

## Unique properties of cell wall-associated $\beta$ -glucosidases

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Two maize (*Zea mays* L.) root cell wall-associated  $\beta$ -glucosidases were compared to two cellular forms. The ionically bound cell wall enzyme was more difficult to dissociate from the cell wall compared to artifactually bound cytosolic  $\beta$ -glucosidase activity. The ionically bound cell wall enzyme also had an isoelectric pH of 6.8–7.0 compared to the acid *pI* of the cytosolic enzyme (4.8–5.2). A second cell wall enzyme was very tightly bound to the cell wall and could not be extracted with 3 M NaCl or LiCl. Both the ionically bound cell wall  $\beta$ -glucosidase and the tightly bound  $\beta$ -glucosidase were more thermostable at 40°C and 50°C compared to the cytosolic enzyme and the particulate intracellular isozyme. The tightly bound cell wall  $\beta$ -glucosidase was the only thermostable enzyme at 60°C.

*Key words:* cell walls; bound enzymes; free enzymes; *pI*; thermostability

### Introduction

Most reports comparing the properties of  $\beta$ -glucosidases have focused on pH optima and enzyme kinetics [1–3]. At least four types of  $\beta$ -glucosidase activity are present in corn (maize) root cortical cells. A cytosolic form (I) and an intracellular enzyme which is bound to an unidentified particulate fraction (II) have been reported [1]. Two other  $\beta$ -glucosidases are associated with the cell wall. One enzyme is ionically bound to the cell wall (III) and the second form is tightly bound (IV) and cannot be removed by high ionic strength solutions [1]. Although these various enzymes have slightly different pH optima, they are similar kinetically [1].

Only a few reports [4,5] have indicated that cell wall enzymes have properties that can be used to distinguish them from intracellular isozymes. We report here several properties which are unique to

cell wall-associated  $\beta$ -glucosidases when compared to other cellular forms of the enzyme. These properties can be used to distinguish enzymes which artifactually bind to the wall during the cell disruption process from actual cell wall-associated enzymes.

### Materials and Methods

#### *Plant material*

Corn seeds (WF9  $\times$  MO17) were germinated and harvested as previously reported [6]. The root cortex was physically removed from the stele and frozen in liquid nitrogen [6].

#### *Isolation and separation of various $\beta$ -glucosidases*

Cell walls were purified from isolated corn root cortex as described [6]. The unidentified particulate source of  $\beta$ -glucosidase activity was isolated from a linear sucrose gradient overlaid with a 1000 g pellet obtained from a crude homogenate of primary corn roots [7]. This particulate form (1.24 g/cm<sup>3</sup>) was not identified but could represent cytoplasmic fragments or possibly proplastids [7]. The cytosolic enzyme was purified as recently reported [1].

#### *Salt extraction of purified cell walls*

Cell walls were extracted with 2 M NaCl or 2 M LiCl unless otherwise indicated in the text. The cell walls were exhaustively rinsed in cold deionized-distilled water and the salt extract was dialyzed against cold, deionized-distilled (DD) water for 2 days at 4°C. The extracted cell walls still retained the tightly bound cell wall  $\beta$ -glucosidase activity. In certain experiments, this residual activity was eliminated by further treating the cell walls with 6 M LiCl for 15 h at 4°C. This treatment denatured the tightly bound enzyme since no activity could be found in the extract or in the cell walls.

#### *Biochemical assays*

Enzyme assays were performed as described for soluble enzyme activity [7] and cell wall-bound activity [6]. Assays were performed at 38°C with *p*-nitrophenyl (PNP)- $\beta$ -glucose or 4-methylumbelliferyl (MU)- $\beta$ -glucose as substrate. Protein estimation was done by a modified Lowry procedure [8].

#### *Determination of isoelectric pH*

The pI for the various  $\beta$ -glucosidases was determined by column isoelectric focusing (IEF) [9], IEF gels using a Pharmacia Phast System according to the manufacturers recommendation, or by chromatofocusing on a Polybuffer Exchanger 94 column (Pharmacia) and eluting with Polybuffer 74. To detect  $\beta$ -glucosidase activity in IEF gels, 4-MU- $\beta$ -glucose (3 mM in pH 5.5 sodium citrate buffer) was applied directly to the gels for 1–5 min at room temperature. The substrate was quickly rinsed off and 1 M sodium carbonate was applied to the gels for 1 min and quickly rinsed with distilled water. Fluorescent bands were detected in an ultraviolet (UV) light box at 366 nm. The activity bands were marked with a razor blade and the gels were immediately stained with Coomassie blue using the Phast System. Briefly, the gels were fixed in 20% trichloroacetic acid, washed in 30% methanol in 10% acetic acid, stained with 0.02% PhastGel Blue R solution (containing 30% methanol, 10% acetic acid and 0.1% CuSO<sub>4</sub>) and destained with the wash solution. The activity bands were compared to known pI standards.

#### *Dissociation curves of cell wall-bound proteins*

Aliquots (1 ml) of purified cell walls were mixed with 1 ml of increasing NaCl concentration (0–2 M). Samples were gently stirred with a glass rod every 15 min and after 1 h at room temperature, samples were centrifuged and the supernatant fluid was checked for solubilized enzyme activity. The pelleted walls were washed twice with cold water and suspended in a 1-ml volume and rechecked for  $\beta$ -glucosidase activity. This was necessary to insure that the starting total activity could be recovered after salt treatment.

In rebinding experiments, cell walls treated with 6 M LiCl were washed six times with DD water and then stirred in 10 mM ethylenediaminetetraacetic acid, sodium salt (EDTA) containing 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer at pH 7.5 for 30 min at room temperature. The cell walls were then washed an additional six times with cold DD water. Cytochrome *c* (horse heart, type III from Sigma) and the salt-extracted  $\beta$ -glucosidase were incubated with these cell walls at 4°C for 1 h in water to allow binding (or rebinding). The cytosolic  $\beta$ -glucosidase would not bind to the cell walls under these conditions but would adsorb if the binding mixture was made acidic (pH 4.0–4.3) with a sodium citrate buffer (25 mM final concentration). Cytochrome *c* was quantified by reducing an aliquot of the binding mixture (after centrifugation) with sodium dithionite. Results were compared to a reduced cytochrome *c* standard curve at 550 nm [10].

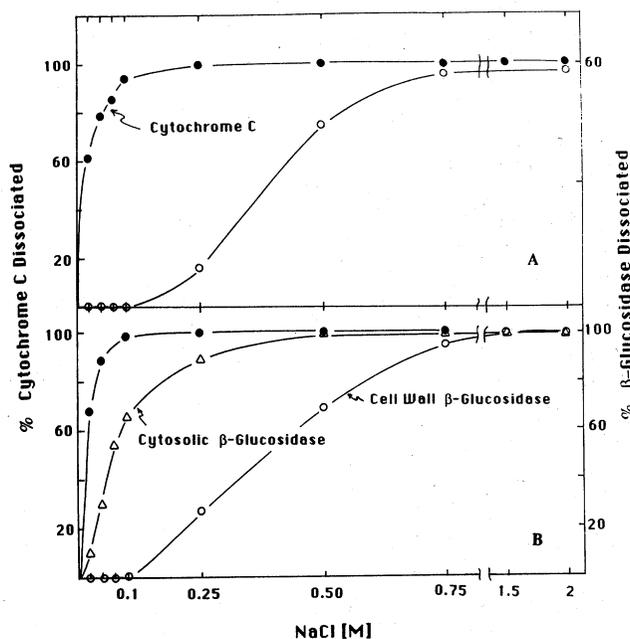
#### *Thermostability curves*

Aliquots of the various  $\beta$ -glucosidases were incubated at 40°C, 50°C and 60°C. At specified time periods, samples were removed and tested for residual  $\beta$ -glucosidase activity. When cell walls were used, 15–20-ml aliquots were placed in 30-ml beakers during the incubation period. All samples were capped or covered during the extended preincubation period to prevent a change in volume due to evaporation at high temperatures. Cell wall samples were stirred with a magnetic stirring bar before removing an aliquot.

## Results

### Salt dissociation curves

The salt dissociation curve of the endogenous cell wall-associated  $\beta$ -glucosidase was compared to an artifactually bound protein. Cytochrome *c* was chosen for this experiment because it had a high *pI* (10.5) and was not present in purified cell walls. Cytochrome *c* (5 mg/ml) was allowed to bind to purified cell walls in water for 1 h at room temperature. The cell walls were then washed to remove unbound cytochrome *c* and salt dissociation curves were measured. Using 50 mM salt, over 80% of the bound cytochrome *c* protein was solubilized from the cell wall but no endogenous bound  $\beta$ -glucosidase was released at this concentration (Fig. 1A). Using 500 mM salt, 40–50% of



**Fig. 1.** (A) Salt dissociation curves of the ionically bound corn root cell wall  $\beta$ -glucosidase (O—O) in comparison to artifactually bound cytochrome *c* (●—●). These experiments were performed on untreated, purified cell walls. (B) Purified cell walls were treated with 6 M LiCl to denature the tightly bound  $\beta$ -glucosidase. Cytosolic  $\beta$ -glucosidase ( $\Delta$ — $\Delta$ ), cytochrome *c* (●—●) and the salt-extracted cell wall  $\beta$ -glucosidase (O—O) were allowed to bind to separate aliquots of treated cell walls. Salt dissociation curves were then repeated with increasing concentration of NaCl.

the cell wall enzyme activity could be solubilized. Even at salt concentrations greater than 1000 mM, only 60% of the total activity could be extracted. This residual activity which was not removed by high salt concentrations represented the tightly bound cell wall enzyme.

Because cytochrome *c* was added after cell walls were purified, it could be argued that this protein could not bind to sites already occupied by endogenous proteins and therefore may not bind very tightly. To address this possibility, the endogenous salt-extracted cell wall enzyme, the cytosolic enzyme and cytochrome *c* were allowed to bind to separate aliquots of cell walls treated with 6 M LiCl. This treatment removed all salt-extractable protein and denatured the tightly bound cell wall enzyme activity. All added proteins had the same chance to bind to any available site on these walls during the incubation period. The salt dissociation curves were then repeated and both the cytosolic  $\beta$ -glucosidase and cytochrome *c* rapidly dissociated from the cell walls at low salt concentration (Fig. 1B). The endogenous cell wall enzyme again required higher salt concentrations to be dissociated compared to artificially bound proteins.

### Isoelectric pH

The cytosolic  $\beta$ -glucosidase (Fig. 2A) and the unidentified particulate  $\beta$ -glucosidase (data not shown) both had a *pI* of 5.0 when chromatofocused in a column. In an IEF gel (pH 3–9), a major activity band for both samples occurred at approximately pH 5.0–5.2 (gels not shown) and multiple activity bands between pH 4.8 and 5.2 were observed when an IEF gel containing a pH 4.0–6.0 gradient was used.

The ionically bound cell wall-associated enzyme had a major peak at pH 6.85 in a chromatofocusing column and several minor peaks between pH 5.1 and 5.3 (Fig. 2B). This distribution was consistently observed if the extracted protein sample was suspended in pH 7.4 buffer, applied to the column and eluted with pH 4.0 buffer. The minor peaks between pH 5.1 and 5.3 could be breakdown products of the *pI* 6.85 isozyme as suggested by the following results. If the salt-solubilized protein

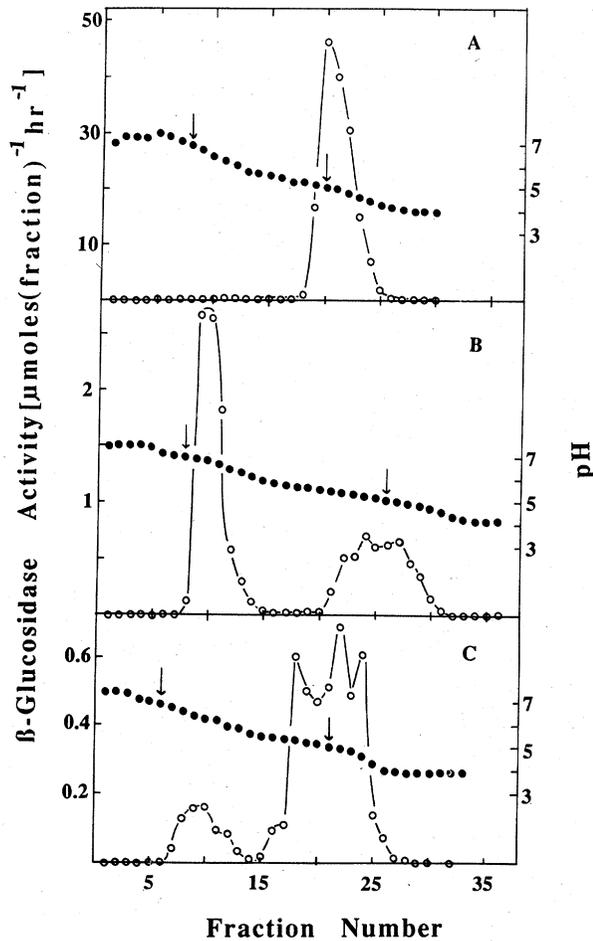


Fig. 2. Chromatofocusing of cytoplasmic and cell wall extracts of corn root cortical cells. (A) Cytosolic  $\beta$ -glucosidase. (B) Salt-extracted cell wall-associated  $\beta$ -glucosidase suspended in buffer (pH 7.4) before chromatofocusing. (C) Salt-extracted cell wall-associated  $\beta$ -glucosidase suspended in eluent buffer (pH 4.0) before chromatofocusing. Arrows mark pH 5.0 and pH 7.0.

was suspended in buffer (pH 4.0), the results of chromatofocusing were considerably different. Only a minor peak of activity was observed at pH 6.8 and this activity was less than that observed between pH 5.0 and 5.3 (Fig. 2C). Only 45% of the starting activity could be recovered from the chromatofocusing column and this suggested that the pH 6.85 isozyme was acid labile. This was confirmed by the fact that in IEF gels, a  $\beta$ -glucosidase activity band comigrated with a  $pI$  standard for

pH 7.0 only when the sample was applied to the alkaline end of the gel. If the sample was applied to the acid end of an IEF gel, no activity band was detected at pH 7.0 (gels not shown). The apparent acid affect on the  $pI$  of the  $\beta$ -glucosidase was specific for this cell wall enzyme. Application of cytosolic isozymes at different ends of a gel did not change the  $pI$  of these enzymes.

#### Thermostability properties

The various  $\beta$ -glucosidases were incubated at 40°C, 50°C and 60°C. Aliquots were removed at specified times and checked for residual enzyme

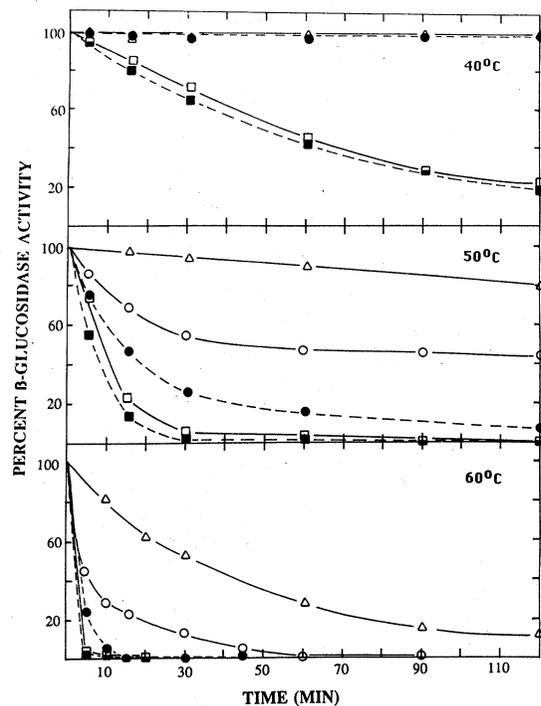


Fig. 3. Thermostabilities of various  $\beta$ -glucosidases isolated from corn root cortical cells. The enzymes were incubated at 40°C, 50°C and 60°C and after specified times, aliquots were removed and assayed for  $\beta$ -glucosidase activity at 38°C with PNP- $\beta$ -glucose as the substrate. Tightly bound cell wall enzyme ( $\Delta$ - $\Delta$ ); ionically bound cell wall enzyme in the free form ( $\bullet$ - $\bullet$ ); ionically bound cell wall enzyme, allowed to rebind to cell walls ( $\circ$ - $\circ$ ); cytosolic enzyme in free form ( $\blacksquare$ - $\blacksquare$ ); cytosolic enzyme in the bound form ( $\square$ - $\square$ ). At 40°C, the ionically bound cell wall enzyme (salt-solubilized) was allowed to rebind to cell walls and its thermostability was identical to the tightly bound and salt-soluble cell wall enzyme in the free form.

activity. At 40°C, the cytosolic enzyme steadily lost activity as the incubation period was extended to 2 h (Fig. 3). The salt-extracted cell wall enzyme (free form), the salt-extracted enzyme rebound to treated cell walls (data not shown) and the tightly bound enzyme were completely stable under these conditions (Fig. 3). As the temperature was increased to 50°C, the salt-soluble cell wall enzyme (bound and free) lost activity but was still more stable than the cytosolic  $\beta$ -glucosidase. At 60°C, the tightly bound cell wall enzyme exhibited the greatest thermostability followed by the salt-soluble cell wall enzyme (bound form) and then the salt-soluble free enzyme (Fig. 3). The unidentified particulate form of  $\beta$ -glucosidase behaved very similarly to the cytosolic enzyme (Fig. 3) and this supported the contention that this particulate enzyme was the bound form of the cytosolic enzyme [7].

The rapid loss of cytosolic enzyme activity at 60°C indicated thermodenaturation but the gradual loss at 40°C could be a result of proteolytic activity in this  $\beta$ -glucosidase preparation. To determine if this loss was due to thermodenaturation or a protease, the 40°C incubation was performed in the presence and absence of protease inhibitors (chymostatin, 5  $\mu$ g/ml; leupeptin, 15  $\mu$ g/ml). These inhibitors are known to inhibit corn root proteases but in this experiment, greater loss of  $\beta$ -glucosidase activity was observed in their presence compared to their absence (data not shown). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels using the samples after the incubation period as well as an untreated control (kept at 4°C) were prepared. The gels of the incubated samples at 40°C with and without inhibitor looked virtually identical and were the same as those from the untreated control after electrophoresis (data not shown). No increase in small peptide fragments or loss of protein bands were observed as would be expected if proteolytic activity was present.

### Conclusions

We report here three different properties which indicate that the cell wall-associated  $\beta$ -glucosidases are unique and can be distinguished

from other cellular isozymes. The salt dissociation curve of the ionically bound  $\beta$ -glucosidase indicates that this enzyme is more tightly bound to the cell wall than artifactually bound proteins. This result is similar to a previous report where the dissociation of a wall-bound saccharase was compared to an artificially adsorbed cytosolic saccharase [5]. Because the enzymes used in this study [5] and in the results shown in Fig. 1B were comparing dissociation curves of isozymes with acidic and basic  $pI$  values respectively, the dissociation of a very basic protein, cytochrome *c* ( $pI = 10.5$ ), was compared to the endogenous cell wall  $\beta$ -glucosidase. The results of these experiments clearly demonstrate (Fig. 1) that the difference in dissociation curves between the cytosolic  $\beta$ -glucosidase and the cell wall-bound isozyme is not simply due to the isoelectric pH of the two enzymes. If this were the case, then cytochrome *c* would have been more tightly bound than the cell wall enzyme. These experiments and those reported earlier [5,11,12] using competitive binding studies plus a current localization study which shows at least two different binding sites for acid phosphatase [13] all indicate that there is more than one type of protein binding site in cell walls. The specificity of the binding sites has not yet been determined.

The higher isoelectric pH of the cell wall  $\beta$ -glucosidase compared to intracellular forms was consistent with previous reports which compared  $pI$  values of cytosolic proteins with salt-extracted cell wall proteins [9,14]. However, for corn root cell walls, the cell wall-bound activity was very unstable at pH 4.0 and indicated that acid buffers should be avoided when isolating cell walls from this tissue. Not only was enzyme activity lost under acid conditions, but a conversion of the more basic form to acidic forms may also occur. This type of phenomenon was reported earlier for peroxidase enzymes of potato tubers [14]. In general, enzymes bound to the cell wall appear to have a more alkaline isoelectric pH when directly compared to their cytosolic isozymes.

Finally, a surprising property of cell wall  $\beta$ -glucosidases was their thermostability at 40°C compared to cellular enzymes. Both the salt-soluble enzyme (III) in the bound or free form and the tightly bound enzyme (IV) were completely

stable for 2 h at 40°C. The cytosolic (I) and unidentified particulate enzyme (II) both lost activity when incubated at this temperature. As the incubation temperature was raised, the order of stability was as follows: tightly bound cell wall enzyme  $\gg$  salt-soluble cell wall enzyme (bound)  $>$  salt-soluble cell wall enzyme (free)  $>$  cytosolic = unidentified particulate (Fig. 3). The stability of a cell wall-bound enzyme at 50°C compared to a cytosolic isozyme has been reported previously [4] so the case for the tightly bound  $\beta$ -glucosidase in corn root cell walls does not appear to be rare. Binding or covalent attachment to the wall may prevent protein unfolding during high temperatures and hence provide stability. The stability of the salt-soluble enzyme (free form) at 40°C is more difficult to explain but clearly indicates that this cell wall ionically bound enzyme is inherently different from the cellular forms. In addition, the thermostability of the salt-extracted enzyme increased when the  $\beta$ -glucosidase was allowed to rebind to cell walls before heat treatment (Fig. 3). The unique properties of cell wall-bound enzymes reported here clearly support the contention that these enzymes do not arise as an artifact of cell disruption. Once the basis for these differences are determined, the biochemical basis for the compartmentation of the ionically bound and tightly bound enzyme forms in the extracellular matrix can be addressed.

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