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Carbon isotope ratios of amylose, amylopectin and mutant starches

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Abstract

Carbon isotope ratios (expressed as $\delta^{13}C$ values) were determined for various sources of starch and the starch fractions amylose and amylopectin. The $\delta^{13}C$ values of amylose were consistently less negative, 0.4–2.3 ‰, than those of amylopectin in kernal starch from maize (*Zea mays*) and barley (*Hordeum vulgare*) and in tuber starch from potato (*Solanum tuberosum*). Kernel starch isolated from the maize mutants *wx1* and *ae1*, with known genetic lesions in the starch biosynthetic pathway, also showed significant differences in $\delta^{13}C$ values. Collectively, these results suggest that variation in carbon isotope ratios in the amylose and amylopectin components of starch may be attributed to isotopic discrimination by the enzymes involved in starch biosynthesis. Published by Elsevier Science Ltd.

Keywords: *Zea mays*; *Hordeum vulgare*; *Solanum tuberosum*; *Oryza sativum*; Carbon isotope ratios; Starch; Amylose; Amylopectin

1. Introduction

Starch is the major storage polysaccharide of many plants and it accumulates to high levels in seeds or tubers as insoluble granules. Potato (*Solanum tuberosum*) accumulates starch to about 75% of the dry weight in tubers (Lee, 1986), whereas maize (*Zea mays*) seeds contain 65–80% starch by weight (Lee, 1986). Starch is a polymer of α-D-glucose. Normal starch is composed of a mixture of 20–30 % amylose and 70–80% amylopectin. The polymer amylose is a mostly linear α-D-(1,4) glucan with only 0.1% α-D-(1,6) branch points and a MW range of 200,000 to 700,000. The polymer amylopectin, by contrast, is an α-D-(1,4) glucan with about 4% α-D-(1,6) branch points and has a MW range of 50–200 million (Nawrath, Poirier & Somerville, 1995). The biochemical pathway used in the production of starch has been inferred from many biochemical and genetic studies (reviewed in Martin and Smith (1995)). In maize, biochemical and/or genetic evidence suggest that ADP-glucose pyrophosphorylases, starch synthases, and starch branching enzymes are involved in starch biosynthesis. However, multiple enzymes with each of these activities have been identified. Hence, the exact role of each isoform of the enzymes in the biosynthetic pathway is unknown. The *waxy* (*wx*1) gene product, a granule-bound starch synthase (Klösgen, Gierl, Schwarz-Sommer & Saedler, 1986), is one of the best-characterized enzymes in this pathway. Genetic modifications to this gene often result in kernels lacking amylose, which suggests that the *wx*1 starch synthase is involved in production of amylose (Nelson & Rines, 1962). A second well-characterized gene in the starch biosynthetic pathway is *amylose extender* (*ae*1). This gene encodes starch branching enzyme II (Stinard, Robertson & Schnable, 1993) and mutations in *ae*1 can result in increased amylose levels in the seed (Shannon & Garwood, 1984).

Application of stable carbon isotope ratio method-

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ology to determine the photosynthetic pathway types (O'Leary, 1981) and to probe water use efficiency (Brugnoli et al., 1988) in plants has long been known. Plants discriminate against the heavier stable isotope ^{13}C during carbon fixation. This results in lower $^{13}C/^{12}C$ isotope ratio in plant products than in atmospheric CO_2.

Carbon isotope ratio studies are, in general, carried out at natural abundance levels of ^{13}C. Because these levels are low and differences in the ^{13}C content of natural materials are small, stable carbon isotope ratios ($^{13}C/^{12}C$) are expressed in relative terms as $\delta^{13}C$ values. A $\delta^{13}C$ value represents the per mil (‰, parts per thousand) deviation of the ^{13}C content of the sample from the international PDB (Pee Dee Belemnite) limestone standard whose $\delta^{13}C$ value has been set arbitrarily at 0‰ (Craig, 1957). Thus the $\delta^{13}C$ value is defined as the isotropic ratio of a sample compared to the isotope ratio of a standard (see materials in methods).

The $\delta^{13}C$ values of the primery products of photosyntesis and carbohydrates formed later are directly correlated with the kinetic isotope effects on the CO_2 fixing reactions catalyzed by ribulose bisphosphate carboxylase or phosphoenolpyruvate carboxylase (O'Leary, 1981). Secondary products are additionally depleted in ^{13}C, for example, due to the kinetic isotope effect of the pyruvate dehydrogenase reaction (DeNiro & Epstein, 1977). Changes in carbon isotope ratio of up to 2‰ can be caused by environmental effects such as fertilization (Bender & Berge, 1979) temperature (Smith et al., 1973) and light intensity (Smith & Jacobsen, 1976).

Stable carbon isotope distributions have been examined in many types of natural products and often give information about the synthesis of these products. Examples include glucose (Rossman et al., 1991) Cyanogenic glucosides (Butzenlechner et al., 1996) and amino acids (Abelson et al., 1961).

Carbon isotope ratios of starch isolated from different plant sources (Brugnoli et al., 1988; Smith & Jacobsen, 1976) and hydrogen isotope ratios of starch from potato (Smith & Jacobsen, 1976) have been previously reported. In this paper, we present the results from carbon isotope ratio analysis of starch fractions and starch from plants with mutations in genes involved in starch production. To our knowledge, this is the first report of variation in the carbon isotope ratios of starch components and mutant starches.

2. Results and discussion

2.1. Amylose and amylopectin have different $\delta^{13}C$ values

Carbon isotope ratios were determined for starch, amylose and amylopectin purified from maize kernels. Maize kernel starch was fractionated into amylose and amylopectin by butanol precipitation (Jane & Chen, 1992; Schoch, 1942). These fractions and the starting material were then analyzed for their carbon isotope ratios. The $\delta^{13}C$ values of the three compounds were clearly different, with amylose less negative and amylopectin more negative than the starting material (Fig. 1). Moreover, an average of the $\delta^{13}C$ values of amylose and amylopectin — weighted to reflect the relative abundance of these two fractions in the seed — approximately equals the $\delta^{13}C$ value of the starch from which they were purified. Amylose and amylopectin are probably derived from the same pool of glucose-1-phosphate, so any differences in their $\delta^{13}C$ values likely result from discriminations that occur in the starch biosynthetic pathway.

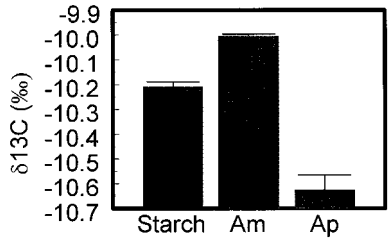

Fig. 1. Carbon isotope ratios (expressed as $\delta^{13}C$‰) of OH43 maize kernel starch and its component fractions amylose and amylopectin. Each sample was measured three times, and error bars represent one standard deviation from the mean. On the *x*-axis, Am stands for amylose and Ap stands for amylopectin.

Amylose preparations tend to contain more contaminating lipids than do amylopectin preparations (Bulin, Welch & Morris, 1982). It is known that the $\delta^{13}C$ values of lipids are more negative than those of carbohydrates due to the isotopic fractionation associated with the decarboxylation of pyruvic acid (O'Leary, 1988). If contaminating lipid contributed significantly to our measured $\delta^{13}C$, we would expect the amylose fraction to be more negative than the starting material. Instead, the $\delta^{13}C$ value of amylose was less negative than that of amylopectin, indicating that contaminating lipids in the amylose preparations do not account for the difference between the $\delta^{13}C$ value of the amylose and amylopectin fractions of starch.

2.2. Mutations in the starch biosynthetic pathway alter the $\delta^{13}C$ value of starch

If the $\delta^{13}C$ values of starch and its components are influenced by isotopic discrimination by starch biosynthetic enzymes, then differences in the $\delta^{13}C$ values should be detected in starch from plants with mutations in genes involved in starch biosynthesis. To test this hypothesis, we analyzed preparations of starch from maize mutants known to have lesions in the

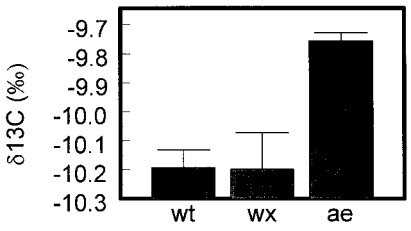

Fig. 2. Carbon isotope ratios of kernel starch from the maize inbred OH43 and mutants in the same genetic background. Starch was prepared in triplicate, and error bars represent one standard deviation from the mean. On the *x*-axis, w.t. stands for wild-type, wx stands for *waxy*1 and ae stands for *amylose extender* 1.

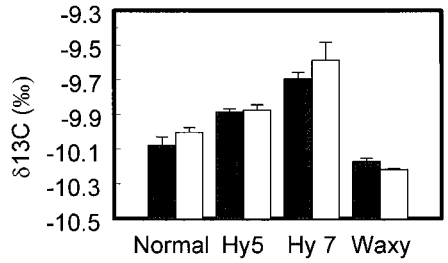

Fig. 3. Carbon isotope ratios of commercial starch. Filled bars represent commercial preparations, empty bars represent the same preparations after defatting. Each sample was measured three times, and error bars represent one standard deviation from the mean. On the *x*-axis, Hy5 and Hy7 stand for the commercial starches Hylon 5 and Hylon 7, respectively.

starch biosynthetic pathway. Many such mutants have been identified, but because our initial observation was that there is a difference in δ^{13}C values of amylose and amylopectin, we decided to concentrate on two well-characterized mutants known to have a large effect on the amylose/amylopectin ratio. Starch was purified from kernels of the maize inbred OH43 containing either the *wx*1 (*waxy*) or *ae*1 (*amylose extender*) mutation and subjected to carbon isotope ratio analysis. The δ^{13}C values of *wx*1 starch were more negative than those of *ae*1 starch (Fig. 2). This result is consistent with the analysis of amylose and amylopectin carbon isotope ratios because *wx*1 starch contains no amylose, whereas *ae*1 starch contains high levels of amylose. The carbon isotope ratios of the mutant starches were similar to the ratios of the starch fraction enriched in each mutant starch (compare Figs. 1 and 2). This lends support to the hypothesis that enzymes in the starch biosynthetic pathway influence the carbon isotope ratios of their end products.

Commercial starch preparations made from maize kernels with mutations similar to those used in the OH43 study were also analyzed for their carbon isotope ratios. Waxy starch (0% amylose), was prepared from lines carrying a *wx1* mutation. Hylon 5 (29.4% absolute amylose) and 7 (43.8% absolute amylose) are commercial preparations made from lines with an *ae*1 mutation. These amylose concentrations are in contrast to normal maize kernel starch, which contains 19.9% absolute amylose (Jane et al., submitted for publication). Carbon isotope ratios of these starches (Fig. 3) were similar to those of the OH43 derived starches.

Defatted commercial starch samples were also analyzed for their carbon isotope ratios to determine the possible influence of lipid contamination. As expected, carbon isotope ratios of defatted starch preparations were slightly less negative than before defatting them (Fig. 3). Overall, the impact of lipids on the δ^{13}C values was small.

2.3. The δ^{13}C value of mutant starches is different from vegetative tissue δ^{13}C values

Subtle differences in carbon fixation caused by pleiotropic effects of the mutation in *wx*1 and *ae*1 plants could cause variation in their tissue δ^{13}C values, even though these mutants were morphologically similar in their appearance. These pleiotropic effects could alter the δ^{13}C value of starch and could account for the observed differences in its components (Fig. 2). If the δ^{13}C differences seen in *wx*1 and *ae*1 starch were due to pleiotropic effects of these mutations, we would expect the δ^{13}C values observed in the starch to be reflected in the vegetative tissues as well. To test this hypothesis, carbon isotope ratios of vegetative tissue harvested from OH43 wild-type, *wx*1 and *ae*1 maize seedlings were compared (Fig. 4). Although there were some differences in their δ^{13}C values, these differences did not reflect the pattern of differences found in the starch of these mutants. On the contrary, carbon isotope ratios of the vegetative tissue from the *wx*1 mutants were less negative than those of the same tissue from *ae*1 mutants, as reflected by their δ^{13}C values (compare Figs. 2 and 4). The differences seen in the starch of these varieties are therefore not due to whole plant differences in carbon isotope ratio, but are due

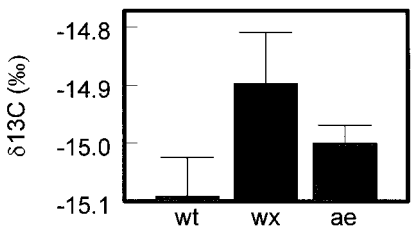

Fig. 4. Carbon isotope ratios of the above-ground portion of plants from the maize inbred OH43 and mutants in the same genetic background. Three plants were ground separately and measured. Error bars represent one standard deviation from the mean. *X*-axis labels are as in Fig. 2.

Table 1
Carbon isotope ratios of starch and starch fractions from various plant storage tissues[a]

	Starch		Starch fractions	
	wild type	wx	amylose	amylopectin
Potato tuber	-24.74 ± 0.06	-25.29 ± 0.01	-24.68 ± 0.03	-25.14 ± 0.03
Wheat seed	-24.33 ± 0.07	-25.51 ± 0.06	N.A.[b]	N.A.
Rice seed	-25.65 ± 0.07	-25.40 ± 0.03	N.A.	N.A.
Barley seed	-24.81 ± 0.10	-25.34 ± 0.11	-23.69 ± 0.09	-25.96 ± 0.01

[a] Data are expressed as $\delta^{13}C$ values in ‰ $\pm$ one standard deviation, $n = 3$.
[b] N.A. means data not available.

to differences in the carbon isotope ratios specific to the starch.

2.4. Carbon isotope discrimination occurs in the biosynthetic pathway of starch from several organisms

Carbon isotope ratios were also measured for starch and/or starch fractions from plants using the C_3 photosynthetic pathway, including wheat (*Triticum aestivum*), rice (*Oryza sativum)*, barley (*Hordeum vulgare*) and potato (*Solanum tuberosum*) (Table 1). Typically, in their dry organic matter, C_3 plants display more negative $\delta^{13}C$ values ($\backsim -27$‰) than C_4 plants ($\backsim -13.0$‰). Carbon isotope ratios of starch and starch fractions are in the range expected for products from these plants based on previous whole-plant analyses of C_3 and C_4 plants (O'Leary, 1988). The relative $\delta^{13}C$ values for amylose and amylopectin from potato and barley followed the trend seen with maize starch, that is amylopectin had a more negative $\delta^{13}C$ value than amylose and starch. Like maize waxy starch, waxy starch from the C_3 plants had a more negative $\delta^{13}C$ value than wild-type starch from the same species with the exception of rice which had a slightly (0.25‰) higher value for waxy starch than for normal starch. This could be due to varietal differences in the waxy and wild-type rice. We were unable to determine the genetic background of the wild-type rice starch, and we have observed that the $\delta^{13}C$ value of maize seed starches varies with the genetic background (data not shown). These data suggest that carbon isotope discrimination in the starch biosynthetic pathway is a general phenomenon, and not unique to maize endosperm.

There may be application for this method in chemical purity testing. Commercial waxy and high amylose corn starches are currently derived from the mutant varieties tested in this study. These starches command a premium in the marketplace, so methods of purity testing are in demand. While fairly expensive (US$12 per sample) this method offers the advantages of accuracy and a lack of hazardous waste products. Also, because carbon isotope ratio analysis readily distinguishes between starch from C_3 and C_4 plants, starch from the two major starch sources, potato and corn, can be differentiated.

Carbon isotope ratio analysis of plants and plant metabolites provides a long-term view of physiology because the carbon isotope ratio is influenced by each enzyme that makes or breaks a bond to a given carbon. This gives it advantages over instantaneous methods of biochemical or physiological analysis such as enzyme or metabolite assays because it gives information about the metabolic history of a product. The inherent drawback of this method is that in order to determine the step in a biochemical pathway where an isotope fractionation occurred, it is necessary to analyze mutants or intermediate metabolites.

We believe that this is the first report of the utilization of this methodology to assess the carbon isotopic signature of the integral components (amylose and amylopectin) of starch. While our data suggest that carbon isotope discrimination occurs in starch biosynthesis, more work is neccesary to clearly identify the source of this discrimination. One possibility is that one or more of the starch biosynthetic enzymes exhibit a kinetic isotope effect. If enzymes in the starch biosynthetic pathway have different kinetic isotope effects, then by correlating $\delta^{13}C$ values of starch fractions with carbon isotope effects displayed by the starch biosynthetic enzymes, it may be possible to determine their exact role in the biosynthesis of a particular starch fraction. For example, if one starch synthase contributes more isotopic discrimination than others do, then starch fractions made by this synthase could be identified by their characteristic isotope ratio.

A second possibilty is that the ADPG contributing to amylose has a different isotopic content than the ADPG contributing to amylopectin. This could be the case because amylose is synthesized within the granule while amylopectin is synthesized at the surface of the granule. Thus, different pools of ADPG are used in the synthesis of these two starch components. Different enzymes or transport processes acting on each pool could result in different isotopic ratios between the pools. We hope to extend this from amylose and amylopectin to include

sub-fractions of these components, for example, the long a chains of amylopectin, in the future.

3. Experimental

3.1. Plant material

In order to minimize environmental effects on carbon isotope ratios, within each experiment we used plant material produced in identical environments wherever possible. This was not possible with the commercially obtained starches. Also starch from different environments was used when comparing data between figures.

Maize whole plant material used in this study was prepared from 20-day-old plants grown in a growth chamber. The above-ground portion of each plant was harvested, lyophilized and ground to a fine powder using an analytical mill. Duplicate samples were taken from different plants.

OH43, OH43*wx*1 and OH43*ae*1 lines were the gift of Dr. Mike Lee, Iowa State University. The mutant lines were back-crossed to OH43 at least six times. Plants were grown in the field during 1997, and kernels were bulk-harvested from the one self-pollinated row of each variety.

Barley seeds were obtained from Dr. W. Newman, Montana State University, Bozeman, MT and wheat seeds were obtained from Dr. Robert Graybosch, University of Nebraska, Lincoln. Waxy rice was obtained from a local market.

3.2. Starch purification

Starch used for the experiments described in Fig. 2 was purified from field grown OH43, OH43*wx*1 and OH43*ae*1 maize kernels using a wet-mill extraction procedure as described by (White, Abbas, Pollack & Johnson, 1990). Maize (field grown OH43), barley and potato starches were fractionated into amylose and amylopectin following standard methods (Jane & Chen, 1992; Schoch, 1942).

3.3. Carbon isotope ratio analysis

Carbon isotope ratio measurements were made following published procedures (Madhavan, 1991). A subsample of the powdered plant or isolated starch (2–3 mg) was combusted to CO^2 and analyzed for carbon isotope ratio using an elemental analyzer (Heraeus, CHN-O Rapid) interfaced with an automated trapping box system and a Finnigan Delta-S isotope ratio mass spectrometer. The isotope ratio of each sample was determined by comparisons with repeated analyses of cellulose and acetanilide which have been calibrated (precision $\pm 0.2‰$) relative to PDB (Pee Dee Belemnite international standard). The ^{13}C content of CO_2 is given as an isotope ratio, R, and $\delta^{13}C$ is given by: $\delta^{13}C = [R_{sample}/R_{standard} - 1] \times 1000$ (Craig, 1957).

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