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Turbidity-Based Assay for Polygalacturonic Acid Depolymerase Activity

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A technically simple and inexpensive discontinuous turbidity assay for qualitative and/or quantitative assessments of polygalacturonic acid depolymerase activity is presented. The enzyme reaction is initiated by the addition of enzyme preparation to a reaction mixture containing 0.02% polygalacturonic acid (PGA) in acetate buffer. The progress of the reaction is monitored by terminating aliquots of the reaction mixture (via heat treatment at appropriate times), subsequently adding poly(diallyldimethylammonium chloride) (PDADMAC) for turbidity development (approximately 30 min), and then measuring the turbidity (typically at 420 nm) of the resulting PGA−PDADMAC complex-containing solution. PGA depolymerase activity causes a decrease in the observed turbidity of PGA−PDADMAC-containing solutions because the stability of the interpolyelectrolyte PGA−PDADMAC complex is a function of the degree of polymerization of the PGA. The rate of turbidity change is herein shown to be proportional to a relatively wide range of enzyme concentrations. The assay is demonstrated using a commercial pectinase preparation and tomato fruit extracts.

KEYWORDS: Polygalacturonase; activity; turbidity; polyelectrolyte

INTRODUCTION

Pectins are among the most abundant polymers in the primary cell walls and intercellular spaces of fruits and vegetables (*1*). The function of pectins in intact plant tissues appears to be to maintain the integrity and rigidity of cell walls, to enhance water retention, and to act as an adhesive between cells (*2*). They play an important role in the ripening of fruit, in determining the rheological properties of solid/semisolid/liquid fruit products, and in the clarity of fruit and vegetable juices (*3*). The major components of commercial pectins are homogalacturonans with variable extents of methylation. Polygalacturonic acids are homogalacturonans that contain no, or minimal, methyl ester groups.

Pectin depolymerase enzymes are produced by both plants and microorganisms. The major depolymerases, i.e., those that split the α-(1 4)-glycosidic linkages in the backbone of the homogalacturonans, are polygalacturonase (PG_{ase}) and pectate lyase (PL). PG_{ase} (EC 3.2.1.15) catalyzes the hydrolysis of the (1 4)-linkages between adjacent galacturonic acid residues within the homogalacturonan backbone. The enzymes normally have pH optima in the acid range (pH 4−6) and can be either exo- or endo-acting, endo-acting PG_{ase} being the most common (*4*). PL (EC 4.2.2.2; sometimes called pectate transeliminase) is a depolymerase that functions via a β-eliminative mechanism (*5*). The pH optima of pectate lyases is generally in the range of 8.5−9.0, and all appear to require Ca^{2+} for activity (*6*).

Pectin depolymerase enzymes are important with respect to the natural course of fruit and vegetable ripening and degradation (*2*), and they are widely used in fruit and vegetable processing (*3*). Hence, it is important to have a relatively simple and inexpensive assay for the routine analysis of such enzymes. In the present paper we present such an assay for polygalacturonase. Polygalacturonase activity is most commonly assayed using either reducing sugar- (*7*−*9*) or viscosity-based (*8*, *10*) methods. The former assays are based on the generation of a new reducing sugar with each catalytic event. The latter is based on the decrease in viscosity that occurs as a result of enzyme-catalyzed depolymerization (*11*). Both reducing-sugar-based and viscosity-based assays are appropriate for many applications, but both have limitations for routine analyses. Direct analysis of enzyme extracts using reducing-sugar-based assays are often hampered by high endogenous reducing compounds which make accurate measurements problematic (*12*). Viscosity-based assays are not limited by background reducing sugar levels, but they are somewhat labor-intensive as traditionally done with capillary or rotating spindle viscometers (*13*), thus making them unattractive for routine multiple analyses. Hence, the objective of the present study was to develop a technically simple turbidity-based assay without the drawbacks of either the reducing-sugar- or viscosity-based methods.

Turbidity-based assays are widely used in the analysis of the major glycan hydrolases, including lysozyme (*14*), amylases (*15*), cellulases (*16*), and xylanases (*17*). In each of these cases a decrease in turbidity is correlated with enzyme activity. A turbidity assay of this type has been proposed for polygalacturonase (*18*). A drawback to the published method is that it requires the use of cetylpyridinium bromide or cetyltrimethylammonium bromide, both of which are relatively toxic (*19*).

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In this paper we describe a relatively simple alternative turbidimetric method for detecting polygalacturonase activity. The assay is based on formation of an insoluble colloidal polyelectrolyte complex between polygalacturonic acid and poly(diallyldimethylammonium chloride) (PDADMAC). The assay works by virtue of the fact that polygalacturonic acid forms the colloidal complex while its breakdown products, monomeric galacturonic acid and lower molecular weight oligosaccharides of galacturonic acids, do not. Highly branched, less anionic pectins, as may be found in native pectins, are not expected to form stable colloidal complexes with PDADMAC.

MATERIALS AND METHODS

Materials. $NaHCO_3$, Na_2CO_3, and NaOH were purchased from Mallinckrodt (Phillipsburg, NJ), bicinchoninic acid was from Pierce (Rockford, IL), and acetic acid was from Fisher Scientific (Pittsburgh, PA). All other chemicals were from Sigma-Aldrich (St. Louis, MO), including the following specific products: $CuSO_4 \cdot 5H_2O$, pectinase (from *Aspergillus niger* (*A. niger*); product P-2736; stock preparation had 8.05 μkat/mL pectinase activity measured as described below for "reducing-sugar-based assay"), low molecular weight poly(diallyldimethylammonium chloride) (35% in water, no. 522376), and polygalacturonic acid (PGA; P-3889, three different preparations were purchased over the 2 year study). The polygalacturonic acid preparations had average degree of polymerization values of 37.2, 37.0, and 27.0 (calculated as number average degree of polymerization from reducing ends per unit mass) and all preparations had degree of methylation values less than 8% (calculated from titratable acidity).

PGA−PDADMAC Turbidity Profiles. To 2.0 mL aliquots of 0.02% PGA in buffer (see "recommended enzyme assay" for buffer composition) were added 200 μL aliquots of PDADMAC-containing solution. The concentration of the PDADMAC solutions was varied such that the PDADMAC-to-PGA ratio (w/w) of the mixed PGA−PDADMAC-containing solutions spanned a range from 0.2 to 3.5. The mixed PGA−PDADMAC-containing solutions were allowed to stand for 30 min for turbidity development. The absorbance of the resulting PGA−PDADMAC suspensions was read at 420 nm.

Recommended Enzyme Assay. The standard assay is initiated by the addition of 1 mL of appropriately diluted enzyme preparation to 99 mL of 0.02% PGA in 50 mM sodium acetate, containing 0.02% sodium azide, pH 5, at 30 °C. At predetermined times, 2 mL aliquots of reaction mixture are transferred to glass tubes and the reaction is terminated by immersion in boiling water for 10 min. To the cooled, terminated, reaction mixture is added 200 μL of 0.4% PDADMAC in water. Turbidity is allowed to develop for 30 min at room temperature; the absorbance is then read at an appropriate wavelength (typically 420 nm). Enzyme activity is determined from the rate of change of turbidity (absorbance) in the initial linear portion of the reaction time course. The "control" assay is done in an analogous manner except the enzyme solution is inactivated by immersion for 10 min in boiling water prior to addition to the substrate solution. A commercial pectinase preparation (source listed above) was used for assay development; the enzyme preparation was diluted with assay buffer prior to use.

Reducing Sugar-Based Enzyme Assay. The reaction conditions were the same as that described for the "recommended" assay with the exception that after terminating the reaction the extent of hydrolysis was determined using the Cu^{++}-bicinchoninate-based reducing sugar assay as described previously (*8*). One unit of enzyme activity (katals) was taken as that amount of enzyme that liberates 1 (mol of reducing sugar)/s under the defined conditions.

Tomato Enzyme Extracts. Three fruits with a combined weight of approximately 225 g were weighed to the nearest 0.1 g and blended together with water (3 parts fruit:1 part water, w/w) at high speed for 30 s. The resulting homogenate was centrifuge at 1500*g* for 30 min at 4 °C. The supernatant was then filtered through Whatman no. 1 filter paper. The resulting filtrate was then immediately used as the "enzyme preparation". The activity of the enzyme preparation was tested using the "recommended" assay as described above.

RESULTS AND DISCUSSION

The presented assay makes use of the fact that the turbidity that results from mixing aqueous solutions containing PGA, a polyanion, and PDADMAC, a polycation, is a function of the degree of polymerization of the PGA. The formation of insoluble PGA−PDADMAC intermolecular polyelectrolyte complexes, those that give rise to the observed turbidity, is consistent with established polyelectrolyte theory (*20*). The stability of intermolecular polyelectrolyte complexes in general, and PGA−PDADMAC complexes specifically, is a function of the degree of polymerization of the constituent macromolecules. This is due to the stabilizing nature of cooperative interactions between sufficiently long polymer segments (*21*, *22*). If the degree of polymerization of PGA is decreased below a critical value, then the necessary cooperative binding is reduced to an extent below that required for stable PGA−PDADMAC complex formation. A result of this relationship is that enzyme-catalyzed reductions in the degree of polymerization of PGA will be observed as a decrease in the turbidity of the corresponding PGA−PDADMAC-containing solutions. This phenomenon is here applied to the detection and measurement of polygalacturonase activity.

The solubility properties of polyelectrolyte complexes are highly dependent on the stoichiometry of the stabilizing intermolecular charge−charge interactions (*22*). The presented assay is based on the use of a 0.02% substrate (PGA) solution. Thus, it was necessary to determine the appropriate amount of PDADMAC to add to the 0.02% substrate solution to maximize the PGA−PDADMAC analytical signal (i.e., optimal stoichiometry for maximum turbidity). This is most easily done in a constant-volume titration experiment (**Figure 1**). The data demonstrate the expected bell-shaped turbidity profile, with relatively low and relatively high PDADMAC amounts corresponding to decreases in the analytical signal. In the present study we evaluated three commercial preparations of PGA. All three preparations showed similar turbidity profiles for the PDADMAC/PGA ratios tested; although the PGA preparation with the lowest degree of polymerization was shifted somewhat upscale. A titration experiment similar to that depicted in **Figure 1** should be done for any previously untested PGA and PDADMAC solutions in order to ensure that the chosen PDADMAC/PGA ratios correspond to maximum signals. On the basis of the data in **Figure 1**, a PDADMAC/PGA ratio of 2 was chosen for this study.

Time courses for typical polygalacturonase-catalyzed reactions, monitored using the presented assay, are shown in **Figure 2**. Rates of reaction were found to be proportional to enzyme concentration (see insert to **Figure 2**). The relationship between catalytic events, determined as the appearance of new reducing ends, and the observed changes in turbidity for a typical reaction are presented in **Figure 3**. The decrease in turbidity is complete prior to the maximum number of reducing ends being attained, implying that complete depolymerization of the PGA substrate

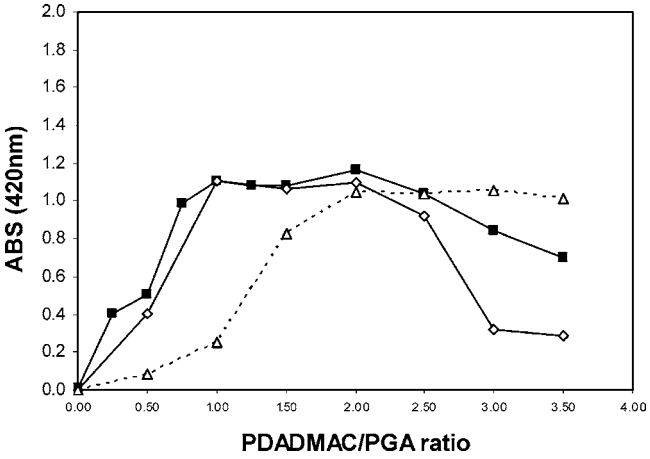

Figure 1. Effect of poly(diallyldimethylammonium chloride) (PDADMAC) to polygalacturonic acid (PGA) ratio on the turbidity of 0.02% PGA solutions. The PGA concentration of all solutions was kept constant at 0.02% (w/w); the concentration of PDADMAC was varied to give the specified ratios (w/w, abscissa); all solution conditions were as follows: 50 mM sodium acetate, 0.02% sodium azide, pH 5.0, 30 °C. Three different PGA preparations were tested (■, △, ◇; see Materials and Methods). The "0" PDADMAC/PGA ratio refers to a 0.02% PGA solution without added PDADMAC.

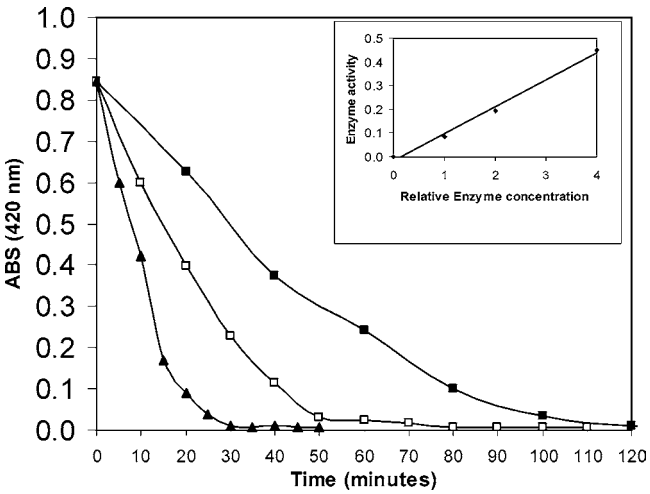

Figure 2. Time course of polygalacturonic acid depolymerase reaction assayed as a decrease in turbidity. Reaction conditions were 0.02% (w/w) polygalacturonic acid (PGA), 50 mM sodium acetate, 0.02% sodium azide, pH 5.0, 30 °C. Relative enzyme concentrations were 1 (■; absolute enzyme concentration, 0.04 *n*katal/mL based on the generation of reducing sugars), 2 (□), and 4 (▲). Turbidity development occurred as a result of the addition of poly(diallyldimethylammonium chloride) (2:1 PDADMAC/PGA ratio, w/w) to terminated reaction mixtures. Insert: Correlation between reaction mixtures' relative enzyme concentrations and corresponding initial velocities calculated from depicted time courses.

is not necessary to eliminate turbidity. Activities obtained using the turbidity assay are not to be interpreted in terms of traditional enzyme units (e.g., katals), as are those obtained using the reducing sugar assay. In the present case, we suggest that enzyme activity be defined with respect to the rate of change in turbidity observed at the initial linear portion of a reaction's time course; e.g. one unit of activity may be defined as that amount of enzyme that yields a change in absorbance of 0.001 (unit/min)/mL reaction mixture under the defined conditions. Such units are consistent with those employed in analogous assays based on measuring a physical property that is itself only

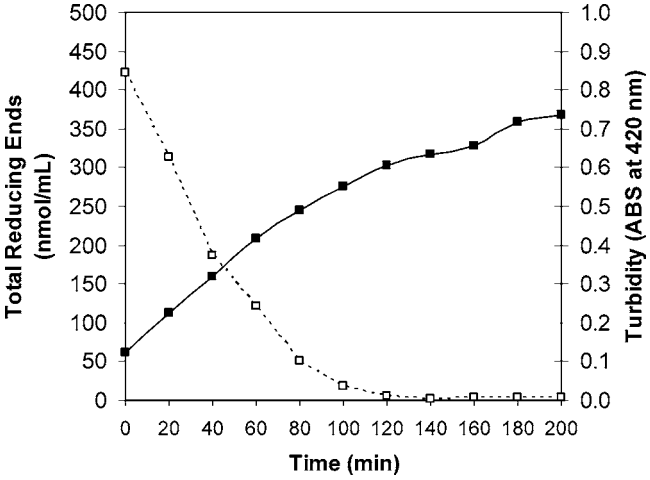

Figure 3. Comparison of time courses of polygalacturonic acid depolymerase reactions followed by the proposed turbidity assay (□) and an alternative reducing sugar assay (■). Reaction conditions were as described in **Figure 2**. The reaction mixture enzyme concentration was 0.04 *n*katal/mL.

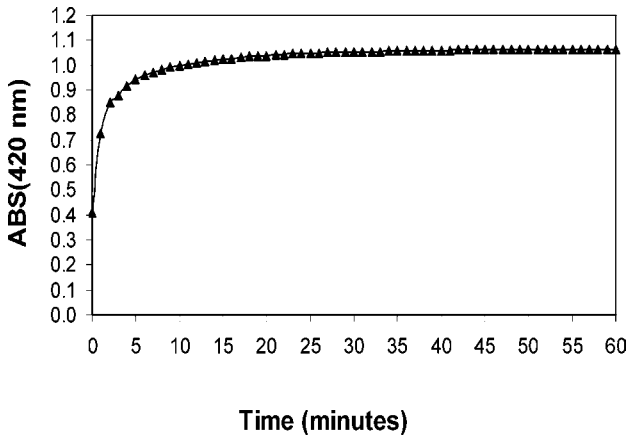

Figure 4. Time course of turbidity development upon mixing PDADMAC with PGA. Final conditions, following PDADMAC addition, were 0.04% PDADMAC, 0.02% PGA, 50 mM sodium aceate, 0.02% sodium azide, pH 5.0, and 30 °C (all percents are on a w/w basis).

indirectly related to the actual number of catalytic events. The reported activity will thus be a function of the rate of PGA depolymerization. Verification that the observed activity is the result of polygalacturonase activity, i.e., the absence of pectate lyase activity, may be obtained by measuring the absorbance of the reaction mixture at 235 nm prior to turbidity development (*7*).

The rate of turbidity development and the stability of the resulting polyelectrolyte complex, following the addition of PDADMAC to the terminated reaction mixture, is shown in **Figure 4**. The PGA−PDADMAC mixture attains >90% of maximum turbidity within 10 min, and the attained maximum value is stable from 30 to 60 min following mixing (longer periods were not tested). Hence, the recommended assay stipulates that the turbidity be measured 30 min after the addition of PDADMAC to the terminated reaction mixture. Formation of PGA−PDADMAC complexes and the associated maximum turbidities can be effected by reaction mixture salt concentrations. Hence, turbidity development assays, as well as the associated calibration curves, should be done using reaction mixture conditions equivalent to those to be used in the final enzyme assays.

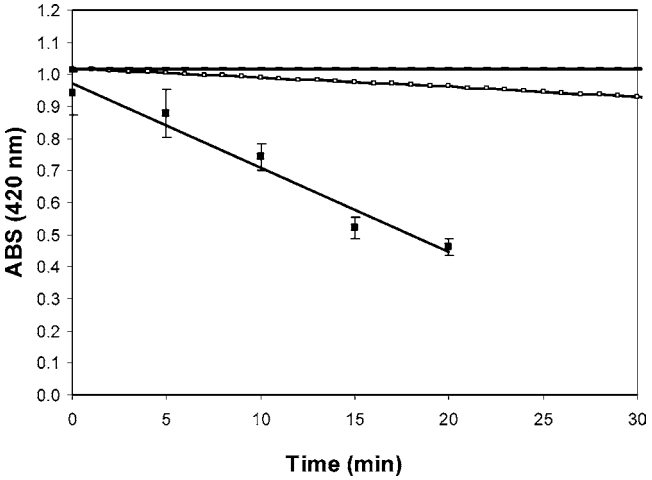

Figure 5. Time courses of turbidity change due to polygalacturonic acid depolymerase activity in the presence of free PGA (■) and PGA–PDADMAC complex (□). Turbidity in the "free PGA" assay was developed, as in **Figure 2**, by the addition of PDADMAC after termination of the PGA/PGA depolymerase reaction. Turbidity in the "PGA–PDADMAC complex" assay was a result of adding the PDADMAC prior to the addition of enzyme, thus allowing it to be run as a continuous assay. Reaction mixture enzyme concentration was 0.08 *n*katal/mL. Control enzyme preparations (——) were boiled prior to their use in the enzyme assay.

The sensitivity of the presented assay, in units of Δ absorbance/(Δ μM) reducing ends (calculation based on data of **Figure 3**), was 0.0044. This sensitivity is lower than expected for reducing-sugar-based assays (*8*). Thus, the advantage of the presented assay is not improved sensitivity, but rather its overall simplicity. As discussed elsewhere, the presented assay is not compromised by the presence of the endogenous reducing sugars that are likely to be present in most crude enzyme preparations—thus making the enzyme workup for this assay considerably simpler. The precision of the method may be considered with respect to the reproducibility of independently obtained calibration curves. Such an analysis of calibration curves obtained from three independent experiments found a mean slope of 0.0083 with a standard deviation of 0.000 28; the corresponding coefficient of variation was ~3.5%.

A possible permutation of the presented method is to form the insoluble PGA−PDADMAC complex prior to the addition of enzyme. In this case, the polyelectrolyte complex would serve as the substrate and the decrease in turbidity could be monitored continuously after the addition of enzyme. A comparison of the recommended method (mix PGA with enzyme, terminate, add PDADMAC, and then read turbidity), and the alternative method (where the PDADMAC complex serves as the substrate) is presented in **Figure 5**. It is clear from the presented data that the presence of active enzyme results in a decrease in turbidity under both experimental scenarios. However, the rate of the reaction is much faster, and thus the assay much more sensitive, when soluble PGA serves as the substrate. Hence, the recommended assay suggests that the enzyme/PGA reaction be allowed to proceed in the absence of PDADMAC; PDADMAC is to be added after terminating the reaction. Note, the demonstrated degradation of the PGA−PDADMAC complex by active enzyme (evidenced by the decrease in turbidity in **Figure 5**) requires that, for stable turbidity readings, the reaction be terminated prior to the addition of PDADMAC. In some cases, particularly when working with high-activity enzyme preparations for which assay sensitivity is not an issue, it may be advantageous to use the alternative continuous assay since it is technically easier (e.g., there is no need for the termination step in the continuous assay).

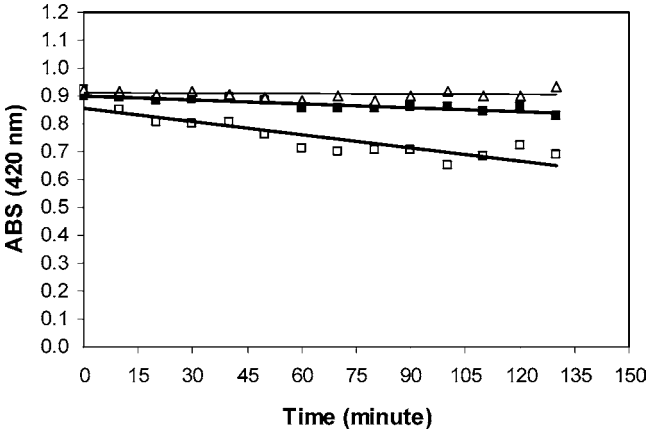

Figure 6. Application of the turbidity assay to ripe (■) and mold-tainted (□) tomato fruit. Enzyme preparations were water extracts of three combined, and homogenized, fruits. Reaction conditions were as in **Figure 2**. Control enzyme preparations (△) were boiled prior to use in the activity assay.

The time courses shown in **Figure 6** illustrate the application of the assay. In this experiment ripe tomatoes and those showing initial signs of mold growth were assayed, along with the appropriate controls (boiled tomato extracts). The data show the enhanced polygalacturonase activity associated with the tainted fruit—and the effectiveness of the heat treatment for enzyme inactivation. The tomato extracts had high enough activity to require dilution prior to assay even though no attempt was made to optimize enzyme extraction (*12*). Thus, the observed activities do not in any way represent the detection limit of the assay. Reaction mixtures contained 0.02% sodium azide as an antimicrobial, with no apparent affect on the turbidity associated with PGA− PDADMAC complex formation. Hence, one may prolong reaction times without potential interference due to microbial growth. For comparative purposes, it is relevant to note that the high reducing sugar content of the tomato extracts would have prevented the use of traditional reducing-sugar-based polygalacturonase assays unless a method, such as dialysis, had been used to lower the "background" reducing sugar content of the enzyme preparations.

The presented assay was developed in response to the need for relatively simple polygalacturonase assays for quality control applications in the fruit and vegetable industries. The assay is appropriate for such application, although it is not limited to qualitative measurements (as demonstrated above). The assay is relatively simple, requires no extraordinary precautions, requires nonsophisticated instrumentation, and is readily adapted for assessing enzyme preparations covering a relatively wide range of activities.

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