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Starch and Its Component Ratio in Developing Cotton Leaves

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ABSTRACT

During cotton leaf development, starch accumulation was characterized by an initial rise to a maximum at the second to the fourth leaf from the apex. Then, starch content progressively decreased with leaf age. Starch accumulation was inversely related to the ratio of amylopectin to amylose. Differences between leaves in this ratio resulted from variations in both amylose and amylopectin levels. Fluctuations in amylose levels were more extreme than those of amylopectin.

During the diurnal cycle, amylopectin was accumulated more than amylose in both young and old leaves during the day. During the night, amylopectin was degraded more than amylose in young leaves and vice versa in older leaves. The rate of change of the amylopectin to amylose ratio during the day was consistently higher than that during the night.

Accumulated leaf starch may restrict photosynthesis in cotton because a negative correlation was obtained between starch content and CO_2 fixation (14). However, measurements were made only on leaves at the third to fourth node below the apex. These leaves were in full sunlight and probably contained more starch than shaded leaves lower on the plants. Information on starch content of lower leaves on plants of various ages was needed to estimate the probability of an effect of leaf starch on photosynthesis.

Starch is composed of two components, amylose and amylopectin. AM[1] is essentially a linear polymer containing α-1,4-linked glucose residues, whereas AP is a multiply branched glucan containing both α-1,4- and α-1,6-linkages (6). The structural differences of the glucans and the action of specific hydrolytic enzymes involved in starch metabolism are mutually dependent (2). Starch, laid down as a temporary reserve in leaves, is enzymically converted into sugars prior to translocation (6). Determinations of the starch component ratios in the developing leaves would provide information as to which glucan contributed more to the total starch. Therefore, changes of component ratios in the residual starch were investigated.

Matheson and Wheatley (13) reported that the iodine affinities of starch increased with increasing tobacco leaf age up to about 22 weeks. The ratio of AP to AM did not vary in senescing tobacco leaves (1). Kakie and Sugizaki (11) reported that the ratio of AP to AM in leaves prior to maturity in tobacco changed from 68%/32% (w/w) in the morning to 78%/22% (w/w) in the afternoon. On the contrary, Mizuno *et al.* (17) demonstrated that the AM fraction of mature tobacco leaves increased in the daytime and decreased at night.

The objectives of this communication were: (*a*) to establish a procedure for the quantitative determination of total starch, AM, and AP from cotton leaves; (*b*) to determine starch and glucan concentrations in leaves at different leaf positions on plants of various ages; (*c*) to present data on the changes of total starch and its components during the diurnal period; and (*d*) to clarify the relative influences of AP and AM on total starch content.

MATERIALS AND METHODS

Glandless cotton plants (*Gossypium hirsutum* L. cv. Coker 100) were grown in a growth chamber in a sand-peat moss-Vermiculite mixture and watered with nutrient solution. Temperature was 32 ± 3 C during the day and 22 ± 3 C at night. A 10-h photoperiod was maintained with a PAR of 710 $\mu E/m^2 \cdot s$. RH was about 75% during the day. Each 19-cm pot contained two uniform plants, from which leaf samples were made to determine starch. One-half to 1.0 g of fresh cotton leaves were cut into small pieces (approximately 1 cm^2) and immediately dropped into a flask of boiling 95% ethanol. A watch glass was placed on the flask and the contents were boiled for an additional 20 min. The leaf samples were stored at −20 C until sugars were extracted. An average of two to three replicates from each plant were made for determinations of compounds in each experiment.

Extraction of Sugars. The leaf sample was homogenized with a VirTis[2] mixer (35% full speed) in 95% ethanol at approximately 70 C for 3 min and centrifuged at 27,000*g* for 7 min. After discarding the supernatant, the pellet was extracted with 30-ml portions of 80% (v/v) ethanol at about 70 C until a test of the extract with anthrone reached a constant low value.

Determination of Starch. The entire process for extracting starch was conducted in a cold room at 4 C. The residue from 1 g of fresh cotton leaves was suspended in 20 ml of 4.8 M $HClO_4$. While the suspension was being stirred, 3 to 5 ml (A) were withdrawn and placed in a beaker. Forty to 50 ml of 4.8 M $HClO_4$ was then added to (A), depending upon the leaf age (the maximum concentration of starch rarely exceeded 100 $\mu g/ml$ in cotton leaves with these ranges of sample fractions and dilutions). The suspension was macerated by stirring with a magnetic stirrer continuously for about 18 h. A fraction of the suspension (approximately 30 ml) was centrifuged at 37,000*g* for 20 min and a small volume of clear sample (approximately 5–7 ml) was removed from the supernatant. The starch was precipitated with iodine, and the starch-iodine complex was decolorized by a standard procedure (9). The starch polymer was dissolved in a volume of 7.2 M $HClO_4$ equal to two-thirds of the sample volume. The solution was then adjusted to 4.8 M by the addition of water. The purified starch volume was then equal to the known volume used for the starch precipitation with iodine. Aliquots of the starch solution (0.2–0.5 ml) containing up to 54 μg starch were pipetted into acid-washed test tubes and diluted with water to 1.0 ml. At the same time, a series of tubes containing 9 to 60 μg D-glucose was prepared in 1.0 ml diluted

[1] Abbreviations: AM: amylose; AP: amylopectin.

[2] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

$HClO_4$ (a concentration similar to the sample to be analyzed). Two ml of anthrone reagent were added to each tube and swirled while immersed in ice-cold water. The tubes were placed in a boiling water bath for 9 min, cooled rapidly in ice water for 3 min, and left at room temperature for 3 min. The samples were read at 630 nm and converted to the amount of starch with a standard curve. (The glucose concentrations were multiplied by 0.90 and by appropriate dilution factors to calculate starch content of leaves.)

Determination of Ratio of AP to AM. One-half ml of I_2-KI reagent was mixed with 2 ml of starch solution (approximately 37 μg). After 15 min, the intensity of the blue color was determined at 660 nm. The observed A at 660 nm was corrected to 37.5 μg ($37.5 \times 10^{-4}/2.5 = 0.0015\%$) of starch and the percentages AM and AP were read from a graph prepared with various ratios of pure AM and AP (Fig. 1).

Total Soluble Carbohydrate and Sucrose. The procedures of Hodge and Hofreiter (10) and Handel (8) were followed for total soluble carbohydrate and sucrose, respectively.

Reagents. Anthrone-H_2SO_4 was prepared by dissolving 60 mg

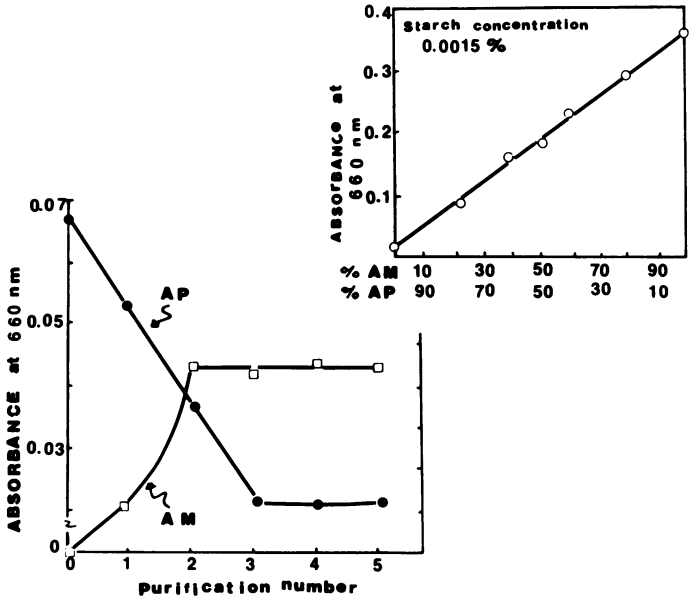

FIG. 1. Fractionation and purification of cotton leaf starch. Starch was extracted from cotton leaves and purified with iodine by the procedure of Hassid and Neufeld (9). Fractionation and purification of starch components were conducted with thymol and butanol according to the method of Banks and Greenwood (3). The procedure was continued until the A at 660 nm reached a constant maximum for AM and minimum for AP (lower, left). Different scales were used for AP (left ordinate) and AM (right ordinate). A standard curve was constructed with 0.0015% starch composed of various per cent ratios of the two components (inset, upper right).

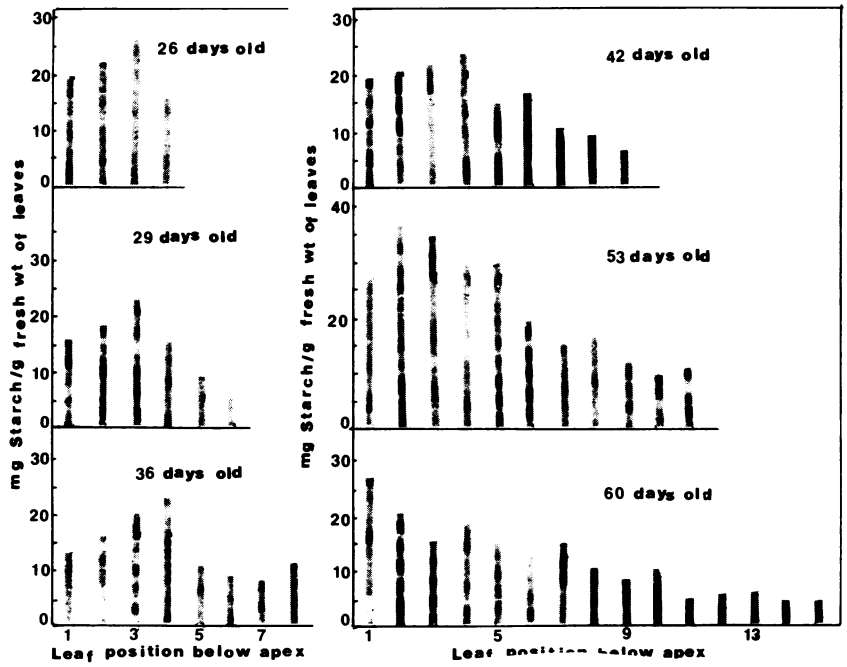

FIG. 2. Changes in starch in various primary leaf positions from plants grown to different ages. Size of youngest leaf (position 1) from apex was approximately 4 cm in diameter. Samplings were made about 2 h prior to end of 10-h light period.

anthrone in 30 ml of cold 95% (v/v) H_2SO_4. A fresh solution was prepared daily. I_2-KI was prepared by dissolving 2 g I_2 in 20 ml of an aqueous solution containing 20 g KI. This was diluted to 1 liter.

RESULTS

Extraction of Starch. At least seven extractions with 80% (v/v) ethanol were required to remove the maximum amount of sugars from 1 g of fresh cotton leaves. This number of extractions contrasts with four required in peas (16).

The extraction of starch by the maceration technique reached a maximum after a period of about 18 h. Prolonged extraction did not change the level of the plateau. Furthermore, dilution of the suspension with 4.8 M $HClO_4$ followed by an additional hour of maceration did not significantly increase the extractability of starch. The constant level of starch obtained after 18 h of maceration was not due to saturation of the suspension, but was instead due to complete extraction. Also, the replicates determined from different amounts of cotton leaves agreed within standard errors of ± 0.202 ($\bar{X}$ = 15.13 mg) for starch and ± 0.034 ($\bar{X}$ = 15.2%) for per cent amylose.

The purities of AM and AP reached maxima after the second and third purifications, respectively (Fig. 1). Artificial mixtures of AM and AP gave A values at 660 nm that fell on a straight line over the entire range from pure AM and pure AP.

Starch and AP/AM Ratios at Various Leaf Positions from Plants Grown to Different Ages. The accumulation of starch with leaf position in plants up to about 53 days old was characterized by an initial rise to a maximum at the second to the fourth leaf from the apex (Fig. 2). Thereafter, starch content progressively decreased with leaf age. The oldest plants (60 days old) contained the highest starch content in the youngest leaves. A consistent relationship of high starch and low soluble sugars existed in the top two to four leaves. In older leaves, the two classes of metabolites changed in a more parallel manner. The results from these experiments were consistent when expressed on either fresh leaf weight or unit area basis.

In plants 29 and 36 days old, the AM content increased to a maximum in the leaves at the fourth node below the apex and decreased sharply in the lower leaves (Fig. 3). As the plants continued to develop, the amount of the AM in the younger leaves fell sharply with a concomitant further gradual reduction of these values in the older leaves. In contrast, the AP content varied less with leaf position. In the oldest plants (60 days old) AM levels declined steadily with leaf age and were approximately 50% lower than AP in the older leaves. The patterns of extreme variation in AM levels relative to AP determined the AP/AM ratio distributions in plants of various ages.

Starch and AP/AM Ratios during a Diurnal Cycle. Starch increased about 4-fold in the younger leaves over the course of the 10-h light period (Fig. 4). The content of AP increased rapidly at the beginning of the light period, whereas the amount of AM increased only during the latter half of the period. Over-all, AP accumulation was greater than that of AM during the entire light period.

Starch accumulation in older leaves (Fig. 4) reached a maximum sooner than in younger leaves. AP accumulated during the first 8 h of light period. AM content also increased during the first 6 h of this period, but thereafter decreased.

At the end of the dark period, starch was degraded to about

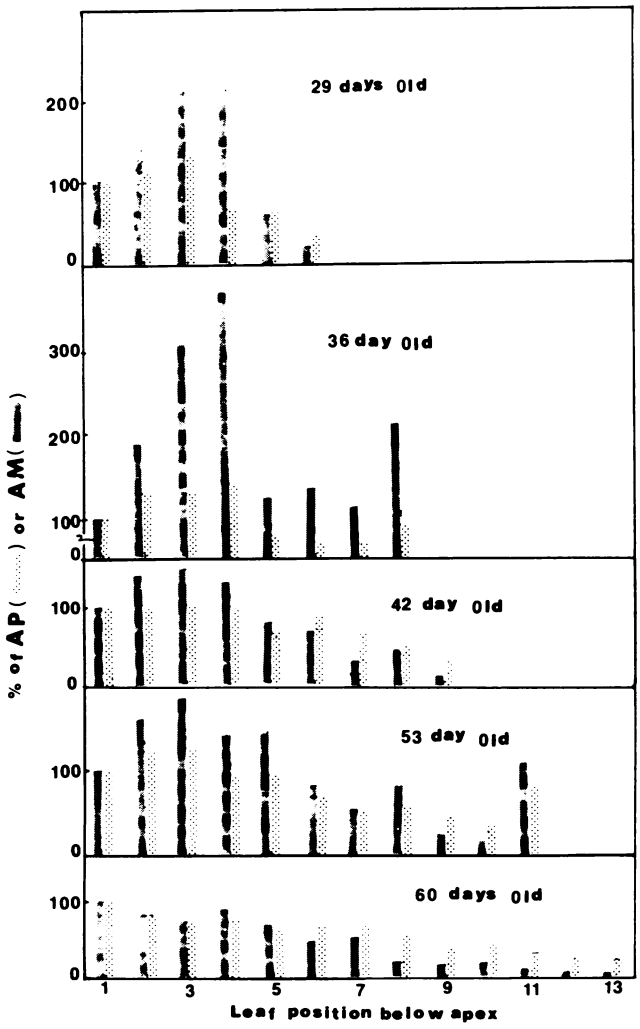

FIG. 3. Per cent changes of AP and AM at various leaf positions from plants grown to different ages. Size of leaf at position 1 from the apex was about 4 cm in diameter. Assigning the amount of each component from first leaf position as 100%, the percentage of AP or AM in each leaf position was calculated.

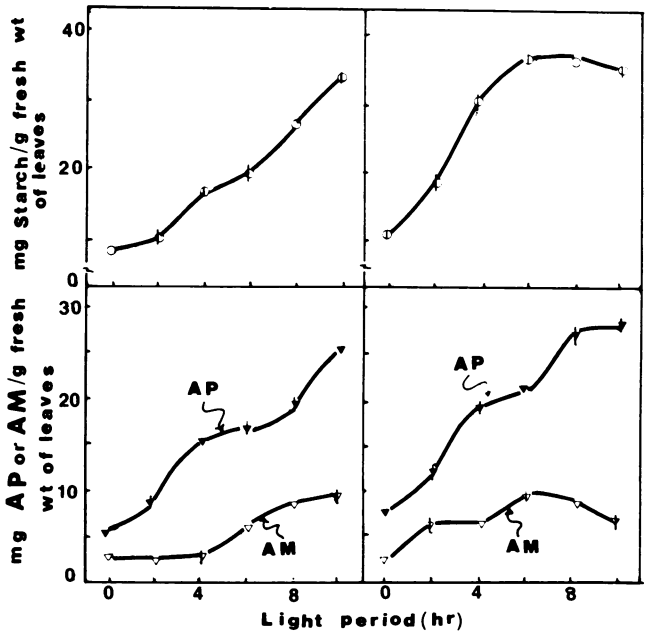

FIG. 4. Time course effects of a light (10-h) environment on starch and its components in young and old leaves from plants 29 days old. Left half (young leaves, first fully expanded leaf from apex): top, total starch; bottom, AP and AM. Right half (old leaves, fourth leaf from the first fully expanded leaf): top, total starch; bottom, AP and AM.

30% (w/w) of its initial content in younger leaves (Fig. 5). AP was degraded more than AM during the entire dark period. However, the variability of AM level was less than that of AP.

Starch content in older leaves (Fig. 5) was degraded to a level similar to that in younger leaves at the end of the dark period. However, the initiation of starch degradation in the older leaves was delayed. The contents of AP and AM changed apparently in a parallel fashion. However, AM was degraded more than AP at the end of the 14-h dark period. This change was due to the significant decrease in the AM level after 3 h dark.

DISCUSSION

There are various methods (5, 12, 15, 16, 20, 21) for quantitative starch extraction. The procedure with $HClO_4$ has been most widely used because it extracts more starch with less interfering substances than other methods. The most common method using $HClO_4$ is the procedure of McCready *et al.* (16). With cotton leaves it was impossible to decant $HClO_4$ extracts quantitatively after each centrifugation because the sediments fell apart readily. A simple maceration process presented in this communication overcame this difficulty.

The range of maximum starch content from the leaves at the third to fourth node below the apex in this study (Fig. 2) was 23 to 36 mg/g fresh weight. To this total starch, AP contributed more than AM, since AM was a minor component (approximately 20% of total starch) in spite of its extreme change with plant age (Fig. 3). The leaves lower on the plants of all ages up to 42 days contained starch contents which were not more than 20 mg/g fresh weight (about 100 mg/g dry weight). Starch content higher than this level was found only from leaves on the top half of the plants which were 53 days old.

According to the correlation ($r = -0.68$, $n = 66$) relating photosynthetic CO_2 uptake to starch accumulation (14), approximately 10 to 15% depression of CO_2 uptake as compared with control might occur in the leaves containing starch ranging from 20 to 36 mg/g fresh weight (Fig. 2). Therefore, the starch levels in the lower leaves on the majority of plants were too low to influence photosynthetic activity significantly.

Maximum starch content (29 mg/g fresh weight) from Coker 100-grown in a growth chamber was comparable with that (27 mg/g fresh weight) from field-grown cotton plants, Deltapine 61 (July 7 to September 7, 1978). The values of average starch content (19 mg/g fresh weight) obtained from Coker 100 in this study were generally lower than those (10–40 mg/g fresh weight) from lemon leaves (4) and higher than the levels (2–12% dry weight) from tobacco leaves (13). The fractions of the two glucans (74–90% AP and 9–27% AM) in cotton leaves (Coker 100) were similar to those (70–90% AP and 10–30% AM) from other plants (2). The increase in AP/AM ratios during the daytime was in agreement with data from tobacco leaves prior to maturity (11) but differed from the ratios of mature tobacco leaves (17). The ratio increase in old leaves at night (Fig. 5) was also observed from mature tobacco leaves (17). The ratio decrease found in young cotton leaves at night was unique. In senescing tobacco leaves, per cent AM values were reported to be constant (1). This finding differed strikingly from the increasing ratios found in leaves of 60-day-old cotton plants (Fig. 3) showing signs of senescence at the lower leaf positions. This difference appeared to be associated mainly with structural and/or enzymic specificities, since the starch component ratios from these two plants, cotton and tobacco, were not greatly different (Fig. 4 and ref. 11).

The mechanism(s) that determine AP/AM ratios *in vivo* is unknown (7). However, involvement of enzymic reactions in these ratio changes is evident, since starch undergoes degradation in the dark (Fig. 5). This degradation may be phosphorolytic and/or hydrolytic (19). In young plants (29 days old) AP was accumulated and degraded more than AM during the initial light and dark periods, respectively (Fig. 4 and 5). *In vitro* experiments have shown that AP is a better primer than AM for glucan synthesis (18). This may account for the preferential accumulation of AP. Also, rapid degradation of AP early in the dark period may be due to the relatively large surface area of the molecule which can come in contact with the enzyme. The relations of glucan dissolution to the action of degradative enzymes are now under investigation.

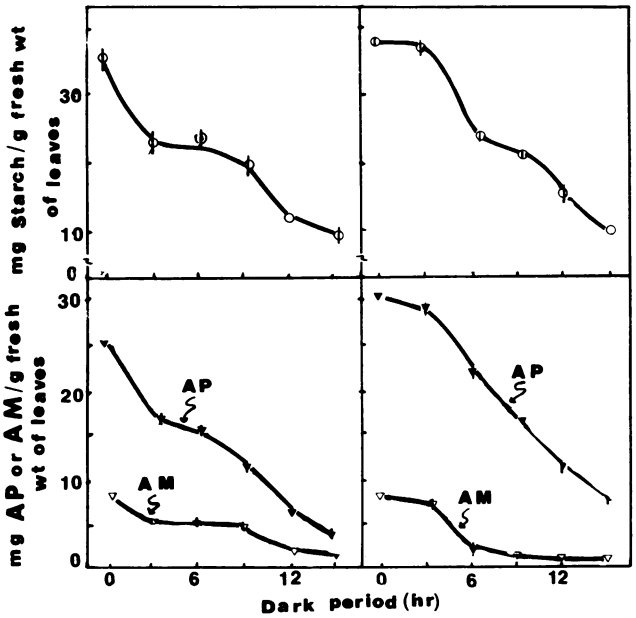

FIG. 5. Time course effects of a dark (14-h) environment on starch and its components in young and old leaves from plants 29 days old. Left half (young leaves, first fully expanded leaf): top, total starch; bottom, AP and AM. Right half (old leaves, fourth leaf from first fully expanded leaf): top, total starch; bottom, AP and AM.

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