Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 238-244

OXIDATION OF CELL WALL POLYSACCHARIDES BY HYDROGEN PEROXIDE: A POTENTIAL MECHANISM FOR CELL WALL BREAKDOWN IN PLANTS

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Received October 7, 1986

Summary: Incubation of cellulose, sodium carboxylcellulose, pectin, polygalacturonic acid, xylan and arabinogalactan with hydrogen peroxide (0.1-10 mM) resulted in rapid breakdown of the polysaccharides when measured by a reduction of solution viscosity or an increase in reducing groups. When the reaction mixtures were precipitated with ethanol or fractionated on G-25-300 Sephadex, low molecular weight reducing groups increased with incubation time indicating that polymer cleavage was occurring and not simply polymer modification. Oxidation was most rapid at pH 6.5 or 7.5, although secondary optima between pH 3.5 and 5.5 were also observed, depending on the polysaccharide. Purified cell walls isolated from various organs of tomato, cucumber and soybean were similarly degraded and the ethanol-soluble reaction products were partially characterized. The data support the hypothesis that hydrogen peroxide generated by peroxidase from NADH may play a role during cell wall breakdown in plants.

Breakdown of cell wall polysaccharides in higher plants is generally thought to be a result of the combined action of several hydrolytic enzymes (1). Polysaccharides, however, can also be oxidized by hydroxyl radical generated by Fenton's reagent (2), ultraviolet light/hydrogen peroxide (3) and Ti^{III}-hydrogen peroxide (4). This oxidation results in the cleavage of glycosidic linkages which leads to depolymerization of the polysaccharides and ultimately the release of low molecular weight products (4,5).

In higher plants, the formation of hydrogen peroxide is catalyzed by several enzymes including cell wall-bound peroxidase (6). Hydrogen peroxide is required for lignin biosynthesis (7), but the possibility remains that it may also play a role during cell wall breakdown in non-lignifying tissues. Hence, the following study was conducted to, 1) determine whether hydrogen peroxide could degrade cell wall polysaccharides in the absence of metallic catalysts; 2) determine pH optima for hydrogen peroxide-mediated

0006-291X/86 $1.50

polysaccharide degradation; and 3) determine whether hydrogen peroxide could degrade purified cell walls isolated from various plant organs and species.

MATERIALS AND METHODS

1. Chemicals: Larch arabinogalactan, larch xylan, orange pectin, polygalacturonic acid and cellulose were obtained from Sigma. Sodium carboxylcellulose was from Nutritional Biochemicals. Remaining chemicals were A.C.S. reagent grade.

2. Cell wall purification: Plant tissue (5 g) was ground at high speed in 15 ml methanol using a Brinkman Polytron homogenizer. The insoluble residue was collected by centrifugation and extracted for 15 min with mixing using 15 ml of each of the following solvents in succession: acetone, hexane, ethylacetate, methanol, methanol-H_2O (1:1), methanol-H_2O (1:19), and H_2O. Cell walls in H_2O were lyophilized before subsequent treatment.

3. Viscosity assay: Reaction mixtures consisting of 2.7 ml of polysaccharide dissolved in 0.3 M Na-phosphate, (pH 6.5) and 0.3 ml hydrogen peroxide (1,10 or 100 mM) were incubated in the dark at 30°C. At given times, the time required for 0.25 ml of the reaction mixture to drain from a 0.5 ml pipet was determined.

4. Reducing group assay: Reaction mixtures consisted of 9.6 ml of 0.5% (w/v) polysaccharide suspended in buffer (0.1 M Na-phosphate, 0.1 M citric acid, 0.1 M boric acid) at the indicated pH and 1.1 ml of 10 mM hydrogen peroxide. Purified cell walls (20 mg) were suspended with sonication in 7.2 ml of 0.3 M Na-phosphate (pH 6.5), then 0.8 ml of 10 mM hydrogen peroxide was added. All samples were incubated in the dark at 30°C. At specified times, 1.5 ml of the reaction mixture was passed through 0.5 x 9 cm column packed with 2.3 g of washed granular activated MnO_2 to decompose remaining hydrogen peroxide and stop the reaction. The eluate was further purified by ethanol precipitation or gel chromatography before the reducing groups were assayed. Ethanol precipitation involved mixing 0.5 ml of eluate with 1 ml of 95% ethanol, then clearing the solution by centrifugation at 5000 xg for 10 min. Gel chromatography was performed by applying 1.5 ml of eluate to a 2.5 x 20 cm column packed with G-25-300 Sephadex and eluting with 0.3 M Na-phosphate (pH 6.5). Reducing groups were determined in 0.5 ml of the ethanol-soluble supernatant or column fractions using potassium ferricyanide (8). Reducing group concentrations were corrected using a zero-time control which contained all reaction components.

5. Gas chromatography of reaction products: Eluates from the manganese dioxide column were dried in vacuo and the residues dissolved in 0.1 ml of 15 M ammonium hydroxide. Alditol acetate derivatives were prepared (9) and separated on a 1.8 m x 2mm ID glass column packed with SP-2330 on Supelcoport 100/120. The column was fitted to a Hewlett-Packard 5890A gas chromatograph, equipped with an FID. High purity helium was used as the carrier gas at 15 ml/min. The initial oven temperature was 210°C and after 9 min was raised at a rate of 5°C/min to 235°C, where it remained for 10 min. The injector and detector temperatures were maintained at 230 and 250°C, respectively.

6. Experimental design: All experiments were performed at least twice with two samples per replicate. Data represents the mean from all experiments.

RESULTS

Effect of Hydrogen Peroxide on the Viscosity of Polysaccharide Solutions.

Hydrogen peroxide at all concentrations tested caused a significant decrease in the viscosity of Na-carboxylcellulose, pectin and polygalacturonic acid solutions (Fig. 1). In the presence of 1 or 10 mM hydrogen peroxide, this

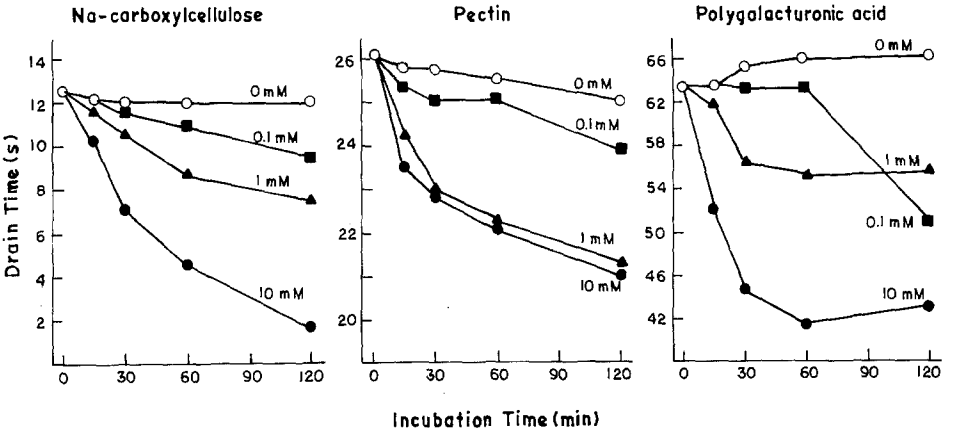

Figure 1. Viscosity of 1.5% (w/v) Na-carboxylcellulose, 12.5% (w/v) pectin, and 12.5% (w/v) polygalacturonic acid incubated at pH 6.5 with hydrogen peroxide at the indicated concentrations. Each point represents the mean of three replicates. SEM was less than 1 for all points.

decrease was evident within 15 min and the reaction rate decreased after 30 min. The decrease in viscosity caused by 0.1 mM hydrogen peroxide occurred less rapidly and the reaction rate did not decrease within the 120 min incubation period.

Effect of Hydrogen Peroxide on the Formation of Low Molecular Weight Reducing Groups From Polysaccharides. To determine whether the decrease of polysaccharide solution viscosity was a result of polymer modification or cleavage, high molecular weight products were precipitated with ethanol, and the presence of low molecular weight reducing groups in the supernatant was determined. As shown (Table 1), incubation of a variety of polysaccharides with 1 mM hydrogen peroxide resulted in the formation of ethanol-soluble reducing groups. The concentration of reducing groups from the respective polysaccharides increased with increasing incubation time, with the largest change occurring between 8 and 24 h. Pectin, Na-carboxylcellulose and xylan were more susceptible to attack by hydrogen peroxide than were arabinogalactan, cellulose and polygalacturonic acid.

Fractionation of arabinogalactan, pectin and xylan on G-25-300 Sephadex after 24 h incubation with 1 mM hydrogen peroxide revealed the presence of two peaks containing reducing groups (Fig. 2). The first peak (fractions 10-14) eluted at the column void volume. The second peak (fractions 20-26)

TABLE 1. Time course for the production of ethanol-soluble reducing equivalents from polysaccharides by hydrogen peroxide [a]

Polysaccharide	Incubation Time (h) 8	24	48
	total μmol glucose equiv. released		
Arabinogalactan	18±11[b]	387±144	738±135
Cellulose	0± 0	342± 34	1143±108
Na-carboxylcellulose	1701±84	4383±441	5286±639
Pectin	99±10	972±297	2124±666
Polygacturonic acid	72± 8	783± 65	1251± 75
Xylan	819±55	2043±113	2565±811

[a] Polysaccharides (0.5% w/v) were incubated at pH 6.5 with 1 mM hydrogen peroxide. Reducing groups were determined as described in "Materials and Methods".

[b] Values represent the mean ± SEM from four experiments, and were corrected for the reducing groups present in zero-time controls.

was eluted at a volume corresponding to a similarly chromatographed glucose standard. Gel chromatography of these polysaccharides after incubation without hydrogen peroxide exhibited no second peak.

Effect of pH on Hydrogen Peroxide-Mediated Polysaccharide Breakdown. Significant rates of degradation of all polysaccharides tested were observed at physiological pH (6.5 and 7.5; Fig. 3). Cellulose, pectin and Na-carboxylcellulose also exhibited breakdown at more acidic pH (3.5-5.5).

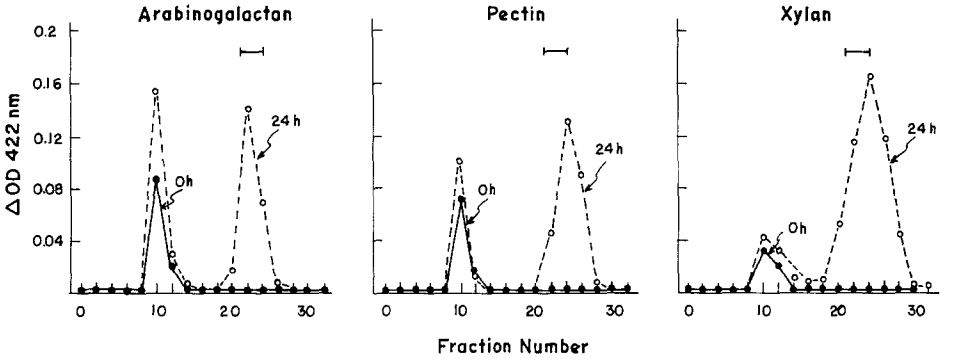

Figure 2. Sephadex G-25-300 gel chromatography of arabinogalactan, pectin, and xylan after 0 and 24 h incubation at pH 6.5 with 1 mM hydrogen peroxide. Samples were eluted with 0.3 M Na-phosphate (pH 6.5). Fractions (3 ml) were assayed for reducing groups as described in "Materials and Methods". Horizontal bar denotes fractions in which a glucose standard eluted.

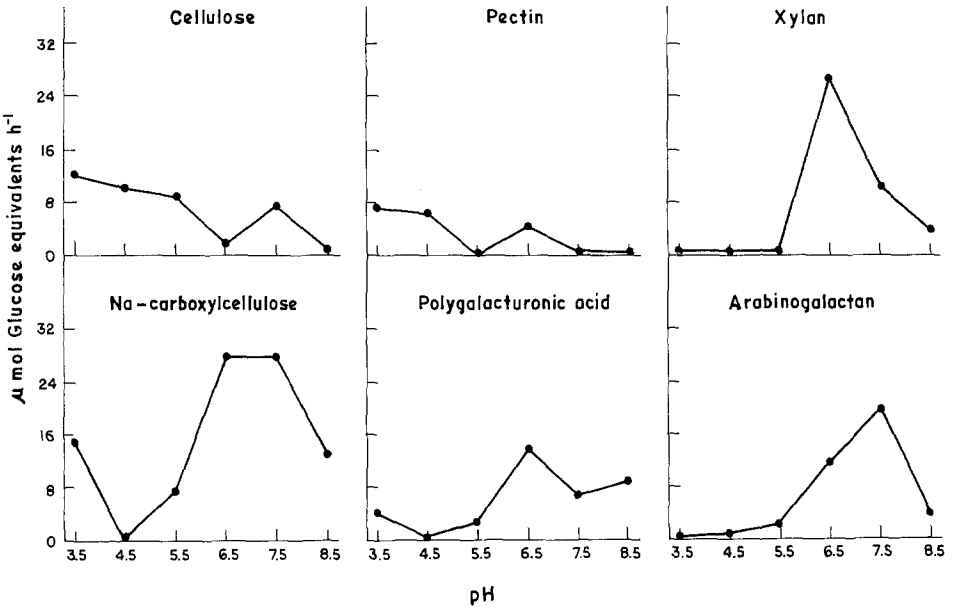

Figure 3. Effect of pH on the rate of oxidation of cellulose, Na-carboxylcellulose, pectin, polygalacturonic acid, xylan and arabinogalactan by 1 mM hydrogen peroxide. Each point represents the mean of duplicate experiments, and values were corrected for reducing groups present in zero-time controls.

At physiological pH, Na-carboxylcellulose and xylan exhibited the highest rates of breakdown.

TABLE 2. Degradation of purified tomato, cucumber, and soybean cell walls by hydrogen peroxide [a]

	Tomato		Cucumber		Soybean	
Organ	Total	h^{-1}	Total	h^{-1}	Total	h^{-1}
	μmol glucose equivalents released					
Leaves	105[b]	4.4	72	3.0	76	3.2
Stem	44	1.8	57	2.4	37	1.5
Fruit	62 green	2.6	30 mesocarp	1.2	52 pod	2.2
	36 red	1.5				
Flower	—	—	37	1.5	—	—

[a] Cell walls were incubated for 24 h at pH 6.5 in 1 mM hydrogen peroxide. Ethanol-soluble reducing equivalents were determined as described in "Materials and Methods".

[b] Values represent the mean of duplicate experiments, and were corrected for the reducing groups present in zero-time controls.

Effect of Hydrogen Peroxide on Purified Cell Walls From Tomato, Cucumber, and Soybean. Upon incubation of isolated cell walls with 1 mM hydrogen peroxide, ethanol-soluble reducing compounds were formed (Table 2). Overall, leaf cell walls were degraded most rapidly and the rate of degradation of cell walls from the other organs depended on the species. Cell walls from green tomato fruit were more easily degraded than those from red tomato fruit.

GC analysis of the low molecular weight products formed after incubation of tomato green fruit cell walls with hydrogen peroxide revealed peaks at retention times corresponding to the alditol acetate derivatives of rhamnose and galactose. Cucumber mesocarp cell walls after incubation with hydrogen peroxide yielded peaks corresponding to arabinose, galactose and xylose. These peaks were absent in the zero-time controls from both species (not shown).

DISCUSSION

Previous work (2,3,4,5) showed that hydrogen peroxide at basic pH in the presence of UV light and/or metallic catalysts would degrade purified polysaccharides. Data presented here shows that oxidation also occurs under physiological conditions (i.e., neutral pH, 30°C, no catalyst, low hydrogen peroxide concentration). Moreover, under these conditions, hydrogen peroxide will attack cell walls isolated from various organs of several plant species. The major mechanism of cell wall degradation has been thought to be through the activity of specific hydrolytic enzymes (1). However, 1 mM hydrogen peroxide degrades polygalacturonic acid and xylan (Table 1) at rates only slightly less than partially purified enzyme preparations from mature cucumber fruit (10). This concentration is within physiological limits as hydrogen peroxide levels in ripening pears range from 0.4-1.8 mmol/kg of tissue (11), and localized levels (i.e., in cell walls) may be much higher. Hydrogen peroxide also reduces the degree of polymerization of cellulose (5).

In vivo hydrogen peroxide could be generated by cell wall-bound peroxidases from NADH (6). In vitro these enzymes can generate hydrogen peroxide at a rate of 0.5 μmol/h/g of cell wall (12). A role for peroxidase during lignification is well established (7), but its activity also increases

dramatically during fruit ripening (13) and organ abscission (14). Both processes involve extensive cell wall breakdown, and peroxidase, whose role has not been defined, may reach maximum activity immediately before or during the period when polysaccharide hydrolysis is observed (13). Hence, it is possible that hydrogen peroxide generated by peroxidase may play a role in cell wall degeneration in plants, and further experiments are in progress to test this hypothesis.

ACKNOWLEDGMENTS

Salaries and research support provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal Article No. 149-86. I thank Christel Velbinger and Thomas Kelley for excellent technical assistance, and Bonnie Beck for typing the manuscript.

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