

Diurnal-Nocturnal Changes of Assimilated Starch Structures in Sweetpotato Leaves

(Received August 19, 2003 ; Accepted November 12, 2003)

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Abstract: Sweetpotato leaves cultivated for three months were harvested at different times for two days, and starch structures in the leaves were examined. The starch content changed in a circadian rhythm, with the minimum at 05:00 and the maximum at 17:00. The starches in the leaves were extracted by maceration of the leaf debris in hot dimethyl sulfoxide and prepared by selective precipitation by iodine. Gel-permeation chromatography of the leaf starches and amylopectins on Toyopearl HW-75F revealed that the leaf starches had a higher amount of material eluted at a low molecular weight fraction compared to that of its root starch, and the low molecular weight fraction contained both amylose molecules and a large amount of small amylopectin molecules. It was also found that both molecular weight distributions of the leaf starch and amylopectin were shifted to high molecular weight during the daytime and to low molecular weight during the period from sunset to sunrise. After debranching the starches and amylopectins, the chain-length distributions and short-chain distributions were analyzed by high-performance gel-permeation chromatography and high-performance anion-exchange chromatography, respectively. The chain-length distributions of the leaf starches were clearly distinct from those of the root starch. The amylose content of the leaf starches, which was evaluated as the difference in long-chain fraction on chain-length distribution between starch and amylopectin, was low during the daytime, whereas the branch aspect of the leaf amylopectin was relatively constant throughout the whole day. Thus, the structural characteristics of the starch in sweetpotato leaf and their diurnal-nocturnal changes have been revealed.

Key words: leaf starch, sweetpotato, amylose, amylopectin

Higher plants generally synthesize starches in both photosynthetic tissues of leaves and storage organs such as seed or root, and the starches often refer to assimilated (or transitory) starch and reserved (or storage) starch, respectively. Since the storage organs have a significant role in agronomy and human nutrition as food, there have been extensive studies on the chemical structural aspects of their starches, but fewer on starches in leaves.

Among studies on leaf starches, starches from tobacco and cotton leaves have been relatively well examined. It was reported that the starch content in tobacco leaves increased during the daytime, and the amylose content was lower during the daytime than at night.^{1,2)} For cotton plants grown in a chamber, it was observed that the starch content increased accompanied by a preferential accumulation of amylopectin during the light period.³⁾ Furthermore, Matheson⁴⁾ investigated the molecular structures of tobacco leaf starch in detail as follows: firstly the amylose content, ranging from 15.7 to 17.2%, was low during the daytime; secondly the amylose fractions had small differences in average molecular size at different times, whereas the average molecular size of amylopectin decreased during the daytime and increased at night; and finally the chain-length distribution of amylopectin was similar throughout the whole day. On the other hand, it has been reported that the leaf starches from pea⁵⁾ and

*Arabidopsis*⁶⁾ contain amylopectin almost exclusively. Thus, it seems that the structural aspects of leaf starches are diverse among the respective plant species and more dynamic than those of starches from storage organs. Since the studies on starch structures in leaves have been limited to a few species, further investigations of leaf starches are required.

In this paper, we describe structural differences between starches from the leaf and tuberous root of the sweetpotato. The leaf starches and their amylopectins were prepared from leaves harvested at different times, and the diurnal-nocturnal changes in structure were examined in terms of both molecular weight distribution and chain-length distribution.

MATERIALS AND METHODS

Materials. Fifty seedlings of sweetpotato (*Ipomoea batatas*, cv. Koganesengan) were planted in an experimental field of Kagoshima University in June 2002. After cultivation for three months, five leaves with similar sizes (13 cm in length) were detached randomly from the middle of the runner of the independent plants at different times over two days. The leaves were immediately frozen in liquid nitrogen and lyophilized. The tuberous roots were cropped at the end of October.

All reagents and solvents, unless otherwise specified, were obtained from Wako Pure Chemical Industries (Japan) and were of analytical grade. Water was treated by

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passing it through a Milli-Q Labo (Nihon Millipore Ltd., Japan) prior to use.

Preparation of starch and amylopectin. The starch content of the lyophilized leaf was measured as a glucoamylase-digestible polysaccharide, as previously reported.⁷⁾

Starch granules were prepared from the tuberous root according to our previous reports.⁸⁾ Leaf starch was prepared using a procedure reported by Matheson⁹⁾ with our modification. The five lyophilized leaves were ground using a MILLSER IFM-750G (Iwatani Ltd., Japan). The powdered leaf (1 g) was immersed in acetone (50 mL) and decolorized overnight. The leaf debris was softened in dimethyl sulfoxide (50 mL) at 100°C for 30 min under bubbling nitrogen gas and macerated in a glass homogenizer (Iwaki, Japan). The extract was collected by centrifugation (2800 × *g*, 10 min), while the residual debris was re-extracted with dimethyl sulfoxide by the homogenizer. The combined extract was poured into ethanol (300 mL) with a slight amount of lithium chloride and left to stand overnight. The precipitate was collected by centrifugation. The crude starch was extracted from the precipitate with 1 M sodium chloride (25 mL) at 100°C for 30 min under bubbling nitrogen gas, and the extraction was repeated twice. After centrifugation, an excessive amount of iodine (3% iodine in 30% potassium iodide solution, 2 mL) was added to the supernatant, which was left to stand overnight. The starch-iodine precipitate was dissolved in a solution of 0.1 M sodium arsenite in 0.2 M sodium phosphate buffer (pH 6.0, 20 mL), and then the solution was dialyzed against water. The dialyzate was poured into ethanol (80 mL) with a slight amount of lithium chloride and left to stand overnight. After centrifugation, the precipitate was washed with ethanol repeatedly and dried in a vacuum desiccator.

Amylopectin was prepared from a supernatant after the removal of amylose by butanol complexing.¹⁰⁾

Molecular weight distribution of starch and amylopectin. The defatted starch⁸⁾ or amylopectin (10 mg) was dissolved in 1 M sodium hydroxide (5 mL) at 4°C. The solution was diluted twice with water and an aliquot (2 mL) was applied to a column (1.3 × 48 cm) packed with Toyopearl HW-75F (Tosoh Corporation, Japan). The column was eluted with 0.2% sodium chloride in 50 mM sodium hydroxide at a flow rate of 60 mL/h. Each eluate (2 mL) was neutralized with 1 M acetic acid (0.3 mL), and the total carbohydrate content of an aliquot (1 mL) was determined by measuring the glucose according to the phenol-sulfuric acid method.¹¹⁾ On the other hand, 1% iodine in 10% potassium iodide solution (20 μL) was added to the remaining eluate (1.3 mL), and the wavelength at maximum absorption of the starch-iodine complex (λ_{\max}) was measured in the range of 400–700 nm using a spectrophotometer (U-3310, Hitachi, Japan).

Chain-length distribution of debranched starch and amylopectin. After debranching starch or amylopectin by isoamylase (Nacalai Tesque Inc., Japan), the chain-length distribution of the debranched sample was determined by both high-performance gel-permeation chromatography¹²⁾ (GPC) using two linked columns of Superose 6 and Sephadex G25SF (Amersham Bioscience K.K., Japan)

and high-performance anion-exchange chromatography⁷⁾ (HPAEC) using a CarboPac PA-1 column (Nippon Dionex K.K., Japan).

RESULTS AND DISCUSSION

Change in starch content in sweetpotato leaves.

The sweetpotato leaves were harvested at 05:00 (before dawn), 09:00, 13:00, 17:00, 20:00 (after dusk) and 00:00 over two days. Figure 1 shows the change in starch content of the lyophilized leaves at different times. The starch content was the minimum at 05:00 and the maximum at 17:00 on both days, and the minimum content amounted to 7% at 05:00, which indicated that the starches do not disappear completely during night period. The diurnal-nocturnal change in the starch content of sweetpotato leaves was similar to the result obtained for tobacco leaves.¹⁾ It was also reported that the starch remaining in leaves at night was different depending on leaf age¹⁾ and leaf position.¹³⁾ Thus, a circadian rhythm of starch content was observed in sweetpotato leaves.

Starch preparation.

Previously we carried out starch extraction from leaves of a wild sweetpotato relative simply by macerating the leaves, and it was easy and successful.⁷⁾ However, the application in this study for leaves of sweetpotato cultivar was unsuccessful, resulting in a low starch yield of less than 10%. A microscopic observation of the residues after starch extraction revealed that the starches were still trapped in the cells. It seems that the leaves of sweetpotato cultivars have a strong cell wall, which causes difficulty in starch extraction by simple maceration. Therefore, the starches in the leaves were extracted by maceration of the leaf debris in hot dimethyl sulfoxide and prepared by selective precipitation by iodine. To check damage to the starch structure by the extraction process, starch granules from the tuberous root were treated using the same procedure as for leaf starch extraction. Both molecular weight distributions and chain-length distributions after debranching were practically identical before and after treatment (data not shown), demonstrating a negligible, if any, degradation of starch molecules during the process. Consequently, the starches were extracted from the leaf samples harvested at different times of the first day, which yielded 60–85% of the respective starch contents.

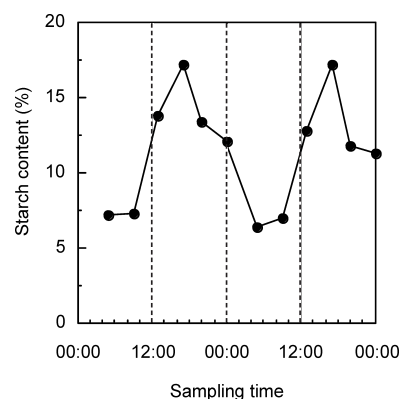


Fig. 1. Diurnal-nocturnal change in starch content in sweetpotato leaves over two days.

Molecular weight distribution.

Figure 2 shows the molecular weight distributions of the root starch and its amylopectin on a Toyopearl HW-75F column, as well as the λ_{\max} of starch-iodine coloration in each fraction. The distribution of root starch showed a typical elution profile having a large amount of high molecular weight fraction and a small amount of low molecular weight fraction. The high and low molecular weight fractions have been shown to mainly correspond to amylopectin and amylose, respectively.¹⁴ As shown in Fig. 2B, the root amylopectin was eluted almost at the high molecular weight fraction.

The elution profiles of the leaf starches were quite distinct from that of the root starch as seen in Fig. 3. The molecular weight distributions of the leaf starches showed a considerable amount of material eluted at the low molecular weight fraction. The material eluted at the lower molecular weight fraction had a higher λ_{\max} , suggesting the existence of long linear glucans in this region. After removing amylose by butanol complexing, the molecular weight distributions of the isolated amylopectins were also examined. The leaf amylopectins distributed themselves over a wide range on the column, unlike the root amylopectin. The λ_{\max} at the lower molecular weight fraction decreased because of the removal of amylose. These results suggest that the low molecular weight fraction of the leaf starches contains both amylose molecules and a large amount of small amylopectin molecules.

Diurnal-nocturnal changes in the molecular weight distributions of the starch and amylopectin were found. On the starch distributions, the amount of material eluted at the lower molecular weight fraction was high at 05:00 and 09:00. The proportion of the low molecular weight fraction decreased at 13:00 and 17:00, and then increased

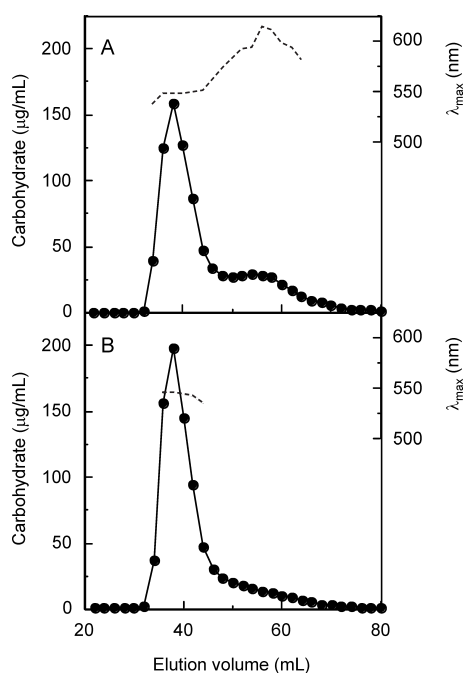


Fig. 2. Molecular weight distributions of starch and amylopectin from sweetpotato root by GPC on Toyopearl HW-75F.

(A) starch, (B) amylopectin. Solid line with closed circles indicates carbohydrates, and dotted line indicates the λ_{\max} in each eluate.

at 20:00 and 00:00. The diurnal-nocturnal change in the molecular weight distribution of amylopectin showed a similar tendency to that of the starches. The distribution of amylopectin was shifted to high molecular weight during the daytime and shifted to low molecular weight during the period from sunset to sunrise. The result indicates that the average molecular weight of amylopectin increases during the daytime. Contrary to our result, it was reported that the average molecular weight of amylopectin from young tobacco leaves decreased during illumination, where it was speculated that low molecular weight materials were preferentially depleted in darkness and preferentially accumulated on illumination.⁴ The reason for this discrepancy is not clear, but since the whole molecular weight distribution would be determined by a relative balance between the creation of fresh molecules and growth of molecules during the starch synthesis, such a balance may be characteristic to the respective plant species or leaf age.

Chain-length distribution.

After debranching the starches and amylopectins with isoamylase, the chain-length distributions and short-chain distributions were determined by GPC and HPAEC, respectively. Figure 4 shows the chain-length distributions on GPC and division of the chromatograms into three fractions, Frs. 1, 2 and 3. The proportion of each fraction is summarized in Table 1. The elution profiles in Fr. 1 of the leaf starches were distinct from that of the root starch,

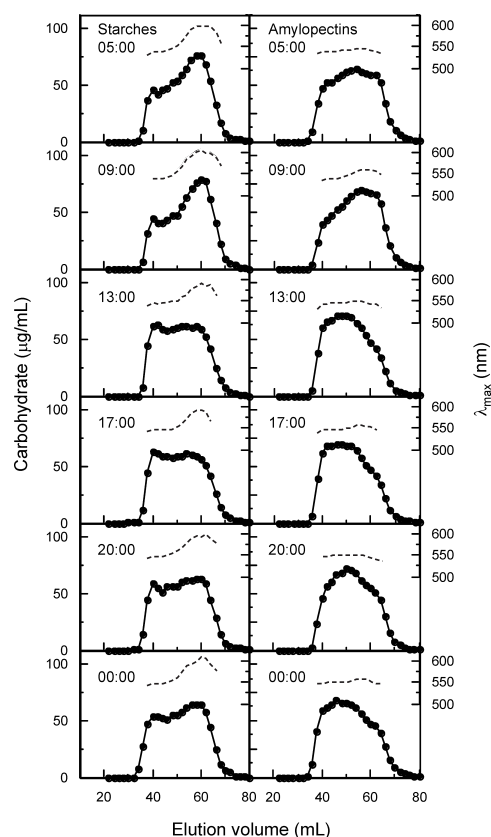


Fig. 3. Molecular weight distributions of starches and amylopectins from sweetpotato leaves at different times by GPC on Toyopearl HW-75F.

Solid line with closed circles indicates carbohydrates, and dotted line indicates the λ_{\max} in each eluate.

showing a lower amount of long chains eluted at void volume and a higher amount of the succeeding intermediate chains eluted at the middle of Fr. 1. A characteristic distribution was also observed in Fr. 3 of the leaf starches. As shown in Table 1, the values of Fr. 3/Fr. 2 of the leaf starches were lower than that of the root starch.

On the distribution of the root amylopectin, most of Fr. 1 disappeared, as a result of the removal of amylose molecules. When the distributions were compared be-

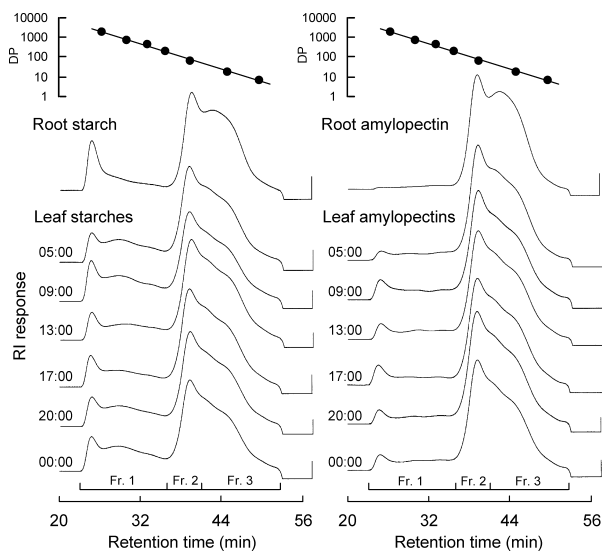


Fig. 4. Chain-length distributions of debranched starches and amylopectins from sweetpotato root and leaves at different times.

The estimated degree of polymerization (DP) from the calibration standards is shown above the elution profiles.

Table 1. Fractions of debranched starches and amylopectins from sweetpotato root and leaves at different times determined by GPC.

	Fr. 1* (%)	Fr. 2* (%)	Fr. 3* (%)	Fr. 3/ Fr. 2	Δ Fr. 1** (%)
Starches					
Root	19.6	30.4	50.0	1.6	15.7
Leaves					
05:00	23.8	31.1	45.1	1.5	14.9
09:00	28.3	29.8	41.9	1.4	14.4
13:00	18.8	32.2	49.0	1.5	7.0
17:00	19.8	31.6	48.6	1.5	7.4
20:00	23.1	31.2	45.7	1.5	12.6
00:00	27.1	29.9	43.0	1.4	14.6
Amylopectins					
Root	3.9	36.5	59.6	1.6	—
Leaves					
05:00	8.9	37.5	53.6	1.4	—
09:00	13.9	36.4	49.7	1.4	—
13:00	11.8	34.0	54.2	1.6	—
17:00	12.4	35.1	52.5	1.5	—
20:00	10.5	36.5	53.0	1.5	—
00:00	12.5	37.3	50.2	1.3	—

* Fractionation of each fraction is shown in Fig. 4. ** Difference in Fr. 1 contents of the starch and amylopectin.

tween the leaf starches and leaf amylopectins, a part of the chains at the void volume and most of the succeeding intermediate chains in Fr. 1 were estimated to be amylose molecules. The existence of intermediate chains of amylose was characteristic of the leaf starches, which indicated that the leaf amylose has a lower average molecular weight than the root amylose. The amylose content of the starches was therefore evaluated as a difference in the respective amounts of Fr. 1 between the starch and amylopectin. As shown as Δ Fr. 1 in Table 1, the leaf starches of sweetpotato had amylose to some extent, although it was reported that leaf starches of pea⁵⁾ and *Arabidopsis*⁶⁾ contain amylopectin exclusively. The amounts of amylose were lower during the daytime than at night, which was consistent with the results from tobacco leaves^{1,2,4)} and cotton leaves.³⁾ Since a selective degradation of amylose molecules is unlikely when the starch content is increasing during the daytime, it is considered that amylopectin molecules are preferentially synthesized during the daytime.

On the other hand, it is known that amylopectins from some reserved starches such as rice and maize¹⁵⁾ as well as sweetpotato^{8,16)} have amylose-like long chains. There were also observed the long chains eluting in Fr. 1 from the leaf amylopectins, and the amounts were rather larger than that of the root amylopectin. The chain-length distribution of leaf amylopectin has been so far determined only for the tobacco leaf.⁴⁾ Although the preparation method for amylopectin and the GPC conditions were different from those in this study, the tobacco leaf amylopectins also had 6% to 9% of the long chains in the molecules. Thus, the existence of the long chains in leaf amylopectins would be of interest in studying organ-specific biosynthesis of starches.

Figure 5 shows the short-chain distributions of the leaf starches with DP 6–40 at different times as compared to that of the root starch. The distributions of the leaf starches were different from that of root starch; in particular, a distinct shoulder from DP 18 was observed in the leaf starches, which was common to the leaves of pea,⁵⁾ *Arabidopsis*⁶⁾ and a sweetpotato relative.⁷⁾ Among the leaf starches at different times, the distributions scarcely changed throughout the whole day. Thus, a diurnal-nocturnal change in amylose content was found, whereas no significant difference in amylopectin structures such as Fr. 3/Fr. 2 or short-chain distribution was found in terms

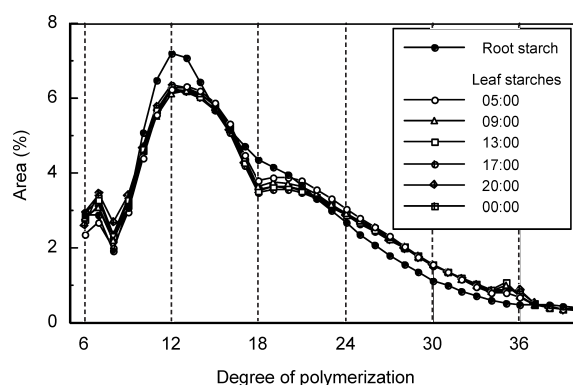


Fig. 5. Short-chain distributions of debranched starches from sweetpotato root and leaves at different times.

of chain-length distribution.

In this study, the structural characteristics of leaf starches from sweetpotato and their diurnal-nocturnal changes have been revealed. The starch content in the leaves changed in a circadian rhythm, showing that the content increased during the daytime and decreased during the night. Analyses by GPC and HPAEC revealed that the leaf starches were composed of both amylose and amylopectin, and that the molecular weight distribution, chain-length distribution and short-chain distribution of the leaf starches were distinct from the respective distributions of the tuberous root starch. With respect to the diurnal-nocturnal changes in the leaf starch structures, it was observed that the amylose content of the leaf starches decreased, and the molecular weight distribution of the leaf amylopectin was shifted to high molecular weight during the daytime, which suggests that amylopectin molecules are preferentially synthesized during the daytime. On the other hand, the branch aspect of the leaf amylopectin was relatively constant throughout the whole day. These findings would contribute to the elucidation of organ-specific biosynthesis of starches.

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サツマイモの葉における同化デンプンの分子構造変化

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3カ月間栽培したサツマイモの葉を時間ごとに2日間に渡って採取し, 含まれるデンプンの構造を調べた. 葉のデンプン含量は, 夜明け前5時を最小, 夕方5時を最大とする概日リズムが認められた (Fig. 1). 葉のデンプンは, 組織を熱ジメチルスルホキシド中で摩砕することにより抽出し, ヨウ素により特異的に沈澱させて調製した. デンプンとアミロペクチンのトヨーパール HW-75F ゲル濾過分析により, 葉のデンプンは, 塊根のものに比べて, 低分子側の溶出量が多く, この低分子画分には, アミロースと多量の低分子アミロペクチンが存在することが明らかになった (Figs. 2, 3). また, 葉のデンプンとアミロペクチンの分子量分布は, いずれも昼間に高分子側に移行し, 日没から明け方にかけて低分子側に移行した (Fig. 3). さらに, 枝きりしたデンプンとアミロペクチンの鎖長分布と短鎖分布を, それぞれ高速液体ゲル濾過クロマトと高性能陰イオン交換クロマトにより分析した. 葉のデンプンの鎖長分布は, 塊根のものとは明らかに異なっていた (Figs. 4, 5). デンプンとアミロペクチンの鎖長分布における長鎖画分の差として評価したアミロース含量は, 昼間が低く (Table 1), 一方, 主にアミロペクチンを構成する短鎖分布は, 昼夜における変動がほとんど認められなかった (Fig. 5). このように, サツマイモの葉の同化デンプンについて, 構造上の特徴と, その昼夜における変化を明らかにした.