

Effects of Pronase on Isolated Chloroplasts¹

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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, glycerate-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 enzyme 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 Na₄P₂O₇. 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 PGA³-induced O₂ evolution as measured with a Gilford oxygraph (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 Pfeleiderer (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 evaluation of treated and untreated chloroplasts 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*

Sample	Counts		% Intact
	Intact	Broken	
Control 1	95	32	75
2	243	74	77
Pronase 1	112	53	68
600 $\mu\text{g}/\text{ml}$			
10 min	157	49	76
Pronase 2	111	33	77
366 $\mu\text{g}/\text{ml}$			
10 min	95	54	69

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 O_2 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

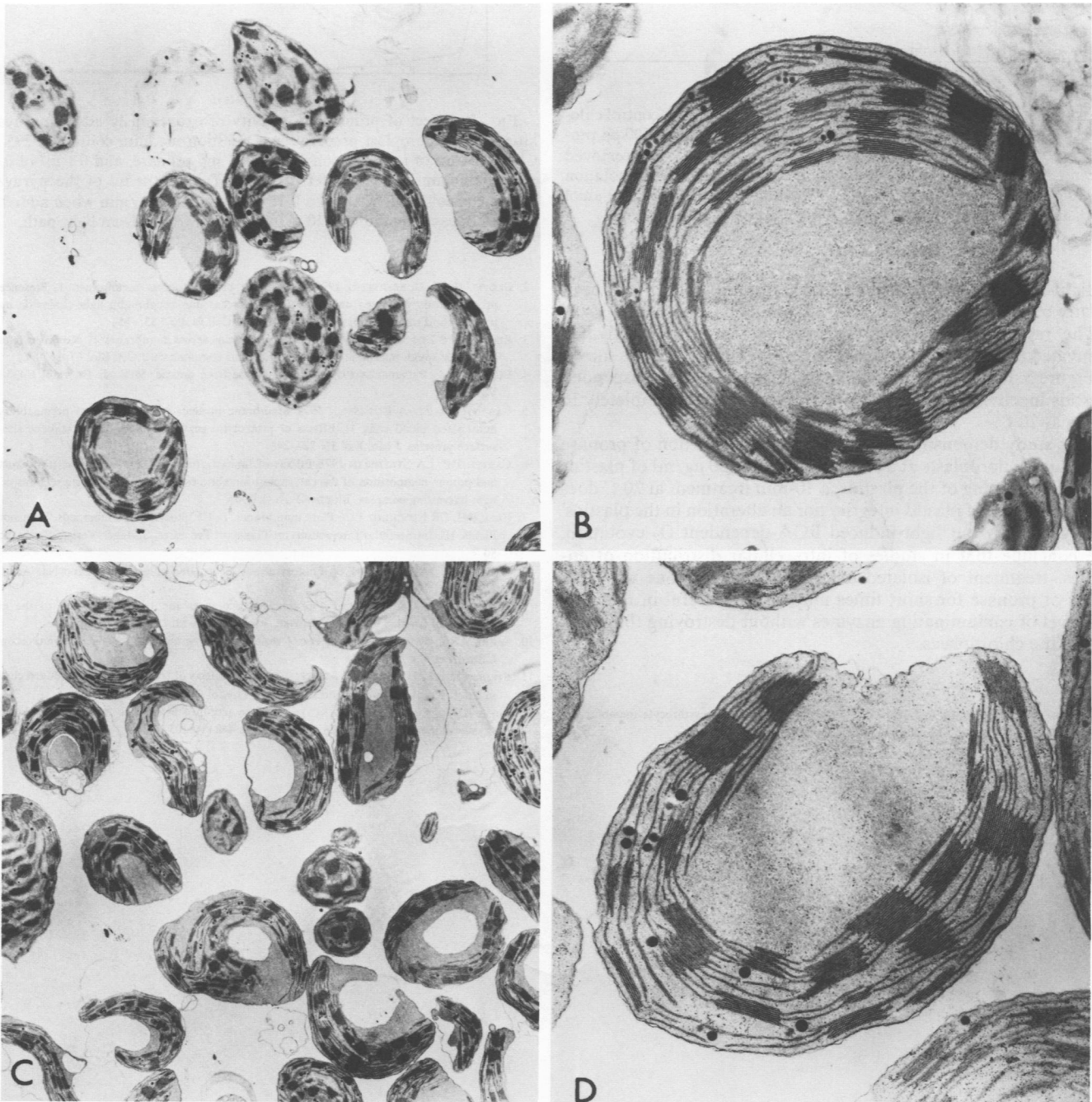


FIG. 1. Electron micrographs of isolated chloroplasts. A: control ($\times 3,879$); B: control ($\times 17,033$); C: plastids treated with 500 μg pronase/ml for 10 min at 20 C ($\times 3,780$); D: pronase-treated plastid ($\times 19,297$).

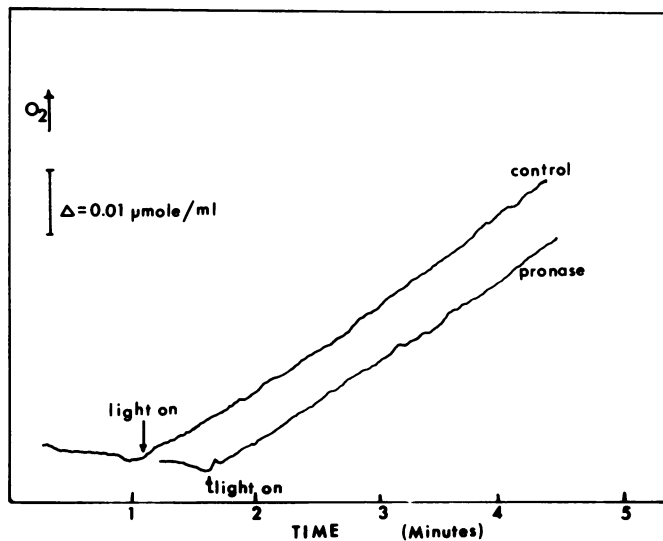


FIG. 2. Light-stimulated PGA-dependent O_2 evolution by control chloroplasts and pronase-treated chloroplasts. Pronase treatment, 500 μg pronase/ml of resuspended chloroplasts for 10 min at 20 C. Pronase removed by centrifugation of the plastids prior to the O_2 test. The reaction solution contained resuspension medium (pH 7.6), 3 μmol PGA, 3 μmol Pi, 3 μmol ADP, and chloroplasts equivalent to 33 μg Chl. Final volume: 2 ml.

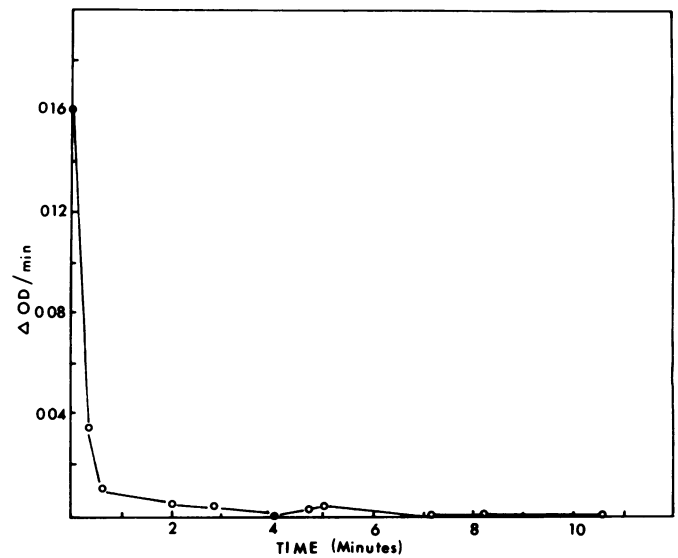


FIG. 3. Effect of pronase on activity of exogenously added pyruvate kinase in a chloroplast preparation. Digestion medium contained 295 μg Chl in 2.1 ml of resuspension solution, 1 mg pronase, and 0.1 ml of 0.05 M triethanolamine-HCl buffer (pH 7.5). Two-tenths ml of the pyruvate kinase control brought about a 0.16 decrease in O.D./min when added to 0.61 ml of assay medium at 20 C in a cuvette with a 1-cm light path.

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 μg 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 $\mu\text{g}/\text{ml}$ 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.

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