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A Spectrophotometric Assay for the Enzymatic Demethoxylation of Pectins and the Determination of Pectinesterase Activity

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A rapid spectrophotometric method for the determination of pectinesterase activity is presented. In this assay, methanol released from pectin by pectinesterase is oxidized with alcohol oxidase to form hydrogen peroxide and formaldehyde. Hydrogen peroxide is then quantitated with peroxidase and the chromogen 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). Since both reactions exhibit the same pH optimum it was possible to couple the methanol assay directly to the action of pectinesterase for the real-time determination of this enzyme. The assay is reliable and sensitive, being capable of quantitating a minimum pectinesterase activity of 0.0625 unit (1 unit = 1 μM methanol released per minute). It is also capable of detecting the enzymatic demethoxylation of galactopyranosyl uronic acid methyl esters of pectin down to a minimum concentration of 1.56 nM of methanol per milliliter using a pectin substrate with a methoxy content of 10% (w/w) at a concentration of 0.5 μg/ml.

Pectin, a major biopolymer of fruits, is composed primarily of essentially linear polymers of D-galactopyranosyl uronic acid units joined in α-D-(1 → 4) glycosidic linkages. This regular structure is interrupted, however, with L-rhamnopyranosyl units and with side chains containing other neutral sugars. The intervening regions of the chain, which are largely composed of galacturonic acid, are esterified with methanol up to 80%. The degree of methoxylation (DM)[3] varies with age and location within the plant tissue (biosynthetic conditions) and with the method of extraction. A good knowledge of the chemical structure of pectins is of major importance from a technological point of view and also aids the understanding of the role of pectic substances in the cell walls (1).

Pectinesterases (pectin pectylhydrolase EC 3.1.1.11; PE) catalyze the hydrolysis of the galactopyranosyl uronic acid methyl esters of pectin, converting high methoxy pectins (HM) to low methoxy ones (LM) and methanol (Eq. [1]). Pectinesterases have been found in all species of higher plants tested and are also produced by a number of plant pathogenic fungi and bacteria. Plant pectinesterases participate in the conversion of protopectin to soluble pectin and pectate and are involved in plant maturation processes as well as in mechanisms that protect the plant from infection (1). Plant pectinesterases do not hydrolyze methyl esters in a more or less random fashion, as do microbial ones, but act processively on the galacturonan chain creating blocks of free carboxyl groups. These blocks are extremely prone to complex formation and precipitation with calcium ions (2). Fractionation of pectins by ion-exchange chromatography is mainly based on the degree of esterification of the pectin. Furthermore, the distributions of free and methoxylated carboxyl groups influence the elution behavior of pectic molecules during high-performance ion-exchange chromatography (HPIEC) (3). Versteeg, who investigated the demethoxylation abilities of PE isoenzymes I and II from orange peel on partially demethoxylated pectins, recently

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[3] Abbreviations used: DM, degree of methoxylation; PE, pectin pectylhydrolase; HM, high methoxy pectins; LM, low methoxy pectins; HPIEC, high-performance ion-exchange chromatography; AOD, alcohol:oxygen oxidoreductase; POD, donor:hydrogen peroxide oxidoreductase; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

showed that pectinesterases were unable to demethoxylate pectin completely and that PE I was able to demethoxylate highly esterified pectin better than PE II (4). A substantial amount of substrate was not demethoxylated by either PE, possibly because of irregularities in the pectin chain. As free carboxyl groups in the pectin are required for optimal enzyme–substrate complex formation, this behavior has been assumed to depend on the distribution of these groups along the polymer backbone. Therefore, the activities of various pectinesterases on a pectin fraction in relation to the degree of methoxylation after partial alkaline saponification may give valuable information on the intramolecular distribution of free and esterified carboxyl groups in pectin. To gain such information, a sensitive and simple assay for pectinesterase is desired.

A unit of PE activity is defined as the amount of enzyme that liberates 1 μM of carboxyl groups from methoxylated pectin per minute under standard assay conditions. Methods for the determination of the enzymatic activity by titrimetry (5), spectrometry (6), and manometry (7) have been described. The most frequently used method of determining the activity of pectinesterase is by real-time titration of the liberated carboxyl groups with dilute alkali (4) while the pH is kept constant (pH-stat). Demethoxylation of pectin by plant pectinesterase exhibits optimum activity at pH 7.5. Using a titrimetric assay at the optimum pH of pectinesterase corrections must be made for partial dissociation of the liberated carboxyl groups, whose pK values vary with the concentration of the pectin, the degree of esterification, and the mode of deesterification (8).

Because of the need to measure pectinesterase activities without any empirically derived corrections for its substrate pectin, much attention has been paid to the analysis of the released methanol by spectrophotometry (9), gas chromatography (10), headspace gas chromatography (11), high-performance liquid chromatography (12), and radioisotope methods (13). Pyrolysis gas chromatography (14), pyrolysis mass spectrometry (15), ^{13}C-NMR (16), and IR spectroscopy (17) have been employed to examine pectins with regard to their DM during pectinesterase-induced plant maturation processes.

One spectrophotometric method often used to determine methanol in relation to pectin methyl ester hydrolysis involves the oxidation of released methanol to formaldehyde with potassium permanganate, reduction of the excess permanganate with sodium arsenite (9), and condensation of the formaldehyde with ammonia and 2,4-pentanedione to yield the colored Hantzsch product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (18). The procedure was modified by Klavons and Bennett (19), who used the enzyme alcohol oxidase (alcohol:oxygen oxidoreductase, EC 1.1.3.13; AOD) (Eq. [2]) instead of permanganate for methanol oxidation to prevent interferences by trace components of nonreduced permanganate and to replace the toxic arsenite.

A major disadvantage of those methods is that they are discontinuous in that aliquots of the reaction solution have to be taken at distinct time intervals and subjected to analysis of the released methanol. The utility of existing methods is also restricted by the fact that the sensitivities of those assays for the determination of methanol are sometimes too low, especially if low amounts of pectin are available, e.g., in fractions generated during the separation of pectic substances by high-performance size-exclusion chromatography or high-performance ion-exchange chromatography (20).

Recently we developed a very sensitive modification of the AOD procedure (21). As with the previous published method (19) it uses alcohol oxidase from the yeast *Pichia pastoris* to oxidize methanol to formaldehyde, but subsequently employs peroxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7; POD) and hydrogen peroxide, the second product of the AOD reaction, to catalyze the oxidation of the chromogen 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to form the colored ABTS radical cation (Eq. [3]). This so-called AOD-POD-ABTS assay offers a 20-fold increase in sensitivity (0.05 μg methanol/ml) over the AOD procedure (1 μg/ml) (19).

We present here an application of the AOD-POD-ABTS procedure to continuously determine the release of methanol from pectin by pectinesterase and we describe its use to determine pectin or pectinesterase levels. Under the conditions of this assay, the equilibrium constants for the enzymatic reactions involved in this procedure all strongly favor the forward reaction as written in Eqs. [1], [2], and [3]. Thus, in the presence of saturating amounts of pectin the rate of generation of the ABTS radical cation, which absorbs strongly at 420 nm, is proportional to the amount of pectinesterase in the reaction. This interdependence is also valid for the determination of unknown amounts of pectin in the presence of saturating amounts of pectinesterase.

$$\text{Pectin-COOCH}_3 + \text{H}_2\text{O} \xrightarrow{\text{Pectinesterase (PE)}} \text{Pectin-COO}^- + \text{H}^+ + \text{CH}_3\text{OH} \quad [1]$$

$$\text{CH}_3\text{OH} + \text{O}_2 \xrightarrow{\text{Alcohol oxidase (AOD)}} \text{HCHO} + \text{H}_2\text{O}_2 \quad [2]$$

$$\text{H}_2\text{O}_2 + 2\ \text{ABTS(H)} \xrightarrow{\text{Peroxidase (POD)}} 2\ \text{H}_2\text{O} + 2\ \text{ABTS}^{\bullet +} \quad [3]$$

MATERIALS AND METHODS

Chemicals

Citrus pectin, methoxy content 10% (w/w) (supplier's note, as received) (No. P-9135), alcohol oxidase from *P.*

pastoris [52 mg protein/ml (Biuret), 33 units/mg protein (pH 7.5, 25°C), No. A-2404], peroxidase [Type VI-A from horseradish, 1380 units/mg solid (pH 5.0, 25°C), No. P-6782], 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (No. A-1888), and pectinesterase from orange peel [130 units/mg solid (pH 7.5, 30°C), No. P-5400] were obtained from Sigma Chemical Co. (St. Louis, MO). Methanol (high purity) was obtained from Burdick & Jackson (Muskegon, MI). All other chemicals were analytical grade and of the highest purity obtainable.

Methods

Basic assay protocol. The following stock solutions were employed: (A) One tablet of ABTS (=10 mg ABTS) was dissolved in distilled water and diluted to 2 ml (approximately 0.1 mg ABTS per assay); (B) 2.17 mg of peroxidase was dissolved in distilled water and diluted to 2 ml, and 20 μl of this solution was diluted to 2 ml with distilled water (approximately 0.3 unit per assay); and (C) 30 μl alcohol oxidase solution was diluted to 2 ml with distilled water (approximately 0.5 unit per assay). Reagents (A), (B), and (C) were stored at 4°C in the dark and mixed shortly before the assay was performed. For (D) 0.77 mg of pectinesterase was dissolved in distilled water and diluted to 2 ml (approximately 1 unit per assay). For the determination of pectinesterase at various enzyme concentrations, the pectinesterase stock solution (D) was diluted 1:1, 1:4, 1:8, and 1:16 with distilled water (D_x). For (E) 1050 mg of pectin was dissolved in distilled water without further purification and diluted with water to 100 ml (pectin stock solution). Aliquots of this pectin stock solution were diluted in 0.2 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl, to give pectin standard solutions with concentrations of 2.1, 4.2, 6.3, 8.4, and 10.5 μg/ml (E_x).

Solution A (20 μl) and solution B (20 μl) were pipetted into an amber-colored Eppendorf microcentrifuge tube (0.5 ml volume), which was light impermeable to prevent light-induced reactions. Aliquots (100 μl) of pectin standards dissolved in 0.2 M potassium phosphate buffer, pH 7.5, and 0.1 M NaCl were added. The tubes were shaken for 3 s with a Vortex mixer (Scientific Industries, Bohemia, NY). An aliquot of this mixture (120 μl) was pipetted into the microcuvette (path length, 10 mm; volume, 200 μl) placed in the light beam of the spectrophotometer. Solution C, diluted alcohol oxidase (20 μl), was pipetted into the cuvette with repeated flushing of the pipet tip to disperse the enzyme and to start the conversion of free methanol contaminants; the kinetic software was started instantly. After 2.0 min pectinesterase solution (20 μl) was added with multiple pipet flushings. Absorbances were measured as a function of time at 20°C and at a wavelength of 420 nm against air (no cuvette). The absorbance of the blank without pectinesterase was subtracted afterward. A Shimadzu Model UV-2101 PC spectrophotometer (Shimadzu Scientific Instr. Inc., Columbia, MD) was used in its single beam modus to measure the absorbance of all samples. Data were analyzed using the Shimadzu UVPC Kinetics Software, Version 2.7.

To construct a methanol calibration curve, solutions of methanol standards (20 μL) from 8.57×10^{-3} to 0.171 μg/ml were added after 2.0 min of preincubation, instead of pectinesterase. To evaluate the contribution of the assay components to false positive reactions, distilled water was added to the pectin standards instead of pectinesterase. Another blank containing 0.2 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl, instead of pectin standard solution, was treated like the pectin standards.

Calculations of pectinesterase activity. The activity of pectinesterase, expressed as micromoles of methanol released per minute, was calculated from the rate of absorbance change of the A_{420} of the reaction mixture, using a molar extinction coefficient ($E_{420\,\mathrm{nm,\,ABTS}}$) of 21,600 $\mathrm{cm^{-1}\,M^{-1}}$ for the ABTS radical cation (22) and allowing for the 1:2 molar stoichiometry between methanol release and the generation of the ABTS radical cation. This resulted in a sensitivity ($E_{420\,\mathrm{nm}}$) of 43,200 $\mathrm{cm^{-1}\,M^{-1}}$ methanol released.

Determination of the enzymatic deesterification of pectin. Pectin standard solutions $E_{2.1}$ through $E_{10.5}$ (100 μl) and solutions of A (20 μl) and B (20 μl) were mixed in light-impermeable microcentrifuge tubes (as described above). Preincubation was started by addition of solution C (20 μl), followed after exactly 2 min by the addition of solution D (20 μl). The spectrophotometric analysis for methanol was then conducted as described above.

Determination of pectinesterase activity. To establish the suitability of the AOD-POD-ABTS assay for the determination of pectinesterase levels a pectin standard solution was used as substrate (8.4 μg pectin/ml, dissolved in 0.2 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl). This solution (100 μl) was preincubated with solutions of ABTS, POD, and AOD (see above), and 20 μl of the pectinesterase stock solution (D) or its 1:1, 1:4, 1:8, and 1:16 dilutions (D_x) was added. The absorbance of each sample was monitored at 420 nm for 5 min and the rate of release of methanol was calculated.

Examination of interferences of alcohol oxidase by alcohols. Methanol or glycerol were added to a pectin standard solution (8.4 μg pectin/ml, dissolved in 0.2 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl) in amounts sufficient to give to 21, 27, and 36% of the molar content of methanol that was esterified in the

pectin. Thus, for methanol 0.0055, 0.0071, and 0.009 μmol/ml were incubated, and for glycerol 0.009 μmol/ml. A blank (8.4 μg pectin/ml) was treated like the samples except that the addition of alcohol was omitted. Reaction was started by addition of solution C (20 μL) to 120 μL of a mixture of the pectin-alcohol solution (100 μL), solutions A (20 μL) and B (20 μL). After preincubation for 2 min, 20 μl of pectinesterase stock solution (D) were added and the change in absorbance at 420 nm was determined.

Examination of potential sources of pectinesterase inhibition. Pectin stock solution D and 2.0 M potassium phosphate buffer, pH 7.5, 1.0 M NaCl, were added to weighed amounts of glycerol. The resulting solutions were diluted with distilled water to give a final pectin concentration of 8.4 μg/ml and glycerol concentrations in the range of 0.1 to 0.6 g/ml in 0.2 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl. Aliquots (100 μl) were assayed as described above (preincubation with solutions A (20 μL), B (20 μL), and C (20 μL) followed by incubation with pectinesterase stock solution (20 μL).

RESULTS AND DISCUSSION

Examination of the Enzymatic Deesterification of Pectin at Various Pectin Concentrations

The demethoxylation of pectin by pectinesterase, as well as the enzymatic determination of methanol with AOD, POD, and ABTS, exhibit optimum activities at pH 7.5 (21). Therefore, this pH was used for the assay described here. Aliquots of solutions of pectin ranging in concentration from 2.1 to 10.5 μg/ml were subjected to demethoxylation with pectinesterase after a 2-min preincubation with AOD, POD, and ABTS to measure basal methanol levels. Under the conditions described here the release of methanol from pectin by pectinesterase was accompanied by an increase in absorbance at 420 nm.

Pectinesterase-independent reactions during the assay. As can be seen in Fig. 1, preincubation of pectin solutions with AOD, POD, and ABTS resulted in a rapid, small increase in absorbance which lasted for about 1 min, and was followed by a much slower rate of increase of about 40 mAbs/min (Fig. 1, $t = 0–2.0$ min). Even blanks containing only the two enzymes AOD and POD in the presence of ABTS in 0.2 M potassium phosphate buffer (pH 7.5), 0.1 M NaCl, exhibited the latter, slow absorbance increase. The cause of this observed blank reaction has not yet been identified, but it might be the result of the autoxidation of formaldehyde, reversibly bound to *P. pastoris* alcohol oxidase, to formic acid and hydrogen peroxide and the subsequent reaction of peroxidase with this hydrogen peroxide (23). In any case, the rate of change of absorbance due to

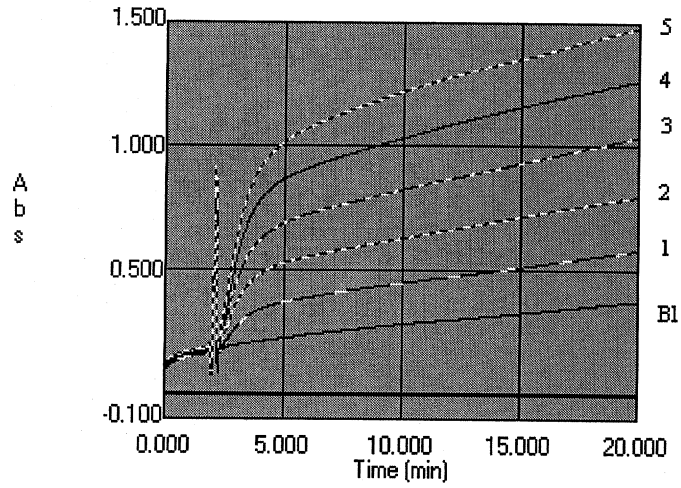

FIG. 1. Demethoxylation of various concentrations of pectin with pectinesterase (PE) using the AOD-POD-ABTS assay. (1) 2.1, (2) 4.2, (3) 6.3, (4) 8.4, (5) 10.5 μg/ml pectin. $t = 0$ min, addition of AOD solution; $t = 2.0$ min, addition of PE stock solution. Bl, blank.

this reaction is small compared to that when pectinesterase is present and can be readily subtracted from the gross rate of change of absorbance.

Addition of pectinesterase to determine demethoxylation of pectin. Pectinesterase was added to the cuvette after 2 min of preincubation of the mixture of pectin, AOD, POD, and ABTS. Opening and closing of the spectrophotometer lid and pipetting and multiple flushing of the pipet tip to disperse the pectinesterase was done in 10 s. A rapid, reversible change in absorbance accompanied this procedure (Fig. 1, spike between $t = 2.0–2.2$ min). After the lid was closed the absorbance reading went back to its initial value. From that point the absorbance change for the blank (no pectin) continued its previously mentioned linear positive increase, which did not reach an end point even after several minutes. The absorbance recording for the pectin samples containing pectinesterase was marked by an immediate increase induced by the release of methanol from pectin by pectinesterase and its participation in the oxidation of ABTS via the action of the AOD-POD enzyme system, resulting in an increase in A_{420} (Fig. 1, $t = 2.2–5.0$ min).

After this initial rapid linear increase in absorbance, the rate of absorbance change decreased, but continued at a distinct, though lower, positive rate, which did not reach an end point even after several minutes (Fig. 1, $t \geqslant 5.0$ min). With increasing concentrations of pectin the slope of the absorbance plot in this region increased proportional to the pectin concentration from 32 mAbs/min (2.1 μg pectin/ml) to 120 mAbs/min (10.5 μg pectin/ml). It has been found recently that this absorbance change is a linear function of the amount of methanol present (21). Since formaldehyde is a substrate for alco-

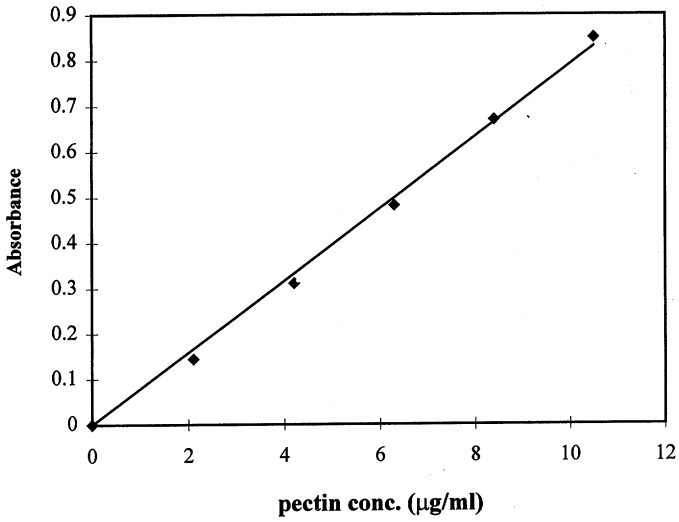

FIG. 2. Calibration curve for the deesterification of pectin standards (average from five values at each concentration) with pectinesterase using the AOD-POD-ABTS assay. Extinctions taken at t = 6.0 min (see Fig. 1).

hol oxidase and is oxidized by this enzyme to formic acid and hydrogen peroxide (23), it is assumed that formaldehyde, as one product of the oxidation of methanol enzymatically released from pectin, is source of additional hydrogen peroxide and is the cause of this observed increase in absorbance change, compared to the blank.

Quantitation of the released methanol. As can be seen in Fig. 1, incubation of increasing amounts of pectin with a fixed amount of pectinesterase led to distinctly higher absorbances in the presence of AOD, POD, and ABTS. When absorbance readings were taken at t = 6.0 min, the measured extinctions were a linear function of the pectin concentration from at least 2.1 to 10.5 μg/ml pectin (Fig. 2). Such pectin levels correspond to concentrations of methanol esterified with the galactopyranosyl uronic acid of pectin in the concentration range from 0.21 to 1.05 μg/ml. Polynomal fitting calculations with intercept zero gave a calibration curve with the formula $y = 0.0007x^2 + 0.073x - 0.0052$ and $R^2 = 0.9994$, where y is absorbance and x is pectin concentration. This validates the subjective impression that the response is a linear one.

A methanol calibration curve was constructed using methanol standards, instead of a mixture of pectin and pectinesterase, ranging in concentration from 0.05 to 1.0 μg/mL. The absorbance was recorded 6 min after the start of the AOD-POD-ABTS assay and plotted as a function of the methanol concentration (Fig. 3). Linear fitting calculations gave the formula $y = 0.8087x$ with $R^2 = 0.9991$, where y is absorbance and x is methanol concentration. It was concluded that, at the very least, the useful range of the assay for methanol was between 0.05 and 1.0 μg/ml. The lower detection limit of 0.05 μg/ml corresponds to the smallest clearly detectable absorbance change (0.030 Abs unit) that is different from the background noise in the presence of pectin, defined as three standard deviations (3σ) of the appropriate blank value. This corresponds to a minimum detectable methanol concentration of 1.56 nmol/ml, which is a minimum detectable amount of 4.2 ng methanol per assay (assay volume 160 μl at a sample volume fraction of 0.536). These results are in good agreement with investigations on the minimal detection limit of H_2O_2, one of the products from the oxidation of methanol, which has been reported for pure solutions of H_2O_2 to be discriminated from blank values by the ABTS method with a probability of 95% at a concentration of 0.2 nmol/ml or 14 ng H_2O_2 per assay (assay volume 2.4 ml at a sample volume fraction of 0.833) (24).

Alkaline demethoxylation of the pectin used here and determination of the released methanol with the AOD-POD-ABTS assay gave an average methanol content of 10.01 μg per 100 μg pectin (21). On this basis the concentration of pectin-bound methanol in a solution of pectin containing 2.1 μg/ml was calculated to be 0.21 μg/ml (6.56×10^{-3} μmol/ml). Employing a sensitivity for methanol of ($E_{420\,\mathrm{nm}}$) = 43,200 $\mathrm{cm}^{-1}\ \mathrm{M}^{-1}$, the measured absorbance of 166 mAbs units (Fig. 2) is significantly lower than the theoretical absorbance of 173 mAbs units to be expected from quantitative demethoxylation. The methanol released by the action of pectinesterase was calculated to be 9.56 μg per 100 μg pectin (=95.6% of the original ester content of the pectin). This less than complete demethoxylation of pectin by

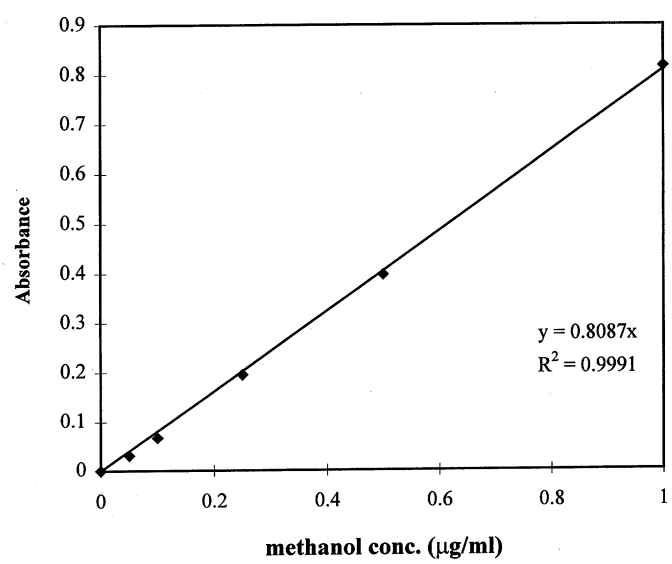

FIG. 3. Methanol calibration curve (average from five values at each concentration). Addition of methanol standards instead of pectinesterase using the AOD-POD-ABTS assay. Extinctions taken at t = 6.0 min.

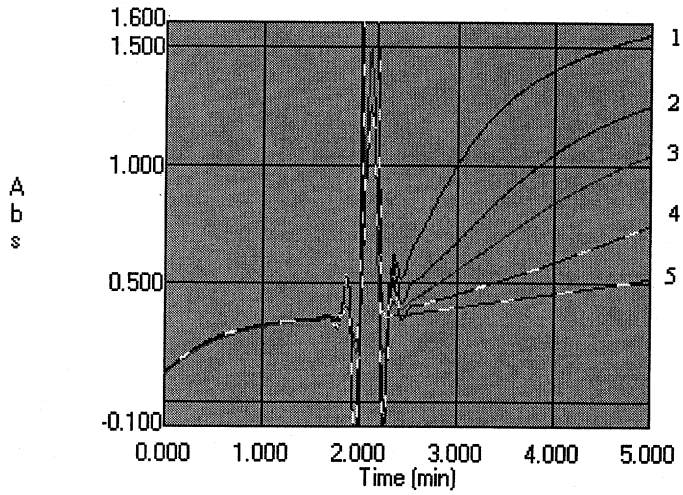

FIG. 4. Determination of pectinesterase (PE) activities in the presence of a given concentration of pectin (8.4 μg/ml) using the AOD-POD-ABTS assay. Pectinesterase concentration: (1) 1.0, (2) 0.5, (3) 0.25, (4) 0.125, (5) 0.0625 unit per assay. t = 0 min, addition of AOD solution; t = 2.0 min, addition of PE solution.

the pectinesterase from orange peel is in agreement with results from other laboratories which have shown that pectinesterases produced by higher plants do not act randomly, as is seen with fungal pectinesterases or alkaline hydrolysis (2). The former primarily demethoxylates blocks of methoxylated galacturonic acids and ester groups next to free carboxyl groups, while the latter agents are also capable of hydrolyzing methyl esters that are not present in blocks. Therefore, methoxylated pectin is only partially hydrolyzed by pectinesterases from higher plants. If the methyl esters are entirely arranged in blocks demethoxylation can be nearly quantitative. As reported here, this was almost the fact with the pectin used in our tests.

The results have been confirmed both by alkaline titration to monitor the appearance of free carboxyl groups as a result of pectinesterase activity and by the well-established permanganate–pentanedione assay (9) to quantitate the release of methanol. Using the AOD-POD-ABTS assay, demethoxylation of pectin at pectin concentrations as low as 0.6 μg/ml (50 ng pectin = 5 ng methanol per assay) could be detected reliably using the pectinesterase concentration employed here.

Determination of Pectinesterase Activity

Solutions of pectinesterase (20 μl) with absolute activities ranging from 0.0625 to 1 unit (activities based on suppliers data) were added to preincubated detection mixtures containing pectin (8.4 μg/ml), AOD, POD, and ABTS in 0.2 M potassium phosphate buffer (pH 7.5), 0.1 M NaCl, and the A_{420} was monitored with a spectrophotometer (Fig. 4). The initial slopes of the absorbance curves following pectinesterase addition were dependent on the activity of the pectinesterase (Fig. 5). The formula for the calibration curve with an intercept zero was calculated to be $y = -0.0273x^2 + 0.8325x$ with an R^2 value of 0.998 using polynomal regression calculations where y is absorbance change and x is pectinesterase concentration. This validates the subjective impression that the response is a linear function of the enzyme concentration. By this method enzyme activities as low as 0.0625 unit per assay could be detected reliably at the given pectin concentration. At pectinesterase levels below this, the amount of methanol required for accurate determination could not be reached in the chosen time interval of 5 min.

A standard pectinesterase assay utilizing a pH-stat to monitor enzyme-dependent deesterification of pectin (4) as well as a conventional methanol assay with permanganate/pentanedione (9) have verified the accuracy of the AOD-POD-ABTS assay. There was agreement between the best estimate of pectinesterase activity obtained from the AOD-POD-ABTS assay and its "true value" as determined by these other methods. Accuracy in comparison to the titrimetric assay was $y = 2.64 + 1.00x$ with a correlation coefficient of $r = 0.992$. The precision of the assay from day to day showed a variation coefficient of 2.2%.

Interferences with the AOD-POD-ABTS Assay

To evaluate the utility of the assay with crude pectinesterase and standardized pectin preparations, potential interferences, their modes of action, and measures for their prevention have to be addressed. In principle, major interferences may be caused by spectral overlapping of colored sample material with the dye formed in the indicator reaction or by competition between the

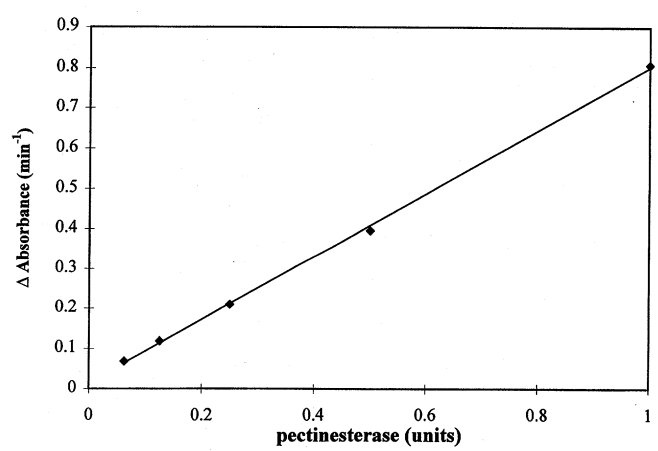

FIG. 5. Pectinesterase (PE) activity as a function of pectinesterase concentration (average of five determinations at each activity). Results evaluated from Fig. 3 (slopes in the time interval t = 2.5–3.0 min).

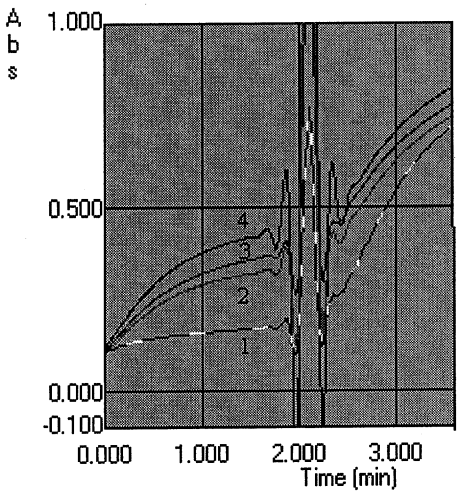

FIG. 6. Interferences of pectinesterase (1 unit/ml) by methanol: (1) blank, (2) 0.0055, (3) 0.0071, (4) 0.009 μmoles methanol/ml. t = 0 min, addition of AOD solution; t = 2.0 min, addition of PE solution.

substrate or its intermediate products and sample components able to serve as alternative substrates.

Interferences with Alcohol Oxidase

It is well known that the substrate specificity of *P. pastoris* alcohol oxidase is not limited to methanol (23). For example, the relative activity toward ethanol has been determined to be 36% compared to methanol (100%), and the K_m values are 0.7 mM for methanol and 9.0 mM for ethanol. Other alcohols such as 1-propanol, 2-propanol, 1-butanol, and allyl alcohol are oxidized at slower rates (23).

Solutions of pectin containing low concentrations of free methanol or glycerol were formulated and the AOD-POD-ABTS assay was conducted, with no added pectinesterase, to test their interferences with the assay. The absorbance curve for glycerol (not shown) was not significantly different than that of the blank, confirming the results of other investigators that polyols at low concentrations do not serve as substrates for alcohol oxidases (25, 26). Free methanol was oxidized to formaldehyde and hydrogen peroxide, resulting in significant linear increases in the initial absorbance (Fig. 6, $t < 0.5$ min). These were proportional to the original concentrations of free methanol. These were followed by much slower rates of increase, similar to that of the blank (Fig. 6, $t > 1.5$ min).

The addition of pectinesterase after 2 min to pectin samples without free methanol (Fig. 6, curve 1) or with various amounts of free methanol (Fig. 6, traces 2, 3, and 4) led to distinct increases in absorbance due to the enzyme dependent release of methanol (Fig. 6, $t \geqslant 2.5$ min). Pectinesterase activity could be calculated by the absorbance change in the time interval t = 2.5–3.0 min without any correction for preexisting methanol in pectin samples. Thus, although free alcohols in pectin samples interfered with the assay, their presence was easily detected because of their reactivity with alcohol oxidase prior to the addition of pectinesterase. With pure aluminum-precipitated pectin, as was used in this investigation, interference by alcohol impurities has not been observed: The absorbance curves in the AOD-POD-ABTS assay prior to the addition of pectinesterase overlap perfectly over the range of pectin concentrations examined (Fig. 1, t = 0–2.0 min).

Errors may arise due to the presence of very high levels of lower alcohols, which can be present in commercial alcohol-precipitated pectin preparations. Due to the high sensitivity of the assay toward such alcohols, purification of those pectins is therefore recommended to remove such alcohol contaminants. This purification can be achieved by using well-accepted procedures, i.e., multiple wetting and drying, dialysis against water, reprecipitation with acetone, or column chromatography.

The presence of free alcohols in pectinesterase preparations would lead to artifactually elevated calculated activities for pectinesterase. Nevertheless, determination of pectinesterase activity could be achieved by subtraction of the absorbance change due to low concentrations of contaminating free alcohol from the apparent pectinesterase activity. The contribution of free alcohols to the AOD-POD-ABTS activity could be determined by conducting a pectinesterase assay with no added pectin, by using heat denatured pectinesterase preparations, by adding pectate (a pectinesterase inhibitor), or by omitting sodium chloride, a pectinesterase activator from the assay. In our investigations with pectinesterase, pectin-free blanks did not exhibit an increase in absorbance after addition of enzyme, which proved its purity with regard to free alcohols.

Interferences with Peroxidase and Hydrogen Peroxide

While peroxidase is specific for the hydrogen acceptor, hydrogen peroxide is not specific for the hydrogen donor. Large numbers of phenols, amino acids, and ascorbate are able to serve as alternative electron donors for hydrogen peroxide (27). Therefore, in the assay method presented here a 100-fold molar excess of ABTS over concentrations of the analyte hydrogen peroxide (22) were employed to suppress reactions in the presence of minor concentrations of competitive hydrogen donors. Reductive reversal of the dye color is of particular concern when ascorbic acid, a strong reductant, is present. Interfering ascorbate could be eliminated by dialysis or by the use of ascorbate oxidase, which does not produce hydrogen peroxide (28). It is important that the pectinesterases contain no or low concentrations of catalase, because catalase destroys hydrogen

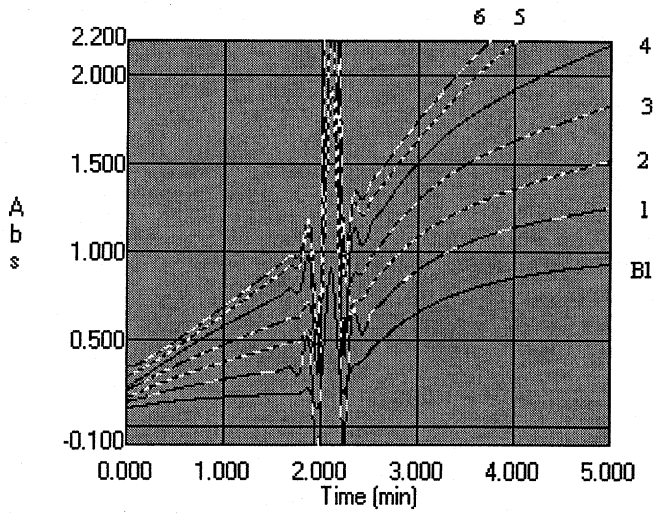

FIG. 7. Inhibition of pectinesterase (1 unit/ml) by increasing concentrations of glycerol: (B1) blank, (1) 0.1, (2) 0.2, (3) 0.3, (4) 0.4, (5) 0.5, (6) 0.6 (g glycerol/ml). t = 0 min, addition of AOD solution; t = 2.0 min, addition of PE solution.

peroxide and can lead to an underestimate of methanol in the assay. Sodium azide may not be included in the assay medium to inhibit contaminating catalase, since alcohol oxidase from *P. pastoris* is strongly inhibited by free azide anions (23).

Potential Interferences with Pectinesterase

High concentrations of sugars inhibit pectinesterase, probably due to their abilities to reduce water activity. Noncompetitive inhibition of pectinesterase was found with sucrose and with glycerol (29). Therefore, glycerol was used here as a reference standard for water activity reducing compounds to study its effect on pectinesterase. Solutions of pectin (8.4 μg/ml) containing glycerol concentrations from 0.1 to 0.6 g/ml were incubated with solutions of AOD, POD, and ABTS. With increasing concentrations of glycerol an increase of absorbance occurred after addition of AOD (Fig. 7, t = 0 min), compared to the blank, which first was presumed to be due to oxidation of glycerol to its aldehyde at high glycerol concentrations (Fig. 7, $t < 2.0$ min). However, previous examinations of the substrate specificity of methanol-oxidizing enzymes with alcohol oxidases from the yeast *Candida boidinii* (25) and from a basidiomycete belonging to the family *Polyporaceae* (26) and our own studies with *P. pastoris* have clearly shown that the enzymes exhibit no activity toward glycerol (0%), compared to methanol (100%). Although data about the substrate specificity of *P. pastoris* alcohol oxidase toward sugars are not available, it has been shown that the *P. pastoris* enzyme is similar to other yeast alcohol oxidases reported in the literature with regard to such features as molecular weight, number of subunits, cofactor requirements, temperature optimum, specific activity, etc. (22). This makes it seem improbable that the *P. pastoris* alcohol oxidase was acting directly on the added glycerol. Even blank samples without pectin and alcohol impurities exhibited changes in absorbance in the presence of glycerol after the addition of alcohol oxidase. It is conceivable that this was caused by an exaggerated blank reaction due to a glycerol-mediated change in reaction conditions which increased the autoxidation of alcohol oxidase. Solutions of purified alcohol oxidase from *P. pastoris* are known to produce low levels of hydrogen peroxide in the absence of added substrate if exposed to air (22). Glycerol in excess might have accelerated the release of formaldehyde normally found bound to the purified enzyme. Another reason for the observed increase in absorbance might be an increase in the dissolved oxygen levels with increasing glycerol concentration, due to a change of the oxygen solubility, which might have affected the kinetic properties of alcohol oxidase as well as the rate of the apparent autoxidation reaction (22). Thus, glycerol at very high concentrations interferes with the indicator assay described here. Nevertheless, addition of pectinesterase to pectin samples containing glycerol concentrations up to 60% (w/v) led to a distinct increase in absorbance (Fig. 7, $t \geqslant 2.5$ min), both compared to the initial absorbance change and compared to a sample lacking glycerol, which enabled determination of its activity.

Figure 8 shows that both the absorbance change in the time interval t_1 = 1.6–1.8 min (□) (initial indicator activity) and the absorbance change in the time inter-

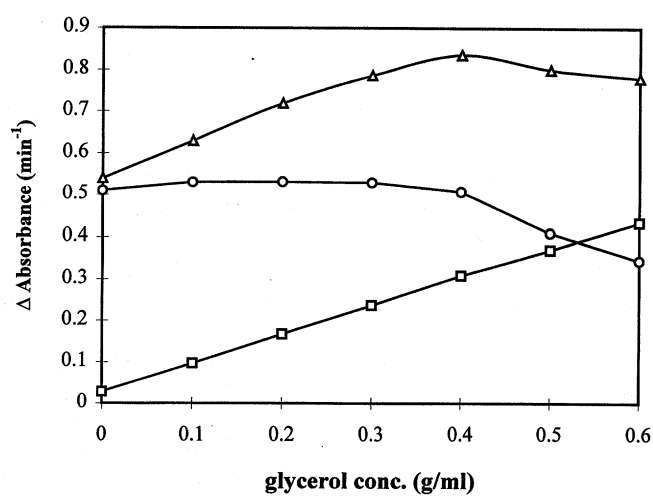

FIG. 8. Pectinesterase (PE) activity as a function of glycerol concentration. Results evaluated from Fig. 7 [slopes in the time intervals (t_1 = 1.6–1.8 min and t_2 = 2.6–2.8 min)]. Initial indicator activity (□) (t_1 = 1.6–1.8 min), apparent activity (△) (t_2 = 2.6–2.8 min), "true" PE activity (○).

val t_2 = 2.6–2.8 min (△) (apparent PE activity) are linear functions of the glycerol concentration with similar slopes, differing only in their ordinate interception. While there was no deviation from linearity with the AOD-POD-ABTS assay throughout the range of glycerol concentrations examined, the apparent activity of pectinesterase reached its maximum at 0.4 g/ml and decreased afterward. Subtraction of the initial indicator activity from the apparent pectinesterase activity resulted in the "true" PE activity (○) which was linear from zero to 0.4 g glycerol/ml and dropped distinctly at higher glycerol concentrations.

The examined glycerol concentrations in the final assay volume were 10 to 60 times higher than the concentration of simple sugars in samples of common fruit pulps. Therefore, it is unlikely that sugar concentrations in pectinesterase samples from the plant matrix would interfere considerably with the assay.

Comparison with Other Methodology

Pectinesterase activity is typically determined by titration of the free carboxyl groups generated by incubation of a pectinesterase sample with pectin (4). Pectinesterase can also be measured by determining the amount of methanol released by the enzyme during this incubation, avoiding corrections for partial dissociation of the free carboxyl groups at the pH optimum of pectinesterase. Such a method is described here.

Here, for the first time, the action of pectinesterase was monitored directly by following the release of methanol with a conventional analytical method without taking samples for subsequent methanol analysis. This decreases the number of analytical steps, and reduces analysis time and the consumption of reagents substantially. The procedure described here is very rapid: with an analysis time of less than 10 min it is one of the fastest methanol assays reported to date. The titrimetric pH assay requires relatively large solution volumes to cover the electrodes and, therefore, large amounts of substrate are needed. In contrast, the AOD-POD-ABTS method requires only small amounts of substrate. Also, in contrast to the pH-stat assay, there is no need to discriminate between the multitude of hydrolytic enzymes, responsible for demethoxylation, deacetylation (30), dephosphorylation (31), and proteolysis (4), such as frequently occurs in fruit juices. Compared to the well-established permanganate–pentanedione assay with a molar absorbance coefficient of the colored Hantzsch product of 7,700 cm^{-1} M^{-1} (18), the AOD-POD-ABTS is almost six times more sensitive. The reduction of the dilution factor of the sample by 2.25, compared to the procedure of Wood and Siddiqui (8), led to a further improvement in sensitivity. Finally, the reduction of the absolute volumes led to a procedure which was capable of allowing the rapid assay of small volumes of column eluant fractions of pectin or pectinesterase, such as those that are generated by chromatography, without further concentration.

The assay has been used to quantitate purified orange peel pectinesterase at levels from 0.0625 to 1.0 unit per assay at a pectin standard concentration of 8.4 μg/ml, methoxy content 10% w/w, and to quantitate pectin levels from 0.5 to 10 μg/mL using 1 unit of pectinesterase. Thus, this method offers not only a higher relative sensitivity then current titrimetric methods, which require approximately 5 mg of pectin per milliliter or 8 units of pectinesterase (32), but moreover a tremendous increase in absolute sensitivity. The AOD-POD-ABTS assay combines a high sensitivity toward methanol with the continuous assay feature of the titrimetric pH-stat assay, making it to a powerful tool for the real-time determination of pectinesterase activity.

The presented assay is a very useful analytical method for the determination of the activities of purified and semipurified pectinesterases. Plant matrix compounds such as polyols have been shown to not substantially interfere with pectinesterase unless they greatly exceed naturally occurring concentrations. We have also shown here that pectinesterase activities could be detected without any corrections for low concentrations of preexisting methanol contaminants in pectin samples. Nonetheless, we have found that the assay described here can only be accurately applied to the determination of pectinesterase activity in crude fruit juices lacking alcohol contaminants. Investigations on the modification of the basic AOD-POD-ABTS assay protocol and the use of this modified assay for the determination of activities of pectinesterase in juices are currently being carried out in our department (manuscript in preparation). When it is desired to study crude pectinesterases, substances interfering with alcohol oxidase and peroxidase-ABTS can be reduced significantly or even eliminated by simple physical, chemical, or enzymatic procedures.

Applications of the AOD-POD-ABTS Assay

With regard to pectin, the AOD-POD-ABTS assay was primarily developed to determine the activity of purified or partially purified pectinesterase preparations on pectins by measuring the methanol released. Comparison of the rate and degree of demethoxylation by pectinesterase with the value for the original total methoxy content of the pectin, determined by alkaline saponification, can give valuable information on the distribution of methoxy groups along the D-galactopyranosyl uronic acid backbone of both high- and low-methoxylated pectins. This provides useful information regarding the gelling behavior of various pectins (2). In conjunction with pectinesterase treatment the method

can also be used to characterize pectin oligomers resulting from treatment of pectin with pectin lyases and endo polygalacturonidases.

With regard to pectinesterase, the AOD-POD-ABTS assay has been used to determine pectinesterase activities in partially purified and purified commercial preparations.

The data presented here show that with state-of-the-art computer-controlled spectrophotometric equipment, the enzymatic demethoxylation of pectin can be determined sensitively and rapidly.

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