An Improved Diffusion Assay for Quantifying the Polygalacturonase Content of *Erwinia* Culture Filtrates

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ABSTRACT

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The agar diffusion procedure for quantifying pectolytic enzyme activity was modified to optimize assay sensitivity and simplify its implementation. The revised assay was run in 100 × 50 mm petri plates containing 20 ml of 1% agarose (Type II), ammonium oxalate (0.5%), and sodium azide (0.2%) in phosphate buffer (0.2 M, pH 5.3) with polygalacturonic acid (0.01%) as the substrate. Samples (35 μl) were pipetted into 4.1 mm diameter wells punched in the agarose with a #1 cork borer. After incubation at 37 C for 17 hr, the gel was developed with 10 ml of 0.05% ruthenium red for 30 min, and the diameter of the clear zone of activity was measured microscopically. Polygalacturonase equivalents as low as 2.3×10^{-4} units were detected. The modified assay required less sample and reduced the problem of gel dehydration associated with the standard assay.

Additional keywords: cup plate assay, pectinase.

The agar "cup plate" diffusion assay of Dingle et al (7) can be used to quantify the activity of a variety of enzymes, including amylase, arabanase, cellulase, lipase, pectinesterase, polygalacturonase, protease, and xylanase. Although it was developed over 30 years ago, this assay is still used to determine enzyme activity. Since 1980, Dingle's original paper has been cited approximately 50 times as a source for polygalacturonase assays. The procedure has been utilized to determine the pectolytic activity of fungal (2,9,10,12,15,16,19,20) as well as bacterial (5,11,13) culture filtrates. This system has also proven useful in evaluating host tissue for the presence of components that inhibit pectinases (1,3,4,12). The major criticism of the agar diffusion technique is its relative inaccuracy when compared with more sophisticated quantitative procedures (8).

We have made several refinements in the standard assay procedure (18) to simplify its implementation and optimize its sensitivity. The assay is easy to run and has the advantage of being able to be performed with minimal equipment. The modified procedure provides consistent results when crude *Erwinia* culture filtrates are assayed.

MATERIALS AND METHODS

Assay procedure. The assay medium contained ammonium oxalate (0.5%), sodium azide (0.2%), Type II agarose (1.0%) (Sigma Chemical Company, St. Louis, MO) in 0.2 M phosphate buffer (adjusted to pH 5.3), with the sodium salt of polygalacturonic acid (0.01%) (United States Biochemical Corporation, Cleveland, OH) as the substrate. The medium was heated to dissolve the polygalacturonic acid and agarose, then transferred to 100 × 15 mm petri plates (20 ml per plate). A #1 cork borer was used to punch five holes, 4.1 mm in diameter and 2.5 cm apart, in the solidified medium. The holes were arranged in three rows in a 1 × 3 × 1 matrix, and the wells were filled (35 μl) with standard, control, or unknown (filtrate) solutions. The assay was incubated at 37 C for 17 hr.

The gel was developed after incubation by flooding the assay plate with 10 ml of 0.05% ruthenium red (Sigma Chemical Co.) for 30 min at 25 C. Excess dye was removed by washing the plate several times with deionized water (dH_2O). The diameters of the resulting clear areas (rings) of activity were measured at 7× magnification with a standard dissecting microscope containing a calibrated ocular micrometer. Two diameter measurements (at right angles) were taken for each well from duplicate plates and averaged.

Preparation of the polygalacturonase standard curve. A standard curve (ring diameter vs. concentration of standard) was prepared with polygalacturonase, poly [1, 4-α-D-galacturonide] glycanohydrolase: EC 3.2.1.15 (Sigma Chemical Co.) at the following concentrations: 5.0, 0.5, 0.05, 0.005, and 0.0005 mg/ml in dH_2O. According to Sigma Chemical Co. analysis, the lyophylized polygalacturonase had a specific activity of 455 units (U)/gm. (One unit of polygalacturonase liberates 1.0 umole of galacturonic acid per min from polygalacturonic acid at pH 4.0 and 25 C.) Pectolytic activity was expressed as U/ml.

Preparation of samples. Control samples were prepared at high and low enzyme levels by arbitrarily mixing an *Erwinia* culture filtrate with commercial polygalacturonase. There was no specific relationship between the high and low controls. The culture filtrates were prepared by growing *Erwinia carotovora* subsp. *carotovora* (strain 71) in Chatterjee's (6) minimum salts medium (MinS) with citrus pectin (Sigma Chemical Co.) directly substituted for glucose. Bacteria were rinsed from a 24-hr nutrient agar slant culture and diluted to 7×10^8 colony-forming units in sterile dH_2O. One ml of the suspension was added to 250 ml of MinS in a 1-L Erlenmeyer flask. After culture at 24 C for 72 hr, the bacteria were removed by sequential filtration through membrane filters VM-1 (5.0 μm), BA-6 (0.45 μm), and GA-8 (0.2 μm) (Gelman Sciences, Inc., Ann Arbor, MI) and the remaining filtrate mixed with polygalacturonase. The high and low control samples were segregated into 1-ml aliquots and frozen at −20 C. An aliquot of each sample was thawed and included as an internal control with each enzyme assay.

Interassay variation was estimated by assaying the high and low samples over several assays at approximately 2-wk intervals. Variation within the assay was examined by assaying the low control several times within a single assay.

RESULTS

Typical standard curves obtained by using the original assay procedure and the modified cup plate assay are given in Figure 1. The modified assay was linear from 2.28×10^{-4} to at least 45.5

U/ml. The equation for the regression line of the standard curve compares favorably with the average regression line based on 10 assays ($y = 4.18x \times 24.92$). Standard errors of the mean slope and mean Y intercept of the 10 standard curves were 0.08 and 0.13, respectively. The standard curve from the original assay was also linear to at least 45.5 U/ml, but the lower limit of resolution was only 2.28×10^{-3} U/ml. Although ring development did occur below this concentration, the margin of the ring was too close to the edge of the well and not distinct enough to be measured accurately. In addition, the slope of the standard curve from the original assay was not as great as that of the revised assay.

Mean polygalacturonase concentrations obtained from the standard samples are listed in Table 1. When low control was tested 12 times within a single assay, the mean polygalacturonase value was 4.50×10^{-3} U/ml. This control was also tested in seven separate assays with a mean approximately twice that of the intraassay mean. A mean concentration of 5.18 U/ml was obtained when the high control was analyzed in nine separate assays, but the interassay standard deviation, variance, and standard error of the mean were not as great as those obtained for the control with the lower pectolytic activity.

Decreasing the substrate (polygalacturonic acid) concentration caused an upward shift in the standard curve, but the slope remained virtually unaffected (Table 2). Even at a low concentration of enzyme standard (2.28×10^{-3} U/ml), there was a good correlation between substrate concentration and ring diameter (Fig. 2).

Storage conditions may affect enzyme stability, so we also monitored pectolytic activity in samples that were frozen and thawed numerous times and samples that were stored for an extended period of time at refrigerator temperature (10 C). An *Erwinia* filtrate taken through eight freeze thaw cycles lost 77% of its pectolytic activity. Assayable enzyme activity of two other filtrates was reduced by 82% and 71% after refrigeration for 110 days.

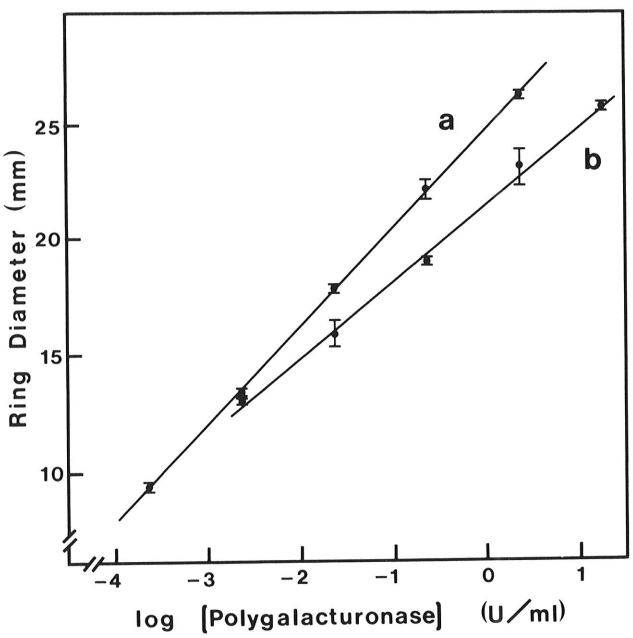

Fig. 1. Comparison of standard curves from the revised "cup plate" assay (**a**) and the assay using original diffusion plate parameters of Dingle et al (7) (**b**). Regression lines: **a**, $y = 4.25x + 24.76$ $r = 0.9999$; **b**, $y = 3.18x + 21.30$ $r = 0.9975$. Polygalacturonase concentrations range from 2.28×10^{-4} to 2.28×10^{0} U/ml, and data points represent means of two determinations from each of two duplicate samples. Bars indicate standard error of the mean.

TABLE 1. Variation in activity values obtained from two control solutions containing pectolytic enzymes

Control	Number of times assayed	Poly-galacturonase equivalents (mean)[a]	Standard deviation	Variance	Standard error
High	9	5.18[b]	0.73	0.53	0.26
Low	7	8.53[b]	3.78	14.94	1.46
Low	12	4.50[c]	0.37	0.14	0.11

[a] High = U/ml, low = U/ml $\times 10^{-3}$.
[b] Interassay.
[c] Intraassay.

DISCUSSION

The sensitivity of the diffusion cup plate assay is related to factors such as the substrate used, concentration of the substrate, pH and nature of the gel, temperature, and length of the incubation period. Changing the substrate from citrus pectin to polygalacturonic acid, reducing the substrate concentration, using agarose as the gel, and developing the plates with ruthenium red stain all contributed to optimizing the sensitivity of the modified assay. The revised diffusion assay is actually more sensitive than the original assay because both the slope of the standard curve and the lower limit of resolution have been increased.

The modified assay is apparently linear to 2.28×10^{-4} U/ml polygalacturonase equivalents; however, sensitivity ultimately depends upon accurately measuring the diameter of the rings marking the zone of enzyme activity. Reducing the substrate concentration promotes greater ring development, as indicated by an increase in the Y intercept of the regression line (Table 2). The increase is consistent throughout the range of enzyme concentrations used in the standard curve. Accurate measurement of ring diameter is particularly important at the lowest portion of the

TABLE 2. Effect of substrate (polygalacturonic acid) concentration on the standard curve parameters

Polygalacturonic acid mg/ml	Slope	Y intercept	r value
1.0	4.43	19.44	0.9877
0.5	4.48	20.96	0.9942
0.25	4.49	22.39	0.9969
0.1	4.42	24.31	0.9995

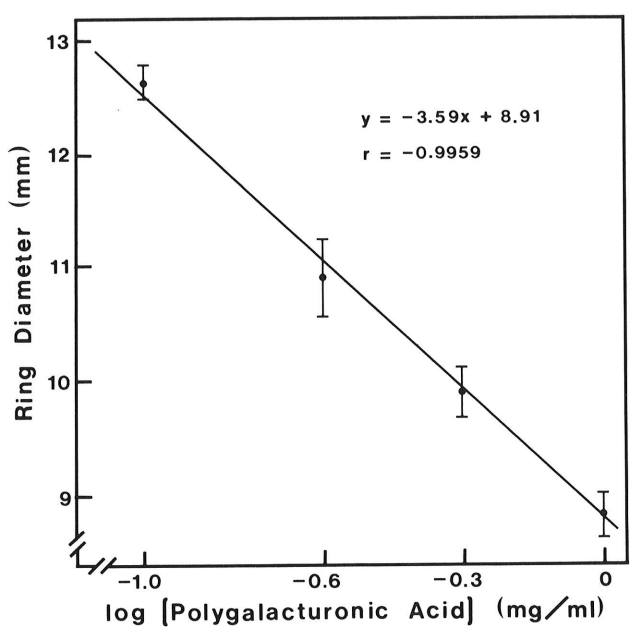

Fig. 2. Effect of substrate (polygalacturonic acid) concentration on ring development in the "cup plate" assay (polygalacturonase = 2.28×10^{-3} U/ml). Polygalacturonic acid concentrations range from 0.1 to 1.0 mg/ml. Data points represent means of two determinations from each of two duplicate samples. Bars indicate standard error of the mean.

curve. When the substrate concentration was decreased from 1.0 mg/ml to 0.1 mg/ml (at 2.28×10^{-3} U/ml of polygalacturonase), the ring diameter increased from approximately 9 mm to 12.5 mm (Fig. 2). An increase of this magnitude is essential for increased assay sensitivity. Rings that are less than twice the diameter of the wells are very difficult to see, and subsequent measurements are subject to a high degree of error.

The modified assay, which uses a well diameter of approximately 4.1 mm and substrate concentration of 0.1 mg/ml, produces a zone of activity greater than twice the well diameter with the lowest polygalacturonase standard. Assay resolution is also enhanced by the procedure used to develop the plates. Ruthenium red was selected for use in the revised diffusion assay because it provides a rapid and sensitive means of detecting pectolytic activity (14). The original cup plate assay (7) is developed with 5 N HCl. Plates stained with ruthenium red have much sharper ring development, particularly at lower pectinase concentrations. Rings could not be detected when the modified assay was developed by the HCl method.

The smaller well size used in the modified assay allows for a smaller sample size. Smaller wells also allow the assay to be run in standard petri plates. The plates can easily be sealed with Parafilm, thus reducing the problem of gel dehydration associated with the standard assay. Because the plates are sealed, they can be stored for long periods of time before using. Assay plates stored for as long as 8 wk produced standard curves identical to those obtained from freshly prepared plates.

The high degree of interassay variation observed for the low control (Table 1) may be partially attributed to a reduction in enzymatic activity associated with reuse of the standards. Three of the assays used to determine the interassay mean of that control were based on recycled standard curves. Because intraassay variability is apparently lower than variation between assays (Table 1), all samples from a particular experiment should be grouped and analyzed together in a single assay. Samples should be stored frozen before the assay. If control samples are to be included with each assay, they should be prepared as a single lot and stored frozen in small aliquots (1 ml). A single aliquot can be thawed for inclusion in each assay and then discarded.

The modified diffusion assay should be relatively specific for polygalacturonases, pectinases that break glycosidic bonds by hydrolysis. These enzymes possess a pH optimum around pH 5.0 and are inhibited by Ca^{++}. Specificity is enhanced because the modified assay is run at pH 5.3 and ammonium oxalate is included to bind any calcium present in the assay solutions. The revised assay provided satisfactory results when crude filtrates produced from *Erwinia carotovora* liquid cultures were analyzed (17). It should be readily adaptable for studies involving other microorganisms that produce pectolytic enzymes.

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