A Method for Estimating the Nature and Relative Proportions of Amorphous, Single, and Double-Helical Components in Starch Granules by ^{13}C CP/MAS NMR

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An improved method to analyze the ^{13}C NMR spectra of native starches, which considers the contribution of the V-type conformation and the nature of the amorphous component, has been developed. Starch spectra are separated into amorphous and ordered subspectra, using intensity at 84 ppm as a reference point. The ordered subspectra of high amylose starches show the presence of both V-type single helices and B-type double helices. Relative proportions of amorphous, single, and double-helical conformations are estimated by apportioning intensity of C1 peak areas between conformational types on the basis of ordered and amorphous subspectra of the native starch. Quantitative analysis shows that the V-type single-helical component increases with amylose content of starches. Different amorphous subspectra are needed to provide a consistent analysis of granular starches from diverse sources. The method of preparation was found to be more important than the starch botanical origin in determining ^{13}C NMR spectral features of amorphous samples.

Starch is the major polysaccharide reserve material in plants and is composed of two main polysaccharides: amylose and amylopectin. Starch occurs in nature as semicrystalline granules and the crystalline organization in starch granules has been studied extensively in recent decades with a variety of measurement techniques such as optical and electron microscopy, X-ray diffraction, neutron scattering, and solid-state NMR spectroscopy.[1−7] X-ray diffraction provides an elucidation of the long-range molecular order, typically termed as crystallinity, which is due to ordered arrays of double helices formed by the amylopectin side chains.[8] Two different polymorphic forms are commonly observed in native starches, namely, the A-type and B-type polymorphs, which consist of parallel-packed, left-handed double helices.[9−12] The differences in the X-ray diffraction pattern of the A- and B-type crystalline forms are mainly attributed to differences in their crystal symmetry and details of the association of water molecules.[9−12] A third form reported for starch molecules is the V-type polymorph, which is a single, left-handed helix often with a complexing agent included in the helical channel.[13] This V-type polymorph has been evidenced in native starch granules (on the basis of ^{13}C chemical shifts for included lipids)[14] and is attributed to complexation between lipids and a fraction of the amylose component in several starches. Crystalline forms of the V-type polymorph have also been observed in high amylose starches by X-ray diffraction[15]

The use of ^{13}C CP/MAS NMR in starch characterization provides information on the molecular organization at shorter distance scales than those probed by X-ray diffraction.[7] The different crystalline polymorphic forms of starch (A-, B-, and V-type) as well as amorphous starch have been reported to exhibit different ^{13}C NMR spectral features.[8,16−18] The spectra of native starches are typically analyzed as a composite of spectra from the amorphous (single chain) and the ordered double-helical components.[8] Accordingly, the relative proportions of amorphous and ordered double-helical components in starch can be estimated by simulating the spectra of native starch as linear combinations of the subspectra because of the amorphous and appropriate crystalline polymorph.[8] An alternative approach to estimate the double-helix content in starch granules has been proposed by Bogracheva et al.[19] In this approach, the proportion of the peak area for C4 resonances relative to the total area of the spectrum (abbreviated as C4-PPA) for native starch is divided by that of a standard amorphous starch. The result is expressed as a percentage to indicate the relative amount of amorphous material in native starch granules.

However, two key assumptions in these methods are (1) that native starch granules are composed of only two components, namely, the double-helical structure and the amorphous phase, and (2) that the amorphous phase is the same for all starches. These assumptions, although generally accepted in the literature, require further examination especially when characterizing starches with high amylose content and which may contain a substantial amount of the V-type polymorph. The results of this current study demonstrate (1) the significant contribution of the V-type polymorph to the ^{13}C NMR spectra of native starches particularly (but not exclusively) high amylose starches and (2) the variation in spectral characteristics for both laboratory-prepared amorphous samples and the amorphous phase of starch

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granules. Accordingly, an improved method of analyzing the ^{13}C NMR spectra of native starches to estimate the relative proportions and nature of amorphous, single V-type, and double-helical (A- or B-type) components within granules and other starch samples is proposed.

Experimental Section

Four types of commercial maize starches with different amylose contents (waxy, regular, Gelose 50, and Gelose 80) were obtained from Penford Australia Limited (Lane Cove, NSW 2066, Australia). Rice starch was a gift from Jody Higgins (CSIRO Plant Industry, Canberra, Australia) and was dried by lyophilization. The amylose content of starches was estimated by the iodine-binding method.[20] Amorphous standards based on waxy maize, regular maize, wheat, and rice starches were prepared by heating starch suspensions (1% w/v) for 30 min at 95 °C. These solutions were then lyophilized. A second amorphous waxy maize starch standard material was prepared by heating a 5% starch suspension for 30 min at 95 °C and drying in a vacuum oven at 60 °C for 48 h. The dried gelatinized starch sample was then crushed and passed through a 180-mm sieve to obtain amorphous starch powder. X-ray diffraction analyses of these amorphous samples gave a typical broad "amorphous" halo, indicating the absence of crystalline structure in these samples.

The water contents of all starch samples were adjusted via vapor-phase isopiestic equilibration over saturated K_2CO_3 salt solution at 20 °C. Starch samples were initially conditioned at a relative vapor pressure of zero to ensure a uniform starting point for all starches following the method of Spiess and Wolf.[21] The preconditioned starch powders were placed in desiccators for one week. The desiccators contained a saturated solution of K_2CO_3 at 20 °C, providing an environment with a relative humidity (RH) of 44%.[22] The resulting moisture content was similar for all conditioned starch samples (within the range 9−11% w/w) as determined by the AOAC standard method of moisture content determination.[23]

X-ray diffraction measurements were performed on a Bruker AXS D8 Advance X-ray Diffractometer operating at 40 kV and 30 mA. Cu Kα1 radiation ($\lambda = 0.15405$ nm) was used. The scanning region of the diffraction angle 2θ was 1−50°. A step interval of 0.02° and a scan rate of 0.5°/min were employed for all measurements. The degree of crystallinity was determined according to the general procedure of crystallinity determination for semicrystalline polymers.[24,25]

The solid-state ^{13}C CP/MAS NMR experiments were performed at a ^{13}C frequency of 75.46 MHz on a Bruker MSL-300 spectrometer. Approximately 200−300 mg of starch samples were packed in a 4-mm diameter, cylindrical, PSZ (partially stabilized zirconium oxide) rotor with a KelF end cap. The rotor was spun at 5−6 kHz at the magic angle (54.7°). The 90° pulse width was 5 μs and a contact time of 1 ms was used for all starches with a recycle delay of 3 s. The spectral width was 38 kHz, acquisition time 50 ms, time domain points 2 k, transform size 4 k, and line broadening 50 Hz. At least 2400 scans were accumulated for each spectrum. Spectra were referenced to external adamantane. The rate of cross-polarization was determined in a variable contact time experiment for amorphous waxy maize starch and the major signal intensities of C1 (103 ppm), C2, 3, and 5 (73 ppm) and C4 (82 ppm) sites were similar for contact times from 0.2 to 2 ms, hence, the spectra are considered quantitative, as had been shown previously for granular starches.[8] The intensity due to C6 at 61 ppm, however, differed from these other peaks, presumably because of a lower rate of cross-polarization reflecting the partial averaging of the heteronuclear dipolar couplings as a result of motion of this exocyclic group.

The analysis of the NMR spectra involves the decomposition of the spectrum of native starch into its respective amorphous and ordered subspectra. The contribution from the amorphous component was determined by adjusting the intensity of a separately determined amorphous subspectrum with a scaling factor so that zero intensity was obtained at a particular chemical shift within the C4 site. This step was performed using the Solver data analysis tool in Microsoft Excel software. The ordered subspectrum was obtained by subtracting the subspectrum due to the amorphous component from the native starch spectrum. The program PeakFitTM version 4.11 (SYSTAT Software Inc., CA) was used to peak fit all spectra including the subspectra due to the amorphous and ordered components as generated from the subtraction method described above. Repeat NMR experiments on a single sample gave identical (±2%) component analysis results.

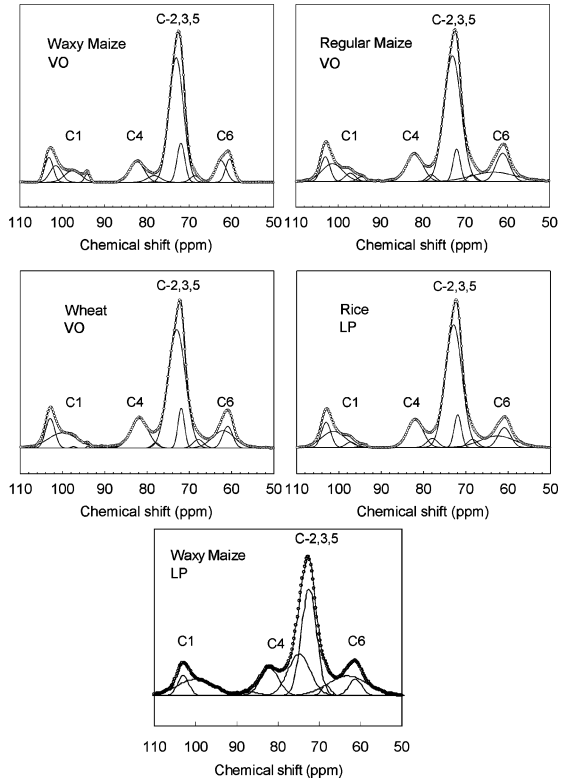

Figure 1. The ^{13}C CP/MAS NMR spectra for amorphous starches from different botanical sources and their peak-fitted profiles. LP = lyophilized, VO = vacuum-oven-dried. Experimental data are presented as circles. The peak-fitting correlation coefficient values were at least 0.999.

Results and Discussion

Method Development. The ^{13}C CP/MAS NMR spectra for the amorphous starch materials are presented in Figure 1. All amorphous starches exhibit broadly similar spectral features; computer peak fitting of each amorphous spectra yielded 9−11 peaks, each with a Gaussian profile (Figure 1). The peak fitting performed on amorphous spectra was aimed at the simulation of spectra to aid the deconvolution of native starch spectra rather than for assignments of chemical shifts or for elucidating the conformational origins of the chemical shifts. Close inspection of C1 signals for amorphous (as defined by the absence of Bragg peaks in X-ray powder diffraction patterns) waxy maize starch prepared by two different processes reveals small but significant differences. For example, the intensity at ca. 95 ppm differs for the two forms. Following drying of a heated waxy maize starch suspension within a vacuum oven, the NMR spectrum shows greater intensity at this chemical shift than after lyophilization of the same heated starch suspension (Figure 1). This difference between amorphous spectral features because of preparation conditions was found consistently for a range of starches from maize, barley, and rice sources. As it cannot be

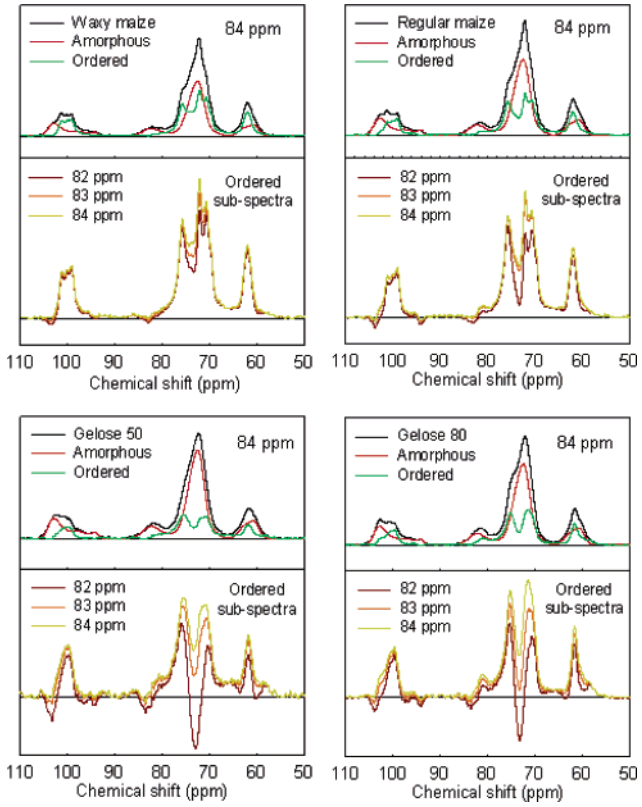

Figure 2. The decomposition of the ^{13}C CP/MAS NMR spectra of maize starches into contributions from the amorphous and ordered phases by subtraction of the subspectrum for the amorphous phase obtained after vacuum oven drying of gelatinized waxy maize starch. The subtraction was performed by scaling the intensity of the amorphous subspectrum so the resultant intensity was zero at 82, 83, or 84 ppm.

determined which type of amorphous sample preparation would be representative of the amorphous phase of starch granules, both types of spectra were used in subsequent analyses of starch granule spectra.

The NMR spectra of the native starches were resolved into subspectra for the amorphous and ordered phases using a subtraction technique. The intensity of the selected amorphous subspectrum was adjusted by a scaling factor so that on subtraction the intensity at a particular chemical shift was zero. The position of this point was chosen to be within the peak due to the amorphous C4 carbon because it is relatively isolated from other resonances and does not overlap with the chemical shifts of the A- and B-type crystalline materials.[8,18,19] The resultant spectra due to the nonamorphous (ordered) component for all maize starch samples examined in this work are shown in Figure 2. Four chemical shifts were tested to determine the best reference position, namely, 82, 83, 84, and 85 ppm. As can be seen from Figure 2, the reference chemical shift of 84 ppm provided a better subtraction than 82 or 83 ppm, with no negative peaks appearing in the ordered subspectra. When either 82 or 83 ppm was used as the reference position, negative intensities were noted in the resulting ordered subspectra, particularly for those of high amylose maize starches (Gelose 50 and Gelose 80). Spectral intensity at 85 ppm was very low leading to an unacceptably high variability in subtracted spectra. The integrated areas under the subspectra for the amorphous and ordered phases generated by subtraction of an amorphous spectrum to zero at 84 ppm can be used to provide a measure of the total amorphous content of the starches.

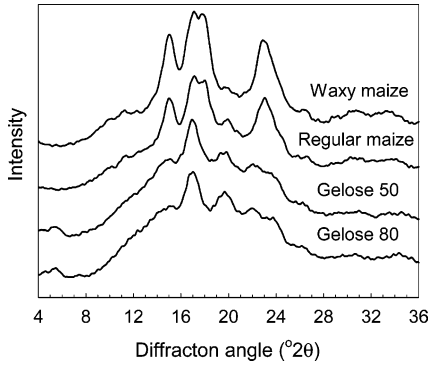

Figure 3. X-ray diffraction patterns for the maize starches studied.

A closer examination of the resulting crystalline subspectra obtained by spectral subtraction using 84 ppm as the reference point indicates the occurrence of distinct shoulders at chemical shifts of around 81 and 102 ppm, for all starches except for waxy maize, and increasing intensity with increasing amylose content. The positions of these peak shoulders, that is, 81 and 102 ppm, are coincident with the C4 and C1 chemical shifts of the V-type polymorph, respectively,[18] which suggests the presence of single-helix V-type polymorphs in starches that exhibit these peak shoulders in their ordered subspectra. This consequently suggests that the negative peaks obtained on subtraction using 82 and 83 ppm as the reference positions can be attributed to the presence of the V-type polymorph. In support of this, reference to the spectra of amorphous and highly crystalline starch model materials[16−18] indicates that only amorphous starch contributes significant signal intensity at 84 ppm.

The ordered subspectra obtained by the subtraction method (Figure 2) show distinct signal multiplicities in the C1 region (94−105 ppm) as a consequence of differences in the symmetry of helix packing between the two crystalline types (the A- and B-type polymorphic forms). A characteristic triplet due to the A-type double-helical conformation, which adopts a 2-fold packing symmetry resulting in three inequivalent residues per turn,[16−18,26] is evident in the C1 region of the spectra for waxy and regular maize starches. This observation is consistent with the X-ray diffraction patterns of these samples (Figure 3), which show diffraction peaks typical for the A-type polymorph.[27,28] On the other hand, the high amylose maize starches (Gelose 50 and Gelose 80) which, according to the X-ray diffraction patterns in Figure 3, show the typical B-type crystalline polymorphic form,[27,28] display a characteristic C1 doublet in the ^{13}C NMR spectra. X-ray intensity at ca. 20° 2θ for Gelose 50 and (particularly) Gelose 80 is also consistent with a minor component of V-type crystallinity.[28] The doublet structure in the NMR spectra of the B-type crystalline form can be attributed to the threefold symmetry of adjacent helices leading to two inequivalent residues per turn.[16−18,26] Therefore, on subtraction, the characteristic triplet or doublet structures due to C1 resonances in ordered environments become well resolved, indicating that the subtraction process has removed the overlapping amorphous signal.[29] This gives confidence that the subtraction procedure is valid.

Additional information on the conformation of the starch polymers can be obtained by fitting of the ordered subspectra to individual peaks with the minimum number of peaks possible. The use of peaks assuming a linear combination of (50/50) Lorentzian and Gaussian shapes provided the best fit (Figure 4) for ordered phases. The positions of these peaks and the peak

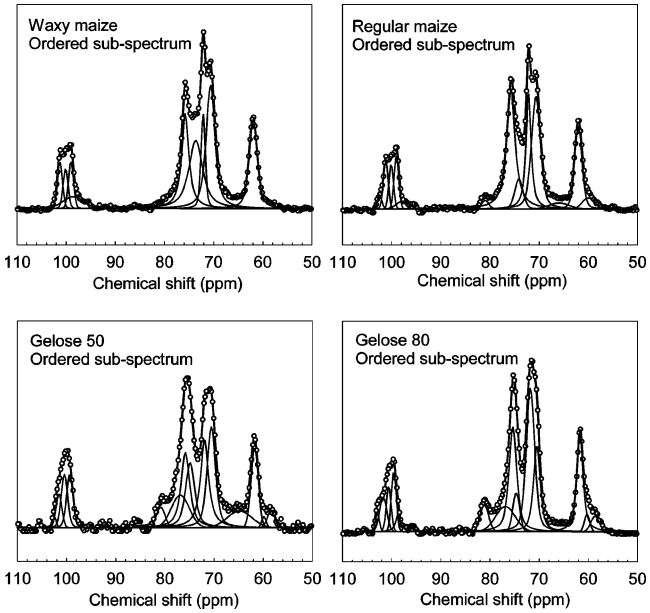

Figure 4. Deconvolution of the ordered subspectra for maize starches into individual peaks. Experimental data are presented as points; the peak fitting simulation yielded a correlation coefficient of at least 0.987.

assignments to the different carbon atoms in the glucose monomer are provided in Table 1. These peak assignments were made in accordance with previous ^{13}C solid-state NMR studies on model crystalline and amorphous starch materials.[16−18]

The data shown in Figures 2 and 4 were obtained using the spectrum of vacuum-oven-dried-gelatinized waxy maize starch as the amorphous reference. As shown in Figure 1, however, amorphous material obtained by lyophilization results in a slightly different signal shape for the C1 peak. To determine if this difference influences the subtraction methodology described above, the analysis shown in Figure 2 was repeated using the lyophilized waxy maize starch as the amorphous reference. Results are shown in Figure 5. For all starches, subtraction at 84 ppm resulted in negative residual peak intensity, notably at ca. 105 ppm. Subtraction at 82 or 83 ppm resulted in greater negative intensities similar to Figure 2. This shows that vacuum-oven-dried-gelatinized waxy maize starch is a better model for the ^{13}C spectrum of the amorphous phase of the specific (commercial) maize starch samples studied than lyophilized material. The spectral similarity of samples prepared by the same technique (vacuum oven drying followed by desiccation and controlled rehydration or lyophilization) suggests that process conditions are more important than botanical origin in determining the detailed spectral form of amorphous starches.

A similar comparison of the effects of amorphous material preparation conditions on suitability for spectral subtraction of native starch spectra was made for a rice starch, which had been lyophilized, not vacuum-oven-dried. In this case, it was found that vacuum-oven-dried amorphous samples resulted in negative peaks, similar to those found for maize starches and a lyophilized amorphous standard (Figure 5), after subtraction using 82, 83, or 84 ppm as a reference chemical shift. Figure 6A compares the ordered subspectra for lyophilized rice starch, obtained by subtraction of a lyophilized amorphous standard, at three different reference points. As shown for maize starch, using 84 ppm as the reference point provides the most realistic representation of the (nominally) ordered signature as negative intensities are avoided. The computer peak fitting of the resulting ordered spectrum for rice starch yielded 12 peaks assuming a linear combination (50/50) of Lorentzian and Gaussian profiles, Figure 6B. The C1 region (94−105 ppm) shows the characteristic triplet signal multiplicity expected for the A-type double-helical conformation. The X-ray powder diffraction patterns of the lyophilized rice starch are also characteristic of the A-type conformation.

Quantitative Analysis. The ability to subtract an amorphous component from a native-starch spectrum with the residual spectrum completely assigned to a combination of double-helical and single-helical polymorphs provides a demonstration of the validity of the proposed analysis method for spectra of granule samples. This method makes no assumptions about the nature of the individual components and provides test criteria (e.g., absence of negative peaks after subtraction) for establishing the relevance of amorphous model spectra. There is also no assumption made about the peak widths or resolution of signals for ordered components, in contrast to an earlier method involving linear combinations of spectra for model amorphous and crystalline material.[8] As relative intensities for C1−5 signals are insensitive to duration of applied cross-polarization contact time,[8] there is confidence that computation of the integrated area of the amorphous and ordered subspectra relative to the total area of the native starch spectrum yields the percentage of amorphous and ordered components, respectively. The areas underneath the amorphous and ordered subspectra at C1 regions (94−105 ppm) were used in this study since, in this isolated range, there are no overlapping signals from other carbon sites and the correlation between chain conformation and glycosidic torsion angles is more straightforward.[18,29] Although it is possible to determine the relative proportion of double helices in starch granules using the C4-PPA method,[19] this method includes the V-type polymorph in the calculation of the amorphous (non-double-helical) content because C4 chemical shifts for the single-helical V-type conformation and amorphous subspectra overlap. As shown in Figures 4 and 6 and detailed in Table 1, it is possible to resolve ordered subspectra in the C1 region into the single-helix V-type component and the double-helix component (A- or B-type polymorph). Comparing the area for C1 signals in the ordered subspectrum relative to the area for C1 signals for the starting starch spectrum, it is then possible to calculate the relative proportions of amorphous, single-helical, and double-helical features within the starch sample. The estimated values for these three components for maize and rice starches are shown in Table 2. There are two internal consistency measurements that can be used to check that the data obtained largely from the C1 signal is valid for other spectral signals. One is to compare the ratio of areas in ordered and amorphous subspectra that is due to C-2,3,4,5 with that due to C1. The second is to check that the combined area of C-2,3,4,5 is 4 times that of C1 in each of the amorphous and ordered subspectra. In all cases in the present study, these internal consistency measurements validated the quantitative data (Table 2) obtained primarily from C1 signals.

The percentage of double helices for waxy and regular maize starches as estimated by this method is comparable to those reported earlier.[8,19] The double-helix contents estimated for the high amylose starches are, however, lower than a previous report;[8] this can probably be attributed to the presence of a substantial proportion of V-type conformation in these starches which was not previously taken into account. The higher values for double-helix contents compared with X-ray crystallinity suggests that not all double-helix chain segments are within crystalline arrays.[8]

Table 1. Positions of the Deconvoluted Peaks for the Ordered Subspectra of Maize Starches and the Assignment of Their Peaks to Different Carbon Atoms in the Glucose Ring

		C atom of glucose		
starch	peak positions (ppm)	A-type polymorph	B-type polymorph	V-type polymorph
waxy maize				C1
	101.3, 100.1, 99.0	C1		
				C4
	75.8, 73.6, 72.1, 70.6	C2,3,4,5		
	61.9	C6		
regular maize	102.4			C1
	101.2, 100.1, 99.0	C1		
	81.0			C4
	75.7, 74.1, 72.2, 70.6	C2,3,4,5		
	65.9, 62.0, 60.0	C6		
Gelose 50	102.4, 101.5			C1
	100.4, 99.3		C1	
	80.8			C4
	76.9, 75.8, 74.9, 71.9, 70.5		C2,3,4,5	
	64.8, 61.7, 59.9, 58.4		C6	
Gelose 80	102.9, 101.7			C1
	100.6, 99.6		C1	
	81.1			C4
	76.9, 75.3, 74.7, 71.8, 70.5		C2,3,4,5	
	61.7, 60.2, 58.6		C6	

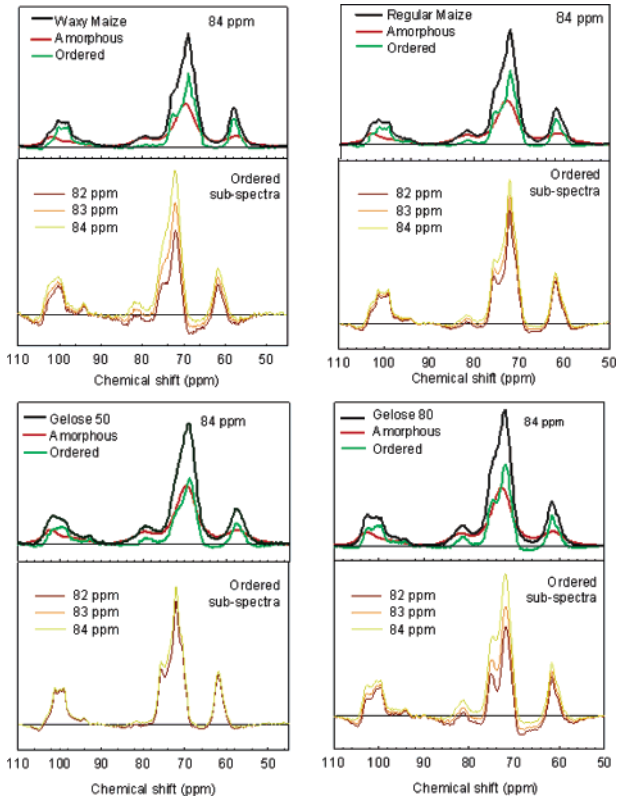

Figure 5. Subtraction of maize starches using lyophilized gelatinized waxy maize starch as the amorphous standard.

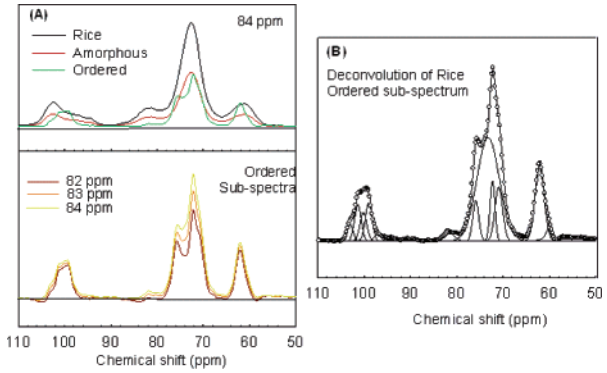

Figure 6. (A) The decomposition of the ^{13}C NMR spectrum into contributions from the amorphous and ordered phases in a rice starch by subtraction at 84 ppm and comparison of the ordered subspectra obtained from subtraction at different chemical shift reference points. (B) Deconvolution of the ordered subspectrum for rice starch into individual peaks using 84 ppm as reference point. Experimental data are presented as points. The peak-fitting simulation yielded a correlation coefficient of at least 0.995.

The Nature of Amorphous Starch. The term amorphous has a negative definition relating to the absence of detectable order. In this study, it was found that the preparation conditions (particularly drying method) used to isolate amorphous starch material resulted in small but consistent spectral differences that were important in accounting for spectral features in granular starches. This suggests that the amorphous phase of the lyophilized rice and commercially air-dried maize starch granules studied here has different spectral signatures. Previous reports have identified that moisture content and temperature as well as process conditions may affect the shape of the C1 peak for amorphous starch[19,29,30] but have not suggested that granular starches may have different amorphous phase ^{13}C spectra. The practical consequence of the current finding that distinct amorphous spectral features are associated with different starch granules is that it cannot be assumed that one type of amorphous material is sufficient to analyze spectra from specific starch samples, particularly if the isolation and drying method is not known. The methodology used in this study, however, allows an assessment of the suitability of a specific amorphous material, as subtraction to zero intensity at 84 ppm should lead to an absence of negative residual signals. As also reported previously,[19,29] amorphous samples prepared by a common method are very similar for starches of different botanical origin.

The spectral difference between the two types of amorphous starch examined in this study (Figure 1 waxy maize LP and VO) is in the distribution of intensity within a common envelope of chemical shifts, particularly the C1 signal between 95 and

Table 2. The Relative Proportions of Amorphous, Single, and Double-Helix Conformations for Starches of Varying Amylose Content along with Their X-ray Diffraction Patterns and Degree of Crystallinity

		relative proportion[b] (%)				
starch	amylose content[a] (%)	V-type polymorph	double helix	amorphous	degree of crystallinity[c] (%)	XRD pattern
waxy maize	3.4	0	47	53	29	A
regular maize	24.4	3	33	64	21	A
Gelose 50	56.3	7	18	75	13	B
Gelose 80	82.9	14	18	68	15	B
rice	13.8	4	38	58	30	A

[a] The maximum error for amylose content determination was 6%. [b] The maximum standard deviation for the ^{13}C NMR analysis calculation was 2.4%. [c] The maximum error for the calculation of degree of crystallinity was 3.5%.

105 ppm. At least 95% of the signal in this region is due to α-(1 → 4) linked glucan, with the residual due to occasional α-(1 → 6) branch points. The very large chemical shift dispersion is considered to be due to a broad range of local chain conformations, characterized by two glycosidic torsional angles that define the relative orientation of adjacent glucose units within the polymer chain.[18,31] On the basis of a combination of chemical shift reference values for crystalline glucans[18] and ab initio chemical shift calculations,[31] the chemical shift dispersion for the C1 signal of amorphous starch is explicable in terms of the presence of all energetically feasible chain conformations. This is a reasonable expectation for an amorphous material. The common range of chemical shifts for different amorphous materials is consistent with them all being devoid of organized structure. The spectral differences would therefore reflect a different population spread across energetically feasible conformations. There is a particular difference for two amorphous spectra in relative intensity around 95 ppm. No crystalline model materials have a signal at 95 ± 1 ppm, the nearest one being a single resonance in crystalline α-cyclodextrin[18] at 97.2 ppm. The proposed assignment for this resonance is a single glucose unit within the cyclic hexamer of α-cyclodextrin that has a distinct 'twisted' conformation in the crystal structure. It seems likely that the intensity at ca. 95 ppm which differs for different amorphous standards and granular starches is also due to relatively high-energy, twisted, conformations remote from those characteristic of single helices (102−103 ppm) and double helices (99−101 ppm), respectively. Further work is needed to identify definitively the origin of intensity at 95 ppm and why the conformational features responsible vary between amorphous samples from different processes and starch granules from different botanical sources isolated by different methods.

Conclusions

We propose an improved method for analyzing the ^{13}C NMR spectra of starch samples to estimate the relative proportions of amorphous, single V-type helix, and double-helical components inside granules or any other physical form of starch. The deconvolution of the NMR spectra into amorphous and ordered subspectra was achieved by choosing a reference chemical shift (84 ppm) which has zero intensity in the crystalline subspectra. Examination of ordered subspectra revealed the presence of shoulder peaks at around 81 and 102 ppm particularly for high amylose maize starches. These chemical shifts are coincident with the chemical shifts for C4 and C1 carbons in the single-helical V-type polymorph. Hence, the previous assumption that the ^{13}C NMR spectra of starch granules are composed only of amorphous and double-helical features is not valid, especially for starches with higher amylose content. The method described in this study provides an approach to the quantitative analysis of the three conformations characteristic of starch granules, namely, amorphous, single-helix V-type, and double-helix (A- or B-type) conformations. In addition, it is shown that starch samples from different sources contain amorphous phases with different ^{13}C NMR spectral characteristics.

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