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Correlative Studies of Cell Wall Enzymes and Growth[1]

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ABSTRACT

If cell wall hydrolytic enzymes are involved in extension growth, a correlation may be expected between hydrolytic activity of the cell walls and growth rate of the tissue from which the walls are prepared. Epicotyl sections from 0 to 5 mm, 6 to 10 mm, and 11 to 15 mm below the apical hook of pea seedlings (*Pisum sativum* var. Alaska) have relative growth rates of 100:15:2, respectively. The relative β-glucosidase activities (units/mg wall) of cell walls from these sections are respectively, 100:24:23, for walls prepared in glycerol and 100:42:23 for walls prepared in aqueous solution. Thus, there is a correlation between growth rate of the tissue and specific activity of the wall-associated β-glucosidase. Similar correlations were found for other cell wall-associated hydrolases.

Relative cell numbers for the above sections, as determined by counting, were 100:25:16, and with these data it could be calculated that the amount of cell wall β-glucosidase activity per cell is essentially a constant. Thus, for epicotyl sections the amount of enzyme per cell does not change during the process of cell elongation but the specific activity declines as the result of deposition of new wall material.

Data from several laboratories suggest that glycosidases play a role in wall plasticization, thus permitting cell elongation during extension growth (9). There is evidence that oligosaccharidehydrolyzing enzymes are localized in the walls (1, 2, 3, 7, 13, 17, 19, 25) and that these enzymes can hydrolyze cell wall oligosaccharides (12, 13, 15, 18, 25). It becomes important to know whether wall-localized glycosidase activity is correlated with the growth rate of the tissue from which the walls are prepared. Previous studies, mainly employing enzymes extracted from wall preparations (7, 13, 19, 25), report that the amount of glycosidase activity correlates with the growth rate of the tissue from which the walls are prepared. In this current study, highly purified cell walls from *Pisum* epicotyls were utilized, thus permitting specific activity determinations. We find that β-glucosidase specific activity is highest in walls from rapidly elongating tissue but that the amount of enzyme per cell remains essentially constant, being diluted as growth ensues. Similar correlations are found for α- and β-galactosidase and acid phosphatase activity. These findings suggest that control of cell elongation depends on factors other than the amount of glycosidase activity.

MATERIALS AND METHODS

Tissue. *Pisum sativum* seeds var. Alaska 28-57 W. R., were surface sterilized in 1% sodium hypochlorite for 15 min, soaked in running tap water for 18 hr, and germinated on absorbent paper in covered plastic trays. Germination was in the dark at 25 C and 85% relative humidity, and seedlings were harvested 84 hr after germination was begun. Growth rates of the seedlings were determined by marking at 2.5-mm intervals and subsequently measuring the spacings. Sequential epicotyl sections were taken from 0 to 5 mm, 6 to 10 mm, and 11 to 15 mm below the apical hook. The sections were either dropped into beakers on Dry Ice immediately after cutting in the case of samples for cell wall preparation by the nonaqueous method, or, in all other cases, they were weighed out in 0.5-g lots and then frozen on Dry Ice as soon as 0.5 g was obtained. The tissue was stored at −80 C until used.

Cell Wall Preparation. Cell walls were prepared by homogenization in glycerol and filtration through a glass bead filter, as described by Kivilaan *et al.* (16). The wall material was dried in an evacuated dessicator over P_2O_5, $CaCl_2$, and paraffin at 4 C for 24 to 48 hr. The resultant white powder was stored at −10 C in a sealed jar over $CaSO_4$. Alternatively glycerol was removed from the pelleted walls by water, rather than by solvent washing, as in the Kivilaan procedure. Washing and collection by centrifugation was repeated three times, the cell wall material was collected by filtration, dried over P_2O_5 *in vacuo*, and stored as described above.

Aqueous Cell Wall Preparation. Cell walls were also prepared by homogenization of 0.5-g aliquots of frozen tissue sections ground at 0 to 4 C in a 50-ml conical glass homogenizer with 3 ml of 0.05 M MES buffer solution. The homogenate was suspended with a Vortex mixer, centrifuged for 10 min at 4*g* (max.), and the supernatant solution removed. The pellet was resuspended in 4 ml of buffer and centrifuged for 10 min at 4*g*. After the second centrifugation the pellet was resuspended and centrifuged at 440*g* for 5 min. This pellet was then resuspended for assays and will be referred to as the 4*g* pellet. The two supernatant solutions from the 4*g* pellet were combined and centrifuged at 130*g* for 10 min. The pellet from this 130*g* centrifugation is referred to as the 130*g* pellet. The supernatant fluid was centrifuged at 10,000*g* for 20 min and the supernatant solution was assayed as the soluble fraction.

Fixation of Tissue for Cell Counts. Tissue sections were fixed in formalin, acetic acid, 70% ethanol (1:1:18) for 24 hr and then transferred to 70% ethanol. The sections were dehydrated, stained with Safranin-O, and embedded in Paraplast for sectioning. Sections 8 μm thick were mounted on slides and the number of cells were counted along the length of the longitudinal sections, exclusive of the stele and vascular bundles, and multiplied by the number of cells in diameter of the corresponding cross section, exclusive of the stele and vascular bundles. This product multi-

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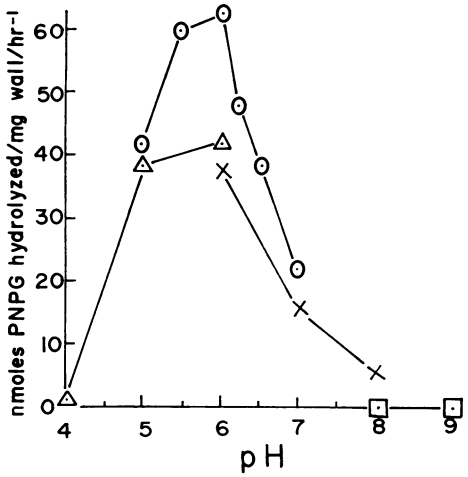


FIG. 1. Effect of hydrogen ion concentration on cell wall β-glucosidase activity. Acetate, phosphate, and tris were at 0.05 M, using 3 mM substrate, wall preparation II, and an incubation time of 45 min. Acetate (△) was used over the pH interval of 4 to 6, phosphate (×) was used from 6 to 8, and tris (□) was used from 8 to 9. MES (○) was at 0.05 M, and incubation time was 30 min, using wall preparation III and 3 mM substrate.

plied by the number of sections per gram, served as an index of relative cell numbers.

Protein Content of Cell Wall Preparations. Cell wall material was hydrolyzed in 6 N HCl under N_2 at 105 C for 18 hr in sealed tubes, the samples were evaporated to dryness, and the residue was taken up in 0.1 N HCl. After neutralization with 0.1 N NaOH, α-amino acid content was determined with ninhydrin (4). Glycine was used as a standard and an average amino acid molecular weight of 100 was used for calculation of protein content.

β-Glucosidase Assay. A weighed aliquot (usually 20 to 30 mg) of cell wall material was suspended in 0.05 M MES buffer at pH 5.8 and allowed to hydrate for 4 hr at 0 C before being assayed. Cell wall suspensions were pipetted with an automatic micropipette because rapidity in transfer is necessary to avoid settling of the wall particles. The reaction mixture contained 0.5 mg of cell wall and 1 μmole of PNPG[3] in a total volume of 1 ml of .05 M MES buffer at pH 5.8. Tubes were incubated at 37 C and the reaction was stopped by adding 0.4 ml of 0.2 M Na_2CO_3. The mixture was clarified by centrifugation. In the case of the crude homogenate or soluble fractions, the reaction was terminated by addition of 1 ml of 5% trichloroacetic acid, and the homogenate or fractions were centrifuged. Sodium carbonate was then added to an aliquot of the clarified solution. Controls were boiled for 2 min before the addition of substrate. Absorbancy was read at 400 nm to determine free PNP, and a molar extinction coefficient of 1.83×10^4 was used based upon that obtained with recrystallized PNP.

Other Glycosidase and Acid Phosphatase Assays. Other glycosidases were assayed in the same manner as β-glucosidase except that appropriate PNP substrate was used, in all cases D-glycopyranosides were used. In the case of aqueous cell wall preparations, all isolation procedures were carried out at the pH optimum of the enzyme being assayed.

Chemicals. The ρ-nitrophenyl glycosides and ρ-nitrophenyl phosphate were purchased from Pierce Chemical Co., Rockford, Ill. ρ-Nitrophenol was purchased from Calbiochem., San Diego, Calif., and MES was a gift from Dr. N. E. Good.

[3] Abbreviations: PNPG: ρ-nitrophenyl-β-D-glucopyranoside; PNP: ρ-nitrophenol; PNP(^{14}C)G: ρ-nitrophenyl-β-D-(U-^{14}C)-glucopyranoside.

RESULTS

The pH optimum for the cell wall β-glucosidase is between pH 5.5 and 6 (Fig. 1), and thus all β-glucosidase assays were conducted at pH 5.8 in 0.05 M MES buffer. This optimum is higher than that reported for *Avena* (11). The slight activity observed at pH 8 is completely inhibited by tris as has previously been observed for glucosidases with transferase activity (24). Cell wall β-glucosidase activity was found to be linear with time for up to 4 hr, and with wall concentration up to 5 mg of wall per 1 ml of reaction mixture, provided substrate concentration was not depleted by more than 30%.

Hydrolysis of PNPG is linear with respect to amount of wall material added (Fig. 2). Variability was experienced from preparation to preparation. The data presented in Figure 2 represent different cell wall isolations done on different days with different lots of tissue. Agreement between replicates was, in general, better than 5%, and agreement between wall preparations made on the same day was excellent. There remains the variability between lots of tissue, and because the seedlings were grown under constant conditions, this is difficult to account for. Although not specifically investigated, the amount of enzyme activity of the walls seems to be responsive to unknown variables of growth conditions or harvesting procedures, or both.

The effect of substrate concentration on β-glucosidase activity is shown in the data (Fig. 3, a and b). The Lineweaver-Burk plots of Figure 3b were derived from the data of Figure 3a. The enzyme is not saturated at the concentrations of substrate used (1 to 12 mM), but because only 5% of the substrate was hydrolyzed during a typical experiment, the error is small. The apparent K_m values for the β-glucosidase activity for the cell walls from the 0- to 5-mm and 11- to 15-mm sections were determined and found to be identical for both sections as illustrated in Figure 3b.

Figure 4 illustrates the origin of the tissue sections used for cell wall preparations, their relative growth rates at the time of harvest, the percentage of protein in the walls, and the relative number of cells in the section. The protein content shown is for cell walls isolated by the Kivilaan procedure (16). Cell walls isolated by the Kivilaan procedure but then water washed were similar having 7.9, 6.7, and 6.1% (w/w) protein, respectively, for the 0- to 5-mm, 6- to 10-mm and 11- to 15-mm sections. These results are also in agreement with those of King and Bayley (14) for walls prepared by the same method and with the same tissue but using Kjeldahl analysis. Interestingly, walls prepared by an

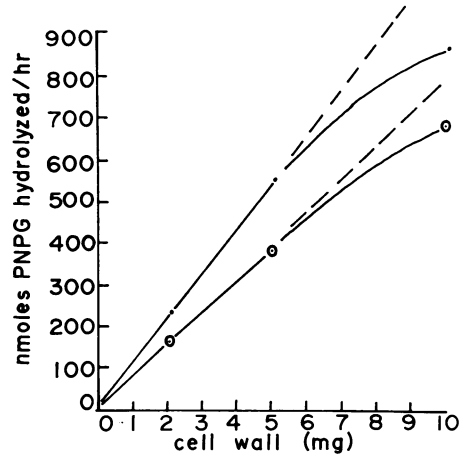


FIG. 2. Hydrolysis of ρ-nitrophenylglucose as a function of amount of cell wall. The indicated amount of wall was incubated in 1 ml of 0.05 M MES, pH 5.8, with 3 mM substrate for 15 min. The curves shown are for two different cell wall preparations.

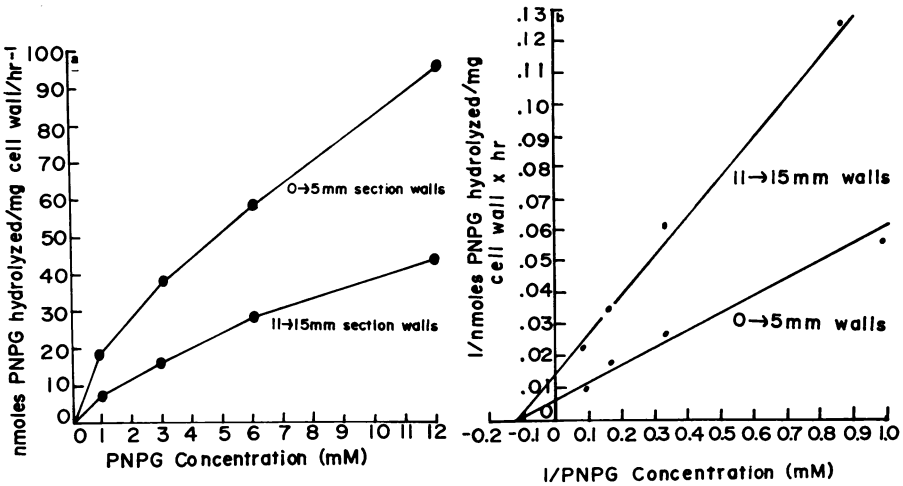
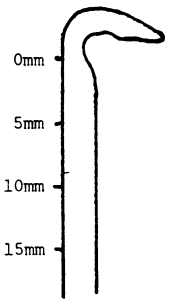


FIG. 3. Rate of β-glucosidase reaction as a function of substrate oncentration. Incubation time was 2 hr using 0.5 mg of wall in 1 ml with the indicated substrate concentrations. Figure 3b is a Lineweaver-Burk plot of the same data.



Section	Growth Rate	Relative Growth Rate	Protein Content
0→5mm	0.47	100	7.2 %
6→10mm	0.07	15	6.0 %
11→15mm	0.01	2	6.0 %

FIG. 4. Growth rate of sections, relative cell number, and protein content of glycerol-prepared, solvent-washed walls as a function of the epicotyl portion utilized.

aqueous homogenization and centrifugation procedure (the procedure commonly used in cell wall studies) gives protein contents of 14, 9, and 14% (w/w), respectively, for the 0- to 5-mm, 6- to 10-mm and 11- to 15-mm sections. This indicates the degree of contamination by membrane or cytoplasmic proteins experienced in the commonly used methods.

The enzymatic activities of cell walls isolated from sequential tissue sections are shown in Table I. In all cases, the specific activity on a weight basis is highest in the walls from the 0- to 5-mm section, in most cases intermediate in the walls from the 6- to 10-mm section, and lowest in the walls from the 11- to 15-mm section. The specific activity was expressed on a wall weight basis because this is more meaningful for wall fragments than is a protein basis. However, if activities are expressed on a protein basis, the ratios of the specific activities for the various sections are not changed significantly. The specific activities of β-glucosidase on a protein basis are 0.78, 0.22, and 0.23 nmoles of PNP liberated/μg of protein·hr for water-washed glycerol-prepared walls and 10.9, 8, and 5 nmoles of PNP liberated/μg of protein·hr from the 0- to 5-mm, 6- to 10-mm, and 11- to 15-mm epicotyl sections, respectively. The reason for the lower specific activity of walls prepared by the glycerol procedure is not known to us. The β-galactosidase is predominantly a soluble enzyme and thus indicates cell wall contamination with cytoplasmic proteins, particularly for the 4g pellet. The β-glucosidase activity per cell does not correlate with growth rate. The relative activities of β-glucosidase per cell were 100, 92, and 152 for glycerol-prepared walls and 100, 168, and 143 for aqueous-prepared walls from the 0- to 5-mm, 6- to 10-mm, and 11- to 15-mm sections, respectively, whereas the relative growth rates for the same regions were 100, 15, and 2, respectively.

Table I. *Enzymic Activities of Cell Walls Isolated from Tissue of Differing Growth Rates*

Walls were isolated by the Kivilaan *et al.* procedure (16) or in buffer, as described in the text. Incubation was as described in the text and was usually for 30 min but was adjusted for high or low activity preparations to a time that yielded a suitable absorbance. The values for aqueous walls refer to the activity observed for the 4g pellet obtained from 1 g fresh weight of tissue. Values in parentheses are relative to the activity of the apical (0–5 mm) section when it is set at 100.

Activity	Section	Glycerol Walls	Aqueous Walls
	mm	*nmoles PNP liberated/mg·hr*	*nmoles PNP liberated/g fresh wt·hr*
β-Glucosidase	0–5	62 (100)	2940 (100)
	6–10	15 (24)	1230 (42)
	11–15	14 (23)	676 (23)
α-Galactosidase	0–5	60 (100)	2680 (100)
	6–10	29 (48)	1260 (47)
	11–15	23 (38)	1150 (43)
Acid phosphatase	0–5	168 (100)	7640 (100)
	6–10	42 (25)	4130 (54)
	11–15	60 (35)	3530 (47)
β-Galactosidase	0–5	4 (100)	3270 (100)
	6–10	2 (45)	1930 (59)
	11–15	1 (28)	1280 (39)

The relative distribution of β-glucosidase, α-galactosidase, acid phosphatase, and β-galactosidase activities in the various fractions from the aqueous cell wall preparations are shown in Table II. It is evident that the β-glucosidase activity is associated with the cell wall fraction. More β-glucosidase activity is found in the 4g pellet than in the crude homogenate, indicating an inhibitory substance for β-glucosidase activity. The inhibition caused by an undiluted supernatant amounts to about 40% (Table III). No attempt was made to isolate and identify the substance inhibitory to the β-glucosidase activity, although it was noted that 40% of the inhibitor was bound by Amberlite MB-3 or Dowex-50 resins. Inhibition of glucosidase activity by amines and by lactones has been described previously (6, 24).

Table II. *Relative Distribution of Enzymic Activities as Percentage of Total Activity*

Fractions and assays are as described in the text. All activities were assayed at their pH optimum of 5.8, 5.1, 5.5, and 4.8, respectively, for β-glucosidase, α-galactosidase, acid phosphatase, and β-galactosidase using 1 mM substrate in 0.05 M MES.

Fraction	β-Glucosidase	α-Galactosidase	Acid Phosphatase	β-Galactosidase
	% of total activity			
0- to 5-mm Section				
Homogenate	100	100	100	100
4g pellet	121	13	2	5
130g pellet	40	9	3	6
10,000g supernatant	14	41	85	46
6- to 10-mm Section				
Homogenate	100	100	100	100
4g pellet	126	14	2	7
130g pellet	42	9	3	7
10,000g supernatant	12	50	88	123
11- to 15-mm Section				
Homogenate	100	100	100	100
4g pellet	101	16	2	4
130g pellet	40	10	3	4
10,000g supernatant	18	46	99	90

Table III. *Inhibition of Cell Wall β-Glucosidase by a Heat-stable Factor*

Assay and incubation conditions are as described in text. The 4g pellet was from 0.5 g of tissue suspended in 6 ml. The boiled 10,000g supernatant was clarified, after boiling, by centrifugation.

Incubation Mixture	p-Nitrophenol Liberated	Inhibition
	nmoles/hr	%
0.5-ml suspension 4g pellet	142	
0.3-ml 10,000g supernatant	24	
0.5-ml suspension 4g pellet + 0.3-ml 10,000g supernatant	95	43
0.5-ml boiled 10,000g supernatant	0	
0.5-ml suspension 4g pellet + 0.3-ml boiled 10,000g supernatant	72	49
0.5-ml suspension 4g pellet + 0.3-ml boiled 10,000g supernatant after Amberlite MB-3	97	32
0.5-ml suspension 4g pellet + 0.3-ml boiled 10,000g supernatant after Dowex-50	99	30

DISCUSSION

Our present knowledge of cell wall structure is insufficient to determine which components of the wall are responsible for the rigid structure of the wall and, as a consequence, what types of linkages must be broken to permit extension growth. Enzymic studies of the wall should be helpful in elucidating mechanisms of extension growth, because a high enzymic activity correlated with high growth rates may suggest which structural linkages are altered. Because many enzymic reactions may increase during rapid growth, studies such as the present one only indicate reactions that are concomitants of growth and do not necessarily identify a rate-controlling growth-linked reaction.

That glucosidases acting on cell wall glucans are involved in extension growth is suggested by many lines of evidence. Changes in the levels of certain polysaccharides in cell walls occur (5, 16, 18, 21, 26) and the acid-soluble, noncellulosic glucose polymers of the cell wall decrease during extension growth (5, 20, 26). There is also evidence that glucanases play a role in the case of auxin-induced extension growth of excised *Avena* coleoptile sections (5, 11). A recent study, utilizing glycosidase inhibitors, presents evidence against the involvement of β-glucosidase or β-galactosidase in the short term auxin- or acid-promoted growth of excised *Avena* coleoptile sections (9). However, this work deals with short-term growth which may not serve as a complete model for the long-term and very large increase in cell size studied in this report. Cell walls from rapidly growing tissues have been reported to have more glucanase or cellulase activity than walls from slowly growing tissue as is reported here and in a preliminary communication (23), and in other work (1, 7, 10, 25). In addition to the inhibition by tris, a further indication that the β-glucosidase studied in this report has glucosyl transferase activity was provided by experiments utilizing PNP(^{14}C)G^3 (22). Upon incubation of cell walls with this radioactive substrate, a substance with the properties of a disaccharide was formed (21, 22). β-Glucosidase activity has been reported to be localized in the cell wall (13) or associated with the cell surface external to the plasmalemma (13). ^{14}C-labeled cell wall polysaccharides can be hydrolyzed first to oligosaccharides and then to free glucose by a cell wall preparation from *Avena* coleoptiles (12). Further, cell wall preparations from corn coleoptiles are capable of autolysis *in vitro* (15, 18), and during autolysis there is an enzymic solubilization of a lichenan type, β-1→3- and β-1→4-linked glucan. Clearly then, cell wall glucans can be hydrolytically cleaved by wall-bound enzymes and thus there is the possibility that such cleavage would alter the mechanical rigidity of the wall.

From this work, the specific activity of β-glucosidase in walls, isolated from rapidly growing tissue, is higher than the specific activity of that in walls isolated from slowly growing tissue. However, the activity per cell is essentially a constant. Each cell has a fixed amount of enzymic activity, and lower specific activities result as the cell elongates with dilution of the enzyme by more wall material. Nonetheless the cell wall-associated β-glucosidase could exert an indirect control over cell wall extension. Nonvacuolated cells in the apex may not enlarge for osmotic reasons—that is, they may be unable to exert the necessary five or so, atmospheres of pressure—or they may, as in roots, lack sufficient glycosidase activity (1). Cells in the elongation zone would possess both vacuoles for high turgor and the proper enzyme concentration to permit elongation. Cells in the lower zones would have undergone secondary irreversible wall thickening, or as observed here and in roots (1), the concentration of glycosidase may fall too low to hydrolyze simultaneously a critical number of bonds.

The Lineweaver-Burk plot of the β-glucosidase activity of the cell walls from the fastest growing and the non-elongating sections shows that in both cases the enzyme has the same apparent *Km* (8.3 mM). This is within the range of those reported for other β-glucosidases. Thus, the properties of the enzyme do not change during wall maturation.

Thus, our work shows that there is a correlation between the specific activity of β-glucosidase associated with isolated cell walls and the growth rate of the tissue from which the walls were isolated. Most of the β-glucosidase activity is associated with the cell wall and the amount of glucosidase per cell remains essentially constant.

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