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MODES OF ACTION OF CARROT AND PEACH EXOPOLYGALACTURONASES

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; *Prunus persica*; Rosaceae; peach; exopolygalacturonases.

Abstract—Exopolygalacturonases from carrots and peaches were extracted and partially purified. The enzymes differ in molecular weight, pH optimum and response to cations. Neither enzyme, however, completely hydrolyzed citrus pectate and polygalacturonates and the limits of degradation were similar. Cleavage of digalacturonate was very slow by both enzymes, but the rates of hydrolysis increased with substrate size. Rates of cleavage were maximum for polygalacturonate with a degree of polymerization of 20. The peach exopolygalacturonase exhibited greater affinity for the substrates, but the extent of binding to both enzymes increased with substrate chain length.

INTRODUCTION

The term "pectic substances" refers to a group of heterogeneous polysaccharides found in all higher plants [1]. The main constituent is α-D-(1 → 4)-linked galacturonic acid in which the carboxyl groups may be esterified with methanol. Highly esterified polymers are known as pectin and unesterified polymers as pectic acid. Evidence has been presented that neutral monosaccharides may interrupt the polygalacturonate chain [2] and, in other cases, form branch points [3]. The galacturonate-rich polysaccharide is usually accompanied by neutral polysaccharides such as arabinans and galactans [1, 4]. It has long been thought that the difficulty in separating the neutral polysaccharides from the polygalacturonate was evidence for covalent linkages between them. Keegstra *et al.* [5] recently showed that this is indeed the case in walls of suspension cultured sycamore cells.

Studies of partial hydrolysis of pectin have produced much of the evidence concerning its structure. The availability of pectic enzymes has circumvented some of the problems associated with acid hydrolysis and provided the advantage of specificity in degradation. Most of the enzymes used in structural studies on pectin have been isolated from microbial sources, but pectic enzymes with useful specificities are also found in higher plants. The plant enzymes function only on pectate and are usually endopolygalacturonases hydrolyzing interior bonds of the substrate in more or less random manner. We recently described an exopolygalacturonase from peaches which hydrolyzes the pectate molecule in a uniform manner, beginning at the nonreducing end of the polymeric chain. Subsequently, we observed that this enzyme does not hydrolyze citrus pectate completely and limit products are formed. The peach enzyme is, therefore, similar to carrot exopolygalacturonase [7] in this regard. The present study was undertaken to compare the properties of the two exopolygalacturonases and, in particular, their action on oligogalacturonates prepared from citrus pectate.

RESULTS

Characterization of substrates. The oligogalacturonates were tentatively identified on the basis of their elution order from the DEAE-Sephadex A-50 column. Their degrees of polymerization were confirmed by PC and the ratios of anhydrogalacturonic acid (AGA) to reducing groups were determined (Table 1). A plot of $\log_{10} R_{Ga} \times 10$) against

Table 1. Chromatographic and reducing properties of the substrates

Substrate	R_{Ga}*	AGA†/reducing groups
Digalacturonate	0·75	2·1
Trigalacturonate	0·58	3·2
Tetragalacturonate	0·45	4·1
Pentagalacturonate	0·35	5·0
Hexagalacturonate	0·26	5·9
Heptagalacturonate	0·19	6·8
Octagalacturonate	0·14	8·2
Polygalacturonate III	0	13
Polygalacturonate II	0	20
Polygalacturonate I	0	79
Pectate	0	201

* Descending PC on Whatman 3M paper using the epiphase of ethyl acetate–AcOH–H_2O (10:5:6). R_{Ga} = ratio of the distances moved by the oligomer and galacturonic acid. †Anhydrogalacturonic acid.

the degree of polymerization yielded a straight line, indicating a homologous series of oligosaccharides. R_{Ga} is the ratio of the distances moved by the oligomer and galacturonic acid on paper chromatograms.

The polygalacturonates (PGA I, II and III) represent fractions of enzymatically hydrolyzed pectate. They are numbered in the order of decreasing MW's and increasing solubility. The solubility properties of these preparations were described earlier [8]. It should be emphasized that, in contrast to the oligogalacturonates, each polygalacturonate fraction was polydisperse, i.e. consisted of a range of molecular species.

Properties of the enzymes. We described some of the properties of peach exopolygalacturonase earlier [6]; the carrot enzyme was partially characterized by Hatanaka and Azawa [7]. The two enzymes are similar in some respects, but have important differences. Both enzymes cleave galacturonate monomers from the nonreducing ends of the substrate molecules. The enzymes used in this study were free from endopolygalacturonases, judging from the low rates of change in the viscosity of pectate solutions. The pH optima were 4·7 and 5·5 for the carrot and peach enzymes, respectively, and were independent of substrate size. The MW's of the carrot and peach enzymes were 55000 and 68000, respectively. The enzymes also differed in their responses to cations in the reaction mixture. The peach exopolygalacturonase was activated by Ca^{2+} and Sr^{2+} while the carrot enzyme was not activated by low concentrations of a large number of monovalent and divalent cations. Relatively low concentrations of salts were inhibitory to the carrot enzyme with 30% inhibition by 0·06 M NaCl.

Kinetics. Values of K_m and V (maximum velocity) for the enzymes acting on the homologous series of the oligogalacturonates and the four polygalacturonates were determined at the optimum conditions for each enzyme (Table 2). The rates of cleavage were slowest for digalacturonate and fastest for PGA II for both enzymes. The substrate size for maximal activity would be slightly smaller than PGA II. The K_m's for peach exopolygalactur-

Table 2. Kinetic parameters of the enzymatic hydrolysis of oligogalacturonates and polygalacturonates

Substrate	Carrot exopolygalacturonase K_m	Carrot exopolygalacturonase Maximum velocity	Peach exopolygalacturonase K_m	Peach exopolygalacturonase Maximum velocity
	M × 10^6		M × 10^6	
Digalacturonate	2800	0·9	2200	0·3
Trigalacturonate	970	2·2	630	2·0
Tetragalacturonate	352	3·3	197	4·0
Pentagalacturonate	238	4·6	124	4·6
Hexagalacturonate	187	6·6	91	5·1
Heptagalacturonate	159	6·8	80	5·3
Octagalacturonate	140	7·7	74	5·6
Polygalacturonate III*	120	9·1	68	8·3
Polygalacturonate II†	29	10·0	38	9·0
Polygalacturonate I‡	16	4·3	28	7·1
Pectate§	12	3·3	15	2·9

* Assuming D.P. = 13 (D.P. = degree of polymerization). † Assuming D.P. = 20. ‡ Assuming D.P. = 79. § Assuming D.P. = 201.

onase were generally lower than for the carrot enzyme, but the extent of substrate binding to both enzymes increased with substrate chain length.

Extent of hydrolysis of pectate and the polygalacturonates. The pectate used in these studies was only 70% AGA and therefore contained a substantial amount of other polysaccharides. This substrate was hydrolyzed 18 and 24% by the peach and carrot polygalacturonases, respectively. The reactions were complete after several hr with the enzymes employed. Because of the possibility that reaction might have been terminated by enzyme inactivation, additional enzyme was added after the reaction ceased. The new enzyme did not increase the extent of hydrolysis, however. This was verified by conducting a large scale reaction to apparent limit hydrolysis and then isolating the undegraded substrate by addition of 3 vols of 95% EtOH and centrifugation. The precipitate was dissolved in H_2O and served as the substrate in a new reaction; neither enzyme produced further degradation.

The extent of hydrolysis was much higher for the polygalacturonates prepared by the action of fungal endopolygalacturonase on pectate (Table 3). Degradation was greatest for PGA II rather than for PGA III. This may be explained by the fact that in the case of PGA III, a rather shortchain substrate, the accumulation of digalacturonate becomes an important factor. Nevertheless, chromatographic examination of the reaction products of PGA III hydrolysis revealed that about 24% of the original substrate had not been cleaved. This was confirmed by adding fungal polygalacturonase to the reaction mixture after exopolygalacturonase action stopped. Incubation with the fungal enzyme increased the reducing groups by 20%. The limit products of PGA I and PGA II cleavage by carrot exopolygalacturonase were also further hydrolyzed by the fungal enzyme. This is evidence that carrot exopolygalacturonase degrades the polygalacturonates to limit fragments which are susceptible to hydrolysis by an endopolygalacturonase.

Table 3. The limits of hydrolysis of pectate and polygalacturonates by carrot and peach exopolygalacturonases

Substrate	Exopolygalacturonase cleavage (%)	
	Carrot	Peach
Pectate	24	18
Polygalacturonate I	57	54
Polygalacturonate II	83	76
Polygalacturonate III	74	73

Extent of hydrolysis of the oligogalacturonates. The entire series of oligogalacturonates were enzymatically hydrolyzed to apparent completion and the products were analyzed by chromatography on DEAE-Sephadex A-50. The reaction products formed from each substrate were similar for the two enzymes. The major product of hydrolysis of the higher oligogalacturonates was galacturonic acid, but relatively high levels of the trimer and low levels of the dimer also accumulated. The ratio of trimer to monomer increased as the chain length of the substrate decreased. However, the trimer and dimer were hydrolyzed by both enzymes as indicated by the accumulation of low levels of galacturonic acid in the reaction solutions of these substrates.

It was difficult to determine whether cleavage of the trimer and dimer proceeded to completion because the reactions were very slow. There is the possibility that portions of these oligogalacturonates represent enzyme limit products containing anomalous linkages or chain branching. To test the hypothesis, a large scale reaction mixture of tetragalacturonate and carrot exopolygalacturonase was incubated for 6 hr. The rate of galacturonate appearance had decreased to almost zero. Chromatographic analysis of an aliquot of the reaction mixture showed that all of the tetramer had been hydrolyzed. The reaction products consisted of 45% monomer, 19% dimer and 36% trimer (% of the total AGA content). These levels of the three products are possible only if some of the original tetragalacturonate had been completely hydrolyzed to galacturonate.

The remaining solution was treated with $SrCl_2$ and 2 vol. of EtOH to precipitate the dimer and trimer and thereby separate them from the large amount of galacturonate. The ppt was collected and dissolved in H_2O. The two components were then separated by chromatography on DEAE-Sephadex A-50 and isolated according to the procedure for the preparation of oligogalacturonates. Solutions of these oligogalacturonates were deionized and tested as substrates for carrot exopolygalacturonase. The rates of their cleavage were identical to those for standard dimer and trimer.

Another factor that might be involved in the reaction mechanism, and that might explain the slow cleavage of di- and tri-galacturonate, is the possibility of transferase action. Reaction mixtures of carrot exopolygalacturonase and digalacturonate did not produce higher oligogalacturonates, however. Similarly, reaction of the trimer did not yield the tetramer. Furthermore, the inclusion of mono-, di-, or tri-galacturonate to reaction mixtures of the polygalacturonates did not inhibit hydrolysis.

DISCUSSION

A serious problem associated with structural studies on pectic substances is the resistance of the galacturonide bond to acid hydrolysis [9]. Treatment of pectate with strong acids at high temperatures leads to decarboxylation of the monomers [10]. For these reasons, enzymatic hydrolysis has become a useful tool. The first enzymes used were endopolygalacturonases which randomly cleave the polygalacturonate chain to oligogalacturonates and ultimately to galacturonate. If other monosaccharides are present in the pectate molecule, these enzymes should yield limit products consisting of neutral sugars attached to galacturonate.

More recently, end-group cleaving pectic enzymes have become available. These enzymes include exopolygalacturonate lyases which cleave dimer units from the reducing ends of the pectate molecule [11, 12]. Exopolygalacturonases have been isolated from microbial sources [13–16] as well as from carrots and peaches. In contrast to the action of exopolygalacturonate lyases, the exopolygalacturonases characterized so far hydrolyze the substrate from the nonreducing ends and usually yield galacturonate; the enzyme of *Erwinia* is an exception which forms digalacturonate [16]. Endo [17] has reported that fungal exopolygalacturonase completely hydrolyzed the glycosidic linkages in citrus pectate. We have now confirmed the results of Hatanaka and Ozawa [7] that carrot exopolygalacturonase does not degrade pectates completely. Furthermore, we have demonstrated that the peach enzyme also forms limit products.

It is reasonable to assume that partial hydrolysis of a polysaccharide by an exoenzyme specific for the predominant linkage in the polymer is due to blockage of enzymatic action by anomalous features. These features could include branching of the main chain or interruption of the main chain by other monomers. Another possibility that has been suggested for enzyme blockage is the presence of intermolecular divalent cation bridges [18]. However, we found that the limits of cleavage were nearly identical for the carrot and peach enzymes, even though Ca^{2+} was added to the peach enzyme reaction mixtures.

A possible physiological function for the exopolygalacturonase in peaches is the degradation of pectin during fruit ripening. This process is preceded by the action of pectinesterase which deesterifies the pectin and renders it susceptible to attack by the polygalacturonases. Presumably the degradation of the glycosidic linkages is initiated by the endopolygalacturonase which is also present in ripening peaches. The oligogalacturonates formed by the random-cleaving enzyme are hydrolyzed to galacturonate by the exopolygalacturonase. A complication in this mechanism is that the exopolygalacturonase exhibits maximal activity on relatively large substrates and cleaves the lower oligogalacturonates slowly. The rate of cleavage of digalacturonate is especially slow and it is, therefore, unlikely that this is a pathway for complete hydrolysis of pectin.

The occurrence of an exopolygalacturonase in carrots is even more difficult to explain because it is not accompanied by an endopolygalacturonase [7]. If the carrot enzyme is involved in cell wall alteration, it must act on terminal blocks of 1, 4-linked galacturonic acid. Possibly the peach exopolygalacturonase has a similar function rather than acting in conjunction with the endopolygalacturonase in pectin hydrolysis. Neither carrot nor peach pectin have been characterized in enough detail to know whether terminal links of galacturonic acid exist. Branching of the main chain has been reported to occur in the pectins of mustard seed [19] and of certain plant gums [4]. Blocks of galacturonosyl residues may also be linked to the main chain through rhamnosyl residues. If galacturonosyl branches exist in pectins, their removal by exopolygalacturonases may be important in phenomena such as pectin solubilization and hence fruit softening.

EXPERIMENTAL

Preparation of polygalacturonates. Pectic acid of citrus origin (Sigma)* was purified by EtOH pptn as described earlier [8]. A series of 3 polygalacturonates were prepared from pectic acid by controlled hydrolysis using fungal pectinase (Sigma) followed by pptn [8].

Quantitative analysis of oligogalacturonates. The method for measuring the individual oligogalacturonates is based on their separation by chromatography on DEAE-Sephadex A-50 [20]. The soln. of oligogalacturonates to be analyzed was adjusted to pH 6 and applied to a 2·5 × 45 cm column of the gel suspended in H_2O and adjusted to pH 6. Elution was performed with 4 l of linear gradient 0·05–0·20 M NaCl. Ten ml fractions were collected and analyzed for AGA.

Preparation of oligogalacturonates. 1% Pectic acid (1 litre) adjusted to pH 5 and warmed to 30°, was treated with 200 mg of fungal pectinase dissolved in 20 ml of 0·15 M NaCl. The soln was immediately heated to 100°, and then cooled and clarified by centrifugation. The oligogalacturonates were then pptd by addition of 10 g $SrCl_2$ and 2 vol. of 95% EtOH. The ppt was collected by centrifugation, dissolved in 200 ml of H_2O and the Sr^{2+} was removed by addition of Dowex 50 (H^+) to pH 2. After removal of the resin, the soln was neutralized with NaOH and applied to a 10 × 70 cm column of DEAE-Sephadex A-50 previously adjusted to pH 6 and washed with H_2O. The column was first developed with 4 l. H_2O and then 4 l. of 0·05 M NaCl. The oligogalacturonates were then eluted by 5 l. of 0·1 M NaCl, 6 l. of 0·125 M NaCl, 6 l. of 0·15 M NaCl, 8 l. of 0·175 M NaCl and finally 0·2 M NaCl.

Fractions (20 ml) were collected and analyzed for AGA by the carbazole method [22]. A total of 8 peaks was obtained. The fractions corresponding to each peak of AGA were combined and evaporated under vacuum to about 200 ml. Oligogalacturonates were pptd by addition of $SrCl_2$ and EtOH, then dissolved in a small vol of H_2O and deionized using Dowex 50 (H^+).

Assay for exopolygalacturonase. Enzyme activity was determined by measuring the increase in reducing groups during the reaction. The reaction mixture consisted of 0·5 ml 1% substrate adjusted to the appropriate pH, 0·1 ml of enzyme in 0·15 M NaCl and H_2O to give a total vol. of 1 ml; 0·1 ml of 0·01 M $CaCl_2$ was included in the soln in the assay for peach exopolygalacturonase. A blank consisting of boiled enzyme was run with each sample. The samples were incubated at 37° for 1 hr and the reactions were stopped by heating at 100° for 3 min. 0·5 ml of each soln was then analyzed for reducing groups by the arsenomolybdate method [21]. A unit of activity is defined as that amount which forms 1 μmol of reducing groups per hr.

Extraction of exopolygalacturonases. Peach exopolygalacturonase was extracted from ripe Cardinal peaches and partially purified according to the procedure described earlier [6]. The sp. act. of the preparation was 16.

The carrot enzyme was prepared by a modification of the procedure described by Hatanaka and Ozawa [7]. Carrot tissue (1 kg) was sliced and blended in 1 liter of 1 M NaCl using an homogenizer. The homogenate was adjusted to pH 6 and stirred 1 hr at 3°. The supernatant obtained on centrifugation at 8000 *g* for 30 min was treated with $(NH_4)_2SO_4$ at 75% of satn. The ppt was collected by centrifugation, dissolved in 50 ml of 0·15 M NaCl and dialyzed 18 hr against 0·15 M NaCl. Proteins in the dialyzed soln were then fractionated with $(NH_4)_2SO_4$; the ppt obtained between 40 and 70% satn. with $(NH_4)_2SO_4$ was collected and dissolved in 10 ml 0·15 M NaCl. After dialyzing this soln against 0·15 M NaCl, it was applied to a 2·5 × 90 cm column of Sephadex G-100 in 0·15 M NaCl. A single peak of activity was obtained. The fractions containing the enzyme were combined and concentrated to about 5 ml. The sp. act. was 18.

Other methods. Anhydrogalacturonic acid (AGA) concs were determined by the carbazole method [22]. Reducing groups were measured by the arsenomolybdate procedure [21]. The standard curves for both methods were obtained using A grade galacturonic acid monohydrate (Calbiochem).

The MW's of the enzymes were determined by gel filtration using a 2·5 × 90 cm column of Sephadex G-100 in 0·15 M NaCl at pH 6. The reference proteins were bovine serum albumin dimer and monomer, ovalbumin and cytochrome *c*.

* Reference to a company or product does not imply approval or recommendation of the product by the USDA to the exclusion of others that may be suitable.

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