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# Characterization of Potato Leaf Starch

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The starch accumulation−degradation process as well as the structure of leaf starch are not completely understood. To study this, starch was isolated from potato leaves collected in the early morning and late afternoon in July and August, representing different starch accumulation rates. The starch content of potato leaves varied between 2.9 and 12.9% (dry matter basis) over the night and day in the middle of July and between 0.6 and 1.5% in August. Scanning electron microscopy analyses of the four isolated starch samples showed that the granules had either an oval or a round shape and did not exceed 5 $\mu$m in size. Starch was extracted by successive washing steps with dimethyl sulfoxide and precipitated with ethanol. An elution profile on Sepharose CL-6B of debranched starch showed the presence of a material with a chain length distribution between that generally found for amylose and amylopectin. Amylopectin unit chains of low molecular size were present in a higher amount in the afternoon than in the morning samples. What remains at the end of the night is depleted in specific chain lengths, mainly between DP 15 and 24 and above DP 35, relative to the end of the day.

**KEYWORDS: Leaf starch; potato starch; starch extraction; starch accumulation; DMSO**

## INTRODUCTION

One of the most abundant and universally distributed forms of storage polysaccharide is leaf starch, also known as transient starch (*1*). Leaf starch is synthesized during the course of a single photoperiod rather than over days or weeks, as in storage organs. It is accumulated during the day in the green plant leaf cells and is used at night to achieve a more or less constant supply of sucrose to the nonphotosynthetic tissues. The dissolution of insoluble leaf starch granule material seems to be performed by endoamylases (*2*). Starch-bound phosphate groups have been suggested to define the initial points of attack and hence provide a signal for starch-degrading enzymes (*3*−*5*).

Leaf starch granules are smaller than granules found in storage organs and vary in size depending on the botanical source, i.e., from 0.2 to 7 $\mu$m for pepper (*6*) and from 1 to 2 $\mu$m for Arabidopsis (*7*). GPC analyses have shown that leaf starch contains both branched glucans of high molecular weight and relatively unbranched glucans of lower molecular weight, corresponding to amylopectin and amylose, respectively (*8*, *7*). Leaf starches generally appear to have lower amylose contents than storage starches. The starches in leaves of, for example, rice and potato contain less than 15% amylose (*9*), whereas for cotton leaves the content varies between 9 and 27% (*10*).

In cotton leaves, it has been found that amylopectin and amylose contents together with the amylopectin/amylose ratio increase during daytime and decrease during night (*10*). An increase of amylopectin/amylose ratio during daytime is in agreement with data from tobacco leaves prior to maturity but differs from the ratio in mature tobacco leaves (*10*).

Debranched amylopectin from Arabidopsis and pea leaf starches shows a more strongly polymodal distribution profile than that of amylopectin of storage starch, which is relatively smooth (*7*, *8*, *11*). Leaf starch differs from storage starch as it has apparent polymodality within the short chain fraction that forms the first population of the polymodal distribution defined by Hizukuri et al. (*12*). The difference between leaf and storage amylopectin implies that factors that determine amylopectin structure differ between these two organs. The divergence appears to lie, at least in part, in differences in starch synthesizing enzymes (*8*, *11*).

Little is known about the starch accumulation−degradation process and the structure of leaf starch in different botanical sources. In the present study, potato leaves with different starch accumulation rates were collected from plants at two different times during the growing season. Leaf starch was investigated by comparison of morning and afternoon samples. Starch was extracted from potato leaves, and its amylose and amylopectin were characterized and compared with those from potato tuber starch by GPC and HPAEC-PAD.

## MATERIALS AND METHODS

**Materials.** All chemicals used were of analytical grade. Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa*, with an activity of 71 000 U/mg protein, was obtained from Hayashibara Biochemical Labs (Okayama, Japan). Thermostable *Bacillus licheniformis* α-amylase (EC 3.2.1.1) and *Aspergillus niger* amyloglucosidase (EC 3.2.1.3) were obtained from Megazyme (Bray, Wicklow, Ireland).

Potatoes of the cultivar *Producent* were grown south of Uppsala, Sweden. Leaves were picked on two separate occasions on two sunny days at two different times during the cultivation period: on July 12 (sunrise, 3:52 a.m., and sunset, 9:59 p.m.), when the tubers were small

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and the plants had leaves of varying sizes, and on August 20 (sunrise, 5.17 a.m., and sunset, 8:30 p.m.), when the tubers were large and ready to harvest and the plant had only large leaves. For each occasion, 50 small and 50 large leaves (in August only the latter) were picked every third hour over 1 day. Additionally, 250 large leaves were picked at 5:00 a.m. and 5:00 p.m. on July 18 and August 20 for structural analyses. All leaves were transported in a cooling bag and then stored at −20 °C. For starch content analysis, the leaves were freeze-dried and milled in a Retsch mill with a 0.5 mm screen (Retsch GmbH & Co. KG, Hann, Germany).

**Methods.** *Starch Content.* Leaf dry matter content was determined by oven drying at 105 °C for 5 h. Low molecular weight sugars were extracted from freeze-dried leaves with 80% ethanol, and starch content was determined enzymatically according to Åman et al. (*13*). These analyses were carried out in duplicate.

*Isolation of Starch Granules.* Isolation of starch granules from leaves was performed according to Zeeman et al. (*14*). About 20 g of frozen leaves was homogenized using a Sorvall omni-mixer (Du Pont Instruments, E. I. Du Pont De Nemours & Co., Newtown, CT) in 100 mL of 100 mM 3-(N-morpholino)propane sulfonic acid (MOPS, pH 7.2) with 5 mM EDTA and 10% (v/v) ethanediol. The homogenate was filtered through a 25 μm sieve and washed with an additional 100 mL of the MOPS buffer. The fiber fraction was spread on paper, dried at room temperature, and stored for further analyses. The liquid fraction was centrifuged at 3000*g* at 4 °C for 10 min, and the pellet was resuspended in 30 mL of the same buffer plus 0.5% w/v of SDS. The starch granules were pelleted and washed twice with the SDS-containing buffer and then five times with 30 mL of deionized water. The enriched starch granules were then dried at room temperature and stored in a desiccator for SEM analyses.

*SEM.* Isolated starch was evenly spread out on a specimen stub covered with double adhesive tape. The specimens were coated with gold/palladium in a Polaron SC 7640 sputter coater, before examination in a JEOL 5600 scanning electron microscope at an accelerating voltage of 10 kV using secondary electron detector.

*Extraction of Starch with DMSO.* Freeze-dried leaves were disintegrated to powder using a Wiley mill with a 0.25 mm screen (Arthur H. Thomas Co., Scientific Apparatus, Philadelphia, PA). About 3.2 g of the milled sample was weighed into a screw-capped glass tube, mixed with 10 mL of 90% ethanol (v/v), and placed in a boiling water bath for 15 min. After centrifugation (1000*g* for 10 min), the supernatant was discarded and the washing step was repeated three times more. A next washing step was performed using 10 mL of 99.5% ethanol with incubation in boiling water for 15 min. Afterward, 10 mL of acetone was added, the sample was stirred, and the supernatant was discarded after centrifugation. The residual pellet was mixed with 10 mL of acetone once more, stirred, centrifuged, and left to dry at room temperature. The dry material was disintegrated in a mortar and stored in a desiccator for future analyses.

About 500 mg of the dried residual pellet was weighed into a screw-capped tube of 50 mL capacity, and 25 mL of 0.2% EDTA was added. The suspension was homogenized in a mixer (Polytron PT 3000, Kinematica AG., Littau, Switzerland) at 20 000 rpm for 5 min and thereafter left with stirring overnight at room temperature. The suspension was centrifuged (1000*g* for 5 min), and the supernatant was discarded. The pellet was mixed with 25 mL of 90% DMSO, homogenized, and incubated in a boiling water bath for 30 min. Heating was continued in an oven at 105 °C for 2.5 h. The sample was centrifuged, and the supernatant was saved. The pellet was mixed with 25 mL of 90% DMSO, homogenized, and placed in an oven at 105 °C overnight. After it was centrifuged, the supernatant was saved, whereas the pellet was extracted once more with 25 mL of 90% DMSO during heating in an oven for 2.5 h. The pellet was discarded, and the combined supernatants were mixed with 99.5% ethanol in a ratio of 1:9 (DMSO:ethanol). The solution was left at room temperature for 15 min and centrifuged (1000*g* for 10 min). The supernatant was discarded whereas the starch-containing precipitate was again dissolved in DMSO, precipitated with ethanol, and collected by centrifugation.

*GPC.* The elution profile of debranched starch was obtained by GPC essentially as described by Torneport et al. (*15*). The starch extracted with DMSO was dissolved in 0.5 mL of 90% DMSO (with heating if necessary), and 3.5 mL of Na acetate, pH 3.6, and 5 μL of isoamylase were added and the mixture was incubated at 38 °C overnight. After the sample boiled, the sample was centrifuged (1000*g* for 10 min) and the supernatant was injected on a Sepharose CL-6B (Amersham Pharmacia Biotech, Uppsala, Sweden) column (65 cm × 1.6 cm). Elution was performed with 0.25 M KOH at a flow rate of 21 mL/h. Fractions of 2 mL were collected and analyzed by the phenol−sulfuric acid method (*16*).

To check whether the presence of other carbohydrates interfered in the elution profile, DMSO-extracted starch was degraded enzymatically and concomitantly run on a Sepharose CL-6B. DMSO-extracted starch was dissolved in 1 mL of 90% DMSO. Afterward, 5 mL of 0.1 M Na acetate buffer, pH 5.0, and 50 μL of α-amylase were added and samples were incubated in a boiling water bath for 30 min. The sample was cooled to approximately 40 °C, and amyloglucosidase (14 U) was added before incubation overnight at 60 °C. The enzymatic reaction was stopped by boiling for 5 min, and the pellet was removed by centrifugation. The supernatant was fractionated on a Sepharose CL-6B column (65 cm × 1.6 cm) and analyzed with the phenol−sulfuric acid method as described previously.

*Unit Chain Length Distribution.* Unit chain length distribution of starch was determined by HPAEC-PAD as described by Koch et al. (*17*). The DMSO-extracted starch from 500 mg of leaves was mixed with 1 mL of 1 M Na acetate buffer, pH 3.6, and heated in a boiling water bath for 10 min. To solubilize the starch completely, 300 μL of 90% DMSO was added and the sample was heated in an oven at 105 °C. The pH was adjusted to 3.6 with 2 M HCl prior to addition of 10 μL of isoamylase. Lower amounts of solvents were used in order to increase sensitivity of the detection. The sample was incubated at 38 °C overnight, and the enzymatic reaction was stopped by boiling for 5 min. The sample was centrifuged, filtered, and pH adjusted to a value over 10 using 60 μL of 4 M KOH before injection into the HPAEC-PAD system.

*Isolation and Analysis of Amylopectin.* Starch extracted with DMSO was dissolved in 1 mL of 1 M NaOH, and 9 mL of distilled water was added in steps during 2 h (*18*). The dissolved material was fractionated on a Sepharose CL-2B (Amersham Pharmacia Biotech) column (70 cm × 1.6 cm) using 0.01 M NaOH as eluent at a flow rate of 24 mL/h. The elution profile was monitored by refractive index (RI detector R-403, Waters Associates, Milford, MA), and the high molecular weight amylopectin fraction was pooled, dialyzed, and freeze-dried. To investigate whether materials other than amylopectin were present in the isolate, the freeze-dried material was debranched and thereafter run on a Sepharose CL-6B. The freeze-dried material was mixed with 1 mL of water, followed by 1 mL of 90% DMSO. Afterward, 3.5 mL of Na acetate, pH 3.6, and 5 μL of isoamylase were added and the mixture was incubated at 38 °C overnight. After the sample boiled, the sample was centrifuged (1000*g* for 10 min) and the supernatant was analyzed on a Sepharose CL-6B column (65 cm × 1.6 cm) together with the phenol−sulfuric acid method as described previously.

## RESULTS AND DISCUSSION

The starch content in potato leaves collected at different times on July 12 varied between 1.8 and 10.6% in small leaves and between 2.9 and 12.9% in large leaves (**Figure 1a**). Hence, small leaves contained a slightly lower content of starch than large leaves, but the profiles of the starch content in both leaves over 1 day were similar. Both showed a pronounced minimum around 5:00 a.m. and a maximum at 2:00−5:00 p.m. A significantly lower starch content (0.6−2%) was found in the leaves collected in August (**Figure 1b**). Also, in this case, the morning samples had the lowest content of starch and the afternoon/evening samples had the highest content. Photosynthesis and translocation of photosynthates to the tubers have been shown to increase considerably after tuber setting of potatoes (*19*). This might be the cause of increased starch accumulation in the leaves in July. The diurnal variation of the starch content, specifically in the July samples, showed the influence of sunlight on starch biosynthesis. Leaf starch synthesis began to increase with the

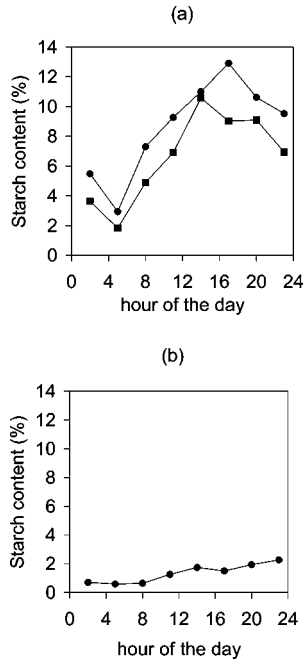


**Figure 1.** Starch content in dry potato leaves collected at different times during 2 days. (**a**) July 12th: ■, small leaves; ●, large leaves. (**b**) August 20th: large leaves.

sunrise, reaching its peak around 5:00 p.m. After this time, the sunlight intensity receded and consequently, the starch synthesis was reduced and the conversion of transient starch into sucrose was increased. This sucrose is transported to the tubers to be incorporated into the growing starch granules or to other sinks until early morning when the leaf starch content is almost depleted and the next sunrise initiates the biosynthesis of leaf starch again.

Attempts to isolate leaf starch granules led to low yields (recovery below 3%) and impure starch fractions. These fractions had only a slightly higher starch concentration than the contents found in dry leaves (data not shown). The low yield may be due to the fact that some of the analyzed starch is not present as granules and is therefore washed out during the isolation procedure. SEM analyses of the isolated leaf starch showed the presence of starch granules in all four samples (July 5:00 a.m., 5:00 p.m. and August 5:00 a.m., 5:00 p.m.). The granules had either an oval or a round shape, and none bigger than 5 $\mu$m were found (**Figure 2**). A large number of prismatic crystals were found in the samples, especially in the three samples with low starch content. These crystals may correspond to calcium oxalate (*6*).

Starch extraction with DMSO showed higher yields than isolation of granular starch, with an average recovery above 80%. The amount of starch recovered by each of the three sequential extractions with DMSO had average values of 43, 31, and 13%, respectively. Debranched DMSO-extracted starches were analyzed by GPC on Sepharose CL-6B and showed the presence of a small peak of amylose between 150 and 200 min elution time (**Figure 3a**,**b**). A material (210−290 min elution time) with chain lengths between those of normal amylose and amylopectin was present in a higher relative amount in the morning than in the afternoon sample from August ($p < 0.05$). Debranched amylopectin of low molecular size (300−360 min elution time) had a higher relative amount in the afternoon than in the morning sample from July ($p < 0.05$) (*20*). A peak between 380 and 430 min elution time was observed in all four samples.

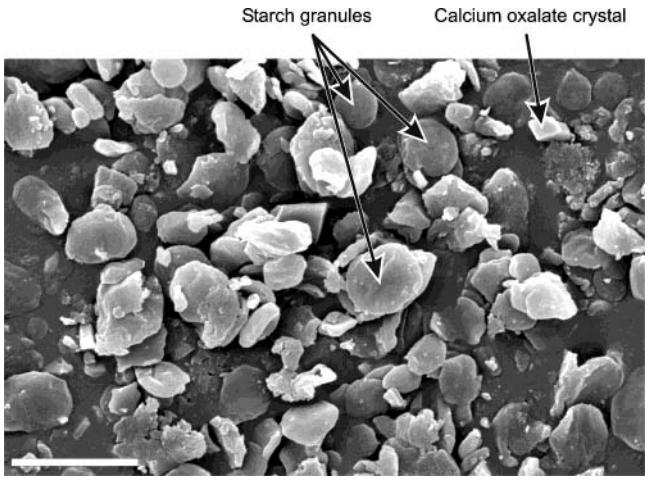


**Figure 2.** SEM image of isolated potato leaf starch from July 5:00 p.m. The bar represents 5 $\mu$m.

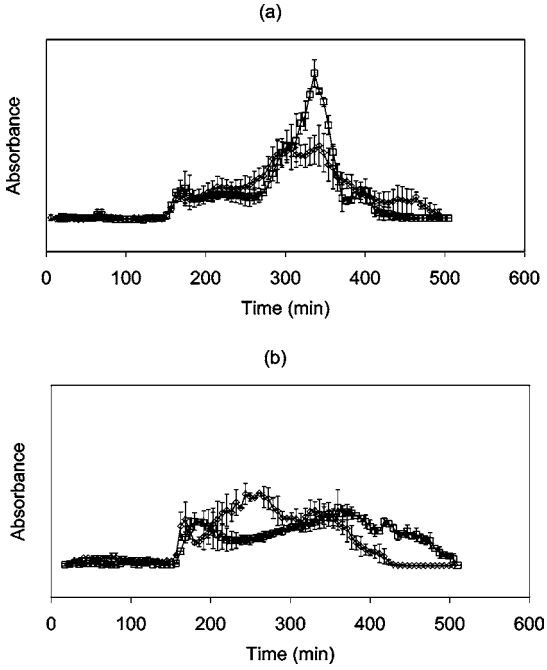


**Figure 3.** Elution profile on Sepharose CL-6B of debranched DMSO-extracted potato leaf starch. (**a**) ◇, July 5:00 a.m.; □, July 5:00 p.m. (**b**) ◇, August 5:00 a.m.; □, August 5:00 p.m. The bars represent the standard deviation of at least two duplicates.

The phenol−sulfuric acid method is a general method for carbohydrates and will detect other polysaccharides besides amylose and amylopectin. The use of EDTA during the extraction helps to reduce the presence of other polysaccharides such as pectin, hemicellulose, etc. in the DMSO-extracted starch that may interfere with the obtained elution profile (*21*). To check if the presence of other carbohydrates interfered in the elution profile, DMSO-extracted starch was hydrolyzed with α-amylase and amyloglucosidase and analyzed with GPC (**Figure 4**). Hydrolysis of the sample from July 5:00 p.m. (**Figure 4a**) showed only one peak from hydrolyzed starch glucose between 380 and 440 min elution time, whereas the sample from July 5:00 a.m. (**Figure 4b**) showed two additional peaks, between 200 and 380 min and above 440 min. The peak between 380 and 440 min corresponded to glucose from hydrolyzed starch, whereas the peaks above 440 min may be due to the presence of glycoproteins or other carbohydrates that have hydrophobic interactions with the column. The material

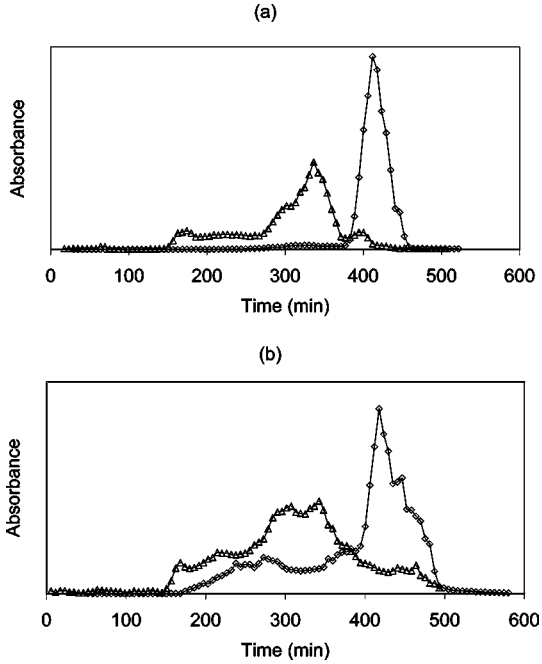


**Figure 4.** Elution profile on Sepharose CL-6B of DMSO-extracted potato leaf starch. (**a**) July 5:00 p.m.; (**b**) July 5:00 a.m.; △, debranched with isoamylase; and ◇, hydrolyzed with α-amylase and amyloglucosidase.

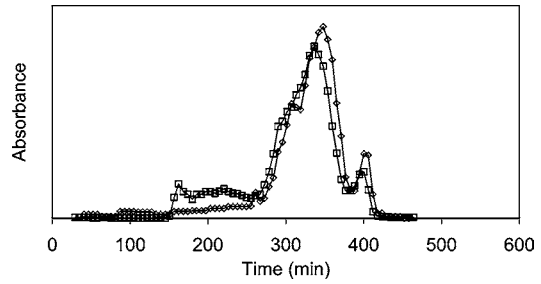


**Figure 5.** Elution profile of DMSO-extracted potato leaf starch from July 5:00 p.m. on Sepharose CL-6B. □, Whole starch debranched with isoamylase; ◇, amylopectin previously isolated on Sepharose CL-2B and debranched with isoamylase.

eluting between 200 and 380 min may correspond to polysaccharides other than starch that were not completely removed with the extraction procedure.

Isolated amylopectin from DMSO-extracted starch (July 5:00 p.m.) was debranched with isoamylase and analyzed by GPC (**Figure 5**). The profile revealed two peaks of low molecular size (270−380 min with a shoulder at about 300 min and 380−430 min elution time, respectively). The late peak between 380 and 430 min elution time corresponded to an oligomer with less than seven glucose units and was also present in the profile from whole starch. This peak is consequently a true part of the leaf amylopectin structure but is not present in potato tuber starch.

HPAEC analyses of DMSO-extracted starch showed the presence of short chains, DP 3−5 (**Figure 6**), which are not present in potato tuber starch (*17*, *22*). The content of chains of DP 3 and 4 was not repeatable between replicates and was present in low amounts. In contrast, chains of DP 5 were present in high amounts, were repeatable between measurements, and were confirmed with an internal standard. Perhaps chains of DP 5 are part of the starch structure, whereas chains of DP 3 and 4 may be traces of short sugar chains remaining after the extraction of starch with DMSO. Populations around DP 5, 10, 19, and 24 were observed for the four starch samples (July 5:00 a.m., July 5:00 p.m., August 5:00 a.m., and August 5:00 p.m.). A higher amount of chains with DP around 19 and above 35 was observed for the afternoon samples in both July and August. A peak with a retention time about 60 min (DP > 70) was also seen, which corresponds to material eluting at 300 min in **Figure 3**. Potato tuber starch showed a similar chain length distribution as the afternoon samples, with a maximum shifted from DP 10 to DP 12, a weaker shoulder at DP 19, and a pronounced peak above DP 35 (*17*).

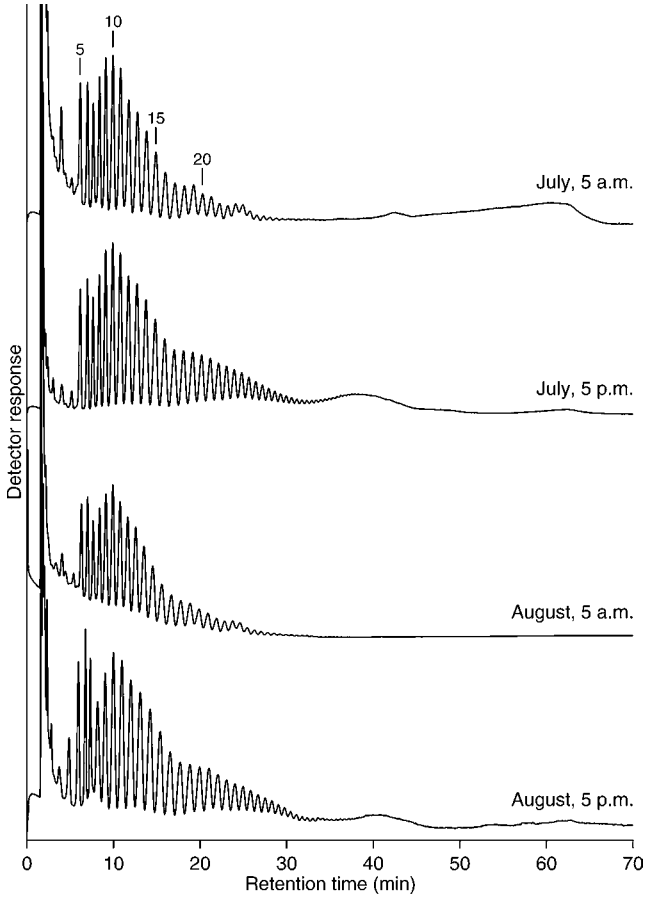


**Figure 6.** DMSO-extracted potato leaf starch debranched with isoamylase and analyzed by HPAEC-PAD. (**a**) July 5:00 a.m., (**b**) July 5:00 p.m., (**c**) August 5:00 a.m., and (**d**) August 5:00 p.m.

A higher expression of SBE II as compared to SBE I in potato leaves (*23*) may explain the high amount of very short chains (*24*) in the DMSO-extracted starch. Comparison of afternoon and morning samples, both from July and August, showed that there was a higher amount of the chains above DP 15 in the afternoon samples, whereas the chain length distribution between DP 5 and 15 remained basically unchanged. This range may correspond to the primer of the starch granule that remained unchanged during the accumulation−dissolution of leaf starch. The present investigation shows that a significant amount of starch is accumulated in potato leaves during the day and that this starch is translocated to the sinks during the night. Similar results have been obtained with soybean leaf starch (*25*, *26*). Some of this starch was present in small starch granules (<5 μm).

Leaf starch contained short unit chains (especially DP 5), which are not present in potato tuber starch, and populations of unit chains around DP 10, 19, and 24. In the afternoon samples, with a high content of leaf starch, the populations around DP 19 and an additional population with DP > 35 became

significant. The results indicate that leaf starch contains a primer of shorter unit chains (DP 5−15) as well as longer unit chains, which are produced during the day and used during the night.

## ABBREVIATIONS USED

GPC, gel permeation chromatography; HPAEC-PAD, high-performance anion exchange chromatography with pulse amperometric system; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SEM, scanning electron microscopy; DMSO, dimethyl sulfoxide; SBE, starch branching enzyme.

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