

THE pH DEPENDENT DISTRIBUTION OF β -GLUCOSIDASE ACTIVITY IN ISOLATED PARTICULATE FRACTIONS

Higher levels of β -glucosidase activity were found in particulate fractions isolated from corn roots under alkaline compared to acid conditions. The greatest pH effect was observed on the crude cell wall fraction (1000 \times *g* for 10 min) where 19% of the total β -glucosidase activity was recovered at pH 7.7 compared to 3% at pH 6.0. Further analysis indicated that the particulate activity recovered in the 1000 \times *g* pellet was dissociated over a narrow range of pH. To determine if the pH effect was on the cell wall or a subcellular component found in the crude cell wall fraction, cell walls were separated from organelles and membranes by sucrose density gradient centrifugation. Most of the β -glucosidase activity in the crude cell wall fraction was not associated with the cell wall but with an unidentified component which equilibrated at 51% (w/w) sucrose.

Key words: crude cell walls, β -glucosidase activity; dissociation pH; localization; subcellular fractionation

Introduction

The binding of hydrolytic enzymes (pectinesterase, β -glucosidase, invertase) to isolated particulate fractions is pH dependent [1-4]. The major particulate binding site appears to be the cell wall [1,3,5,6,] and in these reported cases, the binding of enzymes was greatest at acid pH (4.5-5.0) and least under alkaline conditions (pH 7.0-8.0).

In our previous report [7], we showed that approx. 20% of the total β -glucosidase activity was sedimented with the crude cell wall fraction (1000 \times *g* for 10 min) when corn roots were homogenized at pH 7.7. The purpose of this work was to determine if pH influenced the distribution of particulate associated β -glucosidase activity and to

determine which subcellular component or components were affected by the pH shift.

Materials and methods

Plant material

Corn seeds (*Zea mays* L., FRB-20) were germinated and harvested as described previously [7]. Primary roots were excised and homogenized in grinding medium (containing 5 mM β -mercaptoethanol, 5 mM EDTA, 0.3 M sucrose, and 0.1 M buffer as indicated in the text and illustrations) with a prechilled mortar and pestle at 0-4°C. Four milliliters of grinding medium were used per gram fresh weight. When *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/2-(*N*-morpholino)-ethanesulfonic acid (HEPES-MES) buffers were used, equimolar solutions of HEPES and MES were mixed at the appropriate temperature to obtain the desired pH.

Centrifugation procedures

The crude homogenate was filtered through

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

four layers of cheesecloth and particulate fractions were sedimented at $1000 \times g$ for 10 min in a Sorvall HB-4* rotor (crude cell walls), $13\,000 \times g$ for 15 min in a Sorvall SS-34 rotor (crude organelles), and $120\,000 \times g$ for 40 min in a Beckman Ti60 rotor (Crude microsomes). All centrifugations were performed at 4°C and centrifugal forces were expressed as average g forces.

When linear sucrose density gradient centrifugation was used, the $1000 \times g$ pellet was suspended in grinding medium and re-pelleted at $1000 \times g$ for 10 min. The washed pellet was suspended in 2 ml of grinding medium, overlaid on a 38-ml gradient (containing 15–60% sucrose (w/w) in 1 mM β -mercaptoethanol and 15 mM HEPES–MES at pH 6.0 or 7.7) and centrifuged at 4°C for 15 h (unless stated otherwise) at $84\,000 \times g$ in an SW28 rotor. Linear gradients were made with an ISCO Model 570 gradient former and fractionated into 1.5-ml fractions with an ISCO Model 185 density gradient fractionator. Gradients were fractionated with Fluorinert FC-40 (1.85 g/cc) as the heavy chase solution.

Enzyme assays

β -glucosidase activity was determined with 5 mM paranitrophenyl- β -glucose in 0.1 M sodium citrate buffer at pH 5.5. The 1-ml reaction volume, including 10–100 μl of enzyme (20 μg protein maximum), was incubated at 38°C for 1–10 min in a shaking water bath. Reactions were stopped with 0.2 M sodium carbonate and when necessary, the particulate suspensions were centrifuged before determining the absorbance. All assays were initiated, terminated, and the absorbance determined at timed intervals at 405 nm. A standard curve was made with *p*-nitrophenol (1 mM stock).

*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Results and discussion

Distribution of β -glucosidase activity in particulate fractions isolated at various pH-values

Corn roots were homogenized either at pH 6.0 or at pH 7.7 and particulate fractions were collected by a series of differential centrifugation steps. Table I shows that higher levels of β -glucosidase activity were obtained in all particulate fractions isolated under alkaline conditions. The largest difference occurred in the crude cell wall fraction (6-fold more activity at pH 7.7 compared to pH 6.0) with a progressively reduced pH effect in the crude organelle fraction and crude microsomal fraction. Because the crude cell wall fraction was the major location of the pH effect, the $1000 \times g$ pellet was used for all further analyses.

To determine more precisely the pH range which promoted the dissociation of β -glucosidase activity from the crude cell wall fraction, corn roots were homogenized in grinding medium buffered over narrow increments of pH. The pH of the grinding medium was checked before and after homogenization at 4°C and it never dropped more than 0.02 pH units when 0.1 M buffers were used. Figure 1 (curve A) clearly indicates that β -glucosidase activity associated with the crude cell walls was removed by a small change in homogenization pH. Approximately 18% of the total β -glucosidase activity was associated with the $1000 \times g$ pellet when isolated between pH 6.8 and 7.0 compared to 3% at pH 6.2.

Because three different buffer systems and eight separate homogenizations were used in these experiments, the results were verified by using a modified approach with a single buffer system. Corn roots were homogenized at pH 7.7 (0.1 M HEPES–MES). Aliquots of the crude cell wall fraction were washed at various pH-values as described in Fig. 1 (curve B). Since these cell wall fractions were washed once, the total particulate associated activity was less than the unwashed controls [7]. The results in Fig. 1 (curve B) were similar to those in Fig. 1 (curve A) and confirmed that

Table I. The effect of homogenization pH on the distribution of β -glucosidase activity in fractions isolated from corn roots by differential centrifugation.

Material assayed	pH 6.0 ^a		pH 7.7 ^b	
	Total act. (μ moles/fraction \cdot h)	Percent recovery	Total act. (μ moles/fraction \cdot h)	Percent recovery
Crude homogenate	1587.3 \pm 12.1 ^c		1711.6 \pm 3.4	
1000 \times g 10 min pellet	51.8 \pm 0.8	3.3	319.5 \pm 6.7	18.7
13 000 \times g 15 min pellet	28.9 \pm 0.9	1.8	88.9 \pm 0.7	5.2
120 000 \times g 40 min pellet	18.6 \pm 0.2	1.2	26.8 \pm 2.4	1.6
Supernatant fluid from 120 000 \times g centrifugation	1434.5 \pm 5.5	90.4	1258.3 \pm 16.3	73.5

^a0.1 M potassium phosphate containing 5 mM β -mercaptoethanol, 5 mM EDTA and 0.3 M sucrose.

^b0.1 M HEPES—MES containing 5 mM β -mercaptoethanol, 5 mM EDTA and 0.3 M sucrose.

^c \pm S.E.

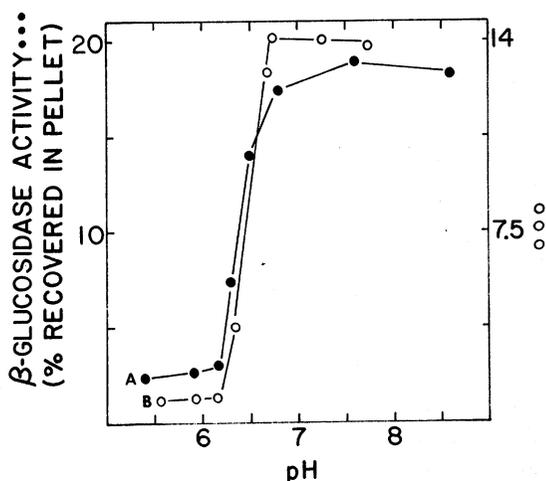


Fig. 1. The effect of homogenization pH (curve A) or washing pH (curve B) on the recovery of β -glucosidase activity in the crude cell wall fraction isolated from corn roots. To obtain curve A, eight separate homogenizations were performed at 0–4° (pH 5.4 and 5.9 with 0.1 M sodium citrate buffer; pH 6.2–7.5 with 0.1 M potassium phosphate; pH 8.5 with 0.1 M Tris—HCl) and β -glucosidase activity was determined in the 1000 \times g pellets and 1000 \times g supernatant fluids. To obtain curve B, one homogenization was performed at pH 7.7. The crude cell wall pellet was suspended in 1.6 ml of homogenization medium (pH 7.7) and divided into eight equal aliquots. Aliquots were suspended in 35 ml of buffer (adjusted to various pH-values by mixing various proportions of 0.1 M HEPES and 0.1 M MES) and centrifuged at 1000 \times g for 10 min. The β -glucosidase activity recovered in the pellets representing the washed crude cell wall fractions was determined.

90% of the crude cell wall associated β -glucosidase was removed as the pH was lowered from 6.8 to 6.2. The dissociation of enzyme activity was not due to an increase in ionic strength since all of the buffer systems used in Fig. 1 decreased in ionic strength as the pH was lowered (calculations not shown).

The results in Table I and Fig. 1 are contrary to those in other reports [1,2,4,6]. This suggested either that the cell walls from corn roots are different from walls isolated from other plant tissues or that the pH effect observed was not on the wall itself but on a subcellular component present in the crude cell wall fraction. To make this distinction, cell walls were separated from subcellular components by sucrose density gradient centrifugation (Nagahashi et al., submitted).

Linear sucrose density gradient centrifugation of the 1000 \times g fraction

The crude cell wall pellet (isolated at pH 7.7) was suspended in homogenization medium (pH 7.7), divided in half, and each half was overlaid on a sucrose gradient. The gradients were identical except that one gradient was buffered with 15 mM HEPES—MES (pH 6.0) (Fig. 2A), and the other was buffered with 15 mM HEPES—MES (pH 7.7) (Fig. 2B). The distribution of β -glucosidase activity in the acid gradient was identical to that reported

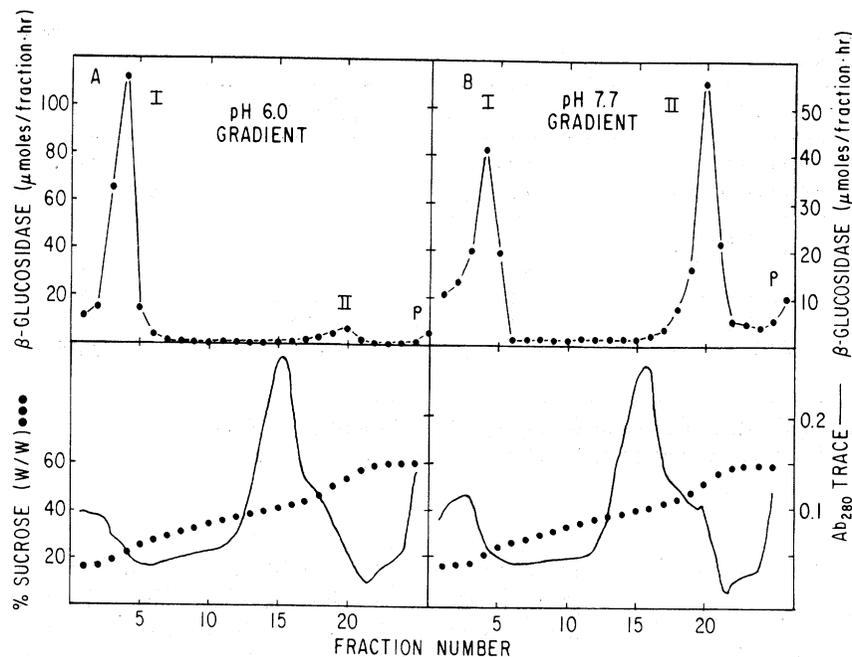


Fig. 2. Distribution of β -glucosidase activity in a sucrose overlaid with a $1000 \times g$ pellet isolated from corn roots. This crude cell wall fraction was isolated at pH 7.7 and washed once in homogenization medium. The suspended pellet was divided in half and each half was overlaid on a sucrose gradient buffered with 15 mM HEPES-MES, pH 6.0 (A) or pH 7.7 (B). The 38-ml gradient was made with 15–60% (w/w) sucrose, centrifuged at 4°C for 15 h at $84\,000 \times g$, and fractionated into 1.5-ml fractions. Cell walls pelleting through the gradient (P) were suspended in 1.5 ml of 15% sucrose and were included as fraction 25. Ab_{280} trace is the absorbance at 280 nm used as an estimate of the amount of protein.

previously for unbuffered gradients (Nagahashi et al., submitted). The major peak of β -glucosidase (Peak I activity) near the top of the gradient (Fig. 2A) was shown to be a 'soluble' enzyme by two criteria. Peak I activity was not isopycnic after 15 h of centrifugation and continued to move further into the gradient after prolonged centrifugation (38 h). This was confirmed by the fact that Peak I was located at different densities in differently composed gradients after 15 h of centrifugation. In a 20–60% gradient, Peak I was found at 22% sucrose compared to 24.7% in a 15–58% gradient. In contrast, a second small peak of activity (Peak II) equilibrated at 51% sucrose (1.24 g/cc) after 15 h of centrifugation regardless of the gradient composition. The source of this particulate activity was not identified previously but it was not associated with micro-

bodies, mitochondria, plastids or nuclei (Nagahashi et al., submitted). A third source of β -glucosidase activity (P) became apparent when the particulate material pelleting through the gradient was suspended and used as a gradient fraction (Fig. 2, Fraction 25). This particulate material had been examined previously by electron microscopy and was shown to consist largely of cell walls (Nagahashi et al., submitted).

When the crude cell wall suspension was centrifuged in the alkaline gradient (Fig. 2B), a surprising observation was made. Over half of the total activity recovered in the gradient equilibrated at 51% sucrose. This increase in Peak II activity was concomitant with a decrease in Peak I activity indicating that Peak I was the solubilized form of Peak II (Fig. 2B). Peak II activity was specifically associated with the unidentified component and was

readily solubilized at acid pH (compare Figs. 2A and 2B). If non-specific adherence were the case, a very broad distribution of enzyme activity would have occurred in the alkaline gradient and the enzyme activity would have coincided with the protein trace [8].

The pellet collected at the bottom of the alkaline gradient also contained slightly higher β -glucosidase activity (7%) compared to the acid gradient (4.2%). The slightly higher level was probably not a pH-effect on the cell wall, but on the cytoplasmic contaminants still present in the semi-purified cell walls (Nagahashi et al., submitted).

The results in Fig. 2 explain why the β -glucosidase activity in our previous report [8] was readily solubilized in sucrose gradients. The gradients used in our previous study were unbuffered; however, the pH was determined to be 6.3 at 4°C. The change in pH between the overlay (alkaline) and the gradient (acid) was sufficient to dissociate most of the β -glucosidase activity from the unidentified component.

The pH-dependent distribution of β -glucosidase activity in sucrose gradients was not unique for corn roots. Similar results were observed when crude cell walls isolated from corn coleoptiles were centrifuged in linear density gradients (Fig. 3). The high shoulder in the $A_{280\text{ nm}}$ trace (Fraction 15–18) next to the mitochondria (Fraction 14) indicated that a large number of plastids (etioplasts) were present in the 1000 $\times g$ pellet isolated from dark grown coleoptiles. Again, the level of Peak II β -glucosidase activity in the gradient was pH dependent.

The potential source of Peak II β -glucosidase activity

Although coated vesicles have a similar density to the source of Peak II activity [9], it is unlikely that they are a source of β -glucosidase activity. The distribution of β -glucosidase activity presented in Table I indicated that the crude microsomal fraction (which should contain most of the coated vesicles) had the lowest yield of particulate

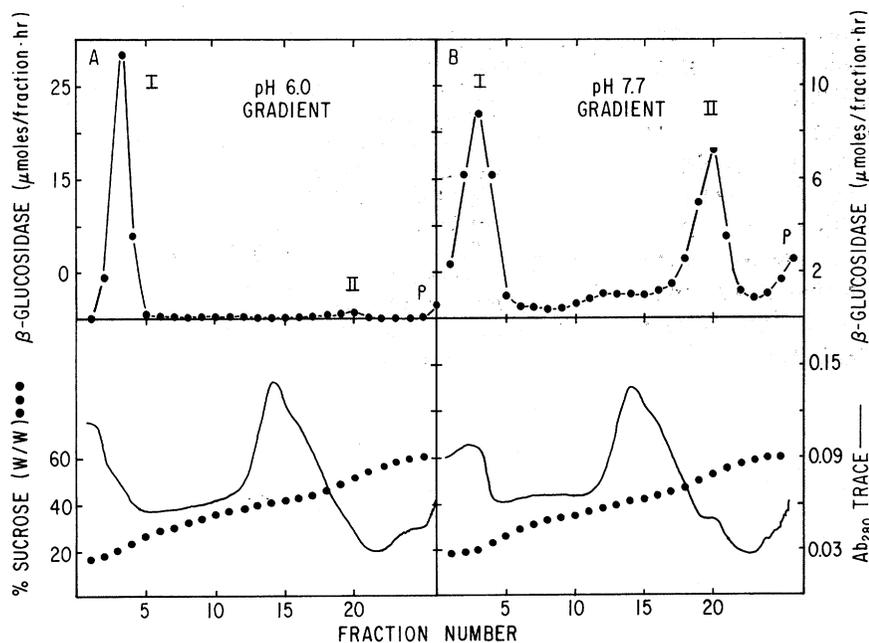


Fig. 3. Distribution of β -glucosidase activity in a sucrose gradient overlaid with a 1000 $\times g$ pellet isolated from corn coleoptiles. Methods were the same as given in the legend of Fig. 2.

activity. It is very unlikely that coated vesicles (12 nm in diameter) would sediment in large quantities in the 1000 × *g* pellet.

One possible source for Peak II activity could be specialized regions of plasma membranes. The plasma membranes typically equilibrate between 35 and 38% sucrose; however, specialized regions or domains of the plasmalemma may have a different composition and, therefore a different density than normally reported. β-Glucosidase activity at the cell surface has been reported [10], but this cytochemical study could not distinguish between cell wall and plasma membrane as the site of activity. The other alternative site of Peak II activity consistent with the cytochemical localization [10] is the possible association of β-glucosidase activity with the microtrabecular lattice. Two recent reports [11,12] have indicated the presence of the microtrabecular lattice in corn root cells. If Peak II activity is associated with this proteinaceous network, it would not be difficult to understand why so much activity in the 1000 × *g* pellet was obtained. This fraction contained many fragments of cytoplasm and these fragments were the most difficult contaminant to remove from cell wall preparations [Nagahashi et al., submitted; 13].

Regardless of the actual site of Peak II activity, the dissociation of β-glucosidase activity from the 1000 × *g* fraction occurred within a physiologically significant pH range (pH 6.8–6.2). However, all attempts to reverse the dissociation have failed. Either the phenomenon observed is not reversible (unlike the binding of enzyme to cell walls) or a portion

of the subcellular component to which the β-glucosidase was originally attached has been dissociated at pH 6.2. If this were the case, then reassociation of the enzyme to the unidentified component may not be possible.

The presence of Peak II β-glucosidase activity has gone unnoticed either because crude cell wall preparations were washed many times before enzymatic analysis and the unidentified source of activity was removed, or homogenizing and washing were performed under acidic conditions (pH 5.0–6.0) which readily solubilized the activity.

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