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Localization and Substrate Specificity of Glycosidases in Vacuoles of *Nicotiana rustica*

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ABSTRACT

Vacuoles isolated from *Nicotiana rustica* var brasilia have been shown to contain significant levels of glycosidase activity when assayed using *p*-nitrophenyl-glycosides as substrates. The substrate specificity for the glycosidases in the vacuolar fraction closely paralleled that found in the protoplasts, and the leaf tissue from which the vacuoles were isolated. The substrate specificity of the vacuolar enzyme(s) was different from glycosidic activity found in the commercial digestive enzyme preparations used to isolate the protoplasts from leaf tissue. It was demonstrated that 70 to 90% of the glycosidases that were found in the protoplasts appeared to be localized within the vacuole, when the *p*-nitrophenyl substrates α- and β-;D-galactose, β-D-glucose, and α-D-mannose were used. Neither the vacuolar nor the protoplast enzymes were active towards the naturally occurring phenolic glycoside, rutin. α-Mannosidase appears to be a valuable marker enzyme for vacuoles isolated from mesophyll leaf cells of tobacco.

Marker enzymes have been used to examine the purity of cell fractions to determine the degree of contamination by unwanted membranes, organelles, or cells, and to determine whether isolated membranes of organelles are functional following isolation procedures (12, 14). In plant tissues specific marker enzymes have been associated with nearly every organelle within the cell with a few notable exceptions. One of the more important exceptions has been the vacuole. Vacuoles which may occupy as much as 80 to 90% of the volume of the cell have only been examined biochemically since mature leaf vacuoles were first isolated on a large scale in 1975 (18).

Vacuoles, isolated from *Nicotiana rustica*, have previously been shown to be hydrolytic in nature (10). This study was undertaken to examine the localization and enzyme specificity of glycosidases in *Nicotiana rustica.* These glycosidases, if present in the vacuole, may function as potential marker enzymes for this organelle in tobacco leaf tissue.

MATERIALS AND METHODS

Plant Material. *Nicotiana rustica* var brasilia was grown under greenhouse conditions and harvested 70 to 90 d after germination. The plants were transferred 1 week prior to harvest to an environmental growth chamber that was maintained on a 12-h day length at 25°C.

Preparation of Protoplasts. In a typical experiment, two to three leaves which weighed approximately 2 g each were painted with 150-mesh carborundum using a 1-cm wide camel hair brush to facilitate infiltration of the digestive enzymes. This procedure has been shown to significantly decrease the time required for the digestion of the cell wall (11). The excess carborundum was rinsed from the leaf tissue by successive rinses in distilled H_2O. The abraded leaf was digested in a mixture containing 1% (w/v) Macerase[1] (300 units/g) and 2% (w/v) Cellulysin (10,000 units/g) in 50 mM Mes-NaOH buffer (pH 5.7) with 0.6 M mannitol. Protoplasts were released within 4 h of incubation at 30°C with a gentle shaking of 40 oscillations/min. The preparation was filtered through two layers of cheesecloth and centrifuged at 100*g* for 1 min in a swinging bucket rotor to pellet the protoplasts. The supernatant was discarded and the pellet washed with 0.55 M Mannitol in 50 mM Tris-HCl buffer (pH 7.5). The protoplasts were repelleted at 100*g* for 1 min and the washing procedure repeated twice. The final pellet was resuspended in a known volume of the washing solution and aliquot taken for protoplast quantitation and the biochemical assays.

Isolation of Vacuoles. Vacuoles were released from the isolated protoplasts by the dilution of the mannitol to a final concentration of 0.2 M with 25 mM Tris-HCl buffer (pH 7.5). In the lowered osmotic condition, the protoplast membrane ruptured with three to four gentle pipetting strokes within a 1-min interval. The suspension was loaded onto the top of a discontinuous gradient composed of 1.5, 8.0, 12.5, and 20.0% (w/w) Ficoll with 0.5 M mannitol and 25 mM Tris-HCl buffer (pH 7.5). The gradient was centrifuged at 90,000*g* for 2 h at 4°C using a Sorvall OTD-50 ultracentrifuge equipped with a AH-627 swinging bucket rotor. At these centrifugation rates, the intact vacuoles banded at the 1.5/8.0% Ficoll interface and unbroken protoplasts banded at the 12.5/20% Ficoll interface. The intermediate 8.0/12.5% interface contained a mixture of both vacuoles and protoplasts. The vacuoles were recovered from the 1.5/8.0% interface using a Buchler Auto-Densi IIC Flow fractionator.

Protoplasts and vacuoles were quantitated by counting on a A/O Spencer Bright line hemacytometer using a Nikon inverted microscope. In preparation for the glycosidase assays, the protoplast and vacuolar samples were subjected to a freeze thaw regime to insure complete rupturing of all cells and organelles.

Enzyme Assays. Substrate specificity was measured using *p*-nitrophenyl-substituted glycosides as substrates. The reaction mixture consisted of 500 μl of enzyme solution containing 50 to 500 μg protein depending on the source, 500 μl of 0.3 M sodium citrate-citric acid buffer (pH 5.5), and 500 μl of 15 mM *p*-nitrophenyl glycoside substituted with the appropriate carbohydrate moiety. The reaction was initiated with the addition of the enzyme solution and multiple reaction mixtures were incubated in a reciprocal water bath shaker at 37°C up to 15-min intervals. With all substrates tested, the reaction rates were linear for the

[1] Mention of a trade name does not constitute an approval by the United States Department of Agriculture to the exclusion of other products that may also be suitable.

first 10 min and all rates of reaction were measured during this time interval. The assay was terminated with the addition of 1.5 ml of 0.5 M sodium bicarbonate-carbonate buffer (pH 10.5) and the change in absorbance recorded at 420 nm on Bausch and Lomb spectronic 710 spectrophotometer. The change in A at 420 nm was expressed as nmol of *p*-nitrophenyl standard solutions incubated under identical conditions.

In the reaction mixture for the rutin assay, the *p*-nitrophenyl substrate was substituted with 15 mM rutin. An aliquot of the reaction mixture was chromatographed by HPLC to separate rutin from the aglycone quercetin and the quercetin-3-glycoside quercitrin. The HPLC system consisted of an isocratic mobil phase of 40% methanol 4% ethyl acetate, and 2% acetic acid using a Water's μBondapak C-18 column. At a flowrate of 0.6 ml/min, all three flavonoids detected at 360 nm are clearly resolved in a 15-min run.

The pH optimum of the glycosidase activity was determined using leaf tissue which was ground in a Ten Broeck homogenizer of the buffer of the appropriate pH range. The filtrate was centrifuged at 10,000*g* for 10 min and the supernatant used as the enzyme source. The reaction buffer was substituted with either 0.3 M sodium citrate (pH range 4.5–6.5) or 0.3 M Tris-HCl buffer (pH 7.0–8.0) to cover the entire active range of the enzyme.

Samples which had been heat denatured in a boiling water bath for 10 min were used as controls in each series of experiments.

Protein Assay. Protein was assayed by the Coomassie blue dye binding technique of Bradford (3) using BSA as a protein standard.

Reagents and Chemicals. The digestive enzymes Macerase and Cellulysin were obtained from Calbiochem-Behring; *p*-nitrophenyl glycosides, *p*-nitrophenol, and Ficoll were obtained from Sigma Chemical Company; and all other reagent grade chemicals were obtained from Fisher Scientific.

RESULTS

Leaf tissue from *N. rustica* has been examined for the presence of *p*-nitrophenyl glycosidase activity in 3-month-old greenhouse-grown plants. Initial experiments with homogenized leaf tissue indicated that the optimal substrate tested was the β-D-glucosidase which had a pH optimum of 5.5. To determine the stability of this enzyme during the protoplast isolation procedure, β-D-glucosidase activity was monitored over a 14-d period. Protoplasts isolated as usual were stored in buffer at −20°C. Aliquots taken at periodic intervals clearly showed that under these conditions the β-D-glucosidase activity was stable for at least 2 weeks. In addition, after 2 d of storage at room temperature, the enzyme retained at least 40% of the glycosidic activity which was present in the freshly prepared protoplast preparation.

To examine the substrate specificity of the enzymes found in the protoplasts, twelve different *p*-nitrophenyl glycosides were added to the reaction mixture as substrates at a final concentration of 5 mM. Table I summarizes the results of these experiments. Little or no glycosidase activity was recorded using fucose, lactose, maltose, and β-D-mannose glycosides. In contrast, significant activity was recovered when α-D- or β-D-galactose, β-D-glucose, or α-D-mannose glycosides were used as substrates. Small but significant amounts of activity were recorded using the *p*-nitrophenyl α-D-glucose, and the α-L-arabinose substrates. The α-L-arabinose and α-L-fucose were the only L-sugars which were effective substrates for the enzyme(s) endogenous to the tobacco protoplasts. It is obvious that both α and β sugars serve as acceptable substrates for the enzymes present in the protoplast and the digestive enzyme mixture.

Commercial digestive enzymes which are used to hydrolyze the cell wall of the leaf tissue are known to contain hydrolytic enzymes which have considerable activity towards a number of diverse substrates (10). It was necessary to closely examine the characteristics of the digestive enzymes to determine whether the glycosidic activity which was recovered in protoplast samples was due to contamination by the digestive enzyme solutions during the isolation procedure or due to endogenous enzyme(s) present in the tobacco leaf tissue.

Table I. *Substrate Specificity for Glycosidase Activity in Isolated Protoplasts and Commercial Digestive Enzymes*

Protoplasts were obtained by incubating tobacco leaf tissue in preparations of the commercial digestive enzymes composed of 2% Cellulysin and 1% Macerase. Using *p*-nitrophenyl-substituted glycosides as substrates, the enzymic activity of both protoplast preparations and digestive enzyme solutions are expressed as the change in nmol/min·mg protein based on *p*-nitrophenol as a standard.

Substrates	Protoplasts	Digestive Enzymes
α-L-Arabinoside	3.5	326.5
α-L-Fucoside	5.0	27.8
β-D-Fucoside	0.1	19.6
β-L-Fucoside	0	5.4
α-D-Galactoside	20.1	524.9
β-D-Galactoside	14.0	22.5
α-D-Glucoside	6.6	94.9
β-D-Glucoside	55.6	2219.3
β-D-Lactoside	1.5	200.9
α-D-Maltoside	0	19.8
α-D-Mannoside	29.4	2.1
β-D-Mannoside	0	0

Table II. *Distribution of Glycosidase Activity in Protoplasts and Vacuoles*

Vacuoles were prepared from isolated protoplasts by gentle lysis and purified by ultracentrifugation on a discontinuous Ficoll gradient. Enzyme activity is defined as the change in nmol of *p*-nitrophenol/min·10^6 cells or organelles.

Substrate	Protoplasts	Vacuoles	% of Activity in Vacuoles
α-D-Galactoside	46.3 (2.4)[a]	31.4 (1.2)	67.8
β-D-Galactoside	32.2 (4.1)	25.4 (3.8)	78.9
β-D-Glucoside	61.8 (3.8)	55.0 (5.8)	89.0
α-D-Mannoside	77.1 (4.3)	69.3 (3.1)	89.9

[a] SE in parentheses; $n = 5$.

Table I lists the specific activity of the digestive enzyme preparations towards the same *p*-nitrophenyl glycosides used for the protoplast samples. It is clear that the digestive enzymes have glycosidic activity for several of the substrates tested. It is of particular interest to note that the patterns of glycosidic activity found in the digestive enzyme preparation was different than that found in the isolated protoplasts. For example, the digestive enzyme sample had low specific activity for the α-D-mannose glycoside which was a very acceptable substrate in the protoplast samples. The digestive enzymes are also active towards β-D-lactose, α-L-arabinose, α-D-maltose, and the β-(D and L)-fucose glycosides which were poor substrates for the protoplast preparations. The differential pattern of the substrate specificities between the protoplast and digestive enzyme samples indicates that the exogenously added digestive enzymes were not the source of the protoplast glycosidases although it does not rule out the possibility of selected absorption of some of the substrates tested.

The localization of the glycosidic activity in the tobacco leaf tissue was investigated by isolating mature vacuoles from the protoplast preparations and comparing the activity recovered in the protoplasts with that found in the vacuoles. The four *p*-nitrophenyl substrates which showed the highest affinity for enzymes from the protoplast preparations were chosen as the

Table III. *Glycosidase Activity in Whole Leaf Tissue, Protoplast, Vacuoles, and Digestive Enzyme Preparations*

Protoplasts and vacuoles were isolated using the digestive enzymes Macerase and Cellulysin in 0.5 M Mannitol and Mes-NaOH (pH 5.7). Using *p*-nitrophenyl-substituted glycosides as substrates, the enzymic activity is expressed as the change in nmol/min·mg protein based on a *p*-nitrophenol standard.

Substrates	Whole Leaf	Protoplasts	Vacuoles	Digestive Enzymes
α-D-Galactoside	34.3 (2.8)[a]	20.1 (4.2)	97.8 (9.7)	524.9 (15.1)
β-D-Galactoside	17.8 (3.4)	14.0 (3.7)	57.0 (7.4)	22.5 (3.4)
α-D-Glucoside	0.6 (0.4)	6.6 (2.6)	16.8 (3.2)	94.9 (4.5)
β-D-Glucoside	3.7 (1.7)	55.6 (6.2)	165.6 (9.5)	2219.3 (36.7)
β-D-Lactoside	0.4 (0.5)	1.5 (0.9)	3.7 (1.3)	200.9 (7.2)
α-D-Mannose	37.4 (2.6)	29.4 (4.1)	104.4 (9.9)	2.1 (1.7)

[a] SE in parentheses; $n = 4$.

appropriate substrates likely to serve as effective substrates in the vacuole. Table II shows the amount of activity for each of these substrates in both the protoplast and vacuole fractions based on the number of cells or organelles in each sample. It can be seen that 70 to 90% of the enzymic activity associated with the protoplasts is retained in the vacuole for these four substrates. It should be noted that glycosidic activity which may be exterior to the plasma membrane would not be considered in these experiments. Thus, any glycosidases in the cell wall, if present, would be lost to the medium during the protoplast isolation procedure. In Table III, any glycosidases in the cell wall would be included in the enzymic activity recovered from the whole tissue.

The vacuoles recovered in these experiments were large mature vacuoles as described previously (10), and all calculations were based on the concept of one vacuole per mature leaf protoplast. In Table II, no statistical differences were observed in the percentage of activity recovered in the vacuoles at the 95% probability level for the substrates listed.

The data from Tables I and II indicated differential selective substrate specificity in the digestive enzyme preparations *versus* the protoplast and vacuole fractions. Six substrates were selected for a comparative analysis of the digestive enzymes, whole leaf tissue, protoplasts, and vacuoles. Table III shows the results of these experiments in which distinct differences were obtained in the substrate specificity for the four enzyme sources tested. The substrate specificity for the whole leaf, protoplast, and vacuole samples showed a similar pattern with increasing specific activities towards the vacuole. The pattern of substrate utilization with the digestive enzyme preparation was quite different from the other enzyme sources. For example, when *p*-nitrophenyl β-D-lactose was used as a substrate, there was considerable activity recorded in the digestive enzyme fraction; however, the leaf, protoplast, and vacuole fractions contained less than 2% of the specific activity of the digestive enzyme sample. In contrast, the digestive enzyme sample had less than 5% of the activity of the vacuole sample when α-D-mannose was used as a substrate. Using the β-D-glucose substrate, the specific activity of the protoplast sample was considerably more than that found in the whole leaf tissue. It is possible that the increase in specific activity could have arisen from the selected absorption of this enzyme from the digestive enzymes. It is clear, however, that the glycosidic activity towards α-D-mannose, and (α and β)-D-galactose were not increased during the isolation of the protoplast. This shift in substrate specificity indicates that the activity for several of the substrates recorded in the protoplast and vacuolar preparations was not a result of contamination by exogenously added crude digestive enzymes.

DISCUSSION

In recent years, numerous reports have described the isolation of vacuoles from yeast and fungal cells as well as from higher plants. There have been several studies which have begun systematic biochemical characterizations of the tonoplast and the vacuolar fluid (6, 9, 10, 12, 16). These investigations have revealed a number of enzymes which have been partially localized either within the vacuole or on the vacuolar membrane, although the search for a marker enzyme which is specific to the vacuole has eluded researchers to date.

In yeast cells, α-mannosidase activity has been suggested as a marker enzyme for the tonoplast (17). This enzyme was reported to be bound tightly to the vacuolar membrane while β-glucosidase activity, which is also present in the same cells, is only partially localized in the vacuole. There have been numerous attempts to confirm this enzyme as a marker system for vacuoles isolated from higher plants, however, these attempts have often resulted in conflicting results. Boller and Kende (2) have indicated that α-mannosidase and β-fructosidase activities were both localized within the vacuoles of cell suspension cultures from tobacco. Although they examined only a few different *p*-nitrophenyl substrates, they concluded that the α-mannosidase represented a good vacuole marker enzyme. Butcher *et al.* (5) have found that vacuoles from *Hippeastrum* do not contain selective glycosidases for several common *p*-nitrophenyl glycosides, although the glycosidic activity is present in the whole cells of the tissue. Using carrot cell suspension cultures, Asamizu *et al.* (1) have investigated the subcellular localization of enzymes which were active towards four *p*-nitrophenyl glycosides. They found that β-galactosidase and β-glucosidase activity is distributed evenly between the cell wall and the interior of the cell. In the same study, they showed that α-galactosidase and α-glucosidase activity was confined to the cell's interior and was not present in the cell wall. They did not further characterize the subcellular distribution of the glycosidic activity; however, the vacuolar system was implicated. Finally, Pohl (9) has also found α-glucosidase intimately associated with the vacuole with β-glucosidase and β-galactosidase only minimally localized in the vacuoles of maize.

Our investigations have shown that the vacuoles from *N. rustica* contain significant levels of glycosidic activity (Table II). We found very little activity either in the protoplasts or in the vacuole preparations apart from these four substrates even though a variety of other *p*-nitrophenyl glycosides were tested (Table I). Of the glycosidic activity which was associated with the protoplasts, at least 70 to 90% of it was recovered in the vacuolar fractions based on an association of one vacuole per protoplast. In support of the suggestion that α-mannosidase may be a marker enzyme for the vacuole (17) we found as much as 90% of the α-mannosidase activity was localized in the vacuole.

Thayer and Conn (15) have suggested that β-glucosidase activity in *Sorghum* leaf tissue is associated with the chloroplasts and not the vacuoles. They and others (8) argue that the artifical *p*-nitrophenyl substrates commonly used in studies of glycosidase enzymes may be yielding information that is entirely different from that which would be obtained using endogenous substrates. In support of this concept, Burmeister and Hösel (4) have shown distinctly different localization patterns for two different β-glucosidases using different endogenous substrates in chick pea.

To address this question, we attempted to use the phenolic glucoside rutin as an endogenous substrate to measure potential glycosidic activity in tobacco protoplasts and vacuole preparations. This phenolic which is the rhamnoglucoside of quercetin, occurs naturally in these cells as one of the major phenolics in tobacco. Our results indicated that rutin was not suitable as a substrate using either whole leaf, protoplast, or vacuolar preparations when assayed by techniques listed in "Materials and

Methods," although there was considerable glycosidic activity in the preparations towards the *p*-nitrophenyl substrates. These results seem reasonable in light of the fact that rutin is normally thought to be stored in the vacuole of the same cells which contained considerable levels of glycosidases. It appears that the identity of the aglycone is an important consideration when comparing data from different reports. Schmitt and Sandermann (13) and others (7, 12) have reported several different types of glycosidic conjugates stored in the vacuoles of higher plants. It would be inconsistent to expect an active glycosidic enzyme and a suitable substrate to be localized within the same organelle without some kind of functional or temporal separation.

The use of protoplast digestion techniques in the isolation of vacuoles from mature leaf tissue necessitates the use of exogenously added proteolytic enzymes generally available from commercial sources. These digestive enzymes are able to completely remove the cell wall from the intact protoplasts in a 3- to 4-h period; however, the enzyme preparations are by no means purified extracts. Any examination on the glycosidic nature of the vacuoles prepared from protoplasts isolated under these conditions must evaluate the possibility that some of the glycosidic activity associated with the vacuole preparations is coming from the exogenously added digestive enzymes. We selected six *p*-nitrophenyl glycosides to examine in close detail the glycosidases in leaf tissue of tobacco as well as the digestive enzymes. The data in Table III clearly indicate that the α-mannosidase activity associated with the whole leaf, protoplast, and vacuolar preparation is virtually absent in the digestive enzyme preparations. Conversely, β-lactosidase activity which is quite strong in the digestive enzyme preparations is negligible in the leaf, protoplast, and vacuolar samples.

In summary, we have shown that vacuoles isolated from tobacco protoplasts contain substantial levels of glycosidases active towards several *p*-nitrophenyl glycosides. Although some of the β-glucosidase activity which is recovered in the vacuoles may have been due to selective absorption of enzyme from the digestive media, it is clear that activity for several of the other substrates was not due to contamination by the enzymes used to digest protoplasts from the mesophyll leaf tissue. It appears that the enzyme which catalyzes the hydrolysis of the *p*-nitrophenyl substrate, α-D-mannose, serves as an important marker enzyme for the vacuole in this tissue.

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