Bot. Bull. Acad. Sin. (1995) **36:** 223-228

Preparation of amyloplasts from sweet potato callus culture

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(Received April 7, 1995; Accepted July 27, 1995)

Abstract. Culture conditions for sweet potato callus were manipulated to enhance starch content. Tissue cultures were frozen in liquid nitrogen, pulverized in a ceramic mortar, and ground with a buffer and sea sand. Fractional centrifugation in a discontinuous Percoll gradient enabled separation of amyloplasts in a 40% Percoll layer sandwiched between 20% and 50% layers. A latency test of marker enzymes for amyloplast, and a preliminary feeding experiment with radio-labeled substrates for starch synthesis confirmed that the preparation is suitable for use in the study of starch biosynthesis.

Keywords: Amyloplast; Callus; *Ipomoea batatas*; Latency test; Starch metabolism; Sweet potato; Tissue culture.

Introduction

Starch synthesis in sink organs occurs in the amyloplasts. The precursor sucrose from the photosynthetic organ must be transformed into appropriate transportable forms to enter the organelle through the amyloplast membrane. Isolated amyloplasts are a desirable research system in which to relate sucrose metabolism in the cytosol to starch synthesis in the amyloplast. To that end, we applied many known methods (Gaynor et al., 1983; McDonald and ap Rees, 1983; Sack et al., 1983; Journet and Dounce, 1985; Macherel et al., 1985; Echeverria et al., 1985; Entwistel and ap Rees, 1988; Mohabir and Hohn, 1988) to tuberous sweet potato root, but were unsuccessful. We noticed that the large size of starch granules in root amyloplasts caused breakdown of the plastid membrane during isolation. To overcome this, we manipulated the culture conditions of sweet potato callus to induce starch synthesis and formation of amyloplasts. We now report our findings.

Materials and Methods

Tissue Culture

Leaves, stems, and young tuberous roots (ranging in size from 1 to 2 cm in diameter) of sweet potato (*Ipomoea batatas* cv. Tainong 57) were obtained from the University farm just prior to use. The tissue sections for callus initiation were sterilized by immersing in 70% ethanol for 30 sec and in 2.5% NaOCl (containing 1–2 drops of Tween 20 or Triton X-100 wetting agent per 100 ml) for 20 min. Tissue sections (about 5 × 8 × 7 mm) were inoculated on 0.8% agar plates containing 3% sucrose, 2 ppm NAA (1-naphthaleneacetic acid), 0.2 ppm Ki (kinetin), and MS (Murashige-Skoog) salts and vitamins. Cultures were kept in the dark at 25°C. Callus formation was evident after one week. Subcultures employed MS medium or B5 medium (Gamborg et al., 1982) containing varying concentrations of sucrose, NAA, and Ki. The starch content of tissues was analyzed at three-week intervals. Growth in a culture medium was estimated by the change in dry weight of tissues.

Enzyme Extraction

The callus tissue was mixed with sea sand, frozen in liquid nitrogen, and ground to a powder with a mortar and pestle. One to one-and-a-half times its weight of a 20 mM HEPES buffer (pH 7.8) containing 2 mM KCl, 2 mM $CaCl_2$, 1 mM EDTA, and 1% polyvinylpolypyrrolidone (PVPP) was added and the mixture was homogenized at 2°C for one min. After centrifugation at 10,000 *g* the supernatant liquid was used for assays of proteins (Bradford, 1976), SP (starch phosphorylase) (DeFekete and Vieweg, 1974), ADPGPPase(ADPG pyrophosphorylase) (Nakamura et al., 1989), PPase (inorganic pyrophosphatase) (Gross and ap Rees, 1986), SS (sucrose synthase) (Su et al., 1977), and amylase (Street, 1974).

Amyloplast Isolation

About 50 g of callus was rapidly frozen in liquid nitrogen and pulverized with a mortar and pestle. The powder was slowly added (with gentle agitation, avoiding freezing) to 500 ml of 50 mM HEPES (pH 7.8) buffer containing 5 mM $MgCl_2$, 2 mM EDTA, 0.1% bovine serum albumin, 5 mM DTT (dithiothreitol), 1 mM $CaCl_2$, and 0.3 M mannitol (buffer A). Subsequent operations were performed at 4°C. The suspension was filtered in succession through 300 μm and 64 μm nylon mesh. The filtrate was centrifuged at 190 *g* for 6 min and the supernatant liquid was discarded. The precipitate was suspended in buffer A, then Percoll was added to 30%. Three milliliters of

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the sample was then incorporated as the 30% layer in a discontinuous Percoll gradient of 200, 50, 40, 30, 20 and 10%. The volumes of the layers were 0.5, 2, 2, 3, 2, and 2 ml, respectively. After a 20 min centrifugation at 2,270 *g*, intact amyloplasts in the 40% Percoll layer were collected as the amyloplast fraction (F40). The F40 fraction was washed with buffer A and sedimented by centrifuging at 250 *g* for 10 min. The pelleted plastids were gently resuspended in a small volume of buffer A, stored in an ice bath, and used on the day of preparation.

Analysis of Marker Enzymes

To determine the integrity and purity of isolated amyloplasts, marker enzymes specific for certain subcellular fractions were analyzed. They were StS (soluble starch synthase), G6PDH (glucose 6-phosphate dehydrogenase), 6PGDH (6-phosphogluconate dehydrogenase) (Fowler and ap Rees, 1970), PGM (phosphoglucomutase), PGI (phosphoglucoisomerase), FBPase (fructose bisphosphatase), ADH (alcohol dehydrogenase), G3PDH (glyceraldehyde 3-phosphate dehydrogenase), aldolase, TA (transaldolase), NiR (nitrite reductase) (Bergmeyer, 1974), GluS (glutamate synthase) (Hecht et al., 1988), fumarase (Stitt, 1989), ADPGPPase, SS, SP, and PPase (loc. cit.). The wholesomeness of amyloplasts was measured by the latency of marker enzymes. Latency (L) is the difference in enzyme activities in ruptured (by adding Triton X-100 to a final concentration of 0.1%, v/v) and intact (I) amyloplasts, relative to that of the ruptured (R) and expressed as percentage—$L = [(R-I)/R]\times100$.

Fluorescence Microscopy

To observe the amyloplast membrane by fluorescent microscopy, we used the method described by Echeverria et al. (1985), using FITC (fluorescein isothiocyanate).

Scanning Electron Microscopy

Purified amyloplasts were fixed in buffer A containing 3% (v/v) glutaraldehyde, for 3 h in the cold and dark. The fixed amyloplasts were thoroughly washed with 50 mM phosphate buffer and spread over several cover-glasses. When dry, two drops of acetone were added. The acetone-fixed amyloplasts were dehydrated through a 50, 60, 70, 80, 90, 95, and 100% ethanol series (15 min per step), and incubated in isoamyl acetate overnight. After critical point drying and gold casting, samples were examined in a Hitachi S520.

Reagents

All chemicals were reagent grade, obtained from Merck, Wako, or Sigma. Enzymes required for coupled-enzyme assays were from Sigma. Percoll from Sigma (designated 100%) was concentrated by evaporation in a 120°C oven until its volume was reduced to one-half; this product was designated 200% Percoll. Percoll concentration was calculated on the basis of percentile content of 100% Percoll by volume.

Results and Discussion

Optimization of Tissue Culture Conditions

The growth rate and starch content of calli grown under different conditions are shown in Table 1. Since callus amyloplasts could be isolated regardless of how much starch they accumulated, the conditions that gave the maximum yield of starch during a fixed period of culture was deemed optimum—25°C for 3 weeks in MS medium containing 0.15 ppm NAA, 3% sucrose, and 0.8% agar. In the absence of Ki, the starch content of calli diminished during the third week. The addition of 2 ppm Ki sustained starch accumulation, although at a much slower rate (data not shown). Because culturing for an additional week in the presence of 2 ppm Ki did not offer a significant advantage, Ki was excluded from subsequent cultures. It should be noted that under approximately equal growth conditions, a higher ratio of NAA to Ki (2 ppm/0.2 ppm) resulted in a smaller accumulation of starch than did a lower ratio (1 ppm/1 ppm) (data not shown).

Table 1. Effects of culture medium compositions on callus growth and starch accumulation.

Medium[c]	Growth rate[e] W4/W1[a]	Starch[e] mg/g fresh wt.	H_2O[e] %
N 0.15, B5, 3% Su	2.53	0.42	91.3
N 0.15, MS, 3% Su	6.57[b]	0.66	95.3
Ki 0.2, MS, 3% Su	2.27	0.99	91.7
Ki 2.0, MS, 3% Su	3.51	0.80	94.4
Ki 2, N 0.15, MS, 3% Su	3.31	0.71	94.8
Ki 4, N 0.15, MS, 3% Su	3.26	0.92	92.0
Ki 4, N 0.15, MS, 6% Su	3.28	0.93	92.6
Ki 4, N 0.15, B5, 6% Su	1.57	1.61	89.6
Ki 0.2 , N 2, B5, 3% Su	7.03	N.D.[d]	94.3

[a]Callus weight at the 4th week/callus weights at the time of transfer.
[b]Callus weight at the 3th week/callus weights at the time of transfer.
[c]N, 1-naphthaleneacetic acid; Ki, kinetin; Su, sucrose. The unit of concentration is ppm.
[d]Not Detected.
[e]Values are means of two experiments.

Table 2. Effects of culture conditions on the compositions of callus cultures.

Medium*	Protein[c] mg/g fresh weight	Starch[c] mg/g fresh weight	H_2O[c] %
N 0.15 (D)[a]	1.26	0.66	95.3
Ki 2.0, N 0.15 (D)[a]	2.47	0.71	94.8
N 0.15 (L)[b]	2.20	0.74	94.1
Ki 2, N 0.15 (L)[b]	2.13	0.70	94.6

[a]D, in the dark. [b]L, in the light.
[c]Values are means of two experiments.
*Calli were grown in MS medium containing 3% sucrose.

Table 3. Effects of callus culture conditions on enzyme activities of carbohydrate metabolism.

Medium*	Activity[ab]				
	SP	Amylase	ADPGPPase	PPase	SS
N 0.15 (D)	5.8	0.26	18.7	15.5	16.4
Ki 2.0, N 0.15 (D)	9.4	0.16	8.7	7.7	5.8
N 0.15 (L)	10.5	0.18	13.8	9.0	16.2
Ki 2, N 0.15 (L)	7.6	0.18	12.9	10.1	18.4

[a]Values are means of two experiments.
[b]Specific activities expressed as nmol product produced / min-mg protein.
*Calli were grown in MS medium containing 3% sucrose.

Influence of Light on Callus Culture

When grown under light, the tissue turned green after three weeks and became dark green after three to four subcultures. The greening was not advantageous for starch accumulation (Table 2).

Enzymes of Starch Metabolism in the Starch Accumulating Calli

Crude extracts of the calli were analyzed for enzymes related to starch metabolism. The addition of Ki to the dark-grown callus caused a dramatic decrease of enzyme activities, except for starch phosphorylase (Table 3). The addition of Ki to light-grown calli did not produce this effect. Cultures grown in media without Ki were better suited to our purpose.

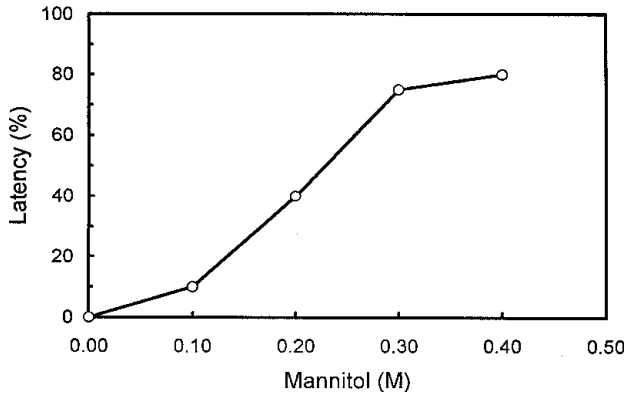

Figure 1. Latency of inorganic pyrophosphatase in the sweet potato amyloplasts prepared under different concentration of mannitol. Buffer A was used.

Optimum Conditions for Amyloplast Isolation

The investigation disclosed that the tissue homogenization procedure, operational temperature, Percoll-gradient-centrifugation conditions, and buffer solutions were satisfactory. We used light microscopy to estimate the yield of amyloplasts and saw that lower temperature and *g* values during centrifugation favored a higher yield, but that overly-low values of either or both hindered the sedimentation of amyloplasts. To maintain the integrity of amyloplasts, mannitol was added to control the osmotic conditions. The optimum concentration of mannitol was determined by the latency of PPase. The results (Figure 1) indicated that 0.3 M mannitol in the buffer solution is sufficient to prevent the plastid bodies from bursting.

Properties of Isolated Amyloplasts

Figure 2 shows a scanning electron micrograph of intact amyloplasts (2A) and one of starch granules (2B). The iodine staining for starch and FITC staining for the membrane of an amyloplast preparation are shown in Figures 3A and 3B, respectively. These images show that the majority of visible particles are spherical bodies surrounded by intact membranes. Results of the analysis of some typical marker enzymes and of enzymes of carbohydrate metabolism of the purified amyloplast are presented in Tables 4 and 5, respectively. The preparations were virtually free of contamination by cytosolic and mitochondrial components and they contained enzymes related to various facets of carbohydrate metabolism. The high latency values for these enzymes are consistent with the intact plastid structures seen by light and electron microscopy. We had previously made rather uniform and smooth 8 μm sections of amyloplasts in sweet potato tuberous roots—the organelle diameter was usually greater than 10 μm. We found

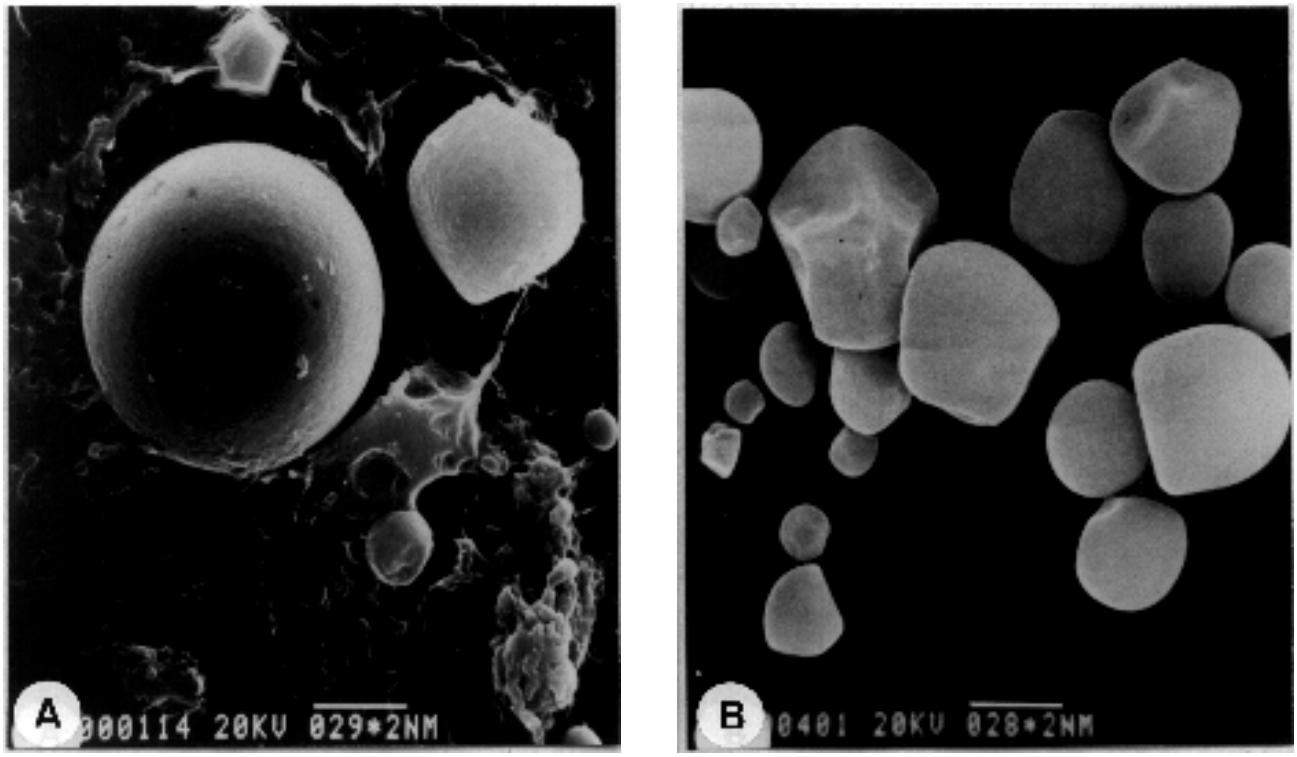

Figure 2. Electron micrograph of amyloplasts (**A**) and starch granules (**B**) isolated from sweet potato callus.

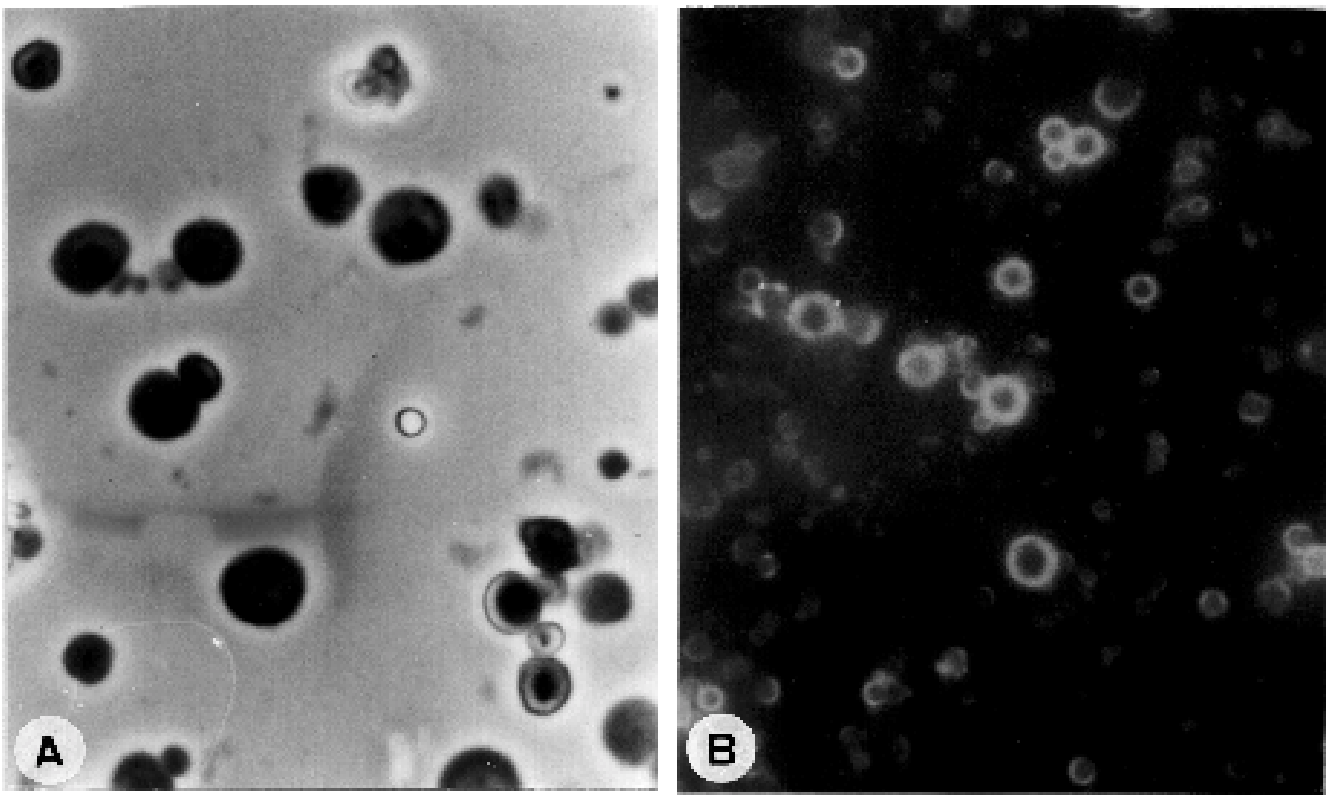

Figure 3. A, Light micrograph of iodine-staining of purified amyloplasts from sweet potato callus. **B**, Fluorescence micrograph of purified amyloplasts. Intact amyloplasts show a FITC-FC fluorescence, but starch granules do not.

Table 4. Enzyme activities of marker enzymes in purified amyloplasts.

Marker enzyme	Associated cell compartment	Activity[ab]		Latency %
		Intact	Ruptured	
6PGDH	Amyloplast	1.5	6.2	76
PPase	Amyloplast	12.2	33.4	64
ADH	Cytosol	0.12	0.20	–
SS	Cytosol	0.23	0.21	0
Fumarase	Mitochondria	N.D.	N.D.	–

[a]Specific activity expressed as nmol products/min-mg protein.
[b]Values are means of duplicate samples.
N.D., not detected.
*Calli were grown in MS medium containing NAA 0.15 ppm and 3% sucrose.

Table 5. Enzyme activities of carbohydrate metabolism in purified amyloplasts from sweet potato callus*.

Enzyme	Activity[ab]		Latency %
	Intact	Ruptured[c]	
Starch synthesis			
St.S	1.3	8.2	83
SP	0.5	1.3	62
ADPGPPase	0.6	1.9	68
Glycolysis			
PGM	30.7	48.3	36
PGI	6.5	13.5	52
Aldolase	2.6	10.1	74
GAPDH	11.5	24.6	59
Pentose-P pathway			
G6PDH	0.3	2.6	88
6PGDH	1.5	6.2	76
TA	2.6	1.3	-

[a]Specific activity expressed as nmol products/min-mg protein.
[b]Values are means of two experiments.
[c]Rupturing of amyloplasts was done by adding 0.1% (v/v) Triton X-100.
*Calli were grown in MS medium containing NAA 0.15 ppm and 3% sucrose.

that 1–3 starch granules filled an organelle, which indicates that the diameter of starch granules was close to 10 μm (Chang et al., 1986). The largest-diameter starch granule shown in Figure 2B is 6 μm. We conclude that the manipulation of tissue culture conditions to obtain amyloplasts containing small starch granules is the key to preparing sweet potato amyloplasts suitable for experiments. How the plastid body so induced in the tissue culture can withstand the drastic treatment of freezing in liquid nitrogen followed by pulverizing in a mortar is an intriguing question.

Acknowledgment. The authors thank the National Science Council of the Republic of China for their financial support.

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甘藷癒傷組織造粉體的製備

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調整甘藷癒傷組織培養條件，可令其堆積澱粉且其顆粒大小適合於製備造粉體之培養物。將組織培養物在液氮中冷凍，在瓷製研缽中加緩衝液及海砂以手工研磨，經不連續 Percoll 梯度進行離心分劃，在 20 %及 50 % Percoll 夾層中之 40 %分劃中分離得到完整的造粉體。進行造粉體標記酵素潛能分析，和使用放射性標幟之澱粉合成系基質進行給與試驗，證明製備所得之造粉體爲適用於澱粉生合成系研究之材料。

關鍵詞：造粉體；組織培養；癒傷組織；潛能分析；澱粉代謝；甘藷。