Immunological Identification of Proteinase Inhibitors I and II in Isolated Tomato Leaf Vacuoles

MARY WALKER-SIMMONS and CLARENCE A. RYAN
Department of Agricultural Chemistry and Program in Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164

ABSTRACT
Proteinase inhibitor I has been identified and quantified in isolated vacuoles from tomato (Lycopersicon esculentum) leaves induced to accumulate inhibitors either by wounding or by supplying excised leaves with the wound hormone, proteinase inhibitor-inducing factor. Proteinase inhibitor II was also identified in the vacuoles but not quantified. Control vacuoles were prepared from unwounded plants that did not contain inhibitors. Vacuole to leaf cell ratios of inhibitors, chlorophyll, and several vacuolar and cytoplasmic enzymes were determined. The inhibitors were found almost entirely in the vacuoles. Acid phosphatase was located in control leaf vacuoles, but was found in both vacuoles and cytoplasm in induced leaves. Carboxypeptidase, induced by wounding, was found distributed between the vacuoles and cytoplasm of induced leaves. Low vacuole to cell ratios of three cytoplasmic markers, triosephosphate isomerase, catalase, and chlorophyll, indicated that the isolated vacuoles were relatively free of intact protoplasts and cell debris.

Two proteinase inhibitors, called inhibitor I and inhibitor II, accumulate in large quantities in tomato leaves that have been physically wounded, attacked by chewing insects, or detached and supplied with the wound hormone PIIF (7-9, 13). The two inhibitors strongly inhibit animal and microorganism proteases having specificities similar to chymotrypsin and trypsin but do not inhibit any known plant proteinases. The wound-induced accumulation of proteinase inhibitors is considered to be a response that may help protect the plants from insect or microorganism attack by inactivating the digestive enzymes of the invading pest (21).

Shumway et al. (15, 16) observed that protein bodies are present in tomato leaves which contain proteinase inhibitor I and later (17) indirectly identified the inhibitor in the protein bodies using immunological methods combined with electron microscope techniques. In this study we have isolated vacuoles from tomato leaves that were induced to accumulate inhibitors I and II and directly confirm that these two proteinase inhibitors are located in vacuoles of the leaf cells.

MATERIALS AND METHODS
Young (15-day-old) tomato plants (Lycopersicon esculentum, var. Bonnie Best) were grown in a growth chamber at 1,000 ft-c with a 17 hr day. Excised leaves were induced to accumulate proteinase inhibitors by supplying them with a crude PIIF solution (13) for 20 min. The leaves were rinsed and supplied with water under 1,000 ft-c for 72 hr at 31 C. Leaves from young intact plants were induced to accumulate inhibitors by crushing the center of the lower leaves between a rubber stopper (No. 000) and a flat file. This was repeated for 2 consecutive days to ensure maximal accumulation when vacuoles were prepared from the leaves.

Proteinase inhibitor I was quantified by the immunoradial diffusion method described by Ryan (12). Purified proteinase inhibitor I from tomato leaves (9) was used as the standard. Inhibitor II was identified by this method but was not quantified because no standard tomato leaf inhibitor II was available at the time of assay.

The number of cells/g in leaf tissue was estimated from measurements of the volume of epidermal, mesophyll, and palisade cells in a young tomato leaf representative of those utilized for this study. An intact leaf was measured and weighed, and the number of cells, based on volume of each type of cell, was calculated for the whole leaf. Approximately 2.5 × 10² cells/g of leaf tissue were calculated for the leaves used in this study.

Vacuole isolation was a modification of the method of Wagner and Siegelman (20) and is similar to that of Strobel and Hess (19). Tomato leaves were sterilized in 70% alcohol and shedded into longitudinal strips 1 to 2 mm wide. The strips were floated on a sterile solution of 1.5 % (w/v) Cellulysin (Calbiochem) and 0.25 M sucrose adjusted to pH 5.75 with NaOH. The sterile leaf mixture was incubated at room temperature with gentle shaking for about 20 hr in small Petri dishes (60 × 15 mm) each containing 12 ml of solution. After incubation, the shredded leaf solution was collected in a beaker, stirred (10 rpm) with an Omni-Mixer for 30 min, and filtered through glass wool and cheesecloth. The filtered solution was centrifuged in Babcock bottles (Kimble No. 1000) (14) in a one-speed Jasco centrifuge (model 58) for 5 min at approximately 500g. During centrifugation, a vacuole and protoplast mixture floated to the surface and collected in the neck of the Babcock bottle. After centrifugation the vacuole-enriched fraction was gently removed from the neck of the Babcock bottle with a Pasteur pipette. The vacuole-enriched fraction was resuspended in 0.195 M sucrose, which released more vacuoles from the protoplasts, and was centrifuged again. The resuspension and centrifugation process was repeated several times until as much as possible of the cell debris and protoplasts were removed from the isolated vacuoles. Vacuoles were counted on a depression slide with a light microscope.

Crude leaf extracts were prepared by macerating leaves with a mortar and pestle with sand and tris buffer (1 g leaves/3 ml of 0.2 M tris buffer [pH 8] containing 0.2 M sucrose and 1.5 mM EDTA). For carboxypeptidase and acid phosphatase assays, β-mercaptoethanol (0.1 % v/v) was added to the extracts. The leaf extracts were sonicated and centrifuged under nitrogen at
 Vacuole extracts were prepared by diluting the final vacuole fraction to a final concentration of 0.2 M tris (pH 8) containing 0.2 M sucrose and 1.5 mM EDTA. For carboxypeptidase and acid phosphatase assays, β-mercaptoethanol (0.1% v/v) was added to the extracts. The extracts were sonicated to lyse the vacuoles and appropriate assays were performed immediately.

Acid phosphatase (2) was assayed after preincubating 10 to 50 μl of sample in 1.2 ml sodium acetate buffer (pH 5) for 5 min at 35 C. The assay was initiated by adding 0.25 ml of 32 mM p-nitrophenyl phosphate followed by incubation at 35 C for 60 min. The reaction was terminated by adding 3 ml cold 1 M tris-phosphate (pH 8.5), and the change in absorbance at 420 nm was measured immediately.

Carboxypeptidase was assayed with the substrate BTPA (1). Fifty to 100 μl of sample was preincubitated with 1.35 ml of 0.1 M sodium phosphate buffer (pH 6.2) for 5 min at 35 C. Two-tenths ml of 3 mM BTPA in N,N-dimethyl formamide was added and the samples were incubated for 120 min at 35 C. The assay was terminated by adding 1 ml of 30% acetic acid and the change in absorbance at 405 nm was measured.

Triosephosphate isomerase was assayed with the substrate glyceraldehyde-3-P and with glycerophosphate dehydrogenase (Sigma) as the coupling enzyme according to Beisenherz (3). DL-Glyceraldehyde-3-P was prepared from the diethylacetal monobarium salt (Sigma). The assay mixture contained (final concentration) 0.75 mM racemic glyceraldehyde-3-P, 0.1 mM NADH, 0.3 M triethanolamine (pH 7.9), and 2 units of glycerophosphate dehydrogenase (sulfate-free). The reaction was initiated at room temperature by adding varying volumes (5-40 μl) of sample and after 2 min, measuring the time required for the absorbance to decrease by 0.1. The enzyme activity was inversely proportional to the measured time.

Catalase (22) was assayed by adding 10 to 50 μl of sample to 3 ml of H₂O₂ substrate (0.30 ml of 30% H₂O₂ in 50 ml of 0.05 M K-phosphate buffer (pH 7)), and measuring the change in absorbance at 240 nm at room temperature. Chl was estimated in 80% acetone extracts of tomato leaves according to the procedure of Strain et al. (18).

### RESULTS AND DISCUSSION

Using the method of Wagner and Siegelman (20), but substituting 0.25 M sucrose instead of mannitol for the Cellulysin digestion, we found that significant amounts of free vacuoles were released along with intact protoplasts. More vacuoles were released by resuspending the protoplast-vacuole mixture two to three times in a lower concentration of sucrose (0.195 M). The vacuolar preparations obtained in this manner appeared to be relatively free of intact protoplasts and cell debris. However, under our conditions of preparation it was not possible to obtain vacuoles completely free of adhering cell debris and the vacuole preparations from PIIF-induced leaves always contained slightly more contamination from protoplasts and cell debris than the vacuoles from noninduced leaves.

A photograph of a typical vacuolar preparation from noninduced leaves is shown in Figure 1. Many of the vacuoles are pink colored, probably due to anthocyanins, and all of the vacuoles take up neutral red dye from the surrounding solution, a property of vacuoles isolated from Hippeastrum and Tulipa (20).

It was essential to demonstrate that proteinase inhibitors from broken cells do not bind to the outside of the vacuoles in a nonspecific manner. An excess of proteinase inhibitor I was added to a crude vacuole preparation from control plants to determine if the inhibitor would bind during vacuole isolation. Shredded leaves (10 g) containing no inhibitors were incubated in Cel Mandal for 20 hr. At this time, 8 mg of purified tomato inhibitor (more than twice the amount of inhibitor that would ordinarily be present in 10 g of induced leaves) was added to the crude vacuole preparation, mixed for 30 min, and the vacuoles were isolated as described under "Materials and Methods." As shown in Table I, the first sucrose wash of the vacuole fraction removed all measurable amounts of proteinase inhibitor I, indicating that the proteinase inhibitors do not bind to isolated vacuoles.

The amount of Chl and four enzymes which are considered to be vacuolar and cytoplasmic markers were measured in vacuolar extracts and in extracts from intact leaves in order to determine their location with respect to inhibitor I. Vacuole to cell ratios for each marker were calculated based on the assumption that the leaves contained about 2.5 x 10⁷ cells/g and that each cell contains but one central vacuole. The vacuole to cell ratios are presented in Table II for control leaves and for both PIIF-induced excised leaves and leaves from intact plants whose lower leaves had been severely wounded to induce inhibitor accumulation.

No proteinase inhibitors were detectable in the control leaves. The vacuole to cell ratio for PIIF-induced leaves was 0.95, which indicates that virtually all of inhibitor I was located in the vacuoles. Inhibitor II was shown immunologically to be present in the induced vacuolar preparations, although not quantitatively. The qualitative results indicate that the majority of this inhibitor is also located in the vacuoles. Acid phosphatase, an enzyme reported to be associated with vacuoles in many plants (10), was found in vacuoles of normal, uninduced leaves, with a vacuole to cell ratio of 0.89. However, in PIIF-induced leaves

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**Fig. 1.** Light micrograph of a vacuolar preparation from leaves of young tomato plants. The largest vacuoles are approximately 60 μm in diameter and the smallest about 20 μm.

**Table I.** Proteinase Inhibitor Binding Control Experiment

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No. of Vacuoles Recovered in Vacuoles (μg)</th>
</tr>
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<tbody>
<tr>
<td>10g shredded non-induced leaves incubated in cellulysin for 20 hr</td>
<td></td>
</tr>
<tr>
<td>+ 8 mg tomato inhibitor I, mixed 30 min filtered centrifuged</td>
<td></td>
</tr>
<tr>
<td>Vacuoles</td>
<td>2.5 x 10^6</td>
</tr>
<tr>
<td>Vacuoles ↓ 1st sucrose wash</td>
<td>2.0 x 10^6</td>
</tr>
<tr>
<td>Vacuoles ↓ 2nd sucrose wash</td>
<td>1.4 x 10^6</td>
</tr>
</tbody>
</table>
Table II. Ratios of Activities of Inhibitors, Enzymes, and Chlorophyll in Vacuoles and Leaves from Control, PIIF-Induced, and Wound-Induced Leaves

<table>
<thead>
<tr>
<th>Activity</th>
<th>Non-Induced Vacuole/Cell</th>
<th>PIIF-Induced Vacuole/Cell</th>
<th>Wound-Induced Vacuole/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase Inhibitor I</td>
<td>4</td>
<td>0.95</td>
<td>0.71</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>0.89</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>0.25</td>
<td>0.34</td>
<td>0.16</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.0</td>
<td>0.15</td>
<td>0.03</td>
</tr>
<tr>
<td>Triosephosphate Isomerase</td>
<td>0.24</td>
<td>0.25</td>
<td>0.11</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>0.20</td>
<td>0.35</td>
<td>0.23</td>
</tr>
</tbody>
</table>

1Vacuole and cell activities normalized to one gram of leaf equivalent. 
2Ratios are an average of 4 separate preparations of vacuoles and leaf extract. 
3Results of one preparation of vacuoles and leaf extract. 
4No activity detected.

The wounding of young tomato plants appears to cause a change in vacuolar enzyme concentrations, which results in a partitioning of acid phosphatase and perhaps carboxypeptidase between the cytoplasm and the vacuole (Table II). It appears from electron microscopy that the proteinase inhibitors are in the form of membraneless protein bodies, and therefore may not be able to move easily through the tonoplast. Thus, in induced tomato leaves, proteinase inhibitors do appear to be more reliable markers for vacuoles than either acid phosphatase or carboxypeptidase. It may be an advantage to the plant to sequester the proteinase inhibitors inside the vacuoles as protein bodies that readily dissolve when the vacuoles are broken. They are apparently not subjected to degradation while in the vacuole (9). This might be advantageous to the plants as a mechanism to provide a long lived defense mechanism against pests.

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