Branching Ratios of Starch via Proton Nuclear Magnetic Resonance and Their Use in Determining Amylose/Amylopectin Content: Evidence for Three Types of Amylopectin

Larson B. Dunn, Jr.* and William J. Krueger[1)]

United States Department of Agriculture/Agricultural Research Service, ational Center for Agricultural Utilization Research, Plant Polymer Research Unit, Peoria, IL 61604, and University of Illinois at Urbana-Champaign, Department of Food Science and Human Nutrition, Urbana, IL 61801, USA

SUMMARY. The branching (α-1,4)/(α-1,6) ratio of starch from a number of sources can be quickly and accurately determined by proton nuclear magnetic resonance (NMR). This NMR ratio, with standard ratios for isolated amylose and amylopectin, can then be used to determine the amylose/amylopectin content of starches. In the course of determining the amylose/amylopectin content of various starches, it was discovered that two different types of amylopectin standards were required to obtain results comparable to those obtained from iodine-binding amylose determinations. These two types were a waxy amylopectin, with a high level of branching, and a potato amylopectin, with a lower level of branching. A third type of amylopectin, with a still lower level of branching, is apparently present in high amylose cornstarches, leading to the conclusion that starches with higher amylose contents generally contain amylopectin with a lower level of branching. The three amylopectin types are referred to as amylopectin I, II and III, with the higher numeral coinciding with higher branching (α-1,4)/(α-1,6) ratio, or less branching.

Introduction

Methods based on iodine binding for determining the amylose/amylopectin content of starches are generally long and tedious, and their dependency on certain assumptions about the iodine binding capacity of amylopectin may lead to doubts about their accuracy. Other researchers have reported the use of nuclear magnetic resonance (NMR) spectroscopy to analyze starch structure. For example, proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy have been used to determine the extent of branching in glycogens, amylopectins and degraded starches[2)]. The anomeric protons involved in α-(1,4) and α-(1,6) linkages are sufficiently resolved for quantification by proton NMR. Gidley[3)] reported α-(1,4)/α-(1,6) ratios of 20 and 26 for waxy maize and degraded starches respectively. Proton and carbon-13

 CCC 1022-1360/99/$ 17.50+.50/0

provide similar information, but the greater sensitivity of proton over carbon-13 NMR led Gidley to prefer the former.

The amylose portion of starch possesses the ability to bind with iodine, forming a blue polyiodine complex. The two most common methods for determining proportions of amylose in starch utilize this iodine binding capacity and measure amylose content by potentiometric titration[4] or colorimetrically[5]. These methods are generally time consuming and have undergone numerous modifications to minimize analysis time[6], account for iodine binding to amylopectin[7], and reduce the interference of monoacyl lipids bound to amylose[8]. Gel permeation chromatography has been used to profile the components of native starch or starch treating with debranching enzymes as a means of fractionation and quantitation of amylose and amylopectin[9]. Others have used differential scanning calorimetry to establish linear relationships between enthalpies associated with the melting of amylose lipid complexes and amylose content[10]. The myriad of iodine absorption and other methods published indicate their inherent sensitivities to experimental conditions and the lack of a convenient, universal method to encompass starches from a variety of sources.

If the NMR determination of the degree of branching, the branching ratio, or branch fraction, can be optimized to give accurate, reproducible results, then the branching ratio should be an indication of the proportion of amylopectin in the starch. The proton NMR method has been optimized in terms of sample size, solvent systems, sample handling and NMR variables, such as signal-to-noise (S/N), line broadening, pulse width, and delay time. We have then made use of these branching ratios in determining the amylose/amylopectin fractions of various starches[11].

Results and discussion

This optimized NMR method was used to determine the α-(1,4)/α-(1,6) ratios of selected maize starches. Table 1 summarizes the ratios and amylose contents of waxy maize, corn, Amaizo V, and Amylomaize VII starches. The ratios increased as the amylose content increased. This is expected because amylose is essentially a linear polymer with only a low level of branching. Waxy maize gave a ratio of 19.0. Gidley[3] found waxy maize to have a ratio of 20.

Table 1. α-(1,4)/α-(1,6) Ratios and Amylose Contents of Various Maize Starches

Source	*(1,4)/(1,6)*[a] *Ratio*	*Amylose (%)*[a,b]
Waxy Maize	19.0 ± 0.5	0
Maize	27.8 ± 0.6	26.7 ± 1.2
Amaizo V	49.9 ± 3.4	50.8 ± 0.7
Amylomaize VII	83.9 ± 4.0	71.7 ± 0.9
Maize Amylose	203.7 ± 19.0	99

[a]Values are averages of triplicate analyses ± standard deviations.

[b]Determined by iodine affinity.

The (1,4)/(1,6) ratios of a variety of starches are shown in Table 2. Potato amylopectin, determined to have a ratio of 20.8, and waxy maize amylopectin with a ratio of 19, were used as standards. Gidley[3] reported ratios of 23 and 20 for potato amylopectin and waxy maize starch, respectively. The purity of the amylopectins were estimated by iodine affinity and determined to be essentially free of amylose. Amylose, obtained via butanol precipation[12] from dent cornstarch, was used as the amylose standard and was determined to have a branching ratio of approximately 203.7 (see Table 1). Analyses of a number of isolated amylose samples indicated little difference between samples that were isolated from a number of starch sources, although it was extremely difficult to obtain reproducible ratios due to the very small size of the α-1,6 peak and problems with integrating it accurately.

Amylose content of starches was determined from the equation:

$$B = F_1(b_1) + F_2(b_2) \qquad (1)$$

where the branch fraction of the sample B is equal to the sum of the products of the weight fractions (F) and branch fractions (b) of amylopectin and amylose, respectively. The branch fraction (B) of the starch was determined by proton NMR and is related to the α-(1,4)/α-(1,6) ratio by the equation:

Table 2. (1,4)/(1,6) Ratios and Amylose Contents Determined by Proton NMR[a]

Source	*1,4/1,6 Ratio*	*IA[b] (% Amylose)*	*AP (21)[c] (% Amylose)*	*AP (19)[d] (% Amylose)*
Maize	27.8 ± 0.6	26.7	24.1 ± 1.7	30.5 ± 1.6
Amaizo V	49.9 ± 3.4	50.8	57.0 ± 2.8	60.7 ± 2.6
Amylomaize VII	83.9 ± 4.0	71.7	74.3 ± 1.2	76.4 ± 1.1
Wheat	25.8 ± 0.5	22.5	18.5 ± 1.4	25.4 ± 1.3
Potato	27.5 ± 0.9	23.3	23.4 ± 2.4	29.8 ± 2.2
Rice	21.7 ± 0.4	13.6	4.0 ± 1.5	12.0 ± 1.4
Pea	27.8 ± 1.0	35.5	24.2 ± 2.7	30.6 ± 2.5
Tapioca	23.4 ± 0.1	18.8	10.7 ± 0.3	18.2 ± 0.3

[a] Values are averages of triplicate analyses ± standard deviation.
[b] Amylose content determined by iodine affinity; CV less than 5%.
[c] Amylose content determined using 1,4/1,6 = 21 as amylopectin standard (21).
[d] Amylose content determined using 1,4/1,6 = 19 as amylopectin standard (19).

$$B = 1/[\alpha\text{-}(1,4)/\alpha\text{-}(1,6) + 1] \qquad (2)$$

The amylopectin degree of branching of the starch must be known prior to the determination. Calculations were made by accounting for the slight branching in amylose or by assuming the contribution amylose to the branch fraction is negligible, in which case equation **1** reduces to $B = F_1(b_1)$. Using these equations, the α-(1,4)/α-(1,6) ratio of normal corn starch analyzed by Takeda, et. al.[13], was estimated to be 27.2. This number agrees with the corn starch ratio (27.8) determined by the optimized proton NMR method.

Calculations accounting for the slight branching in amylose were determined using the branching ratio of maize amylose (1,4/1,6 = 203.7). Accounting for amylose branching resulted in a 10.8% or 11.9% increase in the amylose contents, depending on the amylopectin standard used, 19 or 21, respectively. Wheat, rice, pea, and tapioca amylose contents were best predicted using 19 as the amylopectin standard. An amylopectin standard of 21 under-predicted the amylose content of these starches. Determination of maize and potato amylose

contents were best estimated using an amylopectin standard of 21, as the amylopectin standard of 19 over-estimated the amylose contents when compared to iodine affinity values.

Amylomaize starches have been reported to have an amylopectin fraction with an average chain length near 30 [14]. Therefore, an amylopectin branching ratio of 30 and an amylose standard branching ratio of at least 203 were used for the calculations of the high amylose starches. Amaizo V was determined to have an amylose content of 39.0% or 43.3%, depending on whether or not the branching of amylose was accounted for in the calculation. The values for amylomaize VII starch were 63.4% and 70.4%, respectively. These values were less than those determined by iodine affinity except for the Amylomaize VII starch with the amylose branching included. An amylopectin standard ratio of 21 resulted in a value of 74.3% amylose, which was close to the value of 71% predicted by iodine affinity. However, the accuracy of the iodine affinity method for high amylose starches may be suspect due to the greater iodine absorption of longer amylopectin chains[15]. Therefore, comparisons between values determined by NMR and the iodine affinity method must be made with caution.

Perhaps the most important conclusions to draw from the data in Table 2 are: 1) use of an appropriate standard amylopectin for each starch results in an amylose/amylopectin determination close to, but always lower than the iodine affinity number and 2) one of two amylopectin standards gives acceptable results for each starch, except for the high amylose cornstarches, which apparently require a third type of amylopectin standard. This seems to indicate that, based on branching ratio, there are at least three types of amylopectin present in starch. The branching ratio of the amylopectin present seems to roughly correlate with the amount of amylose present in the starch, with the higher amylose amounts corresponding to starches that may contain a lower branched amylopectin. Pea starch may be an exception to this generalization. Nevertheless, this theory may have important ramifications concerning the biosynthesis of starch, as it is logical that the enzymes that are constructing a starch with higher amounts of amylose would also tend to reduce the amount of branching in amylopectin.

If there are indeed three different types of amylopectin based on its branching, then some terminology should be proposed to describe this situation. We suggest that amylopectin I, II and III be used to describe the three types of amylopectin, with amylopectin I corresponding to waxy amylopectin, with a branching ratio of 19, amylopectin II corresponding to potato

amylopectin, with a branching ratio of 21, and amylopectin III corresponding to the amylopectin present in high amylose cornstarches (>=30), of yet unknown branching ratio, but certainly higher than potato amylopectin. In other words, the higher number amylopectin refers to higher branching ratio. According to this classification system, common starches would be classified as follows:

Table 3. Classification of Starches According Amylopectin Type

Amylopectin I	*Amylopectin II*	*Amylopectin III*
Pea	Maize	Amaizo V
Rice	Potato	Amylomaize VII
Tapioca	Wheat	
Waxy		

Conclusions

Branching ratios can be determined accurately and reproducibly for a variety of starches, and those α-(1,4)/α-(1,6) ratios can be used to determine amylose/amylopectin fractions. The results are comparable to iodine affinity numbers, although almost always slightly lower amylose contents, probably indicating an over estimation of amylose by the iodine method because of amylopectin iodine binding.

The amylose/amylopectin determinations appear to be most accurate for most starches when one of two amylopectin standards are used, with the exception of high amylose cornstarches, which apparently require a third amylopectin standard. Pea, rice, tapioca and waxy maize starches appear to belong to group of starches containing amylopectin with a high level of branching, having a α-(1,4)/α-(1,6) ratio of about 19. Maize (or dent cornstarch), potato and wheat starches appear to belong to group of starches containing amylopectin with a lower level of branching, having a α-(1,4)/α-(1,6) ratio of about 21. High amylose cornstarches appear to belong a group having the lowest level of branching.

As there appears to be three different types of amylopectin, according to its branching, we propose the terminology of amylopectin I, II and III, with the numeral increasing with

increasing branching ratio. The trend that starches with increasing amounts of amylose seem to contain lesser branched amylopectin may have important ramifications concerning the biosynthesis of starch.

Experimental

Samples were prepared by weighing 10 mg starch directly into 5 mm NMR tubes and pipeting 0.7 ml of a 0.6 *N* potassium hydroxide (KOD) solution containing 1% 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS), as internal standard, into the tube. The potassium hydroxide (KOD) solution was prepared by dissolving an appropriate amount of KOH into a limited portion of deuterium oxide and evaporating overnight to dryness. The dried KOD was then dissolved in the proper amount of deuterium oxide. This procedure aides in diminishing the residual HOD signal. The samples were heated in the NMR tubes with a heat gun until the solution was clear and homogeneous. Care was taken not to overheat, or the solution will eject out of NMR tube. The sample was analyzed immediately after heating.

Proton NMR spectra were collected on a Varian U400 Nuclear Magnetic Spectrometer operating at 399.952 MHz., with the variable temperature control set at 90°C. Data was collected using the following instrument parameters: Pulse width, 32 μsec; 256 scans; acquisition time, 4.096 sec; extra delay time, 0.0 sec; line broadening, 1.0 Hz.

After the spectrum was referenced to DSS at 0 ppm, the spectrum was phased and narrowed to 4.7-6.1 ppm. The integrals were measured from 5.42-5.10 ppm for the α-(1,4) C-1 proton and 5.0-4.80 for the α-(1,6) C-1 proton.

It has also been observed that using a solvent system consisting of DMSO-d_6/2-3 drops trifluoroacetic acid (TFA) may make it easier to dissolve starch samples, with little or no change in quantitation. This solvent system does not require the use of DSS as a standard, as the spectra may be referenced to the DMSO solvent peak at 2.49 ppm. In most cases, the DMSO-d_6/TFA system may be preferred, except where molecular weights of intact starches are being determined. In those cases, this system leads to hydrolysis of the starch over the longer data aquisition times required to observe reducing ends.

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[1] Current address: Kelloggs Co., Battle Creek, MI.

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