many adjacent GalA residues are necessary for the enzyme to be able to bind and act on its substrate. For all three DM pectins, the enzyme digestion pattern fit fairly well to that predicted if the enzyme needs four adjacent non-esterified residues to act. As an independent test for the substrate requirements for the enzyme, we determined the maximum cluster size of adjacent non-esterified GalA residues, which remained after complete EPG digestion of the three pectin samples. In each case it was three residues. Thus both methods show that the EPG of *E. carotovora* needs four adjacent non-esterified GalA residues within a partially-esterified region to be able to act. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Plants, plant pathogens, and saprophytes produce a wide range of pectin-degrading enzymes. Some of these enzymes have been very useful in structural studies of pectins and cell walls (McNeil *et al.*, 1984). In structural studies of pectins, endopolygalacturonases (EPGs) have been used to degrade homogalacturonans selectively for solubilization of pectin fragments for further characterization. Most homogalacturonans in plants are methylesterified to some degree, and methylesterification is known to inhibit the activity of these enzymes (Sakai *et al.*, 1993). However, the exact requirements for the structure of their substrates are not known. An understanding of the exact character of sites within methylesterified homogalacturonans at which the EPGs can act should be very useful for determining more about the patterns of methylesterification in these homogalacturonans, and perhaps also for explaining differences in solubilization of pectins by enzymes from different sources. Most of the work on substrate specificities of EPGs has involved determining what is the smallest non-esterified oligogalacturonan which can be degraded by the enzyme (Rexova-Benkova & Markovic, 1976). Sakai *et al.* (1993) in an extensive review of pectin-degrading enzymes also addressed briefly the question of sites suitable for hydrolysis in methylated pectins. They inferred from the reactivities of short non-esterified oligomers that a minimum of three adjacent non-esterified residues was necessary for the action of protopectinases from various fungi, but that in practicality, four were necessary because hydrolysis at sites of less than four was very slow. They also showed a figure depicting the average DP of the digestion products of polygalacturonic acids of various degrees of esterification.

In this report we describe results from two independent methods to determine the nature of the hydrolysis sites in methylesterified pectins. In one of them, we compare the products from limit digests of pectins of

various degrees of methylesterification (DM) with those predicted from a model dependent on the nature of hypothetical cleavage sites. In the other, we presume that there is a minimum number of contiguous non-esterified residues necessary for enzyme activity and looked for the destruction of all such sequences, and longer ones, by the activity of the enzyme.

We made a set of three apple pectins with different DMs by partial saponification of the same pectin. These were digested to completion with the EPG, and the distribution of fragment lengths was determined by chromatography of the completely deacylated fragments after end-labeling with 2-aminopyridine. The distribution of fragment lengths expected can be predicted by a statistical model that takes into account the DM of the pectin sample, the minimum cluster size of adjacent non-esterified residues necessary for the enzyme to be able to act, and assumes a random pattern of esterification. The only unknown in predicted distribution calculations is the cluster size. Thus, the best fit to the experimental data should reveal the cluster size. As a check on these results we used a procedure recently described to quantitate the various GalA cluster sizes present in any pectin sample (Mort *et al.*, 1993). After digestion of the sample with the enzyme, clusters large enough for digestion should all be hydrolyzed. When treated in this way, our high, medium, and low DM pectins did not contain clusters of four or more GalA residues. But clusters of three or fewer remained. Our methods are general and should enable the examination of the substrate specificities of all EPGs and probably also of pectate lyases, and be especially interesting for comparison of specificities of various isozymes. In addition, knowing the exact specificity of the enzymes will be useful for structural studies of pectins.

EXPERIMENTAL

Materials

Trigalacturonic acid, imidazole, and sodium phosphate were purchased from Sigma Chemical Co. (St Louis, MO). Sodium borohydride, 2-aminopyridine, pectic acid, and apple pectin were purchased from Aldrich Chemical Co. (Milwaukee, WI). We calculated from the methoxyl and GalA content reported on the label that the DM of the pectin was 57. Ammonium acetate, sodium hydroxide, ammonium hydroxide, oxalic acid, and potassium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Oligogalacturonic acids of low degree of polymerization (2–25) were prepared by auto-hydrolysis of pectic acid in an autoclave as described by Robertsen (1986) and provided by Dr Edgar Miranda. Small cation-exchange columns ("Extract Clean" cartridges) were purchased from Alltech (Deerfield, IL).

The endopolygalacturonase (EPG) was purified as described by Maness & Mort (1989) from an *E. coli* clone (HB101/pAKC 213::Tn5-2) provided by Dr Arun Chattergee. The *E. coli* clone expresses the *peh* gene from *Erwinia carotovora* subsp. *carotovora* (Willis *et al.*, 1987) and accumulates the enzyme to a high level inside the periplasmic space. The enzyme was released by osmotic shock and then purified by affinity chromatography on crosslinked polypectate.

Preparation of pectins with different degrees of methylesterification

Two aliquots of 1 g of apple pectin were dissolved in 200 ml of water and their pH adjusted to 8 by adding one molar NaOH. From the methoxyl content quoted by the manufacturer, 1 g of the pectin contained 2.45 mol methoxyl groups. We added an additional 1.23 mol NaOH (12.3 ml of 100 mM NaOH) to one sample, and 1.84 mM NaOH (18.4 ml of 100 mM NaOH) to the other. Initially, amounts of alkali sufficient to bring the pH of the solutions to 11 were added and the remaining alkali was added slowly over (10–15 min) to keep the pH around 11. Five to 10 min after all of the alkali had been added, the samples were brought to pH 8 by the addition of acetic acid and then freeze dried.

Determination of the degree of methylesterification of pectins

The DM of the pectins was determined by the gas–liquid chromatographic method described by Maness *et al.* (1990), which is based on determining the percent of GalA residues converted to galactose during a strongly buffered $NaBH_4$ reduction. Non-methylesterified GalA residues are not reduced to galactose under these conditions.

Enzyme digestions

Ten-mg samples of pectins with DM 52, 32, and 16, as determined above, were placed into 20-ml screw cap vials, and suspended in 10 ml of 50 mM ammonium acetate buffer, pH 5.2. The samples were digested by 2 units of EPG for intervals of 24, 48, and 58 h. For the 58-h sample, 2 units of fresh EPG were added after 48 h and the digestion continued for another 10 h. At the beginning of the digestion, 3–4 drops of toluene were added to each sample in order to prevent microbial growth. All the samples were incubated at room temperature.

Formation of oligomers containing clusters of adjacent non-esterified galacturonic acid residues

Methylesterified GalA residues in the enzyme-hydrolyzed pectin samples were converted to galactose by

reaction with excess buffered $NaBH_4$ as described for the determination of the DM of the samples. The galactosyl linkages of the samples were then cleaved specifically using liquid HF (plus 1% water) solvolysis at −15°C for 30 min (Mort *et al.*, 1993). After the reaction mixture had been evaporated to dryness, the samples were hydrolyzed with 100 mM acetic acid overnight at 70°C to remove fluorine from the reducing ends of the oligomers. The resulting oligomers were members of the $GalA_nGal$ series and $GalA_n$galacturonitol series (see Results for explanation).

Chromatography

Before separation of the oligomeric products of the enzyme digestion, the oligomers were first saponified and then labeled with 2-aminopyridine so that they would chromatograph as a homologous series on ion exchange and could be quantitated by fluorescence intensity. Methyl esters in samples (2 mg) of pectin digested with EPG were saponified with 0.2 ml 0.1 M NaOH for 15 min at room temperature, neutralized with acetic acid, and dried by lyophilization. Nonreductive, 2-aminopyridine labeling was done as described by Maness *et al.* (1991), and the derivatives were purified by solid-phase extraction of 2-aminopyridine from the reaction mixtures.

The labeled GalA oligomer series was separated on a Dionex Bio-LC high-performance liquid chromatograph (Dionex Corp., Sunnyvale, CA). The system consisted of a gradient pump module, an eluant-degassing module, and a RF-535 variable excitation and emission wavelength fluorescence detector (Shimadzu, Kyoto, Japan). Separation was carried out on a CarboPac PA1 pellicular anion exchange column (4×250 mm). Labeled galacturonic acid oligomers were detected by fluorescence (excitation wavelength, 290 nm; emission wavelength, 350 nm). Eluant A was water and eluant B was 500 mM oxalate buffer, pH 6.0, prepared from oxalic acid and titrated to pH 6 with KOH. The column was equilibrated with 5% eluant B and a flow rate of 0.8 ml/min. Elution conditions were as follows: Eluant B increased linearly to 20% over 3–9 min, to 40% over 9–40 min, to 56% over 40–95 min, and to 70% over 95–110 min. This gradient was very similar to the one described by Hotchkiss & Hicks (1990). The system was allowed to equilibrate at initial conditions for at least 15 min prior to another injection.

The separation of the $GalA_n$–Gal and $GalA_n$–galacturonitol mixture of oligomers was carried out on the same HPLC apparatus by use of a PA1 pellicular anion exchange column (4×250 mM) and a pulsed amperometric detector. Eluant A was water and eluant B was 1 M sodium acetate, pH 5.2. The column was equilibrated with 3% eluant B and a flow rate of 0.8 ml/min. Elution conditions were as follows: Eluant B increased linearly to 20% over 3–30 min, to 50% over 30–50 min, and to 75% over 55–60 min. The amount of sample injected was 5 μg–1 mg. The system was equilibrated to the initial conditions for at least 15 min prior to another injection. NaOH (1 N) was added to the effluent from the column at 0.8 ml/min before the detector to ensure alkalinity for detection.

Chromatographic data were collected using custom-built data loggers described by Merz & Mort (1992). The data were quantitated and analyzed using Analog Connection Chrom (a software developed by Strawberry Tree Computer, Inc., Sunnyvale, CA) on a Macintosh Quadra 800 computer.

RESULTS AND DISCUSSION

If the distribution of GalA and methylesterified GalA residues in a long polygalacturonan sample is random, it is possible to predict the distribution of fragment lengths, which will be produced from it by an exhaustive digestion with EPG. The distribution of lengths will be determined by the distribution of distances between sites at which the enzyme can digest the pectin. For this prediction one only needs to know the DM of the pectin and the nature of the sites at which the enzyme can act. We assumed that the hydrolysis sites in the pectin consist of a certain minimum number of adjacent nonesterified residues of GalA. Presumably, the character of these sites is independent of the DM of the pectin. However, the abundance of these sites and hence the distribution of oligomer lengths generated by the enzyme depends on the DM. We therefore generated three samples of pectin differing only in their DM by partial saponification of a commercial apple pectin to test whether we could match the predicted range of fragment lengths produced by EPG digestion experimentally in all three cases. The only uncontrolled variable in the predictive model is the number of adjacent residues necessary for hydrolytic activity. Thus, comparing the experimental results to those predicted for a selection of sizes of GalA clusters likely to be required is expected to reveal the actual cluster size necessary for enzyme action.

Predictive model

To be able to predict the distribution of fragment lengths expected for EPG digestion of a pectin of any given DM, the pattern of esterification of the pectin must be known or assumed, as must be the nature of the sites at which the enzyme can act. We propose, as others have, that a minimum cluster size of non-esterified residues is necessary for hydrolysis. Grasdalen *et al.* (1988) and Westerlund *et al.* (1991) suggested that fruit pectins are randomly esterified. However, de Vries *et al.* (1986) proposed a more ordered pattern of esterification. Assuming a random distribution, Mort *et al.* (1993)

proposed a probability model to predict frequencies of cluster sizes of contiguous non-esterified GalA residues for any particular DM pectin. The experimental findings fit the model for some, but not all of the pectins tested. We chose an apple pectin for the present study which gave results that fitted the model quite well and implied random methylesterification. This model was extended as described below to allow prediction of fragment lengths produced from pectins by EPG.

We assume that the pattern of methylesterification is random. Thus, the probability of GalA occurring at any particular position in the polymer is [1−(DM/100)], which we designate p. We assume that the EPG needs a certain number (c) of adjacent non-esterified residues to be able to act. The probability of c non-esterified GalA residues occurring adjacent to each other is p^c. The probability that the stretch of c residues is **not** c contiguous non-esterified residues is $(1-p^c)$. In other words, all combinations of c adjacent residues under consideration that are not potential cut sites for the enzyme, occur with a probability of $(1-p^c)$. If we take a specific example as depicted in Fig. 1, we can test for the probability that a "window" of c residues (in this case $c = 4$) is a site which can be cleaved by the enzyme. We do not know how close to the non-reducing end of the polymer the enzyme can cut. However, we can simplify the problem by assuming that the enzyme, in this example, can digest in any window of non-esterified residues starting four or more residues away from the non-reducing end. To be able to predict the length of the oligomer so produced, we would also need to know where in the window the enzyme made the cut. Let us use the case in which it cuts in the center of the window of four residues. In this example, the shortest oligomer possible would be six residues long. The probability that the hexamer would be produced is dependent only on the probability that window A is a cut site. As explained above, this is p^c. That window A is not a cut site occurs with a probability of $(1-p^c)$. Moving the window one residue to the right, we ask the probability that window B is a cut site. Because of the random nature of the esterification, the answer is the same as it was for window A. The probability for the conditions that window A is not a cut site and window B is a cut site to occur simultaneously and result in formation of a heptamer, is the product of their individual probabilities, $(1-p^c) \times p^c$. The probability that window B is not a cut site for the enzyme would be $(1-p^c)$. Thus, the probability that an oligomer of eight residues is formed would be $(1-p^c)^2 \times p^c$. Upon extending this argument, one concludes that the probability of producing an oligomer n residues longer than a hexamer is $(1-p^c)^n \times p^c$, and that the probability of producing an oligomer of a particular length decreases by a factor of $(1-p^c)$ per residue increase in length. If the logarithms of the ratios of the quantities produced of oligomers of $6+n$ residues compared to oligomers of six residues are plotted against n, one should obtain a straight line with a slope of log $(1-p^c)$. Both the position in the window at which the enzyme cuts, and the proximity to the non-reducing end of the polymer of the closest window in which the enzyme can cut, would only affect the length of the shortest oligomer that could be formed, but would not affect the decrease in probability of formation of oligomers of increasing length. Thus, plots of log (relative oligomer abundance) vs. oligomer length would still have a slope of log $(1-p^c)$.

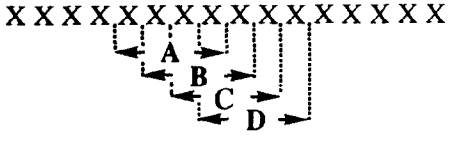

Fig. 1. A diagram of a specific example for the model. Each X represents a GalA residue which may or may not be methylesterified. The arrows represent windows of four residues because in the example we are assuming the enzyme needs four adjacent non-esterified residues for activity. The first window is placed with its start four residues away from the non-reducing end because we are assuming this is as close as the enzyme can bind to the end of the pectin. Our results show that the enzyme does actually need four adjacent non-esterified residues for activity and cleaves between the second and third residues. We do not know how close to the end of the pectin the enzyme can bind, but it is likely, from the ability of the enzyme to produce dimers and trimers of GalA in the limit digest, that it can bind in a window starting at the non-reducing end.

Because of the simplifications that we impose on the model, we cannot expect an exact match between all of the experimental and predicted points for the following reasons:

(1) The pectin chain is not an infinite chain of GalA residues. There are Rha residues interspersed on average every 30 residues.
(2) We do not know how close to the non-reducing end of the polymer a cut can occur. (The limit digest of pectic acid is predominantly GalA trimer for the EPG from *E. carotovora*.)
(3) We do not know how close to the reducing end of the polymer a cut can occur. Cuts close to this end of the polymer could produce fragments too small for additional cuts by the enzyme, but these fragments are not taken into account in the model.
(4) Pectins may not have a random pattern of esterification. De Vries *et al.* (1986) have suggested that newly synthesized fruit pectins may have a repeating pattern of $GalAMe_4$–GalA, but that the pattern loses regularity as methyl esters are lost during pectin maturation and commercial pectin isolation. If the esters are lost randomly, the pattern of esterification will become more and more random as more esters are lost. In the situation suggested by

De Vries *et al.* (1986), every fifth residue would have a probability of 1 that it is not esterified. Each remaining position would have a probability of 1.25 times the DM/100 that it is esterified since all of the esterification is distributed in four out of the five residues. Thus the probability of these residues **not** being esterified will be $(1-1.25\times DM/100)$. For three adjacent residues to be non-esterified, the probability would be $1\times (1-1.25\times DM/100)^2$ as opposed to the $(1-DM/100)^3$ expected for the totally random case. These equations predict that for high DMs, the frequency of cut sites for the enzyme would be lower in the non-randomly than in the randomly esterified pectins, but that in the low DM pectins it would be higher.

There is nothing we can do to change the model to account for non-randomness in the pattern of esterification (if it is present) since we do not know the pattern. To minimize the problem of not being able to account for oligomers formed from unpredictable cutting at the ends of the polymer, we disregarded the shortest oligomers and normalized to the abundance of the pentamer of GalA.

Preparation of pectins of various DMs with otherwise identical structures

The commercial apple pectin was saponified to two different degrees as described in the methods section and the DMs of the products were found to be 32±0.9 and 16±1.4 by the GLC method of Maness *et al.* (1990). By our analysis, the DM of the original sample was found to be 52±1.7. Native pectin in the fruit and pectins extracted carefully would have a higher DM. The deesterification caused during the commercial extraction process and our saponification would be expected to decrease the regularity of any pattern of esterification in the native pectin (de Vries *et al.*, 1986).

Distribution of fragment lengths after EPG digestion

Each of the samples was digested with purified EPG for 24, 48, or 48 h followed by 10 more after addition of more enzyme. The resulting oligomers were then saponified, labeled with 2-aminopyridine, separated on a PA1 column, and quantitated by integrating the fluorescence intensity associated with each peak. The relative abundances of the various members of the resulting homologous series of galacturonan oligomers were normalized to the amount of tetramer present in each sample to correct for differences in recoveries between samples. Figure 2 shows the distribution for the high DM pectin sample after the various times of digestion. No major difference was found between the three times. It was concluded that the pectin digestion by EPG was complete after 24 h.

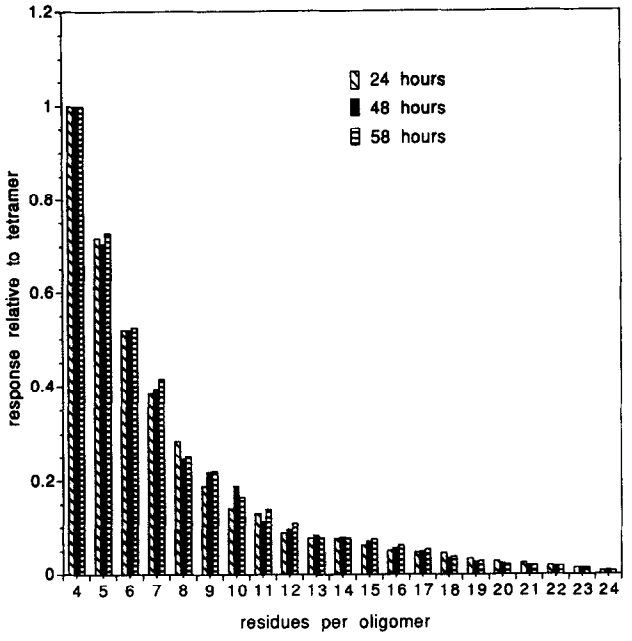

Fig. 2. Distibution of fragment lengths, normalized to the tetramer of GalA, produced by EPG digestion of the high DM pectin for 24, 48, and 58 h.

The distributions of fragment lengths produced from the three different DM pectin samples were plotted as the logarithms of the ratios of abundances of particular oligomers to that of the pentamer vs the number of residues in the oligomer and found to give fairly linear plots as shown in Fig. 3. Plots of the distributions predicted by the model were made for hypothetical pectins of the same DMs by assuming GalA cluster sizes required for enzyme digestion of 2, 3, 4, and 5 residues.

For the low DM pectin (DM = 16), the least squares fit experimental slope (−0.221, correlation coefficient R = 0.993) is closest to that expected for a cluster size of 5. However, the first three experimental points did not fall on a straight line, whereas the last 8 did. The slope of the line through the last 8 points is −0.251 (R = 0.998), which is half way between the predicted slope of −0.235 expected for a minimum cluster size of 5 and −0.299 expected for 4. For the medium DM pectin (DM = 32), the experimental data fit well those predicted for a minimum cluster size of 4. The experimental slope is −0.093 (R = 0.991) and that predicted for a cluster size of 4 is −0.104. The slope predicted for clusters of 3 is −0.164 and for 5 is −0.068. For the high DM pectin (DM = 52), the experimental slope (−0.035, R = 0.987) lies between those predicted for minimum cluster sizes of 3 (slope −0.051) and 4 (slope of −0.024). If only the longest 13 oligomers are plotted, the slope is −0.0263 (R = 0.994). If oligomers 5–12 are plotted, the slope is −0.0447 (R = 0.975). A minimum cluster size requirement of four GalA residues is quite consistent with the results, but is not certain given the divergence of the experimental results from those predicted by the model.

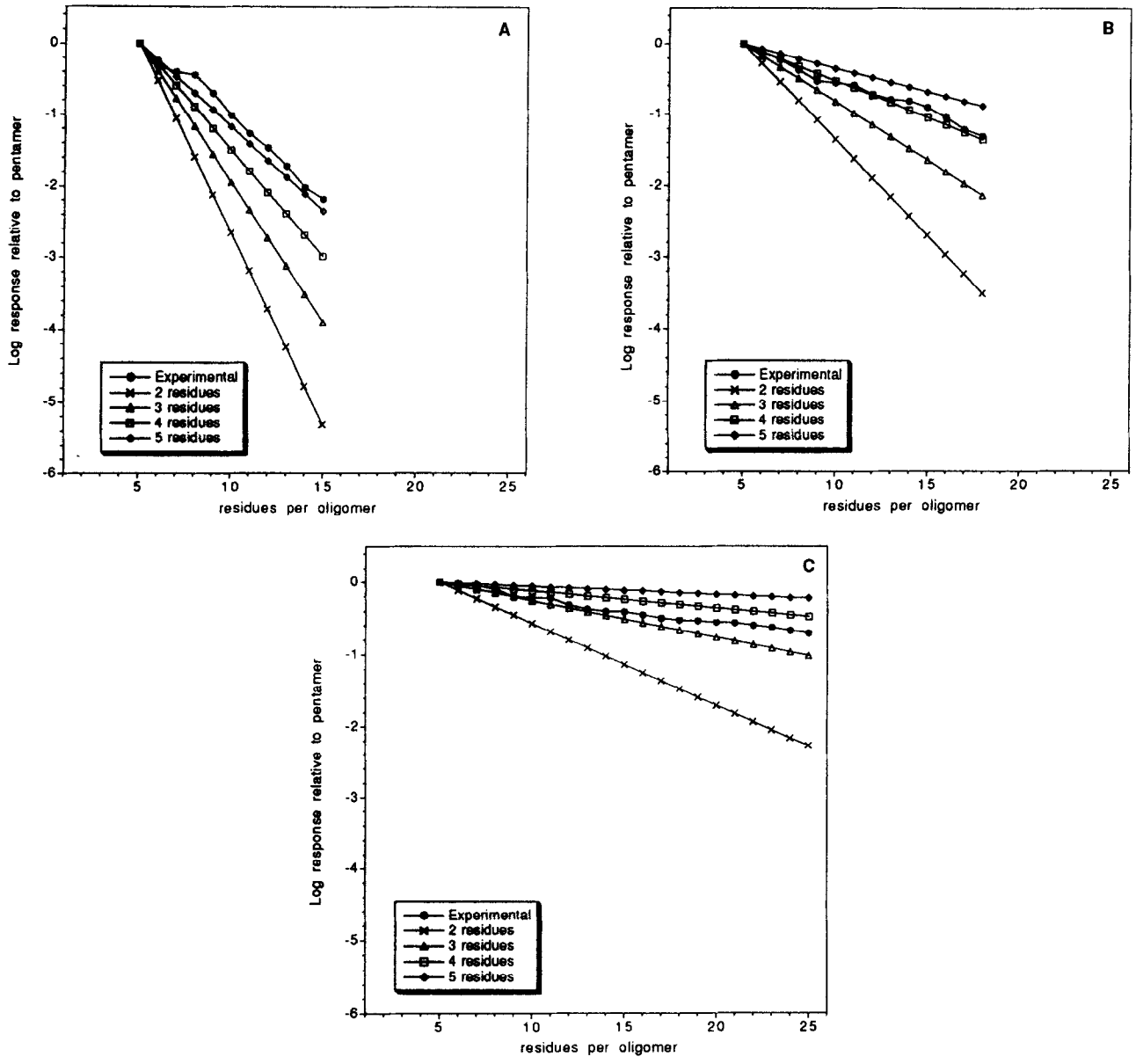

Fig. 3. Log plots of the abundance$_s$ of each fragment length produced by EPG digestion, normalized to the pentamer, predicted from the model and found experimentally, versus oligomer length for the three different DM pectins: (A) Low DM; (B) Medium DM; (C) High DM. The equation which describes the relationship is $\log(\text{Area}_n/\text{Area}_5) = (n-5) \times \log(1-p^c)$, where "Area$_n$" is the area of the particular oligomer peak, "Area$_5$" is the area of GalA$_5$, "p" is 1−(DM/100), and "c" is the number of adjacent non-esterified GalA residues needed for enzyme activity.

Determination of maximum cluster length resistant to EPG hydrolysis

We reasoned that if EPG were allowed to digest a pectin sample to completion, only clusters of GalA residues too short for enzyme activity would remain. A previous report (Mort *et al.*, 1993) described a method to quantitate directly the relative abundances of cluster sizes of adjacent non-esterified GalA residues in pectins. The same pectin preparations used above were digested to completion with EPG and processed to determine which cluster sizes remained. Undigested samples were also processed to show what cluster sizes were present initially. Digested and undigested samples were reduced with excess $NaBH_4$ to convert esterified GalA residues to galactose. This reduction would also be expected to convert the reducing ends of any oligo- or polysaccharide to alditols. In the enzyme-digested samples, one would expect a considerable amount of galacturonitol end groups to be formed. The products after the reduction were then cleaved by liquid HF solvolysis under controlled conditions (−15°C, 1% water in the HF, for 30 min) during which the galactosyl linkages were completely broken with very limited breakage of the galacturonosyl linkages. For the intact, reduced

pectin, this treatment left predominantly oligomers of GalA ending in a galactose residue, which represented the clusters of GalA residues in the original sample. For the digested samples, any oligosaccharide whose reducing end was generated by the enzyme would end in a galacturonitol residue. The number of GalA residues in such an oligomer would depend on where the hydrolysis site was in the window of residues necessary for the enzyme to act. If the enzyme needed four non-esterified residues and the hydrolysis site were between residues two and three, the oligomer would be GalA–galacturonitol. If it cleaved between residues three and four it would be GalA–GalA–galacturonitol.

The HF solvolysis products were separated by chromatography on a PA1 anion exchange column and quantitated by pulsed amperometry. Figure 4A shows the products from the low DM sample in which the pectin was not subjected to the enzyme hydrolysis before the reduction and solvolysis. Figure 4B shows a chromatogram of the products from the low DM pectin sample with enzyme digestion. Comparison of the two chromatograms reveals that the $GalA_n$–Gal oligomers in which $n \geq 4$ are not present after the enzyme digestion, but are present in the undigested sample. This difference indicates that clusters of four or more non-esterified GalA residues are hydrolyzed by the enzyme, and confirms the result of the product analysis described in the previous section.

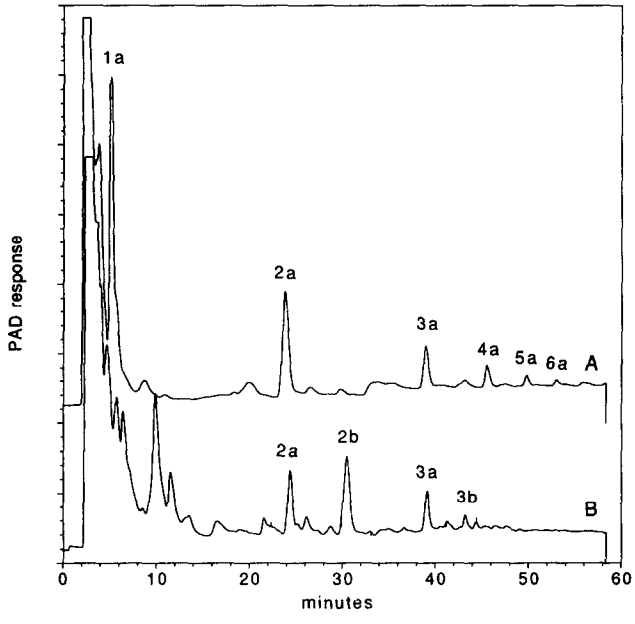

Fig. 4. (A) Chromatogram of the oligomers derived from the low DM pectin, reduced with excess $NaBH_4$, and then cleaved by HF in the presence of 1% water at −15°C. (B) Chromatogram of the oligomers derived from the low DM pectin, digested by EPG prior to reduction and cleavage by HF. The oligomers were detected by pulsed amperometry. Peak 1a: (GalA)–Gal; Peak 2a: $(GalA)_2$–Gal; Peak 3a: $(GalA)_3$–Gal; etc. Peak 2b: $(GalA)_2$–OH; Peak 3b: $(GalA)_3$–OH.

As expected, major peaks 2b and 3b are present in the enzyme-treated sample, but are not in the undigested sample. To confirm that these oligomers were $GalA_n$–galacturonitols, a mixture of $GalA_n$ oligomers was reduced with $NaBH_4$ and then chromatographed under the same conditions as were the non-reduced ones. Non-reduced (Fig. 5A) and reduced (Fig. 5B) oligomers of the same DP were slightly separated, with the reduced oligomers being eluted slightly later. Authentic trimer of GalA had the same retention time as peak 3 in Fig. 5A, and peak 3b in Fig. 5B was identified as $GalA_2$–galacturonitol by its co-elution with reduced pure trimer of GalA.

The results of the procedure described here with all three DM samples are summarized in Fig. 6. In all cases, clusters of four or more GalA residues were not found in the enzyme-digested samples; the enzyme could hydrolyze the pectin wherever four or more GalA residues occurred in sequence. By far the most abundant oligomer ending in galacturonitol in all cases was GalA–galacturonitol. This finding is a strong indication that the active site of the enzyme binds to a stretch of four non-esterified GalA residues and hydrolyses the bond between the two centermost residues. The small amount of digalacturonosyl–galacturonitol could be generated from a cluster of five non-esterified GalA residues or by lack of exact specificity of the enzyme. Unfortunately, any free galacturonitol was undetectable because of interferences in the chromatograms.

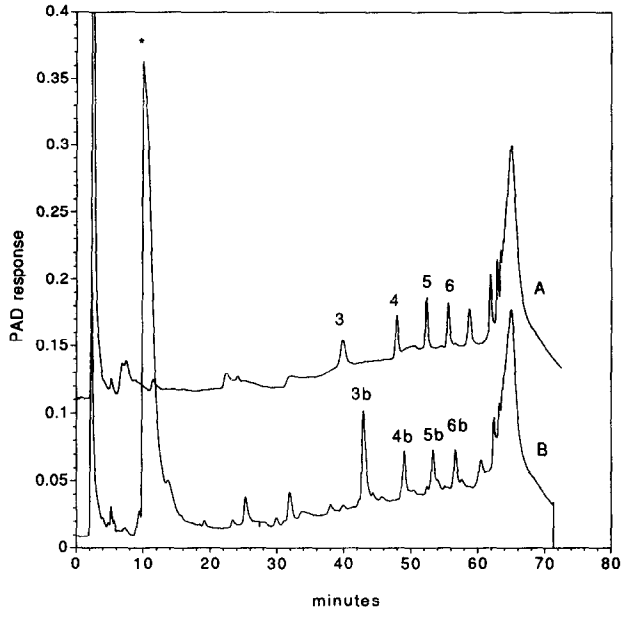

Fig. 5. (A) Chromatogram of a mixture of non-reduced oligomers of GalA. Peak 3: $GalA_3$; peak 4: $GalA_4$, etc. (B) Chromatogram of oligomers of GalA after reduction with $NaBH_4$. Peak 3b: $GalA_3OH$; Peak 4b: $GalA_4OH$, etc.; *unidentified. Chromatogram B was from a mixture of the same sample as in A after reduction plus some authentic $GalA_3$ after reduction.

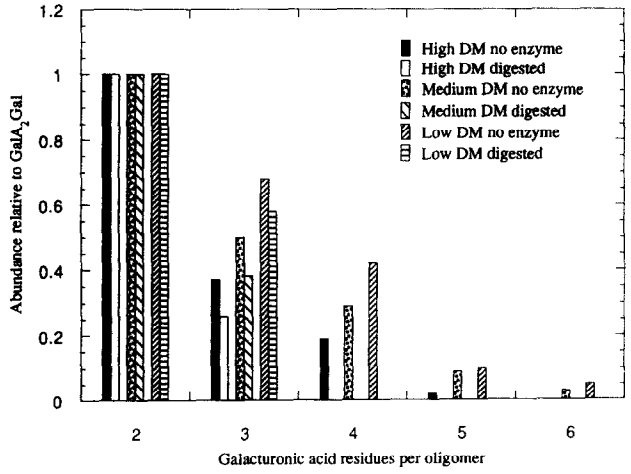

Fig. 6. A plot of the relative abundances of the various $GalA_nGal$ oligomers obtained from the three different DM pectins with and without EPG digestion.

CONCLUSIONS

The distributions of fragment lengths produced by complete digestion of pectin of several DMs with the EPG of *E. carotovora* were close to those predicted if the enzyme needs four adjacent GalA residues for activity.

Clusters of non-esterified GalA residues of four or more within partially-esterified pectins are hydrolyzed by the enzyme.

Thus, two different methods point to the need for four adjacent non-esterified GalA residues in order for EPG of *E. carotovora* to act.

ACKNOWLEDGEMENTS

This research was supported in part by DOE grant DE-FG02-93ER20102 and has been approved for publication by the director of the Oklahoma Agricultural Experiment Station. We wish to thank Dr Margaret Pierce for providing valuable comments on the manuscript.

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