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TWO FORMS OF POLYGALACTURONASE IN TOMATOES

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SUMMARY

Two polygalacturonidases, (poly--1, 4-galacturonide glycanobydrolase, EC 3.2.1.15) (polygalacturonases I and II) have been separated from extracts of ripe tomatoes by chromatography on DEAE-Sephadex A-50. Polygalacturonase II was the predominant enzyme in all of the samples examined. The enzymes differed in stability with respect to both temperature and pH. The molecular weights determined by gel filtration were 84 000 and 44 000 for polygalacturonases I and II, respectively. The activities of both enzymes were shifted to the acid side as the substrate size decreased and on addition of NaCl to the reaction mixture, but polygalacturonase I was less dependent on these factors. At levels of enzymes forming reducing groups at equal rates, polygalacturonase II was much more effective than polygalacturonase I in reducing the viscosity of pectic acid.

INTRODUCTION

The first evidence of more than one form of polygalacturonase (poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15), in tomatoes was the incomplete inactivation of the activity by heat reported by McColloch and Kertesz[1]. Most of the activity in crude extracts was destroyed at relatively low temperatures, while part of it survived heating to 90 °C. Patel and Phaff[2] confirmed the presence of a heat-stable component, but on the basis of limited studies they concluded that it was similar to the heat-unstable enzyme in its action on pectic acid. Both polygalacturonases appeared to be random-cleaving enzymes.

A number of other characteristics of tomato polygalacturonase have been interpreted in terms of several forms of this enzyme. For example, its optimum pH for hydrolysis of pectic acid is about 4.5 (ref. 1). The hydrolysis of oligogalacturonides occurs optimally not only at pH 4.5 but also at about pH 3.0 (ref. 2). The two separate peaks of activity have been attributed to different polygalacturonases. Furthermore, the hydrolysis of low molecular weight substrates is partly inhibited by pectic acid[3]. The inhibition is obtained with low levels of pectic acid, and the residual activity is not blocked even by excesses of pectic acid, suggesting at least two enzymes. McColloch[4] has used electrophoresis to demonstrate the existence of two polygalacturonases

in tomato extracts, but the yields of the enzymes were too low to determine their properties.

We have now achieved separation of two peaks of polygalacturonase activity in crude extracts of ripe tomatoes by column chromatography. This paper describes the method for separating the enzymes and some of their properties.

MATERIALS AND METHODS

1 kg of ripe tomato tissue ('Marion' variety) was homogenized with 1 l of cold water. The insoluble fraction was recovered by centrifugation at 8000 × *g* for 20 min at 3 °C. This residue was washed by dispersing it in 2 l of cold water, with the aid of a Polytron* homogenizer (Brinkmann Instruments). The suspension was centrifuged, and the supernatant was discarded. The residue was resuspended in 1 l of cold 1.0 M NaCl. The pH of the sample was adjusted to 6 by addition to 0.5 M NaOH. After the suspension had been stirred for 3 h at 3 °C, it was centrifuged, and the supernatant was saved. The proteins in this solution were precipitated with solid $(NH_4)_2SO_4$ at 75% of saturation. The precipitate was collected by centrifugation, dissolved in 30 ml of 0.2 M NaCl, and dialyzed overnight against 0.2 M NaCl.

The dialyzed crude extract of tomato polygalacturonase was adjusted to pH 5.5, and the entire sample was applied to a 10 cm × 65 cm column of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals), previously equilibrated with a solution containing 0.15 M NaCl and 0.05 M sodium acetate, pH 5.5. Elution was performed with 0.15 M NaCl and 0.05 M sodium acetate, pH 5.5, and 20-ml fractions were collected. A typical elution pattern is presented in Fig. 1. The second peak was always

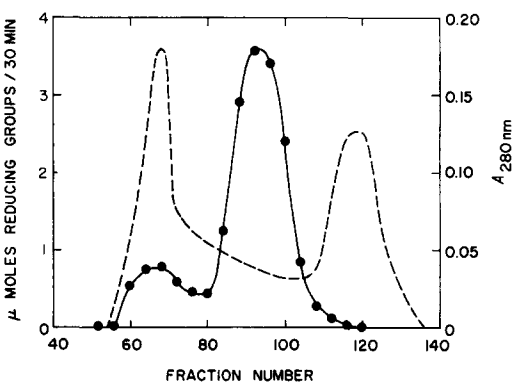

Fig. 1. Chromatography of tomato polygalacturonases on DEAE-Sephadex A-50. ●—●, polygalacturonase activity; — — —, protein concentration.

much larger than the first peak. Fractions 60–70 (polygalacturonase I) and fractions 88–100 (polygalacturonase II) were combined, and each solution was concentrated to about 10 ml by ultrafiltration, using PM-10 membranes (Amicon Corp.). The concentrated enzymes were dialyzed against 0.15 M NaCl.

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Dept of Agriculture to the exclusion of others that may be suitable.

Polygalacturonase was assayed by measuring the formation of reducing groups according to the method described earlier[3]. The reaction mixture contained 0.5% substrate and was incubated at 37 °C.

The stabilities of the enzymes were determined by diluting them to equal levels with 0.15 M NaCl and heating them over a wide range of temperatures for 5 min. The solutions were cooled, and the residual activities were measured. The effect of pH on the stability to heat was determined by diluting the enzymes with 0.1 ml of 0.05 M sodium acetate solutions, with pH over the range of 3–6, before heating. The heated solutions were then further diluted with 0.2 ml of 0.2 M acetate, pH 4.5, before they were assayed.

RESULTS

Stability to heat

The temperatures for 50% inactivation were 78 and 57 °C for polygalacturonases I and II, respectively. Polygalacturonase I was completely inactivated at 90 °C, and polygalacturonase II, after 5 min at 65 °C. When the pH of the enzyme solutions was varied before heating, polygalacturonase I was most stable at pH 4.3, while polygalacturonase II was most stable at pH 5.6.

Molecular weights

The molecular weights of the enzymes were estimated by analytical gel filtration[5] on a 2.5 cm × 95 cm column of Sephadex G-100 equilibrated with 0.2 M NaCl and standardized with bovine serum albumin monomer and dimer, ovalbumin, and cytochrome *c*. Molecular weights of 84 000 and 44 000 were obtained for polygalacturonases I and II, respectively.

Effect of pH and NaCl concentration on hydrolysis of pectic acid

The optimum pH for hydrolysis of pectic acid by tomato polygalacturonase is at about 4.5(refs. 1, 3). When the two enzymes were assayed at pH 4.5 in the absence of added NaCl, only polygalacturonase I hydrolyzed pectic acid (Table I). Maximum activity for polygalacturonase I at pH 4.5 was obtained in the presence of 0.2 M NaCl.

TABLE I

EFFECT OF NaCl CONCENTRATION ON THE ACTIVITIES OF POLYGALACTURONASES I AND II

The reaction mixtures consisted of 0.05 ml of polygalacturonase solution, 0.95 ml of NaCl solution and 1 ml of 1% pectic acid, pH 4.5. Other experimental details are described in the text.

NaCl (M)	*μmoles reducing groups/h*	
	Polygalacturonase I	*Polygalacturonase II*
0	0.34	0
0.025	0.42	0.05
0.05	0.53	0.17
0.10	0.75	0.51
0.15	1.15	0.70
0.20	1.23	0.93
0.25	1.12	1.22
0.30	1.01	1.20

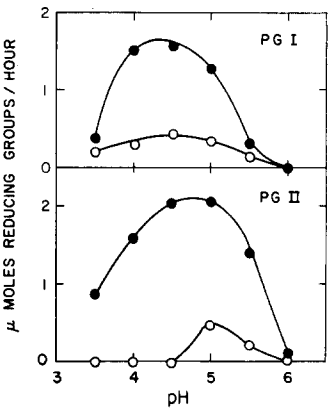

Fig. 2. Effect of pH on the activities of polygalacturonases I and II. ○—○, in the absence of added NaCl; ●—●, in the presence of 0.25 M NaCl.

Activation of polygalacturonase II required higher concentrations of NaCl, with a maximum response at about 0.3 M NaCl. Polygalacturonase I hydrolyzed pectic acid between pH 3.5 and 5.5 in the absence of added NaCl (Fig. 2). Addition of NaCl to the reaction mixture increased the rate of hydrolysis at all pH values and shifted the pH optimum slightly to the acid side. Hydrolysis of pectic acid in water by polygalacturonase II occurred only at pH 5 to 5.5. As in the case of polygalacturonase I, NaCl activated polygalacturonase II at all pH values below 6, but most pronouncedly at low pH.

Effect of substrate size

The rates of pectic acid hydrolysis by the two polygalacturonases were compared to those for the three polygalacturonic acids described previously[3]. The polygalacturonic acids were prepared by partial enzymatic hydrolysis of pectic acid. They have been designated polygalacturonic acid I, polygalacturonic acid II, and polygalacturonic acid III in the order of increasing solubility and, therefore, decreas-

TABLE II

EFFECT OF SUBSTRATE SIZE ON THE RATES OF CLEAVAGE BY POLYGALACTURONASES I AND POLYGALACTURONASES II

The reaction mixtures consisted of 0.05 ml of polyglacturonase solution, 0.95 ml water or 0.45 ml water and 0.5 ml 0.8 M NaCl, and 1 ml of 1% substrate adjusted to pH 4.5. Other experimental details are described in the text. The substrates are listed in the order of decreasing molecular size.

Substrate	*μmoles reducing groups/h*			
	Polygalacturonase I		*Polygalacturonase II*	
	Water	*0.2 M NaCl*	*Water*	*0.2 M NaCl*
Pectic acid	0.35	1.26	0	1.06
Polygalacturonic acid I	1.32	1.51	1.37	1.62
Polygalacturonic acid II	1.80	1.80	1.51	1.76
Polygalacturonic acid III	1.46	1.51	0.65	1.06

ing molecular weight. The activity of polygalacturonase I was less dependent on the molecular weight of the substrate than that of polygalacturonase II (Table II). Furthermore, polygalacturonase II was activated more than polygalacturonase I by NaCl with all of the substrates, but especially pectic acid and polygalacturonic acid III. As the molecular weight of the substrate decreased, the activities of both enzymes shifted to the acid side in a manner described earlier[3] for unfractionated tomato polygalacturonases. Polygalacturonase I was more active in the absence of NaCl at low pH, and activation by NaCl was greater for polygalacturonase II. Broad peaks of activity extending from pH 1.5 to 5.5 were obtained for both enzymes acting on polygalacturonic acid III. The hydrolysis of polygalacturonic acid III was inhibited by pectic acid identically for the two polygalacturonases.

Effects of the enzymes on the viscosity of pectic acid

The rates of appearance of reducing groups were compared to the changes in viscosity of pectic acid during enzymatic hydrolysis. Levels of the two polygalacturonases were selected to yield identical rates of reducing-group formation at a given concentration of NaCl and pH. The results obtained for 0.75% pectic acid at pH 4.5 and in the presence of 0.10 M NaCl are shown in Fig. 3. Polygalacturonase II catalyzed a rapid decrease in the viscosity of the substrate during the first 30 min of reaction. The viscosity of the solution gradually leveled off on continued incubation but was still decreasing after 3 h. While polygalacturonase I catalyzed the formation of

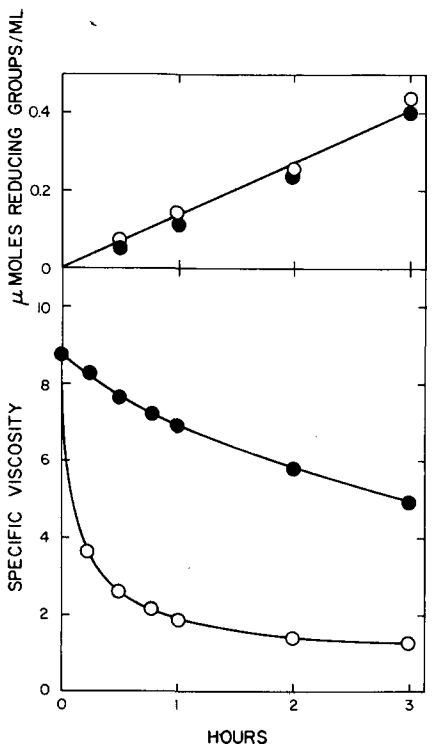

Fig. 3. Changes in the viscosity and reducing groups of pectic acid during cleavage by tomato polygalacturonases. ●—●, polygalacturonase I; ○—○, polygalacturonase II.

reducing groups at the same rate as polygalacturonase II, the viscosity of the solution containing polygalacturonase I decreased much more slowly. After 3 h of reaction, the viscosity was equal to that of the polygalacturonase II solution after only about 5 min.

DISCUSSION

The softening associated with ripening of many fruits is often attributed to enzymatic hydrolysis and the consequent solubilization of cell-wall polysaccharides[6,7]. Changes in the pectin have been of particular interest because it is concentrated within the region of the middle lamella[8]. Pectin-cleaving enzymes have been detected in numerous ripe fruits. In most instances, these have been identified as polygalacturonases rather than pectin transeliminases. One of the questions regarding pectin hydrolysis is whether multiple forms of polygalacturonase are required to degrade a polysaccharide that is heterogeneous in terms of molecular size and degree of esterification. It has long been suspected that there is more than one polygalacturonase in tomatoes[1,2], and the results of the present study demonstrate that this is indeed the case.

The two polygalacturonases isolated from tomatoes are similar in some respects, but they differ markedly in stability to heat and mechanism of pectate cleavage. The difference in their action on pectate is of particular interest because of an earlier suggestion[2] that tomato polygalacturonase may be a mixture of endo- and exo-enzymes. The rapid reduction of the viscosity of pectate by polygalacturonase II, compared to the change produced by polygalacturonase I, indicates that the former cleaves the substrate randomly. But polygalacturonase II is not specific for high molecular weight substrates, as it hydrolyzes pectic acid and a low molecular weight polygalacturonate at nearly identical rates. Polygalacturonase I also does not exhibit substrate-size specificity, but the slower effect on the viscosity of pectate suggests that this enzyme releases smaller products than polygalacturonase II. The change in viscosity is too rapid for a mechanism consisting of cleavage of monomer units, however.

We had reported earlier[5] that pectic acid partly inhibits the hydrolysis of low molecular weight polygalacturonates by crude extracts of tomato polygalacturonase. We interpreted these results in terms of two enzymes differing in susceptibility to inhibition by pectic acid. This view has been invalidated by the identical responses of polygalacturonases I and II to inhibition by pectic acid at low pH. A more reasonable explanation for the partial inhibition of the tomato polygalacturonases is that the enzymes interact with the polysaccharide but retain some of their activity. Pectic acid solutions become very viscous and tend to gel on acidification, evidence for extensive intermolecular hydrogen bonding. Adsorption of tomato polygalacturonase on pectic acid gels has been demonstrated[9] and actually used as a step in purification methods. At low ionic strength, therefore, the enzymes may form relatively stable complexes with the acidic polysaccharide.

According to this mechanism, only low molecular weight polygalacturonates are hydrolyzed at low pH, because they remain soluble and unassociated. As the molecular weight of the substrate increases, aggregation occurs as a result of hydrogen bonding between the macromolecules. These aggregates not only are unavailable

for enzymatic attack but adsorb and immobilize the polygalacturonases. Shifting the activity of tomato enzymes to the acid side by NaCl must be due to an ionic effect on this system. A structure that is stabilized by intermolecular hydrogen bonds can be disrupted by addition of ions to compete with the electrical charges forming the hydrogen bonds. This is supported by the fact that high salt concentrations are required to activate the cleavage of pectic acid at low pH.

The partial inhibition of polygalacturonase by pectic acid may also be due to an excluded volume effect in solutions of the polysaccharide. The molecular network resulting from intermolecular hydrogen bonding under acid conditions can be considered analogous to Sephadex which offers resistance to passage of other macromolecules. The addition of pectic acid to a solution of a lower molecular weight substrate would decrease the rate of diffusion of the substrate molecules. Thus, the substrate would be present uniformly throughout the solution but would not be entirely available to the enzyme, with apparent inhibition of hydrolysis by pectic acid.

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