Characterization of Molecular Structure of Starch Granules in Suspension-cultured Cells from *Ipomoea cordatotriloba* Denn.

Kanefumi Kitahara,† Koki Imamura, Yuko Omae, and Toshihiko Suganuma

*Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*

Received May 6, 1998

The molecular structure of starch granules formed in suspension-cultured cells of *Ipomoea cordatotriloba* Denn. was characterized by its chain length distribution, which was compared to those of the starches from the root and leaf of the original plant. The cultured cell starches were spherical and had a very small granule size (about 2 μm). The debranched starches roughly separated into three fractions during gel-permeation chromatography, and the fractions were defined as Fr. 1, 2, and 3, respectively. The chain length distribution of the debranched cultured cell starch showed that the high molecular weight fraction (Fr. 1), referred to as an amylose fraction, was much less than those of the root and leaf starches. The ratio of the two lower fractions (Fr. 3/Fr.2) of the cultured cell starch, which was mainly derived from unit chains of amylopectin, was greatest among the starches, suggesting that the amylopectin from the cultured cell starch has much shorter unit chains. By X-ray diffraction analysis, it was found that both cultured cell and leaf starch granules have low crystallinity.

**Key words:** starch; cultured cell; chain length distribution; biosynthesis; sweet potato

Starch granules are efficiently synthesized in storage organs such as roots or seeds, therefore such starches have been well studied and characterized among various plant sources. Generally, higher plants also synthesize starch in their leaves, but the storage is temporary. According to recent studies on starch biosynthesis, it seems likely that there are qualitative and quantitative differences in biosynthetic enzymes among respective plants and also among respective organs. Furthermore, it was reported that undifferentiated cells also form starch granules during culturing. However, there is no information on the molecular structure of starch granules formed in cultured cells. It is interesting, therefore, to analyze the starch structure and compare it with those of other organ starches. The characterization of each starch is considered to be important in plant physiology.

Wild relatives of the sweet potato are expected to introduce a certain new character into sweet potato (*Ipomoea batatas* (L.) Lam.). Shiotani et al. examined the starch properties of *Ipomoea trifida* (H.B.K.) Don. and its hybrids with sweet potato cultivars, in which they indicated the utility of the relative for sweet potato breeding to modify the starch properties. Thus, further investigation of the starch properties of other wild relatives is required. Among the wild relatives of sweet potato, the diploid *Ipomoea cordatotriloba* Denn. also has small tuberous roots, but it is cross-incompatible with common cultivars of sweet potato. However, to overcome this incompatibility problem, somatic hybridization through protoplast fusion has been attempted within cross-incompatible groups.

In this study, suspension-cultured cells were prepared from the petiole of *I. cordatotriloba*. Starch formation in the cells was confirmed under culturing with 2,4-dichlorophenoxyacetic acid, and the molecular structure of the cultured cell starch was characterized mainly by the chain length distribution of the debranched starch. The characteristics were then compared to those of the starches from the root and leaf of the original plant.

**Materials and Methods**

Preparation of suspension-cultured cell. An *I. cordatotriloba* plant grown in vitro was donated from the Laboratory of Plant Breeding, Faculty of Agriculture, Kagoshima University, Japan. The petioles of the plant were cut into 3-mm lengths and put on Murashige-Skoog (MS) medium containing 0.05 ppm 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 ppm kinetin, 3% sucrose and 0.8% agar. After 4 weeks, the induced callus was cut into small pieces and transferred to liquid medium (MS medium containing 1.1 ppm 2,4-D, 30 mM potassium chloride and 3% sucrose at pH 5.8). The suspension was cultured at 27°C under rotary shaking (MNS-3000, Eyela, Tokyo, Japan) at 100 rpm in the dark, and subcultured for a period of 2 weeks.

All cultures were done under aseptic conditions.

Growth of cultured cells and their starch content. After the suspension cells were cultured for 2 weeks, they were collected on a Nalgene filter holder (Inox Toei Co., Tokyo, Japan) with a 42-mesh screen, and washed with a 3% sucrose solution. The collected cells (1–2 g, wet basis) were inoculated into 150 ml of the same liquid medium with sucrose (3–7%) in a 500-ml Erlenmeyer flask. The exact weight of the cells inoculated was estimated as the difference in the total weight of the flask between before and after inoculation. A sample of the fresh cells was lyophilized to measure the moisture content, and the dry weight of the inoculated cells was calculated.

† To whom correspondence should be addressed. FAX: +81-99-285-8639; E-mail: kitahara@chem.agri.kagoshima-u.ac.jp
The suspension cells were cultured at 27°C under rotary shaking in the dark. After culturing for 1–4 weeks, the cultured cells were collected using the same procedure as above, washed with distilled water and then lyophilized. Growth of the cells was expressed as the ratio of dry weight of inoculated cells to that of grown cells.

The starch content of the cultured cells was measured as a glucoamylase-digestible polysaccharide. The lyophilized cells (50 mg) were boiled in distilled water (5 ml) in a glass homogenizer for 20 min and homogenized for 5 min. The contents were treated by adding 1% Termamyl 120L (1 ml) (Novo-Nordisk A/S, Denmark) for 10 min at 95°C. They were filtered up to 10 ml and centrifuged at 12,000 rpm for 10 min. The supernatant (0.1 ml) was treated by glucoamylase (1 U/ml, 50 mM sodium acetate buffer, pH 5.0) (Rhizopus niveus, Seikagaku Kogyo Co., Ltd., Tokyo, Japan) for 6 hr at 37°C. After heating to stop the reaction, glucose in the reaction mixture was measured by the glucose oxidase method. The procedure in the absence of glucoamylase was used as a blank.

Starch extraction. The lyophilized cells (cultured for 2 weeks, about 0.5 g) were homogenized in a glass homogenizer with distilled water (10 ml) for 3 min in an ice bath. The homogenized cells were passed successively through 200 and 400-mesh screens, and the through fraction was centrifuged at 4,000 rpm for 10 min. The precipitate was washed with cold distilled water and recollected by centrifugation. The precipitate was suspended in distilled water with a small portion of toluene, agitated vigorously, and centrifuged. The precipitate was washed with distilled water twice. The white starch fraction in the precipitate was put on the top of Percoll (1 ml of suspension per 10 ml of Percoll, Pharmacia Biotech, Tokyo, Japan) in a centrifuge tube and centrifuged at 12,000 rpm for 10 min. The Percoll treatment was repeated twice. The precipitated starch fraction was washed with distilled water and lyophilized. The yields of the starch granules based on the starch contents were generally 60–70%, and the preparation was repeated several times to obtain more than 50 mg of the starch granules.

Seedlings of *I. cordatotriloba* were planted in a pot which was filled with vermiculite and monthly supplemented with liquid fertilizer (Takeda Aid 2, Takeda Garden Products Co., Ltd., Tokyo, Japan). The green plants were cultivated in a greenhouse of our university in a natural environment (from May 10 to August 27 in 1997). The leaves were homogenized in aqueous 70% ethanol by an Ace-Homogenizer (Nihon Seiki Seisakusyo, Tokyo, Japan), and it was passed successively through 200 and 400-mesh screens. After centrifugation at 4,000 rpm for 10 min, the precipitate was suspended in distilled water at below 4°C and centrifuged. The precipitate was treated with toluene and Percoll as mentioned above. The fresh leaves (3.6 g) yielded about 80 mg of the starch. The roots (15.3 g), including fibrous roots, were homogenized in distilled water. The starch was purified as mentioned above, and about 100 mg of the starch was obtained.

Starch proprieties. The chain length distribution of the starch after debranching by isoamylase was measured through two linked columns of Superose 12 (Pharmacia Biotech) and Sephadex G25 (Pharmacia Biotech) using HPLC. The chromatogram was divided into three fractions. The column system was calibrated using synthesized linear amylloses (DP = 728, 438, 165, 63, Ajinoki, Aichi, Japan), Amylose EX-1 (DP = 17, Hayashibara Biochemical Laboratories, Okayama, Japan) and maltopentaose (Nacalai Tesque Inc., Kyoto, Japan). The debranched starch sample was also analyzed using high-performance anion-exchange chromatography (HPAEC). The debranched starch (500 µg) was dissolved in 1 ml of sodium hydroxide solution to make a 150 mM alkali concentration. The sample (25 µl) was injected onto a Dionex DX-500 with a pulsed amperometric detector (PAD, Nippon Dionex K.K., Osaka, Japan). A column of CarboPac PA-1 (Nippon Dionex K.K.) was eluted at a flow rate of 1.0 ml/min with a gradient elution of 150 mM sodium hydroxide and 150 mM sodium hydroxide containing 500 mM sodium acetate solutions as follows: the percentage of 150 mM sodium hydroxide containing 500 mM sodium acetate solution was 40 at 0 min, 50 at 5 min, 70 at 25 min, 80 at 55 min, and 100 at 60 min.

A scanning electron micrograph was obtained using a Jeol JSM-5300 (Jeol Ltd., Tokyo, Japan) at 20 kV after gold coating of the starch sample. An X-ray diffractogram was obtained using an X-ray diffractometer (RotaFlex RU-200B, Rigaku, Tokyo, Japan) as previously reported.

Results and Discussion

Formation of starch granules in suspension-cultured cells

Figure 1 shows the growth of the suspension-cultured cells and the change in starch content of the cells at different sucrose concentrations. The cells grew well in all liquid media, and the dry matter of the suspension cells increased to about 15-fold those of the inoculated ones at the end of culturing. On the other hand, the starch content in the cells increased in a week and then decreased. It appeared that the starch granules are synthesized for storage before cell proliferation under these culture conditions. The production of the starch was greatest in 7% sucrose medium. The increase in starch content induced by increasing sucrose concentration agreed with the results of sweet potato callus. Thus, the formation of starch granules was confirmed in the case of suspension-cultured cells prepared from the petiole of *I. cordatotriloba*.

Structural features of starch granules in each organ

Iodine staining and microscopic observation found starch granules in the roots and leaves of *I. cordatotriloba*. Starch granules in leaves have been reported for spinach, but there is no structural information. In addition to such organ starches, starch granules were also prepared from cells cultured for 2 weeks. Figure 2 shows scanning electron micrographs of the starches extracted from the root, leaf, and cultured cell in 3% sucrose.
medium. The starch granules in the cultured cell were very small (1–3 μm) and spherical. The starch granules of the root were large (5–15 μm) and bell- or polygonal-shaped, while those of the leaf were flat and irregular in shape (3–7 μm).

Figure 3 shows the chain length distributions of debranched starches from root, leaf, and cultured cell. The elution profiles can be divided into three fractions, the high molecular weight fraction (Fr.1) and the two lower fractions (Fr.2 and Fr.3), which are generally referred to as amylose fraction and unit-chain fractions of amylpectin, respectively. The proportion of each fraction is summarized in Table 1.

<p>| Table 1. Chain Length Distributions of Debranched Starches from Root, Leaf, and cultured Cells |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Starch Sample</th>
<th>Fr.1 (%)</th>
<th>Fr.2 (%)</th>
<th>Fr.3 (%)</th>
<th>Fr.3/Fr.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>19.0</td>
<td>31.2</td>
<td>49.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Leaf</td>
<td>25.3</td>
<td>21.1</td>
<td>53.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Cell</td>
<td>3% Sucrose</td>
<td>4.6</td>
<td>25.3</td>
<td>70.1</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>5.5</td>
<td>24.9</td>
<td>69.6</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>6.0</td>
<td>24.6</td>
<td>69.4</td>
</tr>
<tr>
<td>a: Fractionation of the distribution is shown in Fig. 3.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b: Cultured for 2 weeks.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It was interesting to note that the amount of Fr.1 of the cultured cell starch was much less than those of the root and leaf starches, regardless of sucrose concentration in the medium. The ratio of Fr.3 to Fr.2 (Fr.3/ Fr.2) of cultured cell starches (2.8) was greatest among the starches, indicating that the cultured cell starch has an abundance of shorter unit chains. The high value for Fr.3/Fr.2 of cultured cell starch was rather similar to those of normal cereal starches on the same gel-permeation chromatography (GPC). Fr.1 of the root starch amounted to 19%, which was comparable with those of common sweet potato cultivars (21.0±1.2). However, the value of Fr.3/Fr.2 (1.6) was smaller than those of sweet potato starches (2.2) and was rather similar to potato starches (1.3–1.5, unpublished data). In the case of leaf starch, the amount of Fr.1 was high (25.3%) and the value of Fr.3/Fr.2 (2.5) was larger than that of root starch, in which a distinct multiple distribution was found in Fr.3 (Fig. 3B).

The short unit chains [degree of polymerization (DP) 3–35] of the debranched starch were also analyzed using a HPAEC-PAD. The distribution is expressed as the relative area of each peak and is shown in Fig. 4. Among the cultured cell starches at different sucrose concentrations, no difference in distribution was found. The distri-
Fig. 3. GPC Profiles of Debranched Starches from Root, Leaf, and Cultured Cells.
A: root starch, B: leaf starch, C: cultured cell starch (2 weeks after culturing in 3% sucrose concentration), D: cultured cell starch (2 weeks after culturing in 5% sucrose concentration), E: cultured cell starch (2 weeks after culturing in 7% sucrose concentration)

Distribution for the cultured cell starch at 3% concentration is shown in the figure. In all organ starches, distribution peaked at DP 13 with a hollow at DP 8, which is common for sweet potato amylopectins. On the other hand, chains with DP 3 and 4 were found in the leaf and cultured cell starches. It was also observed that the distribution of leaf starch had a distinct shoulder at DP 18.

The difference in the distribution of the respective organ starches was estimated by subtracting the relative area of each chain of root starch from those of leaf and cultured cell starches. As can be seen in Fig. 5, the differences in the distributions of the leaf and cultured cell starches from that of the root were clearly seen. In both starches, the chains around DP 6 were rich, while those around DP 13 and 18–20 were poor in amount as compared with those of the root starch, although the absolute values of each peak from the two starches were different. Hanashiro et al. examined the chain length distribution of amylopectins from various plant sources, in which they found the same periodicity from different sources by the same analysis as above. In the paper, they discussed that the same periodicity may suggest that the respective isomers of branching enzymes have the same or close action specificities and the characteristic distribution may be caused by variable proportions of the enzymes by sources. The similar periodicity found in the organ starches may also suggest a quantitative difference in the starch-biosynthetic enzymes in the respective organs.

In general, sweet potato starch has the Ca type on an X-ray diffractogram, as shown in Fig. 6A. Interestingly, only weak peaks were found in the diffractograms from the leaf and cultured cell starches, but judging from the peaks at 5.6 and 17–18°, they had faint B type and A type crystallites, respectively. The result indicates that the starch molecules in the leaf and cultured cell starches are packed into granules in an amorphous state. On the other hand, the root starch of I. cordatotriloba showed the Cc type as characterized by the diffraction peaks at 2θ=17–18°, and the intensity was similar to that of the sweet potato cultivar. Unusual sharp peaks were found in the diffractogram, which were identified as those from crystallites of vermiculite and calcium oxalate monohydrate upon comparison with authentic samples. Vermiculite was used as a material in plant cultivation, while calcium oxalate monohydrate was reported as a
Thus, each organ starch of *I. cordatotriloba* has been characterized in this study. It was found that the root starch of *I. cordatotriloba* was structurally distinct from those of sweet potato cultivars. Asante *et al.* reported that several clonal strains of diploid *I. trifida* have starch granules with higher amyllose contents. These wild relatives are expected to be used for sweet potato breeding to modify its starch properties.

Taking into account the general concept for the chain length distribution of debranched starch as well as the elution profiles of sweet potato amylose and amylpectin, it is concluded that the cultured cell starch is low in amyllose and abundant in short chains of amylpectin as compared with the root starch. In addition to these structural properties, the cultured cell starch also showed low crystallinity. The cultured cell starch appears to be similar in structural properties to leaf starch both in having low crystallinity and abundant short chains of amylpectin, but this cannot be said of their amyllose contents.

The findings of the structural differences among each organ starch would be important to analysis of the organ-specific biosynthesis of the starch granules. The discovery of the mechanism of starch biosynthesis has proceeded, but several unclear points remain. The studies on starch granules in cultured cells are interesting because the dynamic metabolism of starch granules, that is, its synthesis and degradation, can be observed during culturing. Further works are in progress to investigate relationships among productivity of starch in the cells, culturing conditions, starch properties, and the related enzymes.

**Acknowledgments**

This study was done through Special Coordination Funds for Promoting Science and Technology (Leading Research Utilizing Potential of Regional Science and Technology) of the Science and Technology Agency of the Japanese Government.

We thank Professor T. Kokubu for kindly donating plants of *I. cordatotriloba*, and Mr. T. Sakai for his technical assistance in culturing the suspension cells.

**References**


