

Short Communication

Vacuolar Localization of Ethylene-Induced Chitinase in Bean Leaves¹

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ABSTRACT

The localization of ethylene-induced endochitinase was studied in bean (*Phaseolus vulgaris* L. cv Saxa) leaves. The specific activity of chitinase in mesophyll protoplasts isolated from the leaves was as high as in tissue homogenates, indicating that most of the enzyme was located intracellularly. Vacuoles isolated and purified from the protoplasts were found to contain most of the intracellular chitinase activity.

Ethylene induces a highly active endochitinase in bean leaves and in many other plants (1, 6). The enzyme increases 30-fold in activity and constitutes up to 1% of the total leaf protein after an ethylene treatment of 24 h (6). Chitinase has no apparent function in the primary metabolism of the plant. Since it readily attacks chitin in fungal cell walls and also acts as a lysozyme against bacterial cell walls (6), it may play a role in the plant's defense against pathogens (3, 12). The present communication deals with the localization of ethylene-induced endochitinase.

MATERIALS AND METHODS

Plant Material. Bean plants (*Phaseolus vulgaris* L. cv Saxa) obtained from Samen Mauser, Zürich, Switzerland, were grown as described previously (6). Trays of seedlings (12-14 d old) were incubated in a tightly closed plastic chamber (100 L) in the presence of 10 nl ml⁻¹ ethylene for 12 or 24 h, or used without pretreatment.

Preparation of Protoplasts. Primary leaves of appropriately treated bean plants were immersed in 0.5 M mannitol, cut into segments (1-2 mm width) with a sharp razor blade, and washed in 0.5 M mannitol. Portions of 3 g of tissue slices were immersed in 15 ml of an enzyme mixture containing 2% (w/v) cellulysin (Calbiochem) and 0.4% (w/v) macerace (Calbiochem) in 0.5 M mannitol, adjusted to pH 5.5. The preparation was gently shaken (40 strokes min⁻¹) at 30°C for 4 h. Control samples of the tissue were incubated under the same conditions in 0.5 M mannitol without enzymes.

Protoplasts were separated from undigested tissue pieces by filtration through a 100- μ m mesh nylon cloth and collected by centrifugation (5 min, 250g). For purification, the protoplasts were resuspended in 0.5 M sucrose containing 10% (w/v) ficoll, and overlaid with 0.5 M sucrose containing 3% (w/v) ficoll and then with 0.5 M mannitol. The step gradient thus formed was centrifuged at 1000g for 5 min. Clean protoplasts floated to

the interphase between the layers of 0.5 M sucrose containing 3% ficoll and of 0.5 M mannitol. The protoplasts were harvested with a Pasteur pipette, diluted with 0.5 M mannitol, and collected by centrifugation (250g for 5 min). All solutions used for protoplast purification were adjusted to pH 6.5 with 10 mM K-phosphate.

Preparation of Vacuoles. Vacuoles were prepared by lysis in a gradient containing DEAE-dextran, essentially as described (7). Isotonic sucrose was used instead of ficoll to obtain density gradients. The gradients were prepared in 12-ml centrifugation tubes and contained 3 ml of 0.5 M sucrose at the bottom. This was overlaid with 4 ml of a 3:1 (v/v) mixture of 0.5 M mannitol and 0.5 M sucrose, containing 1 mg ml⁻¹ dextran sulfate adjusted to pH 8, followed by 2 ml of a 5:1 (v/v) mixture of 0.5 M mannitol and 0.5 M sucrose, containing 1.4 mg ml⁻¹ DEAE-dextran adjusted to pH 6.5. The pH of the solutions was adjusted with 10 mM K-phosphate. For lysis of protoplasts, 1-ml portions of the protoplasts (3×10^6 ml⁻¹) were pipetted onto the freshly prepared gradients and centrifuged immediately at 2,000g for 30 min. The vacuoles liberated in the gradient were found at the interphase between dextran sulfate and the sucrose layer at the bottom of the tube; they were carefully harvested with a Pasteur pipette. For enzyme blanks, gradients were centrifuged without protoplasts, and the interphase corresponding to the vacuolar fraction was harvested.

The initial preparation of vacuoles from the gradients was further purified by flotation in a density gradient. The preparation was gently mixed with 2 volumes 0.5 M sucrose, pH 8, and then overlaid with a 4:1 (v/v) mixture of 0.5 M mannitol and 0.5 M sucrose, pH 8, and further with 0.5 M mannitol, pH 8. The step gradient thus formed was centrifuged at 2,000g for 10 min. Clean, purified vacuoles floated to the interphase below the mannitol layer at the top.

Analysis of the Preparations. Tissue slices incubated without cell-wall digesting enzymes were homogenized in 0.5 M mannitol containing 100 mM K-phosphate, pH 8 (3 ml g⁻¹ fresh weight) with a little sand using a mortar and pestle. The homogenate was centrifuged at 1,000g for 5 min to eliminate sand and tissue debris; then, the extract was diluted 10-fold with unbuffered 0.5 M mannitol. Preparations of protoplasts and vacuoles were used directly for enzyme assays; they were osmotically lysed in the assay mixtures during preincubation.

Chitinase was determined by the radiometric assay described (6). α -Mannosidase, acid phosphatase, and glucose-P isomerase were assayed as described (5). Published methods were used for the determination of Chl (2) and of protein (8). Enzyme blanks contained 0.5 M mannitol or the interphase corresponding to the vacuolar fraction from gradients run without protoplasts. The latter preparation was also added to complete enzyme assays with tissue extracts. It did not interfere with any of the enzyme

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assays. However, because of its content of dextran sulfate, it interfered with the protein assay. Therefore, the protein content of the vacuolar preparation could not be adequately determined.

RESULTS

Localization of Chitinase in Protoplasts. As expected from previous work (6), treatment with ethylene strongly increased the chitinase activity in bean leaf tissue (Table I); the activity of α -mannosidase and of glucose-P isomerase, used for comparison, remained constant. Protoplasts were obtained with similar yields from ethylene-treated and from control tissue, typically about 10^7 protoplasts/g fresh weight of tissue, corresponding to about 20% of the total amount of cells in the tissue. This is in contrast to a recent report on oat leaves where ethylene was found to cause an increased resistance to cellulolytic digestion of mesophyll cell walls (10).

The specific activity of chitinase in the protoplasts was slightly higher than in the corresponding tissue, indicating that most or all of the enzyme found in tissue extracts was located intracellularly (Table I). Similar results were obtained for α -mannosidase, used as a vacuolar marker enzyme (4, 5), and for glucose-P isomerase, an enzyme located in the cytosol and in chloroplasts (see Ref. 5).

The enzyme mixture used for protoplast preparation was found to contain no glucose-P isomerase and only small amounts of chitinase and of α -mannosidase activities; the problem of a contamination by fungal hydrolases (5) was therefore insignificant.

Localization of Chitinase in Vacuoles. Vacuoles were obtained from the protoplasts by lysis in a gradient containing the polycation DEAE-dextran in the top layer, followed by the polyanion dextran sulfate in the layer below (7). In preliminary experiments, the amount and concentration of these two layers was varied in order to optimize the yield of vacuoles. Under the conditions selected, the initial preparation of vacuoles isolated from a gradient run with 3×10^6 protoplasts contained 0.6 to 0.75×10^6 vacuoles; thus, the yield was 20 to 25%. A contamination by protoplasts and chloroplast aggregates was found in this fraction. The contamination could be eliminated to a large extent by flotation of the vacuoles in a second gradient. However, the yield of this purified fraction was quite low; only about 15% of the vacuoles from the initial preparation were covered.

In preliminary experiments, it was found that 10^6 vacuoles contained approximately the same activity of α -mannosidase as 10^6 protoplasts. Therefore, this enzyme was assumed to be localized exclusive in the vacuoles and was used as a marker for the vacuolar contents. Hence, counting of vacuoles, which often

yields variable results (5), could be avoided.

On the basis of α -mannosidase activity, chitinase was found to be located exclusively in the vacuoles (Table II). Similarly, the acid phosphatase present in protoplasts was found to be located in vacuoles. Two markers for the cytosol and for the chloroplasts were almost completely absent from the purified vacuoles, indicating a high purity of the vacuoles. Other markers were not assayed; earlier work on the isolation of vacuoles by this technique had shown that most of the contamination was due to protoplasts that had survived lysis, so that any extravacuolar constituent could be taken as a representative marker for contaminants (7). Glucose-P isomerase was selected because of its high activity and stability in homogenates.

DISCUSSION

Earlier work on the vacuolar enzyme composition has shown that the plant vacuole is analogous to the animal lysosome (4, 5). Our results show now that chitinase is among the hydrolases located in the central vacuole of plant cells. This enzyme has a high potential to attack and degrade fungal and bacterial cell walls and, therefore, may play a role in defense against pathogens (6, 12). Thus, our finding supports the notion that the vacuolar contents of higher plants have a protective function against parasites and pathogens (4, 5, 13).

The question arises as to how a vacuolar enzyme can come into contact with attacking fungi or bacteria, organisms which invade plant tissue from the extracellular space. One possibility is the breakage of vacuoles and the discharge of their contents. This may occur in the hypersensitive reaction of the plant where a small group of plant cells around an invading pathogen die and release their contents.

An alternative possibility would be a more specific transport of a vacuolar enzyme to the extracellular space. No precedent for such a process is known in higher plants. However, in yeast, chitinase was found to be located in about equal proportions in vacuoles and in the extracellular space, and experiments with protoplasts indicated that chitinase, initially located in the vacuoles, was later secreted (9). Note that, in yeast, chitinase probably was a primary function in the fission of septa which leads to cell division (9).

In ethylene-treated plants, the enzyme chitinase accumulated

Table II. *Activities of Chitinase and of Vacuolar and Extravacuolar Markers in Preparations of Tissue, Protoplasts, and Vacuoles from Bean Leaves Treated for 24 Hours with Ethylene*

For tissue and protoplasts, specific activities are presented. The results for the vacuole preparations are given in terms of relative activities and are compared to the contents of the protoplasts on the basis of α -mannosidase. The activity of each enzyme and the chlorophyll content were expressed in units per unit α -mannosidase, and the amount found in protoplasts was taken to equal 100%.

Enzyme	Specific Activity		Relative Activity in Vacuole Preparations	
	Tissue	Protoplasts	Initial	Purified
	<i>nkat mg⁻¹ protein</i>		<i>% of protoplasts</i>	
α -Mannosidase	0.12	0.13	100	100
Phosphatase	11.9	7.9	127	105
Chitinase	9.3	11.5	113	106
Glucose-P isomerase	8.8	9.0	20	3
	<i>μg mg⁻¹ protein</i>			
Chlorophyll	ND ^a	260	7	2

^a Not determined.

Table I. *Specific Activities of Chitinase and of Two Other Enzymes in Tissue and Protoplasts from Untreated and Ethylene-Treated Bean Leaves*

Bean plants were exposed to 10 nl ml⁻¹ ethylene for 0, 12, or 24 h. Primary leaves were harvested, cut into segments, and incubated either in 0.5 M mannitol or in a cell-wall-digesting enzyme mixture in 0.5 M mannitol for 4 h.

Plant Material	Ethylene Treatment	Chitinase	α -Mannosidase	Glucose-P Isomerase
	<i>h</i>	<i>nkat mg⁻¹ protein</i>		
Tissue	9	0.64	0.15	8.5
	12	7.9	0.14	9.6
	24	10.3	0.13	8.3
Protoplasts	0	0.84	0.17	9.8
	12	10.0	0.14	9.1
	24	11.3	0.16	8.2

to a level of about 1% of the total protein of the tissue (6). Since it is easily and reproducibly induced by ethylene and accumulates to such high levels, it should be an excellent model to study its transport from the place of protein synthesis to the mature plant vacuole. In this regard, it is interesting that chitinase synthesized by translation of mRNA *in vitro* has a slightly greater mol wt than the enzyme isolated from the plant (Vögeli, unpublished). This may indicate processing of chitinase during intracellular transport. A similar result has been obtained for proteinase inhibitors I and II in tomato leaves (11), two proteins that accumulate upon mechanical stress and are also located in the vacuole (13).

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