phenol:acetic acid:water slurry. This slurry was blended for 3 min with a Tekmar homogenizer (Tekmar-Dorhmann, Cincinnati, OH) and held in the refrigerator for 2 h before filtering on a Miracloth filter (Calbiochem Corp., La Jolla, CA). To minimize the possibility of any residual enzyme activities in the isolated cell walls, blending with phenol:acetic acid: water was repeated twice until protein was not detectable in the filtrate, based upon lack of a precipitate in ammonium formate/acetone (Fry, 1988). Extractions with phenol and acetic acid must be done in a fume hood. Isolated cell walls were stored in a desiccator over phosphorus pentoxide (P_2O_5) until use.

The water-soluble pectin (WSP) and chelator-soluble pectin (CSP) from 10 g of cucumber cell wall were extracted under refrigeration following the procedure of Sajjaanantakul et al. (1989). The extracted WSP solution was concentrated with a YM-10 membrane in an Amicon thin channel ultrafiltration unit (Amicon, Inc., Beverly, MA). Sodium azide (100 ppm) was added to the concentrated pectin solution, which was stored at 4 °C. CSP was dialyzed and concentrated by ultrafiltration and also stored at 4 °C with 100 ppm sodium azide.

Pectin Hydrolysis. The ionic strength and pH used for determination of the effect of temperature on pectin hydrolysis were the same as used for previous studies of temperature effects on cucumber tissue softening (McFeeters and Fleming, 1990). WSP and CSP isolated from cucumbers, citrus pectin (70% DM), and apple pectin (72% DM) were hydrolyzed. Pectin solutions (10 mL) containing 11.4 mM galacturonic acid in 1.5 M NaCl and 0.1 M formic acid at pH 3.0 were heated at 100, 90, 80, 70, and 60 °C in 10 mL Reacti-vials (Pierce Chemical Co., Rockford, IL). Duplicate vials were prepared for all pectin samples at all the temperatures. Samples were periodically taken, and reducing group assays were done.

To determine whether pectin demethylation occurred during hydrolysis reactions at pH 3.0, samples from reactions carried out at 100 °C were taken after 2.5 and 8 h incubation and analyzed directly for methanol. The effect of 20 mM added calcium chloride on glycosidic bond hydrolysis rates was determined at 100 °C.

The effect of pectin methylation on the rate of pectin hydrolysis was evaluated with citrus pectin samples from <5% to 70% DM. Pectin solutions were prepared as above except NaCl was added at 0.1 M in addition to 1.5 M. Hydrolysis was done only at 100 °C.

The effect of pH on the rate of pectin degradation was determined utilizing citrus pectin with <5% DM, 35% DM, and 70% DM. Heating was done in a model D8 oil bath (Haake, Paramus, NJ) at 100 °C. The pH was varied over the range of 2−6 by using a three-component buffer solution. Concentrated pectin and buffer solutions were heated separately, and then equal volumes of the two solutions were combined to give reaction solutions that contained 11.4 mM galacturonic acid residues in 0.1 M formic acid, 0.1 M acetic acid, and 0.05 M BES with an ionic strength of 0.3. Duplicate reactions were done at each pH for each pectin sample. Samples were taken periodically for measurement of reducing groups and β-elimination.

Reducing Group Assays. The effect of the degree of pectin methylation on the rate of pectin hydrolysis was determined by measuring the release of reducing groups using the bicinchoninate assay of Waffenschmidt and Jaenicke (1987). This method required demethylation of pectin hydrolysis samples prior to the assay to prevent formation of reducing groups during heating of the reagent solution. Demethylation was done by cooling pectin samples taken from the Pierce hydrolysis vial in an ice bath, addition of a small volume of cold 3 N NaOH to give a final NaOH concentration of 0.03 M, and then incubating the samples at 15 °C for at least 4 h. This procedure demethylated the pectin without causing a measurable increase in reducing groups. Samples taken for this assay ranged from 50 to 200 μL.

Reducing sugar assays for evaluation of the effect of pH, temperature, and calcium on the rate of pectin degradation were done using a modification of the Nelson reducing sugar assay procedure (Nelson, 1944) that is specific for reducing groups on hexuronic acid residues (Milner and Avigad, 1967; Keijbets and Pilnik, 1974). Sample sizes taken for this assay varied from 100 to 500 μL, depending upon the extent of reducing group formation.

β-Elimination Assay. β-Elimination, which results in the formation of a double bond in a galacturonic acid residue when a pectin glycosidic bond is broken, was monitored by taking absorbance readings at 235 nm. Samples with any visible precipitate were centrifuged to remove the precipitate, and the absorbance was measured on the supernatant. The amount of unsaturated uronic acid residues formed was calculated using an absorbance coefficient of 5412 M^{-1} cm^{-1} (Voragen, 1972). Results were expressed as micromole of unsaturated residues/micromole of uronic acid.

pH Measurement. pH was measured at 100 °C using the Orion Ross combination pH electrode with an epoxy body (Orion Research, Cambridge, MA). The pH meter was calibrated at 100 °C using pH 4 and pH 7 standard buffers. The pH of the standard buffers at 100 °C was calculated using the temperature coefficients determined by the National Bureau of Standards (Dawson et al., 1986). Samples were heated and maintained at that temperature with a Reacti-therm heating block (Pierce Chemical Co., Rockford, IL) using Reacti-vial containers.

Pectin Methylation and Uronic Acid Analysis. The method of McFeeters and Armstrong (1984) was used for pectin methylation analysis of isolated cucumber pectin samples, except a colorimetric rather than a gas chromatographic procedure was used to determine methanol. Quantitative determination of methanol was done with the colorimetric procedure of Wood and Siddiqui (1971), as modified by Hudson and Buescher (1986). Uronic acid determinations were done using the colorimetric procedure of Scott (1979) on hydrolysates of pectin fractions (Blakeney et al., 1983; McFeeters and Lovdal, 1987). Analyses were run in duplicate. Percent methylation was calculated as the molar ratio of methanol/uronic acid × 100.

Data Analysis. First-order rate constants for pectin hydrolysis were calculated by linear regression analysis using the formation of reducing groups as the indicator of glycosidic bond hydrolysis (Huang and Bourne, 1983; McFeeters and Fleming, 1989). The amount of potential reducing groups present in pectin samples at the start of hydrolysis reactions was calculated based upon 11.4 mM galacturonic acid residues in the pectin solutions. Calculation of values for the enthalpy and entropy of activation of pectin hydrolysis was done using the equation derived from transition state theory (Eyring, 1935):

$$\ln(k/T) = \ln(k_B/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT$$

In this equation, k is the first-order rate constant for hydrolysis expressed in s^{-1}, T is absolute temperature in K, k_B is the Boltzman's constant, h is Planck's constant, R is the gas constant, $\Delta S^{\ddagger}$ is the entropy of activation, and $\Delta H^{\ddagger}$ is the enthalpy of activation. Linear regression analysis of $\ln(k/T)$ vs $1/T$ allowed calculation of the enthalpy of activation from the slope and the entropy of activation from the intercept of the line (Whitaker, 1972).

RESULTS

Figure 1 shows that at pH 3.0 the rate of glycosidic bond hydrolysis decreased as the degree of methylation of pectin increased. This pattern was observed at both high and low ionic strength. The effect of degree of methylation on the rate of glycosidic bond hydrolysis was more pronounced at higher ionic strength.

Figure 2 shows changes in the rate of formation of reducing groups as a function of pH when polypectate (<5% DM) and pectin with two levels of methylation (35% DM and 70% DM) were degraded by heating at 100 °C. Polypectate, which is not subject to β-elimina-

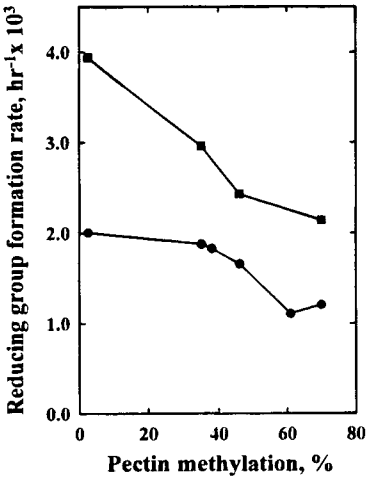

Figure 1. Effect of pectin methylation on the first-order rate of pectin hydrolysis at pH 3.0, 100 °C in 0.1 M (●) and 1.5 M (■) NaCl.

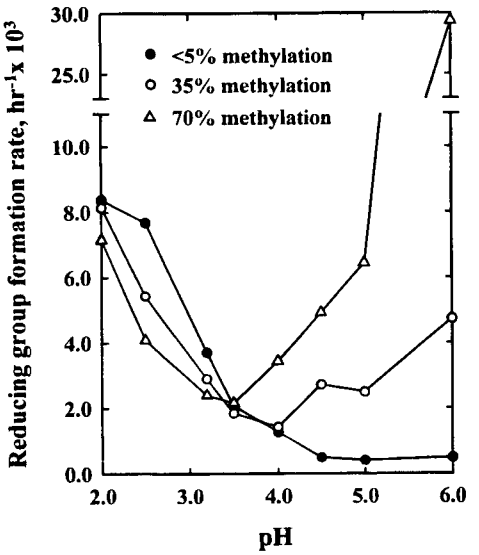

Figure 2. Effect of pH on the first-order rate of reducing group formation with <5%, 35%, and 70% methylated pectin. Reactions were done at 100 °C in 0.1 M formic acid, 0.1 M acetic acid, and 0.05 M BES; $\mu = 0.3$ with sufficient pectin to give 11.4 mM galacturonic acid residues in the reaction mixtures.

tion, decreased in the rate of hydrolysis from pH 2.0 to pH 4.5 and then remained nearly constant from pH 4.5 to pH 6.0. Pectin with 35% DM degraded more slowing than polypectate from pH 2.0 to pH 3.5, but by pH 4.0 the rate of reducing group formation became slightly faster than for polypectate degradation. From pH 4.0 to 6.0, the rate for formation of reducing groups increased. Pectin with a higher level of methylation (70% DM) showed a similar but more pronounced change in the rate of reducing group formation as the 35% DM pectin. Below pH 3.5, degradation was slower than either polypectate or 35% DM pectin. The rate of reducing group formation reached a minimum at pH 3.5, although this minimum rate was slightly greater than for either the polypectate or 35% DM pectin at that pH. Above pH 3.5, the rate of degradation of the 70% DM pectin increased rapidly until at pH 6.0 it degraded nearly 60-fold faster than polypectate at pH 6.0 and more than 3-fold faster than polypectate at pH 2.0.

Degradation of the 35% and 70% DM pectin samples was also monitored at 235 nm to determine the rate of

Table 1. Activation Parameters for Glycosidic Bond Hydrolysis of Cucumber, Apple, and Citrus Pectins at pH 3.0 in 1.5 M NaCl

	enthalpy (kJ mol^{-1})	entropy (J deg^{-1} mol^{-1})	hydrolysis rate at 60 °C × 10^9 (s^{-1})	Q_{10}[a]
cucumber WSP	112 ± 9	−69 ± 26	4.8	3.3
cucumber CSP	141 ± 4	16 ± 10	3.7	4.5
apple pectin, 72% methylation	124 ± 3	−39 ± 9	2.3	3.8
citrus pectin, 70% methylation	117 ± 5	−58 ± 14	2.9	3.5

[a] Ratio of reaction rates at 70/60 °C.

Table 2. First-Order Rate Constants (×10^3) for Glycosidic Bond Hydrolysis of Cucumber WSP and CSP Fractions at pH 3.0 in 1.5 M NaCl at 100 °C with and without Added Calcium[a]

	0 mM calcium (h^{-1})	20 mM calcium (h^{-1})
cucumber WSP	1.64 ± 0.17	1.45 ± 0.10
cucumber CSP	4.02 ± 0.33	4.05 ± 0.87

[a] Results are the means of duplicate reactions.

the β-elimination reaction spectrophotometrically. Below pH 3.5, the rate of β-elimination estimated by this technique was less than the rate of reducing group release as would be expected if acid hydrolysis were the major degradative reaction. However, as the pH was increased above 4.0, visible browning developed in the reaction mixtures and the calculated β-elimination rates from absorbance measurements were greater than the rates of formation of reducing groups. Due to this browning problem, spectrophotometric measurements of β-elimination rates were not reliable in these reactions.

The effect of temperature on the rate of hydrolysis of pectin samples was determined at pH 3.0 in 1.5 M NaCl. For this experiment, WSP and CSP fractions isolated from cucumbers were hydrolyzed. These pectin fractions were 52% and 36% methylated, respectively. The WSP fraction accounted for 6.3% of the total of 180 mg of uronic acid residues per gram of isolated cell wall material from cucumbers. The CSP fraction accounted for 29.8% of total uronic acid residues. Citrus pectin and apple pectin samples were also hydrolyzed. In these reactions no detectable methanol release was observed during hydrolysis at 100 °C. This indicated that pectin demethylation did not occur during the hydrolysis reaction. Table 1 shows the enthalpy and entropy of activation for degradation of these pectin samples as determined by the rate of formation of reducing groups. The WSP from cucumber, apple pectin, and citrus pectin all gave negative entropies of activation for the hydrolysis reaction with enthalpies of activation ranging from 112 to 124 kJ mol^{-1}. The CSP from cucumber cell walls had a slightly positive entropy of activation (+16 J deg^{-1} mol^{-1}) as well as a somewhat higher enthalpy of activation for hydrolysis as compared to the other pectin fractions.

Low concentrations of calcium ions were found to greatly inhibit the rate of cucumber tissue softening (McFeeters and Fleming, 1989). To evaluate whether this effect of calcium on tissue softening might be related to its ability to inhibit pectin hydrolysis, hydrolysis reactions were done with and without 20 mM added calcium chloride. The results (Table 2) showed

that calcium ions had little or no effect upon the rates of reducing group formation.

DISCUSSION

The effect of pH on splitting glycosidic bonds in pectin has not been clearly defined. Albersheim et al. (1960) reported that β-elimination could occur as low as pH 4.5, but it was not determined whether acid hydrolysis also occurs at a significant rate at that pH. Methylated carboxyl groups in pectin molecules are required for the β-elimination reaction to occur. Therefore, the rate of pectin degradation due to the β-elimination reaction will be greater when the degree of methylation is higher (Sajjaanantakul et al., 1989). Pectin can be degraded with the formation of reducing groups by acid hydrolysis as well as β-elimination (Bochkov and Zaikov, 1979). Methylation of carboxyl groups is not required for acid hydrolysis since polypectate, which lacks detectable levels of methyl groups, can be degraded in acid (Smidsrod et al., 1966). However, the effect of methylation on the rate of acid hydrolysis has not been reported. These results showed a consistent decline in pectin hydrolysis rates at pH 3.0 as the degree of methylation increased. High ionic strength had the effect of both increasing the rate of acid hydrolysis and somewhat accentuating the decline in hydrolysis rates, as measured by reducing group release, as pectin methylation increased.

Smidsrod et al. (1966) found that acid hydrolysis rates for polypectate declined as pH increased. In strongly acidic conditions (pH < 1), polypectate was hydrolyzed more slowly than neutral polysaccharides, but in the pH range of acid foods (pH 2.5–4.5) polypectate degraded more rapidly by acid hydrolysis than the neutral polysaccharides studied. An opportunity to define the pH ranges where acid hydrolysis and β-elimination would be the dominant degradative reactions for pectin was provided by the observation that the degree of pectin methylation has an opposite effect on the rate of degradation by acid hydrolysis as compared to its effect on the rate of β-elimination. The effect of pH on the rate of reducing group formation was determined for nonmethylated polypectate, 35% DM pectin, and 70% DM pectin. Figure 2 shows that first-order acid hydrolysis rates were very low, $<8.0 \times 10^{-3}$ h^{-1} at pH 2 for polypectate. As expected, considering the results in Figure 1, the 35 and 70 DM pectin samples hydrolyzed more slowly than polypectate at low pH. However, their degradation rates based upon reducing group formation became faster than polypectate above pH 3.7 for the 70% DM pectin and pH 3.8 for the 35% DM pectin. Since polypectate is not susceptible to β-elimination and it was found to degrade by acid hydrolysis more rapidly than pectin, its hydrolysis rate provided an upper limit for the rate of acid hydrolysis of the 35% and 70% DM pectin samples. Therefore, when their degradation rates, based upon the rate of reducing group formation, exceeded that of polypectate, β-elimination must have become the primary mechanism for degradation. Figure 2 shows that this was pH > 3.7 for the 70% DM pectin and pH > 3.8 for the 35% DM sample.

The effect of temperature on hydrolysis of WSP and CSP fractions isolated from cucumber cell walls was compared to pectin from apple and citrus with high degrees of methylation. The same conditions (pH 3.0, 1.5 M NaCl in formate buffer) had been used previously to investigate the effect of temperature on cucumber tissue softening. Q_{10} values between 60 and 70 °C for these pectin fractions were calculated using the enthalpy and entropy of activation to calculate reaction rates (Table 1). The cucumber CSP had a somewhat higher Q_{10} than the other samples in addition to the slightly positive entropy of activation. This pectin fraction had considerably lower DM than the other pectin samples. Despite the differences in the thermodynamic parameters, at 60 °C the calculated hydrolysis rates of the four pectin samples were within 2-fold of each other (Table 1).

The chemical reaction(s) responsible for nonenzymatic plant tissue softening in acidic conditions is not known. It has been commonly assumed that tissue softening of low acid fruits and vegetables, generally with tissue pH from 5.0 to 6.5, which occurs during heat processing, is due to the β-elimination reaction (Van Buren, 1979; Sajjaanantakul et al., 1989). However, direct evidence of the occurrence of β-elimination during plant tissue softening in low acid conditions is lacking. The results obtained here on the effect of pH on pectin degradation indicate that the β-elimination reaction would become the primary reaction causing breakage of pectin molecules at a lower pH than previously thought (Albersheim et al., 1960). However, the rates of pectin degradation near pH 4 where the β-elimination reaction becomes dominant were very low.

Previous studies on nonenzymatic softening of cucumber tissue at pH 3.0 (McFeeters and Fleming, 1990) has shown that softening is an entropy-driven reaction similar to protein denaturation. That is, in the absence of calcium, the softening reaction has a large enthalpy of activation (165 kJ mol^{-1}) and a large positive entropy of activation (170 J deg^{-1} mol^{-1}), which has the effect of increasing the reaction rate. Values for these thermodynamic parameters are quite different than those obtained for hydrolysis of pectin from the different plant sources (Figure 2). The Q_{10} from 60 to 70 °C for tissue softening calculated from the enthalpy and entropy of activation is 5.8, which is considerably greater than for any of the pectin samples. At 60 °C, the first-order reaction rate for tissue softening is over 19 000-fold greater than the rate of pectin hydrolysis for the cucumber CSP, which has enthalpy and entropy of activation values for the hydrolysis reaction closest to that for tissue softening. The differences in the thermodynamics of pectin hydrolysis as compared to previous results obtained for tissue softening suggest that pectin hydrolysis is unlikely to be the primary reaction responsible for tissue softening in acidic conditions.

Calcium has been shown to be an effective inhibitor of nonenzymatic cucumber tissue softening at low pH and high salt concentration where its ability to form 'egg box' cross-links between pectin molecules would be too small to make that type of cross-linking a likely mechanism for its textural effect (McFeeters and Fleming, 1989, 1990). Therefore, the possibility was considered that it might inhibit softening by inhibiting the acid hydrolysis of pectin. Twenty millimolar calcium ion, which would be sufficient to inhibit cucumber tissue softening by more than 15-fold at 60 °C (McFeeters and Fleming, 1990), had no significant effect on the rate of pectin hydrolysis. The conclusion from this observation is that calcium could not inhibit tissue softening by inhibition of pectin hydrolysis if acid hydrolysis were involved in nonenzymatic softening of plant tissue in acidic conditions.

McFeeters and Brenes-Balbuena (1995) found that fermented cucumber tissue, where the pectin is extensively demethylated during fermentation (Tang and McFeeters, 1983; Hudson and Buescher, 1986), showed broken first-order softening curves. This contrasted with the softening of blanched mesocarp tissue with the degree of methylation similar to that of the fresh cucumber, where softening is described by a single first-order rate constant (McFeeters et al., 1989). A possible explanation for the broken softening curves in fermented cucumbers is that the softening mechanism observed in blanched (high pectin methylation) tissue may be responsible for the initial, more rapid softening observed in fermented tissue. However, the extent of softening that can occur by this still unidentified mechanism may be limited in tissue with low levels of pectin methylation. The slower softening mechanism that was observed in fermented cucumber tissue might be due to acid hydrolysis of the pectic substances.

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