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β-Glucosidase Activity in Corn Roots

PROBLEMS IN SUBCELLULAR FRACTIONATION

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GERALD NAGAHASHI* AND AMY F. BAKER
Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Philadelphia, Pennsylvania 19118

ABSTRACT

Preliminary results from differential centrifugation experiments, washing treatments, and enrichment in linear sucrose gradients at a density of 1.09 grams per cubic centimeter all indicated that β-glucosidase activity in corn root homogenates was associated with a membrane such as tonoplast. A subsequent sucrose density gradient centrifugation time course showed that the β-glucosidase was actually a soluble enzyme which moved into the gradients. The problem of soluble enzymes contaminating light density membranes in sucrose gradients and the question of centrifugation time necessary for membrane vesicles to reach isopycnic conditions are addressed.

The only hydrolytic activity associated with the tonoplast has been phosphatase activity (ATPase, 4, 10, 11; pyrophosphatase, 26). Attempts to find other hydrolytic activities associated with vacuole membrane have not been successful (2, 25). Since several reports have indicated that various types of glycosidases are associated with particulate or membrane fractions, as well as soluble fractions from plant roots (1, 3, 14, 18, 19, 23), it was possible that glycosidase activity was associated with the tonoplast of root cells. Because a reliable marker for vacuole membranes in root cell homogenates is greatly needed, we attempted to localize β-glucosidase activity with a light density membrane distinct from ER or Golgi vesicles.

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays* L., WF9 X Mo17) were placed on filter paper saturated with 0.1 mM $CaCl_2$, germinated in glass trays in the dark at 27°C, and were harvested after 3 d. Primary roots (6–8 cm) were washed twice in cold distilled H_2O and 8 to 16 g fresh weight were ground in a prechilled mortar and pestle at a ratio of 4 ml GM[1]/g fresh weight. GM contained 5 mM EDTA, 5 mM DTT, 5 mM β-mercaptoethanol, and 30 mM Tris-Mes buffer (pH 7.7) in 0.3 M sucrose. Removal of ribosomes from RER was accomplished by the presence of EDTA so that ER was of the light density class (20).

Centrifugation Procedures. Crude particulate fractions were sedimented at 1,000*g* for 10 min, 6,000*g* for 20 min, and 120,000*g* for 40 min as determined previously (16, 17). All centrifugal forces reported are *g* average. The 120,000*g* supernatant was used as a source of soluble enzymes. Crude pellets were washed one or more times (as indicated in the text) and repelleted at their initial isolation force.

When linear sucrose density gradient centrifugation was performed, either a 1,000 to 120,000*g* pellet (crude membranes) was suspended in 2 ml of GM and overlaid on a gradient, or 2 ml of the 120,000*g* supernatant (total volume of 36 ml) was overlaid on a gradient and centrifuged at 84,000*g* in an SW 28 rotor. The range of sucrose used in gradients and the centrifugation time are given in the text. Sucrose gradients were made with an ISCO[2] model 570 gradient former and were fractionated with an ISCO model 185 density gradient fractionator. Per cent sucrose was determined with an ABBE 3L refractometer.

Enzyme Assays. Glycosidase activity was determined by using 5 mM PNP- or 4-MU- sugar derivatives in 0.1 M sodium citrate buffer (pH 5.0). Assays were run at 38°C for 3 to 12 min with 10 to 20 μg of protein and were linear with respect to time and protein concentration. The reaction was terminated with 0.2 N NaOH and read immediately at 405 nm. The PNP standard curve was linear for 2.0 absorbance units (0.1 μmol PNP gave an *A* of 0.525). Enzyme assays were performed to stay between 0.1 and 1.0 OD. Acid phosphatase activity was determined with 2.5 mM PNP-phosphate at pH 5.5 as substrate. MDH was assayed according to Ting (24) using an extinction coefficient of 6.2/mM·cm. NADH Cyt *c* reductase (±1.5 μM antimycin A) and Cyt *c* oxidase activities were assayed as described earlier (6). Catalase activity was measured according to Lück (9). Protein was estimated by a modification of the Lowry procedure (12) using BSA, fraction V (Sigma) as standard.

RESULTS

Differential Centrifugation. The results in Table I confirmed earlier reports which indicated β-glucosidase activity was associated with various membrane fractions and a soluble fraction. The highest specific activity was associated with the crude cell wall fraction (1,000*g* pellet), although the highest total activity was found in the supernatant. The combined crude mitochondrial (6,000*g* pellet) and microsomal (120,000*g* pellet) fractions contained approximately 5% of the total β-glucosidase activity (when the percentage was based on particulate associated activity excluding the supernatant, the per cent total activity was 20%). A low percentage associated with organelles or membranes was not uncommon since triose phosphate isomerase activity associated with plastids only represents 5 to 8% of the total activity (15) and, similarly, membrane-bound cellulase activity represents 5 to 10% of the total activity (7).

Substrate Specificity of Crude Fractions Isolated by Differential Centrifugation. Since it was possible that β-glucose was not

[1] Abbreviations: GM, grinding medium; PNP, paranitrophenol; MDH, malate dehydrogenase.

[2] Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

Table I. *Differential Centrifugation of a Corn Root Homogenate into Subcellular Fractions Containing β-Glucosidase Activity*

All particulate fractions were washed in homogenization medium and repelleted at their initial isolation force.

Fraction	p-Nitrophenyl-β-Glucosidase			Total Protein	
	Specific activity	Total activity			
	μmol/mg protein·h	μmol/fraction·h	%	mg	%
1,000g × 10 min	176.8	309.5	19.2	1.75	6.5
6,000g × 20 min	20.4	41.6	2.6	2.04	7.5
120,000g × 40 min	11.0	37.5	2.2	3.40	12.5
Supernatant	61.6	1227.3	76.0	19.92	73.5

Table II. *The Recovery of β-Glucosidase Activity after Repeated Washing of a Membrane Fraction (1,000–120,000g Pellet) Isolated from Corn Roots.*

The initial pellet (unwashed) was suspended in homogenization medium and divided into four equal aliquots. Membranes were washed in homogenization medium and pelleted at 120,000g × 40 min.

Treatment	p-Nitrophenyl-β-Glucosidase			Total Protein	
	Specific activity	Total activity			
	μmol/mg protein·h	μmol/fraction·h	%	mg	%
Unwashed membranes	20.57	56.37	100	2.74	100
Membranes washed 1×	15.59	35.86	63.6	2.30	83.9
Membranes washed 2×	13.84	30.18	53.5	2.18	79.6
Membranes washed 3×	12.29	24.34	43.2	1.98	72.3

the preferred substrate for all isolated fractions, a substrate specificity was determined. The crude cell wall fraction, crude membrane fraction, and supernatant all gave highest activity with PNP-β-glucose (data not shown) so this substrate was used in further studies. Considerably lower (10-fold) levels of activity were detected with the second preferred substrate (PNP-β-galactose), and only very low levels of α-mannosidase activity were found under our assay conditions (data not shown). Two reports have shown α-mannosidase activity associated with subcellular membranes and these investigators incubated their membrane preparations with PNP-α-mannose for 5 h at 35°C (3, 22) to achieve detectable activity. In contrast, another report indicated that α-mannosidase activity was strictly soluble and was localized inside vacuoles (2).

Extensive Washing of the Crude Membrane Fraction (1,000–120,000g Pellet). Repeated washing of the crude membrane fraction was performed to determine if the β-glucosidase activity was a 'soluble' contaminant which could be removed by this treatment. The first washing removed the greatest amount of enzyme activity (36%) as well as the greatest amount of protein (16%) when compared to the unwashed control (Table II). The first wash removed soluble extraneous protein (7). Successive washings removed considerably less of the total β-glucosidase activity and less protein with a concomitant small decrease in specific activity (successive washing removed some membranes due to the change in sedimentation path length). These results suggested that the residual enzyme was associated with subcellular membranes or organelles since the activity was not readily removed by washing.

Sucrose Density Gradient Centrifugation of the 1,000 to 120,000g Pellet and the 120,000g Supernatant. The washed (1×) membrane fraction was overlaid on a linear sucrose gradient composed of 15 to 50% (w/w) and centrifuged for 15 h. Results in Figure 1 showed that the β-glucosidase activity formed one very prominent peak (1.09 g/cm³) very close to the ER (1.10 g/cm³) as indicated by antimycin A-insensitive NADH Cyt *c* reductase activity but lighter than Golgi membranes (1.12 g/cm³; Ref. 17). The tonoplast was believed to have an average density of 1.11 g/cm³ in corn root tips (4) and, for other tissues, the density was reported very close to the ER (10, 25).

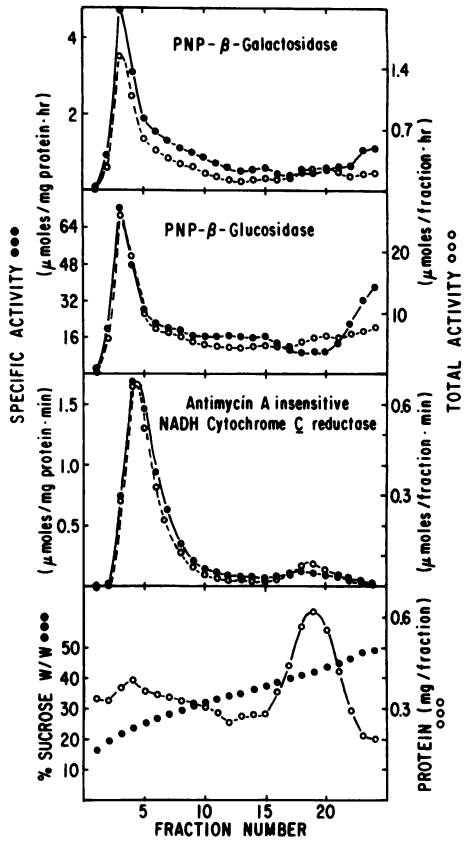

FIG. 1. A washed crude membrane fraction (1,000–120,000g pellet) isolated from a corn root homogenate was overlaid on a 38-ml linear sucrose gradient (15–50%, w/w), centrifuged for 15 h at 84,000g, and fractionated into 1.5-ml fractions. See the text for assay details.

Total activity and specific activity of enzymes were monitored (Fig. 1) in the event the fractions with the highest yield were not the most purified. The major peak of β-glycosidase contained the greatest total activity and was the most enriched. No other peak was detected when total activity was monitored; however, a prominent upward swing in the specific activity of β-glucosidase occurred at the bottom of the gradient. This second site of β-glucosidase activity was likely to be cell walls which pelleted through the sucrose gradient. This interpretation was consistent with the increase in specific activity at the bottom of the gradient.

The distribution of β-galactosidase activity was coincidental with the β-glucosidase activity near the top of the gradient (Fig. 1). This indicated that the β-glucosidase and β-galactosidase were either two different enzymes found in the same particulate fraction, or the β-glycosidase was one enzyme with substrate preference for PNP-β-glucoside. Both β-glucosidase and β-galactosidase activity were enriched approximately 5-fold in the major peak (1.09 g/cm³) isolated in the sucrose gradient (data not shown). Isolated membranes were similarly enriched in sucrose gradients as indicated by the 5-fold enrichment of ER associated marker activity at a density of 1.10 g/cm³ (data not shown).

The observations that β-glucosidase activity was associated with membrane fractions (differential centrifugation) was not readily removed by washing treatments, and showed a 5-fold enrichment in sucrose gradients at a density very near ER were all consistent with the subcellular localization of β-glucosidase on a light membrane such as the tonoplast. To ascertain whether the β-glucosidase activity was membrane bound and not due to soluble enzyme activity adhering to a particulate fraction, a centrifugation time course was performed. Soluble enzymes migrate further into sucrose gradients upon increased centrifugation time (rate sedimentation [13]). If membrane-bound, the β-glucosidase activity should equilibrate at the isopycnic density of the associated membrane and not change in density with prolonged centrifugation time (7).

Figure 2A shows the 'particulate' β-glucosidase activity associated with the 1,000 to 120,000*g* membrane pellet was actually a soluble enzyme which moved further into the sucrose gradient with longer centrifugation time. The ER marker does not change in density with prolonged centrifugation while the β-glucosidase activity moved past the NADH Cyt *c* reductase activity after 40 h of centrifugation. The distribution of protein (Fig. 2B) only changed at the top of the gradient and was consistent with the movement of soluble enzymes into the sucrose gradient during the centrifugation time course.

To confirm that soluble β-glucosidase activity can move into sucrose gradients after extended centrifugation, a 2-ml aliquot of the 120,000*g* supernatant was overlaid on top of a linear sucrose gradient. The soluble activity moved into the sucrose gradient with increased centrifugation time (data not shown). The concomitant movement of β-galactosidase activity was consistent with the presence of a single β-glycosidase activity which showed a preference for β-glucoside linkages. The distribution of protein changed near the top of the gradient and was similar to the movement of protein in Figure 2B with prolonged centrifugation time.

The association of soluble enzyme activity with crude membranes was not unique for β-glucosidase. Other soluble forms of

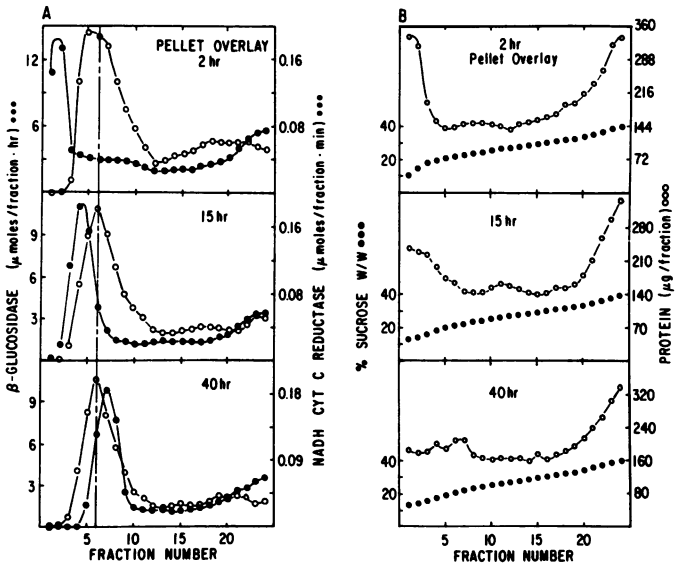

FIG. 2. A washed crude membrane fraction (1,000–120,000*g* pellet) isolated from a corn root homogenate was suspended in grinding medium and separated into three equal aliquots. Each aliquot was overlaid on a separate sucrose gradient (15–40% w/w) and centrifuged at 84,000*g* for the length of time as specified. Gradient size and fractions were the same as in Figure 1. A, The distribution of β-glucosidase activity in relation to the ER marker (antimycin A-insensitive NADH Cyt *c* reductase activity). B, The shape of the gradients and the distribution of protein.

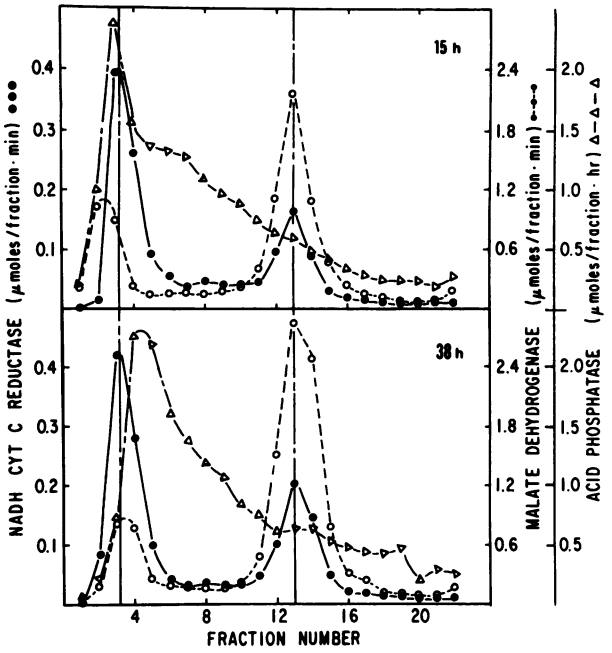

FIG. 3. A crude membrane fraction (1,000–120,000*g* pellet) was isolated from a corn root homogenate and split in half, overlaid on 17 ml sucrose gradients (15–60% w/w), centrifuged at 84,000*g* for 15 (top) or 38 h (bottom), and fractionated into 0.75-ml fractions.

enzymes such as MDH and acid phosphatase (Fig. 3) also move into sucrose gradients with extended centrifugation time. In this case, NADH Cyt *c* reductase was assayed in the absence of antimycin A to show that the mitochondrial associated activity (fraction 13) was isopycnic after 15 h of centrifugation. Cyt *c* oxidase and catalase activity were also coincident with the reductase activity (data not shown). The coincidence of Cyt *c* oxidase and catalase activity was not unique since an earlier report showed that mitochondria and microbodies from pea roots have indentical densities under certain centrifugation conditions (15). MDH activity associated with either mitochondria or microbodies was also at equilibrium after 15 h; however, the soluble MDH activity at the top of the gradient clearly moves into the region of the ER marker by 38 h.

The acid phosphatase activity formed a major peak which clearly moved past the ER marker after 38 h of centrifugation (Fig. 3). Considerable phosphatase activity was detected across the gradient and possibly reflects the presence of ATPases or NDPases which have been reported to be associated with several subcellular membranes (20).

DISCUSSION

Approximately 80% of β-glucosidase activity associated with the 1,000 to 120,000*g* membrane fraction was recovered in the major peak of activity near the top of the sucrose gradient. Although all initial results strongly indicated that β-glucosidase activity was associated with membranes of light density, the centrifugation time course (Fig. 2) clearly demonstrated that the enzyme was a soluble form. The soluble nature of the enzyme was only revealed in sucrose gradients since washing experiments did not readily remove activity. Sucrose gradients have been used by Koehler *et al.* (7) to demonstrate the presence of a soluble cellulase in subcellular fractions isolated from kidney bean abscission zones. Soluble cellulase activity moved further into the sucrose gradient with extended centrifugation time, while the ER marker activity remained isopycnic.

The source of the soluble β-glucosidase activity found in washed crude membrane fractions is not known. It may be due

to a cytosolic enzyme which adheres to particulate material but was removed in sucrose gradients, or due to a soluble enzyme inside a leaky subcellular compartment. The indiscriminate adsorption to membranes by soluble enzymes during subcellular fractionation has been reported (5, 10). The problems which can arise when crude cell homogenates are overlaid directly on sucrose gradients has been discussed (5) and these workers advocated washing organelle pellets prior to sucrose density separation. Our data clearly indicated that soluble β-glucosidase activity can adhere to crude particulate fractions even after several washings (Table II). Whether crude homogenates or crude pellets are centrifuged in sucrose gradients, extreme care must be taken when ascribing a subcellular location for enzymes which may be associated with light density membranes. For example, the data in Figure 3 (38 h) could be erroneously interpreted to mean that MDH was associated with ER. Another case in point was a recent report which suggested that acid phosphatase may be associated with tonoplast vesicles (27); however, their data cannot distinguish between soluble enzyme activity and membrane-bound activity.

Results in Figure 2A suggested that short centrifugation time would be a viable way to minimize the movement of soluble enzymes into sucrose gradients. This approach could result in poor separation of various subcellular membranes because the membrane vesicles may not have reached their respective equilibrium density. The soluble enzyme problem may be circumvented but maximum separation of ER, Golgi, plasma membrane, etc. will not be achieved.

Our results showed that the resolution of the ER marker (Fig. 2A) after 2 h of centrifugation in a linear sucrose gradient was not as distinct as it was after 15 h of centrifugation. A similar but more pronounced effect was reported by Koehler *et al.* (7) for ER isolated from the abscission layer of kidney beans. In their report as well as our data (Figs. 2A and 3), the distribution of the ER marker was broad after short-term centrifugation; however, a sharp peak of marker activity occurred after 15 h with no further change in resolution up to 40 h. We observed similar results with the Golgi membrane marker (detergent-stimulated UDPase activity; see Ref. 18 for assay conditions) during the same centrifugation time course (data not shown).

Although Koehler *et al.* (7) did not comment on their observation, we interpret these results to indicate that not all microsomal vesicles are at equilibrium after 2 h of centrifugation in linear sucrose gradients. Three recent reports have examined the centrifugation conditions necessary for membranes to reach equilibrium density. In one report (2), they claimed no change in marker enzyme distribution occurred between 4 and 8 h of centrifugation (38 ml gradients; 81,000*g*) and concluded that membranes were isopycnic after 4 h. Their data can also be interpreted to indicate that equilibrium conditions were not achieved since the mitochondrial marker exhibited a broad peak even after 8 h of centrifugation. Another report (8) showed that mitochondria and plasma membranes did not reach equilibrium after short-term centrifugation (1.5 h). In a third report (21), the same rotor, gradient size, and centrifugation conditions as in Koehler *et al.* were used except larger fractions (3.6 *versus* 1.2 ml) were collected. No changes in marker enzyme distribution were observed after 3 h; however, the fine resolution of any marker enzyme in sucrose gradients can be diminished by large fraction size as well as nonequilibrium centrifugation conditions. We showed that centrifugation for 2 h was insufficient, 15 h was sufficient, but the minimum time necessary for membrane vesicles to reach equilibrium has not been clearly resolved.

The centrifugation time used in most sucrose density gradient separations has been based on convenience. Fifteen hours provides a convenient centrifugation time for an afternoon preparation followed by centrifugation overnight. Others (2, 3, 7, 10, 21) frequently centrifuge linear sucrose gradients for 2 to 4 h since tissue homogenization and centrifugation procedures can be performed during the same day. We recommend overnight centrifugation to insure that membranes are isopycnic, but appropriate controls must be used before assigning enzymic activity to a particular member of the endomembrane system.

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