Improved Detection of Polygalacturonase Activity due to *Mucor piriformis* with a Modified Dinitrosalicylic Acid Reagent

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ABSTRACT

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An assay for determination of galacturonic acid with 3,5-dinitrosalicylic acid was developed that substantially extends the linear range of detection compared to a previously published method with this reagent. In the improved assay, galacturonic acid was detected with a reagent containing 44 mM 3,5-dinitrosalicylic acid, 4 mM sodium sulfite, and 375 mM sodium hydroxide. The absorbance of the solution after reaction with

galacturonic acid was determined at 575 nm and was linear at concentrations of galacturonic acid up to 50 μ mol, with a lower limit of detection at ~400 nmol. The assay with the improved reagent could be performed in wavelength ranges from 550 to 575 nm, with higher sensitivity at the shorter wavelengths. The new reagent was used in routine assays of polygalacturonase activity in culture filtrates of the important postharvest fungal pathogen *Mucor piriformis*.

Additional keywords: reducing sugar.

The dinitrosalicylic acid reagent developed by Sumner (15) and Sumner and Sisler (16) and modified by Miller (8) provides a sensitive test for determining reducing sugars in a variety of biological fluids. The reaction is based on the reduction of sugar by dinitrosalicylate under alkaline conditions to yield 3-amino-5-nitrosalicylate (8). The reagent is composed of dinitrosalicylic acid, potassium sodium tartrate (Rochelle salt), phenol, sodium bisulfite (sodium sulfite), and sodium hydroxide. During the reaction, color development is stabilized by Rochelle salt and enhanced by phenol, and sulfite protects the reagent and reducing sugar from oxidation (15,16). The method has been widely used in assaying polygalacturonase activity to detect galacturonic acid residues generated during depolymerization of pectin by various fungi (1,2,4,6,12).

Polygalacturonases hydrolyze the α -glycosidic linkages of pectin chains by random action (endopolygalacturonase; EC 3.2.1.15) or by attack at the terminal residues of the chains (exopolygalacturonase; EC 3.2.1.67). This results in the liberation of a series of oligogalacturonates (mono-, di-, and trigalacturonates) (13), which can be measured spectrophotometrically with reagents that react with reducing sugars (1,2,4,6,11,12). In preliminary assays of crude preparations of polygalacturonase from the fungal pathogen *Mucor piriformis* E. Fisch., an important postharvest pathogen of fruits (7), we found that the assay was linear for detection of galacturonic acid between ~0.4 and 1 μ mol. Because this detection range imposes a limitation in the variety of formats possible in our experiments, which often involve many comparisons of polygalacturonase activities over a wide range of conditions, we sought to increase the linear detection range of the reagent and simplify the procedure. We report an improved dinitrosalicylate reagent for detection of high concentrations of galacturonic acid based on modification of the concentrations of sodium hydroxide and Rochelle salt, which eliminates phenol in the reaction mixture. A preliminary report has been published (17).

MATERIALS AND METHODS

Reagents and analytical methods. All chemicals were purchased from Sigma Chemical Co. (St. Louis). Spectrophotometry was performed on a Shimadzu UV-2101PC spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) with 1-cm quartz cells. The dinitrosalicylate reagent described by Miller (8) contained ~44 mM dinitrosalicylic acid, 21 mM phenol, 4 mM sodium sulfite, and 250 mM sodium hydroxide. The modified reagent in this study contained 44 mM dinitrosalicylic acid, 4 mM sodium sulfite, and 375 mM sodium hydroxide. For assays, the reaction mixture contained galacturonic acid solution (1 ml), reagent (1 ml), 1.4 M Rochelle salt (1 ml), and deionized water (1 ml). This mixture was heated in a boiling water bath for 10 min and cooled, and its absorbance at 575 nm was measured. Both reagents were prepared by placing ingredients in the required concentration of sodium hydroxide solution and stirring with a magnetic mixer. Sulfite oxidation was minimized by adding the sodium sulfite to the reagent mix just prior to use (8) or by degassing the reagent immediately after preparation and storing it at 4°C.

Reaction procedure. The standard reaction mixture contained 1 ml of galacturonic acid solution per 1 ml of reagent and was heated in a boiling water bath for 10 min. In the original method, 1 ml of 1.4 M Rochelle salt was added to the test tubes immediately after heating, and the total volume of the solution was adjusted to 4 ml with deionized water before cooling to room temperature (23°C). In the modified procedure, the total volume of the solution was adjusted to 4 ml with deionized water directly before cooling to room temperature. The tubes were cooled to room temperature in a water bath.

The determination of polygalacturonase activity was based on the hydrolytic release of reducing groups from polygalacturonic acid. The reaction mixture contained 0.8 ml of 0.5% (wt/vol) polygalacturonic acid in 100 mM Na-acetate buffer (pH 5.0) and 0.2 ml of a solution containing concentrated enzyme in acetate buffer, pH 5. The polygalacturonic acid was washed with 80% ethanol prior to use. The reaction mixtures were incubated at 30°C for up to 4 h, and the reaction was terminated by the addition of 1

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Fig. 1. Relationship between galacturonic acid concentration and absorbance at 575 nm, using dinitrosalicylic acid reagent with and without phenol.

Fig. 2. Relationship between galacturonic acid concentration and absorbance at 575 nm, using dinitrosalicylic acid reagent, without phenol, containing 250 mM (1%) or 375 mM (1.5%) NaOH.

ml of dinitrosalicylate reagent and immersion in a boiling water bath for 10 min. The final volume of the samples was adjusted to 4 ml with Rochelle salt solution and deionized water for the original reagent or with deionized water only for the modified reagent. After the samples were cooled to room temperature, their absorbances were measured at 575 nm.

Fungal culture. A single-spore isolate of *M. piriformis*, isolate 4B2 (neutral strain), was used throughout our experiments and was maintained on potato dextrose agar. To obtain inoculum for broth cultures, the isolate was transferred to lactic acid agar and incubated at 20°C for 48 h. Mycelial disks (5 mm diameter) were taken from the outer margin of the colonies and transferred to 250-ml Erlenmeyer flasks containing 100 ml of liquid medium. The liquid medium was composed of 2 g of asparagine, 2.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g of KH_2SO_4 , and 5 g of polygalacturonic acid in 1 liter of deionized water. The pH of the medium was adjusted to 5.0 with NaOH or HCl prior to autoclaving.

Extraction of polygalacturonase. After 7 days of incubation at 20°C, the mycelium was removed by filtration, and the resulting culture filtrate was centrifuged at $7,000 \times g$ for 15 min at 4°C. The

Fig. 3. The effect of Rochelle salt on the stability of color developed with dinitrosalicylic acid reagent for detection of galacturonic acid.

supernatant was filtered first through 1.0-μm and then through 0.6-μm polycarbonate membrane filters (Millipore Corp., Bedford, MA). The filtrate was concentrated ~30-fold by freeze-drying and dialyzed at 4°C overnight against 0.5 mM acetate buffer, pH 5. The concentrated enzyme solution was stored at -20°C until use.

RESULTS AND DISCUSSION

The presence of phenol in the reagent increased the color intensity but decreased the upper limit of detection of galacturonic acid. The linear ranges for detection of galacturonic acid for the reagents with and without phenol were 0.4 to 1.1 μmol and 0.4 to 42 μmol, respectively (Fig. 1). Because alkaline pH is required for reaction of reducing sugar with dinitrosalicylic acid (8), the pH of samples can influence the reaction and detection range. Increasing the sodium hydroxide concentration in the reagent to 375 mM (1.5%) was optimal for increasing the color intensity, while maintaining the linearity of detection (Fig. 2); 500 mM (2%) NaOH did not significantly increase the upper limit of detection of galacturonic acid (data not shown).

In the original reagent, Rochelle salt was used to stabilize the color. However, we found that color stability was not significantly affected over a 3-h period when Rochelle salt was eliminated in the new formulation, although color intensity was slightly higher in the presence of Rochelle salt (Fig. 3).

The modified reagent could detect galacturonic acid over a wider range of concentrations than the original reagent, extending the upper limit as much as sixfold (Fig. 4). The improved assay could be performed at wavelengths from 550 to 575 nm, with greater sensitivity at the shorter wavelength (data not shown). When the modified reagent was used to assay the polygalacturonase in the crude fungal culture filtrate, the absorbance of the reaction products increased linearly over a 4-h period (Fig. 5).

The modified reagent and procedure we developed permits analyses of samples with high polygalacturonase activity. The modified reagent also eliminates from the reaction mixture phenol, which is a hazardous chemical that presents additional costs to a laboratory in terms of disposal fees. Other methods are somewhat restrictive in linear range of detection of galacturonic acid, such as 2-cyanoacetamide, which has a reported detection range of 1 to 250 nmol (5), and copper reagent, which has a range of about 10 to 3,000 nmol (3,9,10,14). The latter requires at least two reagents, involving eight chemicals. Use of the copper reagent also can result in high sample turbidity, requiring centrifugation of the

Fig. 4. Relationship between galacturonic acid concentration and absorbance at 575 nm, using original and modified dinitrosalicylic acid reagents.

sample prior to reading. In contrast to the 2-cyanoacetamide reagent, neither the dinitrosalicylate reagent nor the copper reagent is suitable for substrates with a high degree of esterification. Under the alkaline conditions of our assays, glycosidic bonds also could be cleaved by a β -elimination reaction (12). To avoid this systematic error, it is important to include the appropriate control (i.e., with denatured enzyme) in these assays.

Our modified reagent also is simpler in composition compared to the original reagent, and the new procedure requires fewer steps. After termination of the reaction and heating at 100°C, absorbance measurements can be performed immediately after cooling to room temperature. Because the modified reagent can be used to detect a wider range of reducing sugar, it can be used for samples with high background reducing sugars, such as crude culture filtrates, without dialysis.

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Fig. 5. Rate of hydrolysis of polygalacturonic acid by *Mucor piriformis* polygalacturonase, determined with modified and original dinitrosalicylic acid reagents.

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