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Immunocytochemical Localization of ADPglucose Pyrophosphorylase in Developing Potato Tuber Cells[1]

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ABSTRACT

The subcellular localization of ADPglucose pyrophosphorylase, a key regulatory enzyme in starch biosynthesis, was determined in developing potato tuber cells by immunocytochemical localization techniques at the light microscopy level. Specific labeling of ADPglucose pyrophosphorylase by either immunofluorescence or immunogold followed by silver enhancement was detected only in the amyloplasts and indicates that this enzyme is located exclusively in the amyloplasts in developing potato tuber cells. Labeling occurred on the starch grains and, in some instances, specific labeling patterns were evident which may be related to sites active in starch deposition.

ADPglucose pyrophosphorylase catalyzes a key regulatory step in starch biosynthesis in plant cells (14). In leaf tissue this enzyme is located exclusively in the chloroplasts (13) and is allosterically activated by 3-P-glycerate and inhibited by Pi (14). The regulation of starch biosynthesis via allosteric control of ADPglucose pyrophosphorylase is supported by *in vivo* studies using intact chloroplasts. Starch synthesis was suppressed by addition of 1 mM or greater levels of exogeneous Pi (6, 16) and reversed by the addition of 3-P-glycerate (6). Furthermore, the level of CO_2 fixation into starch corresponded directly with the ratio of 3-P-glycerate/Pi in the stroma and agreed well with the *in vitro* activity of ADPglucose pyrophosphorylase at these effector ratios (6).

Tissue specific forms of this enzyme are present in the leaf and seed tissue of maize, wheat, and rice (8). ADPglucose pyrophosphorylase has been purified to apparent homogeneity from potato tubers (15). It was observed to be a tetramer consisting of subunits of 50 kD and its activitiy was highly sensitive to its allosteric effectors, 3-P-glycerate and Pi (15). In contrast to the leaf enzyme, information on the subcellular location of this enzyme in nonphotosynthetic tissues is relatively scant. Echeverria *et al.* (5) have shown that amyloplasts isolated from developing maize endosperm contain enzyme activities of ADPglucose pyrophosphorylase as well as starch branching enzyme. Recently, Mohabir and John (11) demonstrated that amyloplasts from potato tuber protoplasts contain starch biosynthesis enzyme activities, including ADPglucose pyrophosphorylase. In this study, we employed immunocytochemical techniques coupled with light microscopy to demonstrate that ADPglucose pyrophosphorylase is located exclusively in the amyloplasts of developing potato tuber cells and, in at least some instances, may reside at distinct regions within this organelle.

MATERIALS AND METHODS

Plant Material

The potato *(Solanum tuberosum* L.) variety Russet Burbank was used as the source of developing tuber tissue. Developing potato tubers, weighing 40 to 50 g, were utilized in this study.

Western Blotting

Soluble proteins of developing potato tuber were extracted according to the method of Galili and Feldman (4). Total potato tuber proteins were extracted with SDS sample buffer (9) and Western blot analysis was performed as described (2).

Tissue Preparation

The preparation of tissue sections, fixation, and embedding were performed as described by Kim *et al.* (7) with slight modifications. Briefly, 1 to 2 mm potato tuber sections were fixed with 2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 20 mM cacodylate buffer (pH 7.2) for 3 h at room temperature, dehydrated in ethanol (30–100%), and embedded in L.R.White resin (Polysciences, Warrington, PA). Sections (1 μm thick) were prepared with glass knives and stained with Stevenel's blue (3) or incubated with periodic acid followed by the Schiff's reagent for carbohydrate-specific staining (1).

Immunocytochemical Staining

Thick sections (1 μm), mounted on gelatin coated glass slides, were bathed in TBST buffer (20 mM Tris, 500 mM

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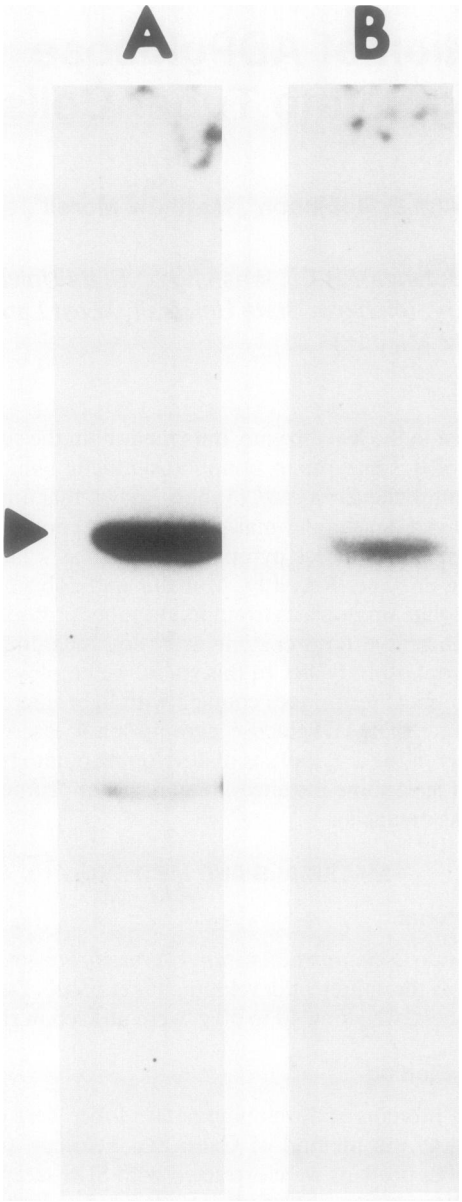

Figure 1. Western blot analyses of potato tuber ADPglucose pyrophosphorylase. Soluble protein (100 μg) of potato tuber was resolved on a 12% SDS polyacrylamide gel, transferred to nitrocellulose filter, probed with either antispinach ADPglucose pyrophosphorylase holoenzyme (A) or anti-51 kD subunit (B), and then incubated with ^{125}I-labeled protein A. Immunoreactive band was visualized by autoradiography. Arrowhead indicates 50 kD potato tuber ADPglucose pyrophosphorylase.

NaCl, and 0.1% Tween 20[4]) containing 1% BSA for 10 min, and then incubated for 4 h with either spinach leaf ADPglucose pyrophosphorylase holoenzyme antibody (8) or 51 kD subunit antibody (12).

For immunofluorescence staining, sections were further incubated with 1:200 diluted fluorescein conjugated goat anti-rabbit IgG (Organon Teknika Corp., West Chester, PA) for 1 h, washed with TBST-BSA, followed by TBST alone and then analyzed with a Leitz microscope equipped for epi-fluorescence with an H_2 filter system (wide band blue). Kodak Tmax 400 black and white film was used for photography.

For immunogold labeling, sections were incubated for 1 h with 15 nm diameter protein-A gold (Janssen Pharmaceuticals, Piscataway, NJ), followed by washing with TBST-BSA, TBST, and then distilled water. Immunogold particles were enlarged by incubation with silver enhancement solution (Janssen Pharmaceuticals, Piscataway, NJ) according to the manufacturer's recommendations. Sections were post-stained with 1% (w/v) aqueous Safranin O. Kodak Technical Pan film was used for photography.

RESULTS AND DISCUSSION

The specificity of the partially purified IgG preparation, raised against either the spinach leaf ADPglucose pyrophosphorylase holoenzyme (8) or 51 kD subunit (12), was examined by Western blot analysis. The holoenzyme antibody cross-reacted strongly with the tuber 50 kD ADPglucose pyrophosphorylase subunit although minor reactions were also evident between the antibody and other proteins which are present at abundant levels in the potato tuber extract (Fig. 1A). One of these reactive polypeptide at about 40 kD was assumed to be patatin, based on its coincident mobility with this protein on polyacrylamide gels. The reactivity pattern of the anti-51 kD subunit was more restricted, with clear binding to only one major band identified as the 50 kD subunit of the potato tuber enzyme (Fig. 1B). However, the capacity of the anti-51 kD subunit to recognize the tuber pyrophosphorylase subunit was much less than that detected between the anti-spinach holoenzyme and potato tuber subunit. In view of the slightly different reactivity properties displayed by these antibodies, both immunoglobulin preparations were routinely employed in this study. Irrespective of the antibody used, however, identical patterns of labeling were evident in our immunocytochemical localization studies as discussed below.

Figure 2 is a low-magnification view of the potato tuber cells showing the general subcellular features of this cell type. These cells have a prominent vacuole and numerous amyloplasts, each filled with a single starch grain. The amyloplasts are up to 30 μm in diameter and most of their volume is taken up by the starch grain. The stroma is thus displaced to the periphery of the organelle and, in 1 μm thick sections presented here, is present as a barely distinguishable dark line surrounding the section through the starch grain. When potato tuber cells were treated with periodic acid followed by Schiff's reagent for carbohydrate-specific staining, only the starch grain contained within the amyloplasts and the cell walls were clearly stained (Fig. 3). These results clearly show that amyloplasts of the potato tuber cells can be easily distinguished

[4] Abbreviations: Tween 20, polyoxyethylenesorbitan monolaurate 20; IgG, immunoglobulin G.

Figure 6. Immunogold/silver enhanced labeling showing ADPglucose pyrophosphorylase distribution in the starch grains. Nucleus, cytoplasm, and cell wall are not labeled; bar = 10 μm. Inset, phase contrast photograph of a large amyloplast demonstrating enhanced labeling along specific layers of the starch grain; bar = 10 μm.

Figure 7. Preimmune control for immunogold labeling; bar = 10 μm.

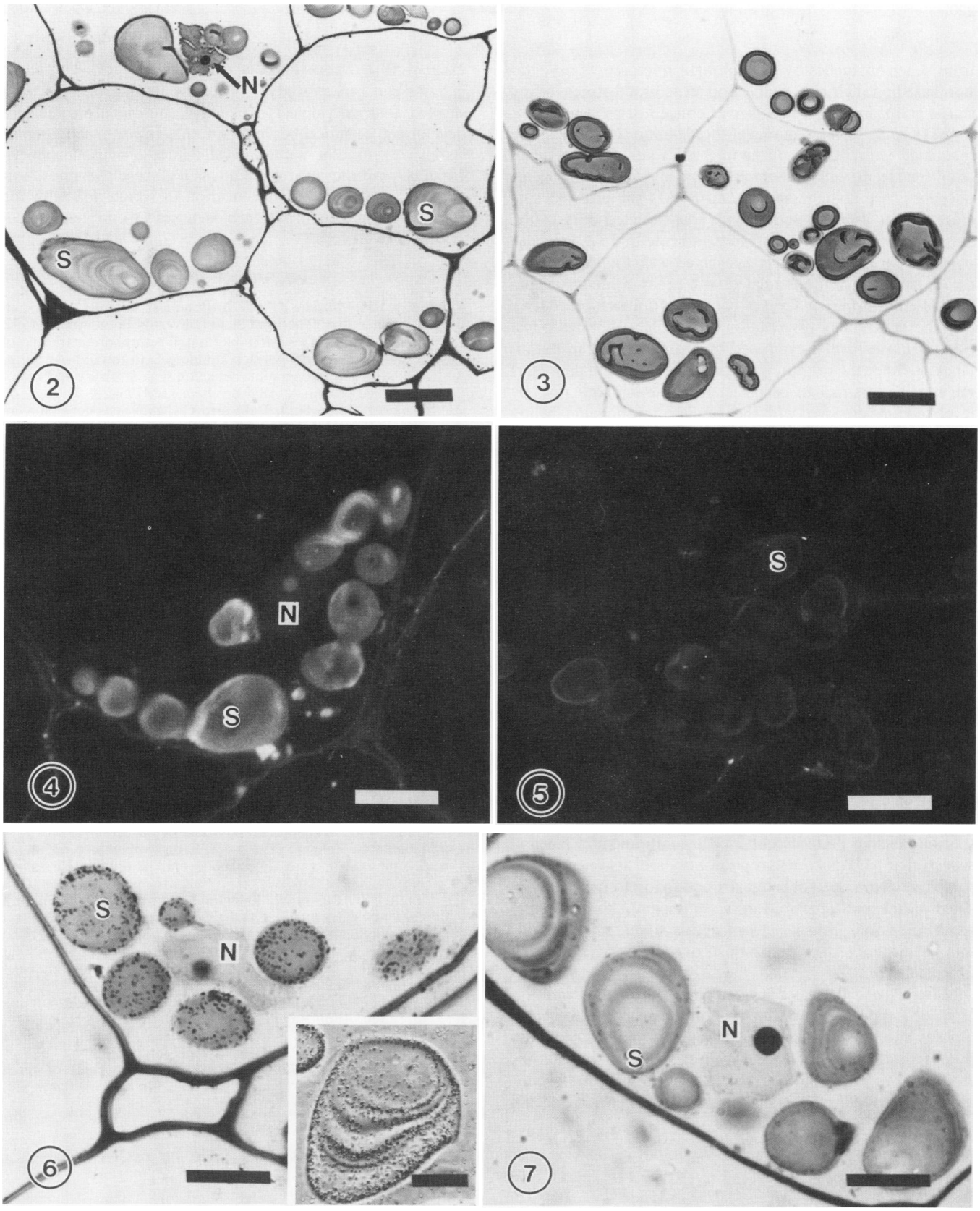

Figure 2. Low magnification view of developing potato tuber cells. S, starch; N, nucleus; bar = 30 μm.
Figure 3. Periodic acid-Schiff's reagent staining of potato tuber cells; bar = 30 μm.
Figure 4. Immunofluorescence labeling for ADPglucose pyrophosphorylase demonstrating the intense and specific immunofluorescence only in the amyloplasts; bar = 10 μm.
Figure 5. Preimmune control for immunofluorescence labeling. Note the low background level of fluorescence througtout the cells; bar = 10 μm.

from other organelles by general, light microscopic observation based on their size, shape, and structural features. Many starch grains had a substructure of concentric light and dark layers (Fig. 2). Thick sections of these potato tuber cells were incubated with fluorescein conjugated goat anti-rabbit IgG after treatment with either antispinach ADPglucose pyrophosphorylase holoenzyme or anti-51 kD subunit and specific, intense immunofluorescence was detected only in the amyloplasts (Fig. 4). These results indicate that ADPglucose pyrophosphorylase is closely associated with the starch granule. In contrast, sections treated with preimmune serum exhibited a uniform, low level of background fluorescence (Fig. 5). The subcellular distribution of ADPglucose pyrophosphorylase was further examined by protein-A gold immunolabeling. Since the immunogold particles (15 nm in diameter) are much too small to be detected at the light microscopy level, sections prepared with the regular immunogold labeling procedure were further treated with silver staining solution, which mediates the specific precipitation of metallic silver around the colliodal gold particles. This silver enhancement technique results in the appearance of highly contrasted, dark brown particles which can be easily detected by the light microscope. By this technique, specific labeling was found only in the amyloplasts (Fig. 6). No significant immunolabeling was found with preimmune serum treatment (Fig. 7). Results from this technique also showed that ADPglucose pyrophosphorylase is not randomly distributed, at least over some of the starch grains. While label could be found distributed across the amyloplasts, there was often an increase in the amount of labeling at the outer periphery of the grains (Fig. 6). This likely reflects a higher concentration of the enzyme in the stromal layer surrounding the edge of the starch grain. Also, in starch grains with highly defined contour patterns, labeling was often most intense along layers that stained darkest with safranin (Fig. 6, inset). Such patterns were not always clear with immunofluorescent staining. The specific labeling patterns obtained by immunogold labeling suggest that ADPglucose pyrophosphorylase, assumed to be a soluble enzyme by biochemical fractionation criteria, is located within specific regions in the starch grain. Such discrete localization may reflect sites of active starch synthesis, although evidence to support this contention has not been obtained.

Mares *et al.* (10) have suggested, based on the common biochemical properties shared by both chloroplasts and amyloplasts and plastid origin of amyloplasts, that ADPglucose pyrophosphorylase is located within the amyloplasts, but direct evidence has heretofore been lacking. Our immunostaining studies conclusively demonstrate that this enzyme occurs exclusively in the amyloplasts of potato tuber cells. This result extends the earlier study of Mohabir and John (11) who showed that amyloplasts isolated from the protoplasts of developing potato tuber contain the enzyme activities of starch biosynthesis, including ADPglucose pyrophosphorylase. The plastidic distribution of ADPglucose pyrophosphorylase is consistent with the location of starch and with the role of this enzyme in starch biosynthesis in the non-photosynthetic plant cells.

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