

The Elevation of Levels of Amyloplastic α -1,4- Glucan Phosphorylase Protein in Cultured Cells of *Acer pseudoplatanus* during Sucrose Starvation

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ABSTRACT

Two types of α -1, 4-glucan phosphorylase isozyme were characterized in the suspension-cultured cells of sycamore (*Acer pseudoplatanus*). The type I, a major fraction of the total extractable phosphorylase, was shown to be localized in the cytoplasm and exhibited a high affinity towards glucans, whereas the low affinity type (type II) resided in the amyloplasts. These two isozymes were distinguishable in their immunological properties : the amyloplastic phosphorylase reacted strongly with the antibody raised against the potato tuber phosphorylase, whereas the cytosolic enzyme did not cross-react with the same antibody. Western blot analysis revealed the molecular mass of the monomeric subunit of plastidic phosphorylase to be 102 kD. The amyloplastic phosphorylase activity and its protein contents increased about 5-fold during 45 hours of sucrose starvation, but significantly declined upon replenishment with sucrose, indicating a possible regulatory role of amyloplastic phosphorylase in breaking down the reserve starch stored in the amyloplast.

Key words: amyloplast, α -1, 4-glucan phosphorylase, sucrose starvation

INTRODUCTION

In plant cells, starch synthesized via the gluconeogenic pathway is stored in the plastid, *i.e.* amyloplast and chloroplast (Dennis and Miernyk, 1982). Although many studies have been carried out on the metabolism in the chloroplast (Beck and Ziegler, 1989) the knowledge on the nongreen plastid, amyloplast, has been limited. However, the amyloplasts isolated from cauliflower bud and soybean suspension culture have been shown to possess all the activities needed for the conversion of triose-P to starch including ADP-glucose pyrophosphorylase and starch synthase. Since amylase and α -1, 4-glucan phosphorylase have been shown to be associated with the plastid, it is postulated that the breakdown of starch occurs *in situ* either hydrolytically or phosphorolytically in nonphotosynthetic tissues (Journet and Douce, 1985).

Phosphorylase (EC 2.4.1.1) catalyses the

reversible phosphorolysis of α -1, 4-glucan. The investigations on plant phosphorylase isolated from spinach and pea leaves have clearly established that there are two types of phosphorylase distinguishable in their properties (Steup and Latzko, 1979). The type I, localized in the extra-chloroplast, has a high affinity towards branched polyglucans, such as soluble starch, amylopectin and glycogen, whereas the type II with a low affinity towards branched polyglucans is inside the chloroplast. Type I and II phosphorylases are also present in nongreen parts of higher plants such as roots or etiolated shoots (Steup *et al.*, 1986; Suda *et al.*, 1987). In most cases, the cytoplasmic phosphorylase is found to be a predominant form. However, in white potato tubers the predominant phosphorylase form is type II enzyme (Fukui *et al.*, 1982).

In contrast to the knowledge concerning the kinetic properties of plant phosphorylase, little is known on mechanism(s) of gluconeogenesis/glycolysis of

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starch in plant cells under various environmental conditions. In this investigation, the two forms of α -1, 4-glucan phosphorylase in the sycamore cells were characterized and the levels of the enzyme proteins were examined during sucrose starvation.

MATERIALS AND METHODS

Cell Culture

The method for the suspension-culture of sycamore (*Acer pseudoplatanus*) cells utilized was that as mentioned by Harinasut *et al.* (1988). For sucrose starvation experiments, cells of 3-day-old were transferred to the sucrose-omitted culture medium and the culture continued for 15, 30 or 45 hours. In some experiments, sucrose was supplemented to 30 hour-starved cells and the culture continued for additional 15 hours.

Isolation of Intact Amyloplasts

Intact amyloplasts were isolated from the suspension-cells of sycamore (Harinasut *et al.*, 1988).

Cell Extracts

Five grams of cells at various stages of sucrose starvation and resupplementation as described above were homogenized in 20 ml chilled buffer A (10 mM Tris-HCl buffer at pH 7.2, 0.1 mM PMSF, 5 mM β -mercaptoethanol, 1 mM EDTA, 15 μ M thymol and 10 % glycerol). The homogenate was centrifuged and then the supernatant fraction was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation, and the precipitated protein (40-50 % saturation with $(\text{NH}_4)_2\text{SO}_4$) was dissolved in buffer A.

Phosphorylase Assay

Phosphorylase activities were assayed as Pi-dependent glucose-1-P forming activity (Journet and Douce, 1985).

Polyacrylamide Gel Electrophoresis

Affinity-PAGE was carried out in the slab or disc gel electrophoresis as described by Steup and Latzko (1979). SDS-PAGE was carried out according to the method of Laemmli (1970).

Western Blot Technique

The Western blot analysis was carried out according to the method of Blake *et al.* (1984) and bands were detected using alkaline phosphatase-

conjugated goat anti-rabbit IgG. The specific rabbit antibody raised against the potato tuber phosphorylase was kindly provided by Dr. T. Fukui, Osaka University.

Analytical Methods

Protein content was determined by Lowry method *et al.* (1951) using BSA as a standard. Starch content was measured as previously reported by Rebeille *et al.* (1985). Carotenoid content was analysed as previous report (Liaaen-Jensen and Jensen, 1971).

RESULTS

Changes of Protein, Starch and Carotenoid Contents during Sucrose Starvation

Three day-old cells were transferred to the new culture medium with or without sucrose. At the selected time intervals, sucrose-depleted and nonstarved cells were collected and subjected to analyses for fresh weight, protein, starch and carotenoid contents. As shown in figure 1A, the fresh weight of the nonstarved sycamore cells per unit volume of the culture medium increased linearly, whereas that of starved cells increased only marginally until 10 hours and declined slightly afterwards. However, even upon supplementation of sucrose at 30 hours of the starvation, the cell fresh weight did not change. Similar pattern was observed for the total protein content per fresh weight (Figure 1B). The starch content expressed on protein was found to decrease dramatically after 10-hour-starvation, whereas it remained almost unchanged in the controls. This result is consistent with that previously reported by Rebeills *et al.* (1985). Upon supplementation of sucrose to the 30-hour-starved cells, however, the starch content increased dramatically (Figure 1C). The carotenoid content, which was assumed to be a marker of plastid membranes (Journet and Douce, 1985), remained constant throughout the experimental period employed (Figure 1D).

Two Types of Phosphorylase in Sycamore Cells

There are two types of (high- and low-affinity forms towards glucans) in spinach and pea leaves as well as in potato tubers (Steup and Latzko, 1979; Fukui *et al.*, 1982). These isoforms are well separable in the affinity-PAGE because of their different affinity to glucan molecule. Thus, in order to characterize the molecular species of the phosphorylase, an aliquot of the dialysed preparation

which was precipitated with 40-50 % saturation of $(\text{NH}_4)_2\text{SO}_4$ was subjected to the glycogen affinity-PAGE. The fraction was found to contain more than 85 % and 70 % of the total plastidic and cytoplasmic phosphorylase forms, respectively. As shown in figure 2A, two enzymatically active bands were detected. Band 1 was retarded by increasing the glycogen concentration, whereas the mobility of band 2 remained unchanged. It was clearly shown that the amyloplast extracts contained only band 2 (Figure 2B). Therefore, it was concluded that band 1 is the high affinity form (cytoplasmic type) and band 2 is the low affinity form (plastidic type).

The two types of phosphorylase were further analysed immunochemically using the specific antibody raised against the plastid type phosphorylase derived from the potato tuber. Only the low affinity form of phosphorylase in the total sycamore cell extracts and the one in the amyloplast extracts gave the positive reaction (Figure 3A). Since the antibody can only recognize the plastidic phosphorylase, molecular weight of the monomeric subunit of the enzyme was determined by the SDS-PAGE followed by the Western blot analysis. As presented in figure 3B, molecular size of amyloplastic phosphorylases estimated to be 102 kD.

Induction of Amyloplastic Phosphorylase Protein during Sucrose Starvation

The pattern of the phosphorylase activity in the cells during sucrose starvation was examined by the zymogram analysis using the affinity-PAGE. As shown in figure 4A, activities of both types of phosphorylase increased during starvation. On the other hand, upon supplementation of sucrose to the culture medium at 30 hours of starvation, there occurred a marked decrease again. Results of the Western blot analysis using the antibody (figure 4 B) show that the level of amyloplastic phosphorylase protein increased about 5-fold during 45-hour-starvation, whereas it increased only 1.4-fold in the controls. Importantly, supplementation of sucrose to the 30-hour-starved cells resulted in a marked decrease in the level of phosphorylase protein within the subsequent 15-hour-period (figure 4B, lane 5). The content of phosphorylase protein in the isolated amyloplasts during the starvation period showed a similar pattern to that of its activity (figure 4C).

DISCUSSION

The effect of sucrose starvation on the levels of carbohydrate and Pi in the sycamore cell have reported by Rebeille *et al.* (1985). They found that when sucrose was deprived from the nutrient medium, vacuolar sucrose was consumed first, while starch, phosphate-ester and Pi contents remained unchanged for a certain period. After 10-hour-starvation, starch began to be consumed and its content decreased to 35 % of the nonstarved cells within 30-hour-starvation. During this period, the content of phosphate-ester also declined progressively in parallel to the increase in the intracellular pool of Pi. The Pi thus released was shown to be rapidly sequestered into the vacuoles. However, it was found that the cytoplasmic Pi pool also increased about two-fold within 30 hours of starvation. Therefore, these workers have hypothesized that an increased level of cytoplasmic Pi promotes the starch degradation. In the present study, phosphorylases in the cultured cells of sycamore were characterized and examined changes in the contents of phosphorylase protein during the sucrose starvation.

As well as spinach and pea leaves (Shimomura *et al.*, 1982; Steup and Latzko, 1979), the cultured cells of sycamore contain two types of phosphorylase, *i.e.* cytoplasmic and amyloplastic forms, which differ in their kinetic and immunological properties (figure 2,3). In contrast to the potato tuber enzyme (Fukui *et al.*, 1982), however, the cytoplasmic enzyme appears to be the predominant form.

Journet *et al.* (1986) reported that some of the cytosolic components containing various cell organelles disappeared after a long term starvation (more than 30 hours) of sycamore cells. It has also been reported that a massive degradation of starch taking place in the senescent potato tubers was accompanied by the disappearance of the envelope membranes of amyloplasts (Brisson *et al.*, 1989). Therefore, there is a possibility that in highly starved sycamore cells the amyloplast envelopes *per se* are structurally disintegrated. However, at least the carotenoid content which was assumed to be a marker of plastid membranes has remained relatively unchanged throughout the period of starvation (figure 1D).

Steup *et al.* (1986) reported the presence of a light-inducible plastidic phosphorylase in addition to the constitutive plastidic enzyme form in the shoot of

Pisum sativum. These two plastidic enzyme forms were shown to differ slightly in molecular sizes and glucan specificities, indicating their characteristic functions *in vivo*. As presented in figure 4, the level of protein content for the plastidic phosphorylase increased five-fold during the sucrose starvation. Thus, the possible induction of amyloplastic α -1, 4-glucan phosphorylase in the liquid-cultured cells of sycamore during the sucrose starvation is thought to show an

important role of the enzyme in the starch breakdown in amyloplasts.

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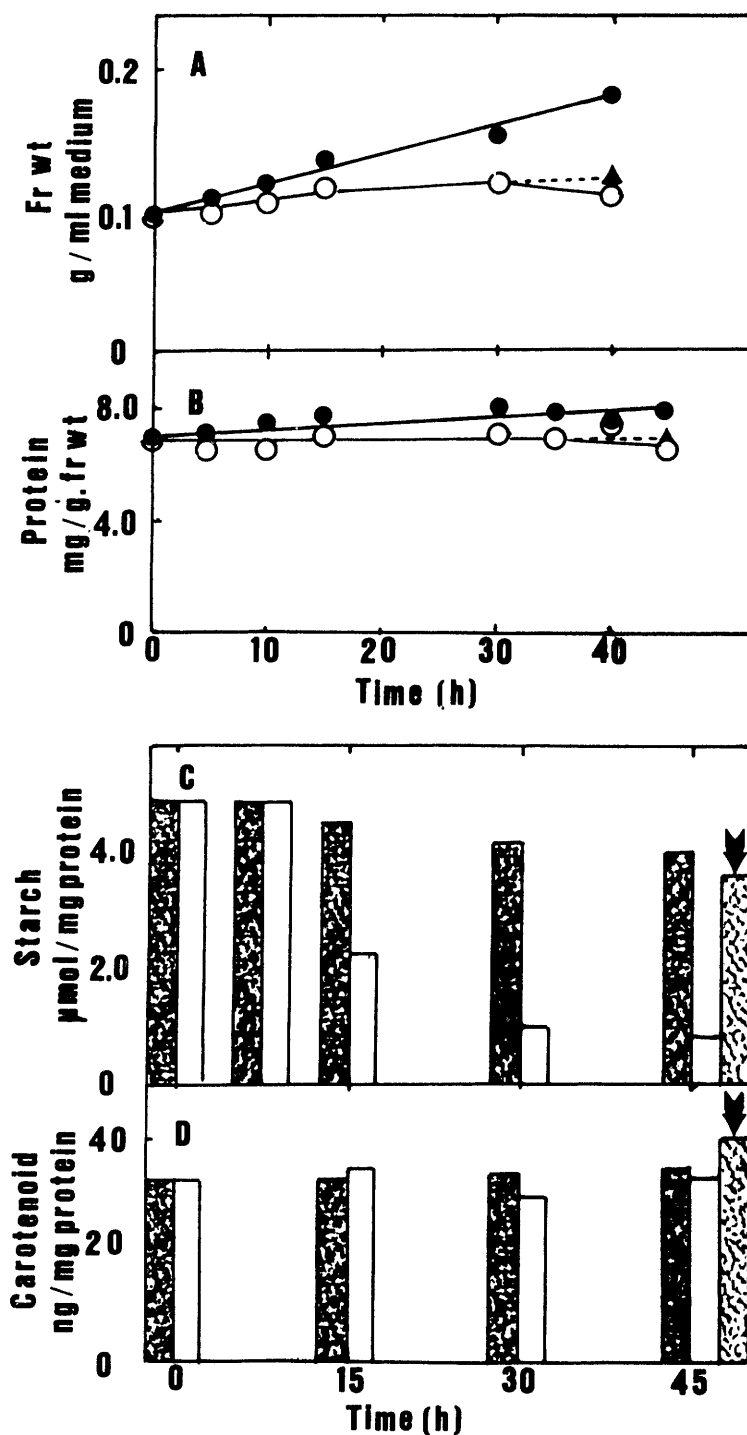


Figure 1 Time course analysis of fr wt (A), total protein (B), starch (C) and carotenoid (D). Three-day-old cells were further grown with (l, z) or without (m,) sucrose. (s) and arrow show sucrose supplementation to 30-hour-starved cells and 15 hours additional culture. Starch content is expressed as μmol of glucose/mg total protein.

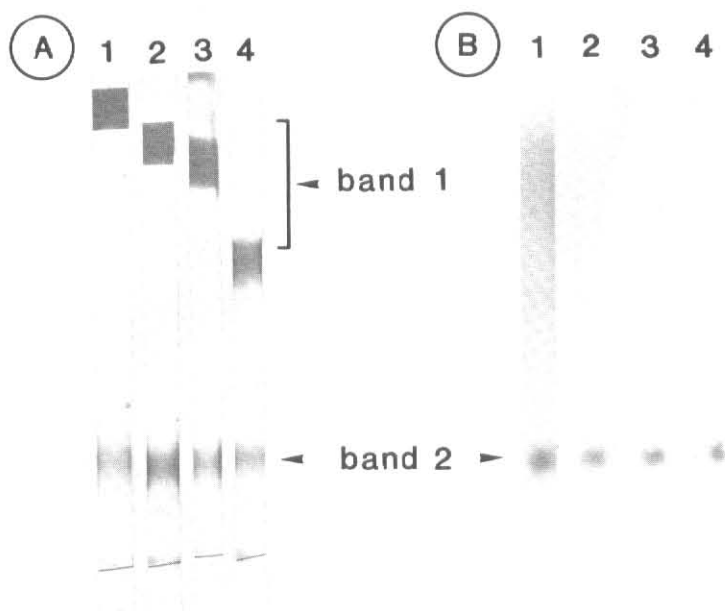


Figure 2 Affinity-PAGE of phosphorylase in sycamore cell extracts (panel A) and amyloplast lysates (panel B). Affinity-PAGE was carried out in 5 % polyacrylamide gels containing glycogen of various concentrations. Lanes 1, 2, 3 and 4 contained 400, 200, 100 and 40 ug/mL of glycogen, respectively. Protein precipitable by 40-50 % $(\text{NH}_4)_2\text{SO}_4$ saturation of total crude extracts was used for the zymogram assay. Band 1, high affinity phosphorylase; band 2, low affinity phosphorylase.

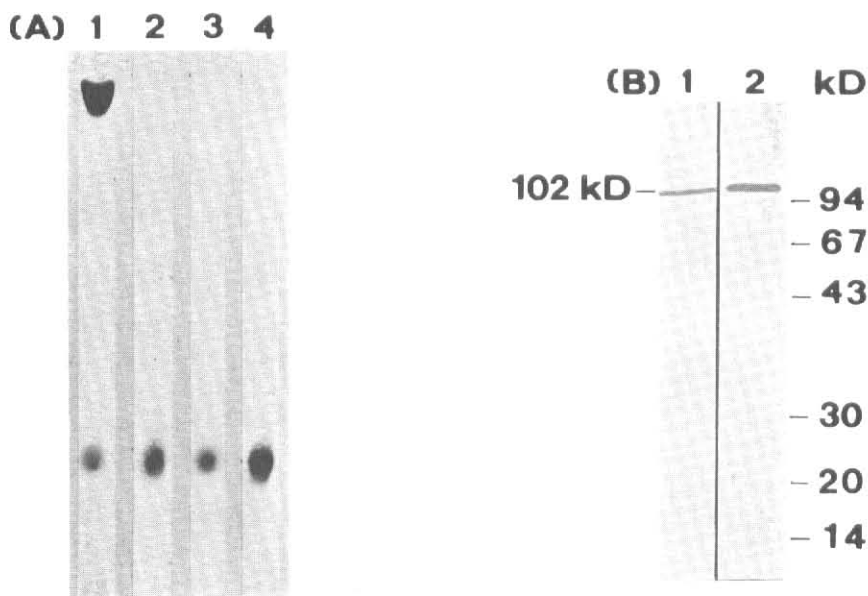


Figure 3 (A) Zymogram (lane1, 3) and Western blot (lane 2, 4) analysis of phosphorylase in protein of $(\text{NH}_4)_2\text{SO}_4$ (40-50 % saturation) fraction in cell extracts (lane1,2) and amyloplast lysates (lane3,4). Affinity-PAGE was carried out in 5% polyacrylamide gels containing 400 ug/mL glycogen. (B) Immunoblotting of $(\text{NH}_4)_2\text{SO}_4$ (40-50% saturation) fraction of cell extracts (lane1) and amyloplast lysates (lane 2) after SDS-PAGE.

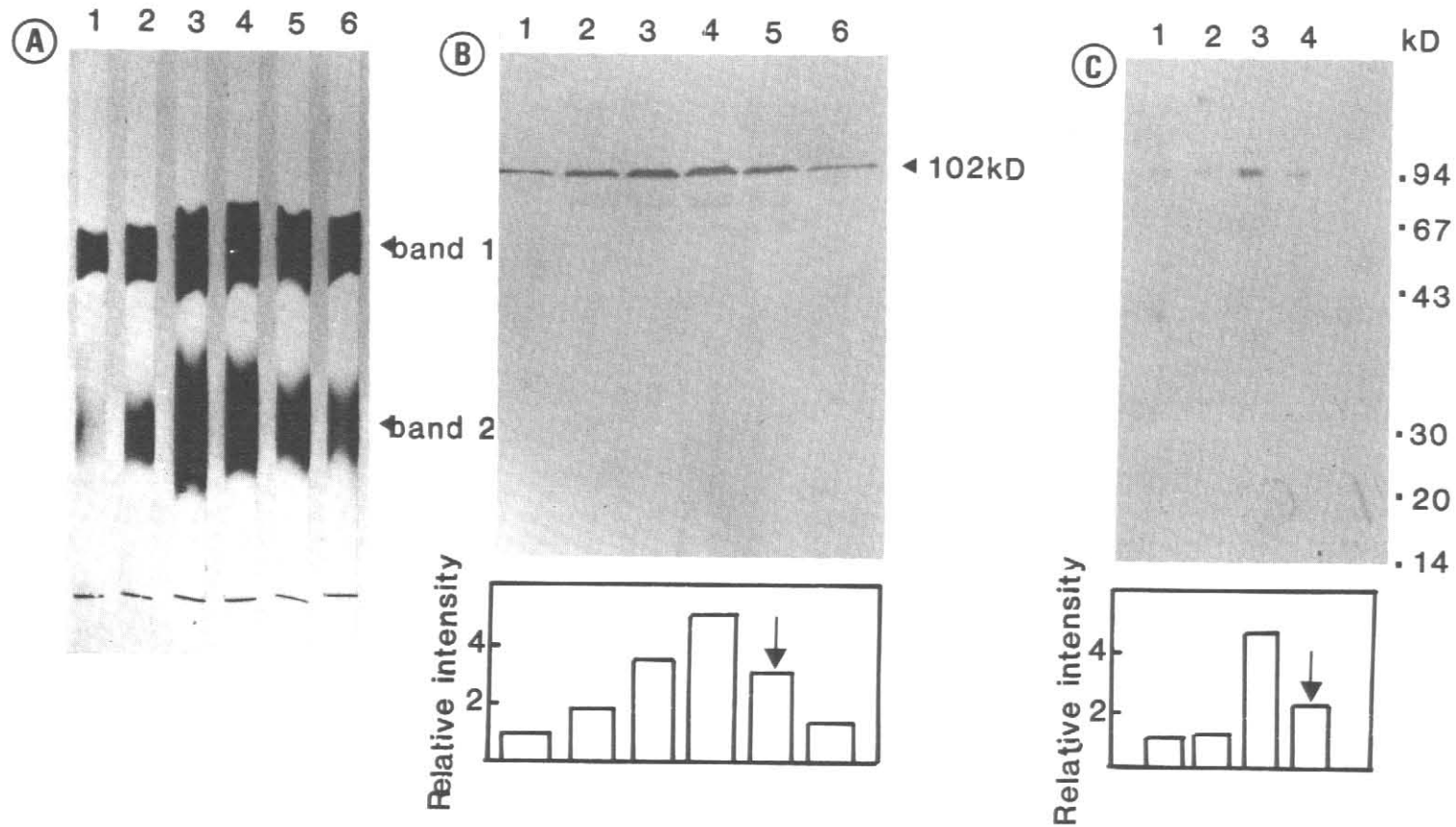


Figure 4 Induction of plastidic phosphorylase in sycamore cells during sucrose starvation. (A) Zymogram analysis of total crude cell extracts which were subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation (40-50 % saturation). Band 1 and 2, high and low affinity phosphorylase, respectively. (B) and (C), immunoblot analysis after SDS-PAGE of cell extracts prepared as for samples in panel A and amyloplast extracts, respectively. Lane 1 and 6 of panel A and B, 3- and 5-day-old nonstarved cells. Lanes 2, 3 and 4 of panel A and B, 3-day-old cells were further grown without sucrose for 15-, 30- and 45-hours, respectively. Lane 5, sucrose was supplemented to 30 hour-starved cells and cultured for additional 15 hours. Panel (C), lane 1-3, isolated amyloplasts from 15-, 30- and 45- hour-starved cells, respectively and lane 4, isolated amyloplasts from cells starved for 30 hours followed by sucrose supplementation for 15 hours (arrow). Histogram in (B) and (C), relative intensity of immunoblot signal for amyloplastic phosphorylase shown in the upper panel.

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