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A Rapid and Sensitive Spectrophotometric Method for Assaying Polygalacturonase Using 2-Cyanoacetamide[1]

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Abstract. **A new assay for nanomole amounts of reducing sugar using 2-cyanoacetamide has been modified and adapted to assay for exo- and endo-polygalacturonase in fruit extracts of tomato (*Lycopersicon esculentum* Mill. cv. Heinz 1350) with increased sensitivity (140%) and simplicity over currently used methods. Linearity was observed with galacturonic acid as a standard up to 250 nmol; the lower limit of detection was 1 nmol. Polygalacturonase product formation was linear for 3 hours and was proportional to the amount of enzyme in the reaction. Thin-layer chromatography of reaction products revealed a range of uronic acid oligosaccharides as well as galacturonic acid. Thus, both exo- and endo-polygalacturonase were active in the extracts.**

Many studies on the biochemistry of fruit softening focus on the hydrolysis and solubilization of the pectic portion of cell walls by polygalacturonase. The concept is that textural changes may occur as insoluble polyuronide, which contributes substantially to cell wall integrity and therefore fruit firmness, is converted to more soluble forms. Polygalacturonase is usually assayed by measuring the formation of reducing groups using the reducing sugar method of Nelson (7) or Luchsinger and Cornesky (6).

Honda et al. (5) recently reported that 2-cyanoacetamide is effective for spectrophotometrically quantifying nanomole amounts of reducing carbohydrate in solution. 2-Cyanoacetamide forms ultraviolet-absorbing products that fluoresce intensely after its direct condensation and cyclization with reducing sugars (4, 5). The procedure represents a significant increase in sensitivity and simplicity over the copper-arsenomolybdate reducing sugar method of Nelson and the dinitrosalicylic acid method of Luchsinger and Cornesky.

This paper reports on the modification and usefulness of the 2-cyanoacetamide procedure of Honda et al. (5) for assaying exo- (EC 3.2.1.67) and endo-polygalacturonase (EC 3.2.1.15) with greater sensitivity than Nelson's copper-arsenomolybdate method (7), a frequently used method.

For enzyme extraction, pericarp tissue was excised from 5 partially thawed and peeled red-ripe fruits (2) of 'Heinz 1350' tomato, which had been stored at −70°C for 4 weeks. Polygalacturonase was then extracted using a procedure similar to that of Tucker et al. (10). Tissue (150 g) was placed in 300 ml of distilled H_2O and homogenized in a Waring blendor for 1 min. The homogenate was filtered through 6 layers of cheesecloth and the residue resuspended in 300 ml of distilled H_2O. The suspension was filtered and the residue suspended in 150 ml of 1 M NaCl. The pH was adjusted to and maintained at 6.0 with 1 N NaOH while the slurry was stirred slowly at 4°. After 3 hr, the slurry was filtered through cheesecloth and the filtrate centrifuged at $9000 \times g$ for 15 min. An aliquot (1.5 ml) of the supernatant was desalted on a Sephadex G-25 column (10-ml bed volume) which was equilibrated in 50 mM Na-acetate (pH 4.4). The desalted extract was used for polygalacturonase assay. All extraction procedures were carried out at 4°.

The assay of polygalacturonase activity was based on the hydrolytic release of reducing groups from polygalacturonic acid. Reaction mixtures (0.2 ml total volume) containing 37.5 mM Na-acetate (pH 4.4), 0.2% polygalacturonic acid (washed with 80% ethanol prior to use), and either 25 µl or 50 µl of enzyme extract were incubated at 30°C for up to 3 hr.

For quantifying released reducing groups with 2-cyanoacetamide, reactions were terminated with 1.0 ml of cold 100mM borate buffer (pH 9.0). Then, 0.2 ml of 1% 2-cyanoacetamide (Aldrich Chem. Co.) were added, and the samples were mixed and immersed in a boiling water bath for 10 min. After equilibration to 25°C, the absorbance at 276 nm was determined using a Beckman Model 25 spectrophotometer. Polygalacturonase products were also measured according to the procedure of Nelson (7). In this method, reactions were terminated by adding 0.5 ml of cold copper reagent. Tubes were placed in a boiling water bath for 20 min and then immersed in an ice bath. One-half ml of arsenomolybdate reagent was added, and the samples were mixed until foaming ceased. Three ml of distilled H_2O were added and the samples were mixed by inversion. Samples were centrifuged at $1400 \times g$ for 3 min and the absorbance at 540 nm was measured.

For product characterization, tubes which had been incubated at 30°C for 3 hr were immersed in a boiling water bath for 10 min to terminate the reaction. Samples were taken to dryness at 40° with a stream of N_2, and the residue was taken up in 0.5 ml of 80% ethanol. Thin-layer chromatography was performed on cellulose plates with *n*-butanol:ethanol:H_2O (4:1.1:1.9, v/v/v) used as the solvent. Uronic acid spots were visualized with 0.05% bromophenol blue in 95% ethanol (pH 6.5).

Honda et al. (5) developed the use of 2-cyanoacetamide for the general detection of reducing carbohydrates with glucose as a sugar standard. At 100 nmol, galacturonic acid gave a 74% greater absorbance at 276 nm than did glucose. However, no standard curve was presented for a range of galacturonic acid concentrations. In the present study, linearity was observed for detection of galacturonic acid at amounts up to 250 nmol, with the lower limit of detection about 1 nmol (Fig. 1).

Since the copper-arsenomolybdate test, which is more sensitive than the dinitrosalicylic acid method (6), is generally used when assaying for polygalacturonase, this method was compared to the 2-cyanoacetamide method modified from that of Honda et al. (5). In

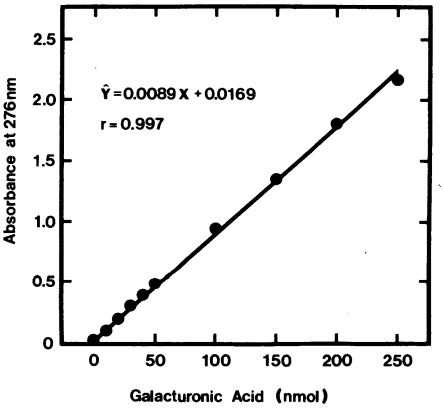

Fig. 1. Relationship between galacturonic acid concentration and absorbance at 276 nm using 2-cyanoacetamide. Samples (0.2 ml) contained the shown amount of galacturonic acid. All other reagent volumes and concentrations were as described for measuring polygalacturonase activity using 2-cyanoacetamide.

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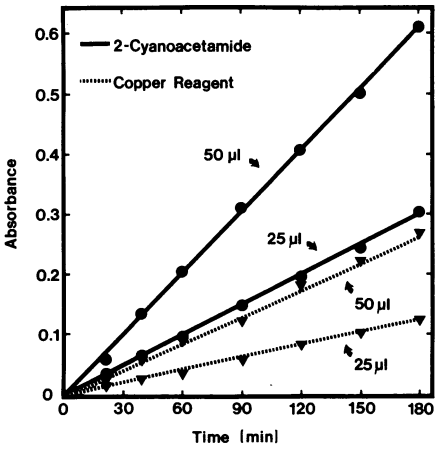

Fig. 2. Comparison between 2-cyanocetamide and Nelson's copper reagent for detecting reducing sugar formed during a 3-hr incubation of tomato enzyme extract (25 µl or 50 µl) with polygalacturonic acid. The absorbance was measured at 276 nm and 540 nm for the 2-cyanoacetamide and Nelson's methods, respectively. The regression coefficient for each line was greater than 0.99. The experiment was repeated twice with similar results.

quantifying polygalacturonase products, the 2-cyanoacetamide method represented a 140% increase in sensitivity over the copper-arsenomolybdate method (Fig. 2). Using thin-layer chromatography, the reaction products were shown to be a range of uronic acid oligosaccharides as well as galacturonic acid (data not shown). Thus, as with other tomato fruit extracts (8), both exo- and endo-polygalacturonase were active in the enzyme preparation. Estimates of polygalacturonase activity using the 2 methods were similar and ranged from 2.2 to 2.7 µmol product/min/gfw. Product formation was linear for 3 hr and was proportional to the amount of enzyme in the reaction. These data indicate that although the molar responses of uronides with various degrees of polymerization may differ slightly in terms of chromophore yield, the assay was suitable for estimating polygalacturonase activity.

In addition to having greater sensitivity, the 2-cyanoacetamide method was also simple, involving only the addition of borate (pH 9.0) and 2-cyanoacetamide. These reagents are easy to prepare and can be stored in the dark for at least a month at 4°C (5). Also, prior to reading the absorbance at 276 nm, the samples did not require centrifugation. Contrary to the 2-cyanoacetamide procedure, the copper-arsenomolybdate method requires 3 reagents involving 8 chemicals (5). The copper reagent must be mixed fresh daily and the arsenomolybdate reagent requires a preparation time of about 24 hr. Also, samples are turbid and require centrifugation prior to determining the absorbance at 540 nm.

Since 2-cyanoacetamide forms products which fluoresce intensely after condensation and cyclization with reducing sugars (4, 5), further increases in sensitivity may be possible by developing a method for measuring polygalacturonase product formation using a fluorimeter. The sensitivity of 2-cyanoacetamide may be particularly beneficial in studies of polygalacturonase in tissues that contain only trace activity, such as apples (1) and green or nonripening mutant tomatoes (2, 3, 9). 2-Cyanoacetamide should also be useful for quantifying product formation when assaying for other hydrolytic enzymes that release reducing sugars from nonreducing sugar substrates, such as invertase and various glycosidases.

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Resistance to Tobacco Mosaic Virus-induced Blotchy Ripening in Greenhouse Tomato Hybrids[1]

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Abstract. **Germplasm sources for resistance to tobacco mosaic virus-induced blotchy ripening of greenhouse tomato (*Lycopersicon esculentum* Mill.) were tested in hybrid combinations using a virus strain belonging to Pelham Group I. Breeding lines possessing *Tm-2* or *Tm-2^a* genes contributed high tolerance against TMV infection based on both leaf and fruit symptoms. 'Perou 2' (*Tm-2*), Ohio MR-13 (*Tm-2^a*), and Ohio MR-12 (*Tm-2^a*) and hybrids involving these lines were tolerant to the virus. Significant dominance for tolerance was observed in most hybrids heterozygous at the *Tm-2* or *Tm-2^a* locus. *Tm-1* gene sources were not very effective in controlling blotchiness.**

Blotchy ripening symptoms may be induced in the tomato fruit when plants are infected with tobacco mosaic virus (TMV) (6, 7) particularly when the infection occurs in the latter stages of fruit development (3). These symptoms have been described as a shock reaction resulting from virus invasion of developing fruits, followed by a hypersensitive host response (3). Resistance breeding appears to offer the most promising method for controlling TMV in greenhouse tomatoes. Resistant genes are mostly dominant and can be readily incorporated in F_1 cultivars by having at least 1 parent homozygous for resistance (4, 5, 8, 9).

Identification of the specific TMV strain involved and corresponding resistance sources are critical considerations for controlling the ripening disorder. In an early study, USDA 63G463, a virescence-free source of resistance, was utilized to reduce both incidence and severity of blotchiness in tomato hybirds (6). Hybrids of 'Perou 2', a source of dominantly inherited resistance (*Tm-2*), and susceptible greenhouse lines were found to

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