residues of this pesticide and its metabolite. Confirmation of the molecular weights of these derivatives by chemical ionization mass spectrometry and supporting evidence from reasonable electron impact mass spectrometry fragmentation patterns gives assurances that the proposed structures are correct.

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Simple Dye Release Assay for Determining Endopectinase Activity

David R. Friend and George W. Chang*

Covalently dyed pectin is used as the basis of a simple spectrophotometric assay for the determination of endopectinase activity. The water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride is used to covalently attach the azo dye N-[1-[4-[(3,6-disulfo-1-naphthyl)azo]-naphthyl]]ethylenediamine (DISANED) to pectin under mild conditions (pH 4.75) to form DISANED–pectin. The dyed pectin is highly soluble in the aqueous buffer solutions used. Conditions for the use of DISANED–pectin to measure endopectinase activities in a variety of crude fungal and bacterial preparations are described. The rate of release of reducing sugars from DISANED–pectin is slower than that from unmodified pectin. Pectin methylesterase (PME) does not release measurable amounts of DISANED from DISANED–pectin. Also, addition of PME to a pectinase enzyme preparation does not alter the rate of release of dyed fragments from DISANED–pectin.

Enzymes that depolymerize pectin (polygalacturonic acid methyl ester) are known collectively as pectinases. They can be classified broadly as endodepolymerases, which act within the polysaccharide chains, and exodepolymerases, which act at the ends. Measurement of the endodepolymerizing enzymes of these two groups has usually involved comparison of the rate of decrease in viscosity with the rate of release of reducing sugars (Lim et al., 1980; Itoh et al., 1980). Although viscometric determination of endopectinase activity using capillary flow methods is quite sensitive, it is also slow and cannot be used with enzyme preparations containing particulate matter. Release of reducing sugars has been widely used to determine pectinase activity, but it cannot distinguish endo- from exodepolymerase activities and it is subject to interference by reducing substances present in crude enzyme preparations. Furthermore, reducing sugar assays are of limited use in the presence of viable organisms that can quickly metabolize the oligo- and monosaccharide products of depolymerization.

Because of these limitations in assay procedures for polysaccharide depolymerizing enzymes, alternative techniques that measure enzyme activity by the release of colored degradation products from covalently dyed substrates have been developed. Such assays are simple, rapid, and free from a great variety of chemical interferences. They have been used successfully for the measurement of α-amylase (Dougherty, 1975; Hejgaard and Gibbons, 1979), cellulase (Poincelot and Day, 1972; Ng and Zeikus, 1980), endo-1,3-β-glucanase (Philpott and Chapman, 1977), dextranase (Huang and Tang, 1976), and β-mannanase (McCleary, 1978). However, the development of a similar dye release assay for pectin and other acidic poly-

saccharides has been hampered by the inability to covalently attach dyes under conditions mild enough to preclude degradation. Another problem of the application of this technique to acidic polysaccharides, such as pectin, is the solubility of the dyed polymer. In preliminary experiments a two-step procedure similar to that of Huang and Tang (1976) was used. First an ethylenediamine moiety was attached to the carboxylic acid groups of pectin via a water-soluble carbodiimide. Then the free amino groups of the ethylenediamine moiety were coupled in a nucleophilic reaction with the vinyl sulfone groups of Remazol Brilliant Blue R, yielding a highly modified polymer. However, this material was nearly insoluble in the buffers used in the enzyme assays. Futhermore, the yields were low due to the rather harsh conditions of the second step (pH 9–10 for 30 min), which resulted in considerable depolymerization of the pectin.

This paper describes the development of a dyed substrate assay procedure utilizing the synthetic water-soluble azo dye DISANED. The dye is coupled to pectin in a reaction with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride to form the water-soluble pectin derivative DISANED–pectin (Figure 1). Endopectinase activity is determined by measuring the rate of release of dyed fragments soluble in 63% (v/v) ethanol.

MATERIALS AND METHODS

Preparation of N-[1-[4-[(3,6-Disulfo-1-naphthyl)azo]naphthyl]]ethylenediamine (DISANED). 1-Aminonaphthalene-3,6-disulfonic acid from Fluka AG, Switzerland, and N-1-naphthylethylenediamine dihydrochloride (NED) from Sigma Chemical Co., St. Louis, MO, were coupled by diazotizing 1-aminonaphthalene-3,6-disulfonic acid, followed by coupling to NED. During the coupling step, 2 N NaOH was added as necessary to keep the pH between 6 and 7. After coupling was complete (30 min), the pH was adjusted to neutrality, NaCl was added

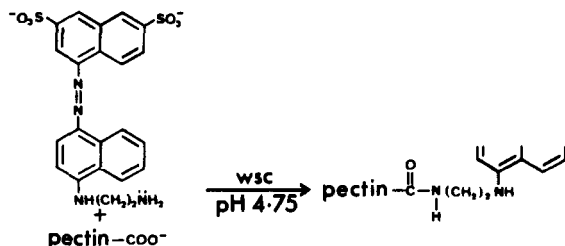Department of Nutritional Sciences, University of California, Berkeley, California 94720.

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Figure 1. Preparation of DISANED–pectin. WSC is the water-soluble carbodiimide, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride.
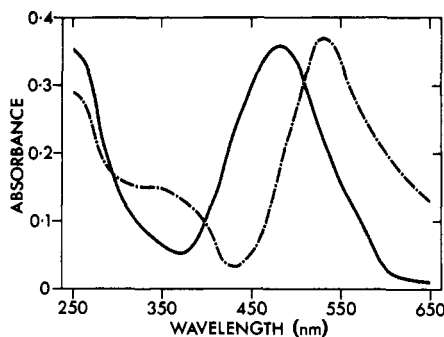

Figure 2. UV–visible spectra of DISANED purified by gel filtration. 12.5 μg/mL in 0.01 M phosphate buffer, pH 7.5 (—), and 12.5 μg/mL in 0.1 M HCl (-·-).

to 0.5 N, and the red-orange azo dye was collected. The dye was recrystallized from a 95% ethanol–0.01 N NaCl solution.

UV and visible spectra were obtained with a Cary Model 210 spectrophotometer. IR spectra were obtained with a Perkin-Elmer Model 137 IR spectrophotometer. The melting point was obtained on a Buchi melting point apparatus and the value reported is uncorrected. TLC was performed on precoated sheets (0.2 mm) of silica gel 60 on aluminum backing (E. Merck, Darmstadt, GFR) by using 1-butanol–95% ethanol–H_2O–acetic acid (30:30:20:1) as the developing solvent. The plates were viewed under natural and UV light (λ = 320 nm). A small amount (10–20 mg) was purified further by gel filtration on a 7.5 × 3.0 cm column of Sephadex G-10 (Pharmacia, Uppsala, Sweden) eluted with water at a flow rate of 0.15 mL/min. The main red-orange band was collected and recrystallized from 95% ethanol: mp >210 °C dec; λ_{max} (0.1 M HCl) 531 nm (ϵ 15 300 M^{-1} cm^{-1}); λ_{max} (0.1 M phosphate, pH 7.5) 485 nm (ϵ 15 000 M^{-1} cm^{-1}) (see Figure 2 for UV–visible spectra); ν_{max} (KBr) 3350, 1625, 1560, 1325, 1250, 1200, 1120, 1060, 820, and 760 cm^{-1}. TLC analysis of DISANED showed the main red-orange spot at R_f 0.43 and two very small red-orange spots with R_f's 0.54 and 0.55. One small spot cochromatographed with and looked the same as NED (R_f 0.51) under UV light. DISANED purified by gel filtration showed only one red-orange spot (R_f 0.43) and no spots visible under UV light.

Preparation of DISANED–Pectin. Pectin (0.5 g, polygalacturonic acid methyl ester from citrus fruits, Sigma, grade 1, ca. 84% galacturonic acid, methoxy content ca. 7.5%) was blended with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) for 3 min at medium speed in water (100 mL). DISANED (4.0 g) dissolved in water (100 mL) was added to the pectin solution. The pH was adjusted to 4.75; and 0.35 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (100 mL) from Sigma was added to the pectin–dye mixture. The reaction mixture was stirred and maintained at pH 4.75 ± 0.1 by periodic addition of 1.0 N HCl. The

reaction was allowed to proceed at room temperature for 24 h, at which time additional DISANED (2.0 g in 50 mL water) and 0.18 M 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (50 mL) were added. The pH was again maintained at ca. 4.75 and the reaction allowed to proceed an additional 24 h. Then the reaction was stopped by addition of 3 volumes of 95% ethanol. After 5 min, the precipitated DISANED–pectin was removed by centrifugation at 5000g for 10 min. The precipitate was suspended in 0.1 M NaCl (100 mL) and blended with the Polytron homogenizer (medium speed, 2 min) and repeatedly reprecipitated and resuspended in the same way until the supernatant was clear. The washed substrate was then suspended in water (100 mL) and dried overnight by lyophilization.

Determination of the Degree of Substitution. DISANED–pectin (ca. 10 mg) was suspended in 0.01 M phosphate, pH 7.5, with the Polytron homogenizer (medium speed, 2 min) to give a concentration of 0.1 mg/mL. The UV–visible spectrum was taken and compared to that of the free, gel-filtered DISANED in the same buffer. The extinction coefficient (ϵ 15 000 M^{-1} cm^{-1}) was used to calculate the DISANED content of the DISANED–pectin solution. The total carbohydrate content of the solution was then measured by the Dubois method (Dubois et al., 1956) and calculated as the number of anhydrohexose units from unmodified pectin. The absorbance reading at 490 nm for the Dubois method was corrected for interference by DISANED, which gave measurable absorbance at 490 nm. From these data the degree of substitution could be determined as the ratio of anhydrohexose units to DISANED units.

Assay of Pectinase Activity with Unmodified Pectin. Enzyme preparations used were Klerzyme Liquid 200 (G. B. Fermentation, Des Plaines, IL), Irgazyme 100 (Ciba Geigy, Greensboro, NC), pectinase (U.S. Biochemical Corp., Cleveland, OH), and cellulase (Sigma, C7377, practical grade), all derived from *Aspergillus niger*. Cecal contents from rats fed one of four diets were also analyzed. One fiber-free diet contained casein (20%) as the protein source. It was diluted with 10% pectin or cellulose to make two fiber-containing diets. The fourth diet was fiber free with soy protein (20%) as the protein source. All other dietary components were equivalent between each diet group.

All the *A. niger* enzyme preparations (0.5 mL) were added to pectin (1.0 mL, 2% w/v in a 0.1 M NaCl–0.03 M acetate buffer, pH 4.5) that had been blended with a Polytron homogenizer (medium speed, 2 min). Incubations were for 1.0 min at 40 °C in a shaking water bath. Cecal enzyme preparations (0.5 mL) were incubated in the same fashion except that the pectin was suspended in 0.1 M NaCl–0.01 M phosphate buffer, pH 7.5. Reactions were stopped by addition of the low alkaline copper reagent (1.5 mL) of Nelson and Somogyi (Nelson, 1944; Somogyi, 1952). These solutions were immediately placed in a boiling water bath for 10 min. The solutions were cooled on ice and the arsenomolybdate reagent (1.5 mL) was added. The solutions were then mixed and diluted 5-fold. Absorbance was measured at 500 nm on a Bausch & Lomb Spectronic 100 spectrophotometer equipped with a flow-cell assembly and the reducing sugars calculated as D-galacturonic acid (Sigma). A unit of enzyme activity is 1.0 μmol of reducing sugar released/min at 40 °C at pH 4.5 for the fungal enzyme preparations and pH 7.5 for the cecal enzyme preparations.

Assay of Endopectinase Activity on DISANED–Pectin. The *A. niger* enzyme preparations (0.5 mL) were
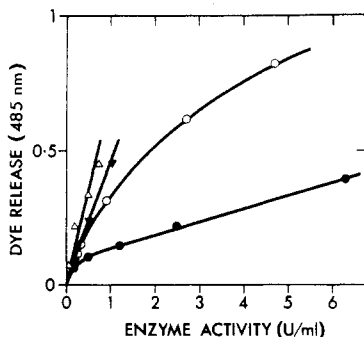
Figure 3. Relationship between endopectinase activity measured at 485 nm and total enzyme activity measured as reducing sugar release for the fungal enzyme preparations Klerzyme Liquid 200 (O), Irgazyme 100 (●), cellulase (▼), and U.S. Biochemical pectinase (△).

added to DISANED–pectin (1.0 mL, 2% w/v suspended in 0.1 M NaCl–0.03 M acetate, pH 4.5, with the Polytron homogenizer at medium speed for 2 min) and incubated for 10 min at 40 °C in a shaking water bath. Cecal enzyme preparations (one cecum per diet) were assayed by the same method except that the substrate was suspended in 0.1 M NaCl–0.01 M phosphate, pH 7.5. The reaction was terminated by addition of 95% ethanol (3.0 mL). Thirty minutes later, the mixture was centrifuged at 5000g for 30 min. The enzyme activity was monitored by the increase in absorbance at 485 nm of the supernatant solution. The reducing sugar equivalents of the supernatant solutions of the A. niger assays were determined by evaporating the solutions (2.0 mL) to dryness under a stream of N_2, redissolving the residues in water (0.5 mL), and analyzing 0.4 mL of this solution by the Nelson–Somogyi method. Absorbance was measured at 660 nm (to avoid interference from the dye) and calculated as D-galacturonic acid.

Determination of the Effect of Pectin Methylesterase (PME) on Dye Release. The dye release assay was performed as outlined in the preceding section for the measurement of endopectinase activity. The enzyme PME (EC 3.1.1.11, Sigma, P6763, 1 unit hydrolyzes 1.0 μmol of methanol from pectin/min at pH 7.5 at 30 °C) was used in place of the pectinase enzyme preparation. The amount of PME assayed ranged from 0 to 2.0 units/mL. In a second experiment, Irgazyme 100 (0–5 units/mL) was incubated with DISANED–pectin (1.0 mL of 2% w/v in 0.1 NaCl–0.03 M acetate, pH 4.5) with and without added PME (1.0 unit/mL).

RESULTS

Preparation and Properties of DISANED–Pectin. Introduction of DISANED into pectin by the use of the water-soluble carbodiimide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, produced a highly substituted polymer. There was very little degradation of the pectin during derivatization. The degree of substitution calculated as the anhydrohexose units to DISANED was found to be 4 to 1. This represents a modification of 65% of the nonesterified carboxylic acid groups present in the pectin used in these experiments. DISANED–pectin was found to be completely soluble in both buffers used in the enzyme assays. The visible spectrum of DISANED, whether free in solution at pH 4.75 or covalently bound to pectin, was identical.

Action of Pectic Enzyme Preparations on DISANED–Pectin. The relationship between solubilization of dyed fragments by the four A. niger preparations and total enzyme activity as measured by reducing sugar analysis on unmodified pectin is shown in Figure 3. Each
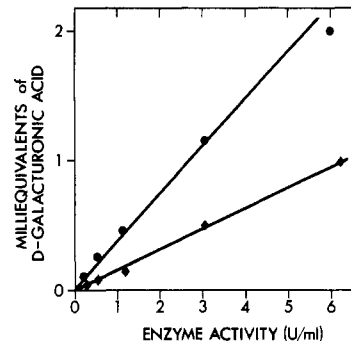

Figure 4. Reducing sugars found in the supernatants when unmodified pectin (●) and DISANED–pectin (♦) are used as substrates for Irgazyme 100. Measurements are at 660 nm and expressed as milliequivalents of D-galacturonic acid.

Table I. Comparison of Reducing Sugars Produced from Unmodified Pectin and DISANED-Pectin

enzyme preparation	RS(unmodified pectin)/ RS(DISANED–pectin)[a]
Klerzyme Liquid 200	3.7
Irgazyme 100	3.2
U.S. Biochemical pectinase	3.0
cellulase	13.0

[a] RS is the slope of the line obtained by plotting reducing sugars produced (measured at 660 nm) against total enzyme activity (units per milliliter) as is shown for Irgazyme 100 in Figure 4.

data point represents the average of four determinations. The coefficient of variation was generally less than 6%.

The rate of release of reducing sugars at a given enzyme concentration from DISANED–pectin was always slower than that from unmodified pectin. Figure 4 shows plots of reducing sugar release form DISANED–pectin and unmodified pectin by the Irgazyme 100 preparation. In Table I, the release of reducing sugars from both substrates by all the A. niger enzyme preparations is compared. Each value represents the average of four determinations, and the coefficient of variability was less than 4%.

When PME (0.2 units/mL) was incubated with DISANED–pectin, no measurable amount of dye was released. Furthermore, addition of 1.0 unit/mL PME to various levels (0.5 units/mL) of Irgazyme 100 had no effect on the rate of dye release: the curve of dye release against total enzyme activity as measured by reducing sugar analysis was identical with that shown in Figure 3.

Assay of Cecal Contents. The cecal contents of rats were analyzed for endo pectinase activity as described under Materials and Methods. Figure 5 presents the dye release data for four different concentrations of the cecal contents for each diet. Values given represent the average of two determinations, and their coefficients of variation were generally less than 6%. Reducing sugar analysis performed after incubating the cecal contents with unmodified pectin showed that an increase in dye release corresponded to an increase in reducing power.

DISCUSSION

Preparation and Properties of DISANED–Pectin. The two sulfonic acid groups make the azo dye, DISANED, highly souble in aqueous buffer solutions. Linking the dye to pectin produces a polymer that is very soluble in both the acetate and phosphate buffers used in these experiments. This obviated problems of insolubility encountered with a two-step procedure similar to that of Huang and Tang (1976). In addition, the use of the one-step reaction
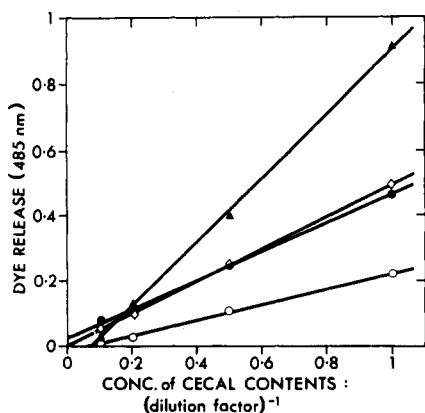
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Figure 5. Dye release (485 nm) against concentration of cecal contents:(dilution factor)$^{-1}$ as assayed under standard conditions for the cecal contents (see Materials and Methods) of rats fed a 10% pectin diet (▲), a 10% cellulose diet (●), a 20% casein diet (◊), or a 20% soy protein diet (O).

at pH 4.75 results in very little degradation of the pectin polymer. This is in contrast to the two-step reaction scheme attempted initially that resulted in considerable pectin degradation.

Action of Pectic Enzymes on DISANED–Pectin. Results from other enzyme systems suggest that dye release is the result of endopectinase activity. The exohydrolases amyloglucosidase (Marshall, 1970) and exo-1,3-β-glucanase (Philpott and Chapman, 1977) sequentially remove D-glucosyl residues from the nonreducing end of amylose and pachyman (a 1,3-β-glucan), respectively. These enzymes are unable to release dyed fragments from Cibacron Blue dyed amylose and pachyman. Thus, it would be reasonable to expect that exopectinases cannot release dyed fragments from DISANED–pectin. If this is the case, a high ratio of dye release to reducing sugar release would indicate a high ratio of endo- to exopectinase activity. Conversely, a low ratio of dye release to reducing sugar release would indicate a low ratio of endo- to exopectinase activity. The data in Figure 3 suggest that the U.S. Biochemical pectinase preparation has the highest ratio of endo- to exopectinase activity, followed by cellulase, Klerzyme Liquid 200, and then Irgazyme 100.

Reducing sugar release from DISANED–pectin is much slower than that from unmodified pectin as can be seen in Figure 4 and Table I. This is not surprising since substitution with DISANED has eliminated 65% of the available carboxylic acid sites for endopolygalacturonase (endo-PG) action. Furthermore, most commercial pectinase preparations contain substantial quantities of PME, which no doubt promotes depolymerization by endo-PG. Although PME activity was not measured, substitution of every fourth sugar residue with DISANED would be expected to severely limit the linear sequential removal of methoxyl groups by PME, which in itself might account for some of the lessened endo-PG activity.

The ability of commercial cellulase to release reducing sugars was much more sensitive to DISANED modification than that of other enzyme preparations (Table I). This may indicate that cellulase is more dependent upon free carboxyl groups or PME than the other preparations are. An alternative explanation, that cellulase has an unusually

low endo-to exopectinase activity, seems unlikely in light of the data in Figure 3.

Pectin methylesterase itself was unable to catalyze any measurable hydrolysis of DISANED from DISANED–pectin. This result is to be expected since PME would have to catalyze the hydrolysis of a hindered amide bond, as opposed to an unhindered ester bond on which it readily acts. Increasing amounts of PME present in a pectinase preparation have been reported (Dahodwala et al., 1974) to affect total pectinase activity by increasing the number of carboxylic acid groups in the substrate. However, when PME was added to Irgazyme 100, the rate of release of dyed fragments from DISANED–pectin was found to be unaltered.

Measurement of endopectinase activity in cecal contents illustrates the use of this assay under conditions where reducing sugar measurements might be biased. As can be seen in Figure 5, excellent linearity between dye release and enzyme concentration was obtained. Although not the purpose of these experiments, the effect of diet can be seen since the pectin diet gives nearly twice the enzyme activity as the cellulase and casein diets and almost 4 times the activity as the soy protein diet.

In conclusion, a simple spectrophotometric assay method for the measurement of endopectinase activity has been developed by using DISANED–pectin as a dyed substrate. This method should be easily adaptable to other acidic polysaccharides such as gum arabic, alginic acid, and gum xanthan. This method of enzyme analysis appears timely because of the increasing interest in dietary fiber and the health and well-being of the individuals who consume it.

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