Effects of Pronase on Isolated Chloroplasts

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ABSTRACT

Subjecting isolated spinach chloroplasts to mild proteolysis (10-minute incubation at 20°C in 500 micrograms per milliliter pronase) caused chloroplast clumping but did not affect their integrity as measured by their ability to carry out light stimulated, glyceraldehyde-3-P-dependent O2 evolution. Transmission electron microscopy revealed no detectable differences between the control and treated plastids. Mild proteolysis inactivated exogenously added pyruvate kinase and should be a useful technique in certain enzyme distribution studies.

Proteolytic enzymes are commonly used in studies of the transfer of polypeptides across membranes. For example, microsomal vesicles appear to be resistant to mild proteolysis and presumably protect processed peptide chains which have been inserted into the intravesicular space (2, 3). Although extensive digestion of red blood cells occurs when the cells are treated with high concentrations (1-4 mg/ml) of pronase for long periods, no lysis occurred when the cells were treated with lower concentrations for shorter periods (100 μg/ml for 10 min or 10 μg/ml for 100 min) (1, 6).

Although the effects of proteolytic enzymes on thylakoid membranes have been studied (5, 11, 12), no observations have been reported on their effects on the integrity of isolated chloroplasts. One of the critical problems in studies of the intracellular distribution of enzymes is the question of the in vivo location of low concentrations of enzymes associated with isolated organelles. The adsorption of the enzyme on the surface of the organelle, or the occlusion of small amounts of enzymes in the pellet during isolation are always annoying possibilities. In connection with our studies of isolated chloroplasts, the feasibility of using proteolytic enzymes to digest nonplastid enzymes that might contaminate a preparation of isolated chloroplasts was investigated.

MATERIALS AND METHODS

Chloroplasts were isolated by a modification of the method of Jensen and Bassham (8) from greenhouse-grown spinach (Spinacia oleracea L.) or spinach purchased at a local market. Tissue rupture was carried out at ice temperature by chopping 10 g of leaves in 25 ml of isolation solution for 1 to 1.5 min with an electric knife fitted with razor blades. The isolation medium contained 0.33 M sorbitol, 2 mM EDTA, 2 mM isoascorbic acid, 1 mM MgCl2, 1 mM MnCl2, 0.05 M Mes buffer (pH 6.1), and 0.02 M NaCl. The chopped material was filtered through nylon mesh, centrifuged at 100g for 1 min and 2,000g for 2 min. The 2,000g pellet was washed once or twice by resuspending the pellet in a solution similar to the isolation solution but containing 0.05 M Hepes buffer at pH 6.7 instead of the Mes buffer and then repeating the centrifugations. The washed pellet was resuspended in a solution similar to the wash solution but at pH 7.6, without NaCl, and containing, in addition, 5 mM Na2HPO4. This solution was also used as the basic reaction medium for O2 evolution studies. The per cent of intact chloroplasts in the isolation was estimated by counting the intact and broken chloroplasts in appropriate aliquots by phase contrast microscopy.

The photosynthetic activity of the plastid preparations was determined as light-stimulated PGA-induced O2 evolution as measured with a Gilford oxigraph (9). Details of the reaction conditions are given in the legend of Figure 2.

Plastid suspensions were fixed overnight at 4°C with 3.5% glutaraldehyde in isolation solution, then pelleted. Pellets were rinsed in Na-phosphate buffer (0.1 M, pH 7.2) and postfixed for 4 h with 2% OsO4. They were rinsed with buffer, dehydrated with acetone, and embedded in Spurr's resin (10). Thin sections were stained with uranyl acetate and lead citrate and viewed on a JEOL JEM-100S transmission electron microscope.

Pronase (Calbiochem pronase B grade) from a freshly prepared stock solution (5 mg/ml of resuspension medium) was added to resuspended chloroplasts to bring the final concentration to 0.5 mg/ml unless otherwise indicated. Incubation was carried out at room temperature (20°C).

Studies with exogenously added pyruvate kinase (Calbiochem A Grade) were made by adding the kinase to the resuspended chloroplast preparation and then, at zero time, adding the appropriate amount of pronase. The rate of inactivation of the added pyruvate kinase was followed spectrophotometrically by measuring the rate of oxidation of NADH in the lactic dehydrogenase coupled reaction as described by Bücher and Pfleiderer (4). NADH oxidase activity of the preparation was estimated by omitting P-enolpyruvate and lactic acid dehydrogenase from the reaction medium. Sufficient exogenous pyruvate kinase was added to the chloroplast preparation so that a 0.5-ml aliquot of the control sample in 0.81 ml of reaction medium brought about a 0.16 O.D. decrease per min at 20°C (cuvette 1-cm light path).

RESULTS AND DISCUSSION

Preparations of untreated and pronase-treated chloroplasts were monitored by phase contrast microscopy, and the per cent of intact chloroplasts was estimated. No significant differences in the number of intact plastids were observed even when the plastids were treated with 600 μg pronase/ml for 10 min at 20°C (Table I). Similarly, electron microscopical examination of treated and untreated plastids showed that in both samples a high percentage of intact chloroplasts existed and no readily identifiable differences between the two samples were observed (Fig. 1). Care was taken to monitor the entire length of the pellets prepared for electron microscopy to take into consideration any differences in stratification that may have occurred during sample preparation (7).

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3 Abbreviation: PGA: glycerate-3-P.
Table I. Effect of Pronase on Integrity of Isolated Chloroplasts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intact</th>
<th>Broken</th>
<th>% Intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>95</td>
<td>32</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>243</td>
<td>74</td>
<td>77</td>
</tr>
<tr>
<td>Pronase 1</td>
<td>112</td>
<td>53</td>
<td>68</td>
</tr>
<tr>
<td>600 µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>157</td>
<td>49</td>
<td>76</td>
</tr>
<tr>
<td>Pronase 2</td>
<td>111</td>
<td>33</td>
<td>77</td>
</tr>
<tr>
<td>366 µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>95</td>
<td>54</td>
<td>69</td>
</tr>
</tbody>
</table>

Although no differences between the pronase-treated samples and the control samples could be observed by either phase or electron microscopy, it should be pointed out that the pronase caused a very intense clumping of the chloroplasts in the sample tube. The plastids tended to stick together and produce a stringy appearance and were difficult to resuspend completely. This phenomenon currently is being studied.

Since the pronase-treated plastids appeared to be intact, their capacity to evolve oxygen was tested. Figure 2 shows that pronase-treated plastids carried out light-stimulated PGA-dependent O₂ evolution at the same rate as the control plastids. If the pronase treatment had significantly changed the permeability of the chloroplasts to internal factors such as NADP and ferredoxin, the rate...
of light-induced PGA-dependent $O_2$ evolution would have been greatly reduced (9).

The rapidity with which the pronase treatment inactivated pyruvate kinase that was added to the plastid preparation is shown in Figure 3. It is seen that 500 $\mu$g of pronase/ml of the resuspended plastids inactivated the exogenous pyruvate kinase completely in 3 min at 20 C.

This study demonstrates that although the addition of pronase to isolated chloroplasts at a concentration of 500 $\mu$g/ml of plastids causes a clumping of the plastids, a 10-min treatment at 20 C does not cause a loss of plastid integrity nor an alteration in the plastids' ability to carry out light-induced PGA-dependent $O_2$ evolution. We conclude that in studies of intracellular distribution of enzymes, treatment of isolated chloroplast preparations with low levels of pronase for short times may be very useful in reducing the level of contaminating enzymes without destroying the integrity of the chloroplasts.

LITERATURE CITED


