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Assessment of the Extent of Starch Dissolution in Dimethylsulfoxide by ^1H NMR spectroscopy

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Summary

Complete dissolution is needed for the separation, characterization or homogeneous labeling of whole starch molecules. A method is presented to quantify the extent of starch dissolution in dimethylsulfoxide (DMSO) for the first time, with ^1H NMR using toluene as an internal standard; it is validated on a commercial rice starch. It is used directly on starch dispersions containing possible undissolved or co-dissolved species. High-amylose maize starches, known to be digested slowly in vivo, only quantitatively dissolve in the presence of high concentrations of an H-bond disrupter, LiBr, although they form clear dispersions at low LiBr concentrations. Starch quantitatively dissolves from waxy rice flours; non-starch components partially co-dissolve but do not interfere with the dissolution quantification.

Introduction

Starch is a major component of human diets, supplying more than 50 % of caloric energy, and is a major component of a large number of food plants, for example wheat, potatoes, maize, rice, peas and bananas.^[1, 2] Furthermore, being renewable, cheap and biodegradable, starch has major applications in the paper, pharmaceutical and textile industry.^[2-5] Starch has an extremely complex (branched) molecular and supramolecular structure, with six identified levels of complexity, on scales ranging from nm to mm.^[6, 7] This structural complexity gives unique functional properties to each starch species, but renders its analysis difficult.^[7] Complete dissolution is required for (i) a successful separation of whole starch molecules (with techniques such as size-exclusion chromatography, SEC, also known as gel permeation chromatography, GPC,^[8-10] field-flow fractionation,^[11, 12] or analytical ultracentrifugation^[11]), (ii) a meaningful characterization (after separation^[13] or off-line such as dynamic light scattering, DLS,^[14-16]), (iii) homogeneous chemical modification of starch.^[8, 17, 18] Dissolution in an aqueous medium is also an important step in the digestion of starch-containing food by higher animals.^[19]

Starch is a polysaccharide formed of anhydroglucose units with two types of linkages: $\alpha(1,4)$ linkages constitute the backbone and $\alpha(1,6)$ linkages the branching points.^[6] Starch has two components: the overall smaller molecules of amylose exhibit a low level of long-chain branching, while the larger amylopectin molecules have a high level of short-chain branching.^[6] Depending on the natural source of the starch, the amylose content can vary from 0 to 85 %.^[1] Many investigations on starch have been made after dissolution or dispersion in aqueous media: in alkaline conditions,^[20-23] with treatment at high temperature and pressure,^[23-27] under microwave irradiation,^[9, 15, 24, 28, 29] with sonication^[24] or under stirring.^[25] It is claimed that pressure and heat treatment allows complete dissolution without degradation^[26, 27], but also that harsh conditions may result in degradation of starch^[8, 24, 25, 28, 30, 31] and thereby change in its original molecular structure and composition. The solubility of starch in aqueous media has rarely been investigated quantitatively, and always indirectly:

via enzymatic digestion (which might enhance dissolution),^[32] via sulfuric acid digestion after ultracentrifugation or filtration,^[15, 28, 33] after SEC elution via apparent recovery^[29] or recovery measurement.^[34] Furthermore, starch dispersions in aqueous media are plagued by spontaneous and non repeatable precipitation (retrogradation).^[16, 21, 35]

To overcome the degradation and retrogradation issues, dissolution of starch in polar organic solvents was investigated by various research groups. Dissolution of starch in dimethylacetamide requires heating at 150 °C for one hour under stirring, which is expected to degrade the starch.^[36, 37] Dimethylsulfoxide (DMSO) has been shown to be a suitable solvent for starch since dissolution experiments carried out in mild conditions give clear and transparent dispersions which are stable for weeks.^[21, 38-42] Dissolution is usually assumed to be complete if a clear dispersion is obtained,^[39, 43] but this does not rule out the presence of small microgels or aggregates. For high-amylose starches, dissolution in pure DMSO was shown to be more effective than in water.^[32] Some studies showed a decrease of viscosity of starch dispersions in DMSO upon standing,^[31] or in DMSO/water 90/10 v/v upon shaking or heating at 100°C.^[41] This could be due to degradation,^[41] but also to dissolution of aggregates.^[31] The mechanism of dissolution of starch granules is radically different in anhydrous DMSO and in water: in water, the granules swell then burst; in DMSO they do not swell but are peeled from their surface (DMSO solvates starch molecules gradually from the surface of the granules, thus granules dissolve layer by layer).^[44] Corn and sorghum granules first fragment into three to five pieces in DMSO.^[41] Gelatinization (i.e. loss of crystallinity, not dissolution) has been shown to occur faster in DMSO/water mixtures than dissolution in anhydrous DMSO in the same conditions.^[44] Furthermore, a study concluded that starch dissolves faster in DMSO/water 90/10 v/v than in anhydrous DMSO by ultracentrifuging the mixture and assuming that the clear supernatant did not contain any undissolved starch.^[41] However, a recent kinetic study of starch dissolution in DMSO conducted in our group using ¹H nuclear magnetic resonance spectroscopy (NMR) showed that actual dissolution (not gelatinization) occurs faster in anhydrous DMSO than in DMSO containing a few percent of water.^[38] Therefore anhydrous DMSO was chosen for starch dissolution with minimal degradation in the present study.

Lithium salts have been used to dissolve and analyze polysaccharides in organic solvents such as dimethylacetamide.^[45] Strong interactions between the solvent and the Li⁺ cation were revealed by NMR studies through signal shifts to lower frequencies and through decrease in solvent longitudinal relaxation times.^[45-47] This leaves a relatively free and strongly basic anion, which can abstract protons from the polysaccharide, thus disrupting hydrogen bonds and enhancing the polysaccharide solubility.^[46, 48]

To our knowledge, only one method has been published for the quantification of starch dissolution in organic solvent.^[41] It is based on the assumption that in a centrifuged, visually clear mixture, the starch is completely dissolved. We demonstrate here a novel method to quantify the extent of starch dissolution in DMSO directly in solution, by ¹H solution-state nuclear magnetic resonance (NMR), using an internal standard. Solution-state NMR detects only dissolved starch: highly mobile solutes exhibit sharp lines while solids are not detected and gels show very broad signals. The principle of the method, sample preparation protocol, NMR experimental parameters and data treatment are described in detail. The method is first validated on a model rice starch, and then applied to maize starches with various amylose contents and to waxy rice flours.

Experimental Part

Materials

Sigma rice starch was purchased from Sigma Aldrich (S-7260, lot 013K0030); its amylose content was found to be 36 % by the iodine binding method.^[8] Maize starches with varying amylose contents: Avon, Gelose 50 and Gelose 80, were purchased from Penford Australia Limited (Lane Cove, NSW 2066, Australia); the amylose content of these starches was determined by the iodine-binding method^[49] to be 24, 56 and 83 respectively.^[50] Flours of the waxy rice varieties Hom (IRGC 107140) and Makfay (IRGC 107649) were provided by the International Rice Research Institute in Los Baños (Philippines). Their amylose content was determined via a standardized test using iodine binding.^[51]

DMSO-d₆ (99.9 % D) was purchased from Cambridge Isotope Laboratories Inc. Toluene (HPLC grade), lithium bromide (LiBr; Reagent Plus, ≥ 99%) and molecular sieves (3 Å) were purchased from Sigma Aldrich.

Molecular sieves and LiBr were dried prior to use for 24 h at 150 °C in a vacuum oven, then stored in a desiccator. Toluene was dried over molecular sieves prior to use. Anhydrous DMSO-d₆ was obtained by drying over molecular sieves. An anhydrous 0.05 wt.-% or 5 wt.-% solution of LiBr in DMSO-d₆ was prepared by drying 10 g of DMSO-d₆ in a glass bottle over molecular sieves overnight; 5 mg or 500 mg of anhydrous LiBr were quickly weighed into another clean glass bottle (note that LiBr is highly hygroscopic); the anhydrous DMSO-d₆ was then transferred into the LiBr containing bottle and the dispersion was sonicated until the salt was dissolved (~ 30 min). This protocol was used to avoid having LiBr and molecular sieves in the same solution, as molecular sieves may trap Li⁺ ions. The absence of water ($\tilde{\nu}(\text{OH}) = 3500 - 4000 \text{ cm}^{-1}$) was checked by solution IR spectroscopy (Perkin Elmer Series 100) checking that the absorbance by water is not more intense than for a freshly opened DMSO bottle (less than 0.2 % water).

Detailed Sample Preparation

All starch samples and flours were kept under a controlled atmosphere to adjust their moisture content to a reproducible value prior to weighing. This is important as the moisture content has to be taken into account to quantify the dissolution (Equation 1). The procedure was to use vapour phase isopiestic equilibrium by storing the samples in a desiccator over a saturated aqueous K₂CO₃ solution at 20 °C, in an environment with a relative humidity of 44 %.^[52] The moisture content had been previously determined for the maize starch samples by the AOAC standard method of moisture determination^[53] and lies in the range of 9-11 %.^[50] The sample preparation procedure is illustrated in Figure 1. A quantity of starch between 9.95 to 10.05 mg was weighed into a 1 mL glass vial (step I), using a precision balance with an accuracy of 0.01 mg. 1 mL of anhydrous DMSO-d₆ (pure or containing 0.05 wt.-% or 5 wt.-% LiBr) was then added with a Gilson precision pipette (II). The pipette

tip was changed after every sample preparation to avoid possible contamination of the DMSO-d₆ with components of the tip.

Note that DMSO-d₆ is hygroscopic, and that anhydrous DMSO-d₆ dissolves starch faster than wet DMSO-d₆;^[38] thus only anhydrous solvents were used in the experiments (see experimental part for solvent preparation). LiBr was added as a hydrogen-bond disrupter in cases where the starch formed a gel in pure DMSO-d₆, or for starches with high amylose contents, for which quantitative dissolution could not be obtained in pure DMSO-d₆.

The mixture was gently stirred with a spatula. Then the vials were closed with a glass stopper, wrapped with Parafilm[®] and briefly shaken by hand to yield a homogeneous dispersion of the unsolubilized starch (III). The vials were then set in a bi-directional Thermomixer Comfort 5355 at 80 °C and 300 rpm overnight (IV). Previous work^[38] has shown that dissolution equilibrium is reached within an hour under the chosen conditions for Sigma rice starch. Solubilization overnight ensures that the equilibrium is reached. To make sure that the starch does not sediment and form a gel at the bottom of the tube, the vials were shaken a few times by hand during the dissolution process. After dissolving starch for 12 h and cooling to room temperature, 11.50 ± 0.5 μL of anhydrous toluene were added with a Hamilton precision syringe (V) as an internal standard, and the mixture was shaken by hand (VI). The complete dispersion was finally transferred with a Pasteur pipette to a 5 mm NMR tube which was sealed and wrapped with Parafilm[®] (VII) before recording an NMR spectrum (VIII, see below). Every experiment was repeated four times to check for repeatability (except for rice flours, for which respectively 2 and 5 repeats were run).

NMR Experiments

NMR spectra were measured on a Bruker Avance NMR spectrometer operating at a Larmor frequency of 500.13 MHz for ¹H, equipped with a TXI5z probe (Bruker Biospin). A temperature calibration of the NMR spectrometer and probe was carried out with a pure ethylene glycol standard (distilled, in a sealed tube) following the procedure recommended by Ammann et al.^[54] ¹H NMR

spectra were recorded at 333 K (60 °C) with an 8 μ s 90 ° pulse, a repetition time of 60 s (composed of an acquisition time of 4 s and a relaxation delay of 56 s) and 16 scans. The chemical shift scale was calibrated using the residual DMSO signal at 2.549 ppm.^[55]

Care was taken to ensure that the magnetization was fully recovered between pulses for both starch and toluene signals, in order to determine true relative signal integrals.^[56] To ensure full magnetization recovery between pulses, ¹H NMR spectra must be recorded with a delay between pulses longer than 5 times the longitudinal relaxation time (T_1) for each signal of interest. One-dimensional inversion-recovery T_1 relaxation measurements (1D T_1) were conducted, each with a single delay in the “indirect dimension”, for delays ranging from 1.34 to 20.8 s, and corresponding delays between pulses of 5×1.44 times that value. Phasing the spectra the same way as a conventional ¹H spectrum recorded in the same conditions, each signal is negative for short delay values, positive for long delay values, with a zero crossing occurring at $T_1 / 1.44$. For a delay of 7.64 s, the toluene signals change from negative to positive, and thus their T_1 relaxation time is less than $7.64 \times 1.44 = 11$ s. All starch samples exhibit T_1 longitudinal relaxation times significantly shorter than 11 s. Measuring all subsequent dissolution experiments with a delay between pulses longer than 5×11 s thus ensured that full magnetization recovery took place and the ¹H spectra were quantitative. The 1D T_1 measurements were carried out both for starch dissolved in DMSO- d_6 with added toluene and for starch dissolved in DMSO- d_6 / LiBr with added toluene. In the case of a 5 wt.-% LiBr solution in DMSO- d_6 , the T_1 relaxation times of all signals used for quantification were shorter than 5 s.

For toluene, the five aromatic protons were chosen and their signal was integrated over the range from 7.8 to 6.7 ppm. For starch, the signals of four protons were integrated from 6.0 to 4.15 ppm: the three OH-protons and the anomeric H-1 proton. The fraction of initial starch actually dissolved $\%S_{\text{diss}}$ can be determined from the relative integrals of starch and toluene signals $\int S_{\text{diss}}$ and $\int T$ using Equation (1):

$$\%S_{\text{diss}} = \frac{100 \cdot \int S_{\text{diss}} \cdot V_{\text{T}} \cdot d_{\text{T}} \cdot H_{\text{T}} \cdot M_{\text{S}}}{\int T \cdot \left(1 - \frac{mc}{100}\right) \cdot m_{\text{Swet}} \cdot H_{\text{S}} \cdot M_{\text{T}}} \quad (1)$$

where $\int S_{\text{diss}}$ and $\int T$ are in the same arbitrary unit, V_{T} (introduced toluene volume) is in μL and m_{Swet} (mass of (wet) starch initially weighed) is in mg. The detailed derivation is given in supporting information. Taking into account the numerical values of the molar masses M_{T} and M_{S} of toluene and the anhydroglucose unit (92.14 and 162.14 $\text{g}\cdot\text{mol}^{-1}$ respectively), the relative density of toluene at room temperature (0.865), the moisture content (10 wt.-% for the maize starches investigated here), and the number of protons of the integrated signals for toluene and anhydroglucose unit (5 and 4 respectively), Equation (2) is determined:

$$\%S_{\text{diss}} = 211.4 \times \frac{\int S_{\text{diss}}}{\int T} \cdot \frac{V_{\text{T}}}{m_{\text{Swet}}} \quad (2)$$

Results and Discussion

Principle of the Experiment and Validation with Model Rice Starch

The internal standard used to quantify the extent of dissolution of a solute in a solvent must satisfy several criteria. It must be soluble in the solvent in which the experiment is conducted, DMSO($-d_6$), and stable at the temperature at which the NMR experiment takes place, 60 °C. It must be non-volatile and available in high purity, to allow a precise measurement of the quantity introduced. There must be two regions of the ^1H NMR spectrum in which the solute (starch) and internal standard can each be quantified independently, without overlap with a signal from any other species. The standard must not interact with the starch: it must not be able to form hydrogen bonds with the hydroxyl groups of starch, and must not contain an alkyl chain with four or more carbons, which would form a complex with starch chains. It should not include protons which exchange easily with deuterons from the solvent; this is relevant for solvents such as D_2O which contain labile deuterons. Toluene was chosen as an internal standard satisfying all those criteria.

The sample preparation procedure is illustrated in Figure 1 and detailed in the experimental part.

A quantitative ^1H spectrum was recorded in which starch and toluene signals were independently integrated. For toluene, the five aromatic protons were chosen and their signal was integrated over the range from 7.8 to 6.7 ppm. For starch, the signals of four protons were integrated from 6.0 to 4.15 ppm: the three OH-protons and the anomeric H-1 proton. These signals are well resolved from any signal from other species, including the water signal at ca 3.3 ppm, arising from moisture in the initial starch and on glassware. Note that this water signal can not be used for quantification of moisture content in the starch, as a major and non-repeatable part of it originates from the glassware. The relevant part of the ^1H spectrum of Sigma rice starch and toluene in DMSO- d_6 with the corresponding integration is shown in Figure 2. The extent of dissolution is calculated from the relative signal integrals of starch and toluene, and their initial quantities, according to Equation (2) (see experimental part for the equation, and supporting information for the detailed derivation).

The method was first validated on a model starch, Sigma rice starch. The sample preparation protocol, which was found to give repeatable results, was the result of optimization using a large number of experiments. Other protocols, for example weighing the solvents (DMSO- d_6 , toluene) or using other equipment (pipette for toluene or imprecise syringes), did not have the same precision and led to a larger scattering of dissolution efficiency values (see supporting information for examples). Clear and transparent dispersions were always obtained after dissolution of starch in DMSO- d_6 for 12 h in the thermomixer. As expected, the viscosity of the dispersions was higher than that of pure DMSO- d_6 . The percentage of Sigma rice starch dissolved in DMSO- d_6 , $\%S_{\text{diss}}$, was calculated for each repeat according to Equation 2. The following values were obtained: 93 %, 97 %, 97 % and 109 %. The average value was then determined to be 97 ± 7 %. The results obtained for Sigma rice starch show that the method developed here gives reasonable and repeatable results. The validated method was subsequently applied to various maize starches and rice flours.

Dissolution of Maize Starches with Varying Amylose Content

The extent of dissolution of various maize starches in anhydrous DMSO-d₆ at 80 °C overnight is shown in Table 1. Numerical values obtained for each repeat are shown in supporting information. Although all samples give clear and transparent dispersions, the percentage of starch actually dissolving %S_{diss} decreases with increasing amylose content.

It could be hypothesized that the incomplete dissolution of Gelose 80, containing 83 % amylose, is due to the possible presence of non-starch material in large proportion (up to 15 %) in the sample. However, its ¹³C solid-state NMR spectra recorded using cross-polarization (CP-MAS)^[50] is the characteristic spectrum of pure starch, strongly indicating that Gelose 80 does not contain a significant amount of non-starch material. Note that highly mobile non-starch materials would be detected not by CP-MAS but by ¹³C SPE-MAS NMR (single-pulse excitation); however, further dissolution experiments confirmed later in this manuscript that Gelose 80 is pure starch. This result clearly confirms that a clear dispersion of starch can be obtained even if the dissolution is incomplete: visual control is a necessary but not sufficient evidence for complete starch dissolution. It has been observed before that high-amylose starches are more difficult to dissolve than regular starches in aqueous media.^[32] Note that a high amylose proportion in starch, among other factors such as starch or food processing, is correlated with increasing resistance to digestion, which has implications in health and nutrition.^[57] Furthermore, the solubilization of starch is a major factor in its digestion in higher animals, although this digestion is a heterogeneous process.^[19] The dissolution behaviour of starch in an organic solvent (DMSO) might possibly be relevant to in vivo digestion, which occurs in aqueous media. Monitoring the kinetics of dissolution of starch and its activation energy shows that it is a complex process. The difficulty in dissolving high-amylose starch in DMSO might however arise from an intrinsic lower solubility due to a difference in crystalline structure or in V-type single helices content,^[50] or from a strong tendency for amylose to rearrange and form networks upon cooling in DMSO.^[32] In each case, the driving force is hydrogen bonding. Although clear, transparent, macroscopically homogeneous dispersions are obtained, the presence of microgels can not be discarded. Therefore, complementary dissolution experiments were carried out on Gelose 80.

In the sample preparation procedure described previously, specific parameters which could cause a microgel formation were modified one by one. In a first experiment, the toluene standard was added to the dispersion at 80 °C, without cooling to room temperature. The dispersion was then immediately transferred to the NMR tube and set into the NMR machine at 60 °C. This would avoid a possible rearrangement of the amylose chains into networks due to the cooling step. In another experiment the concentration of Gelose 80 was lowered from 10 to 5 mg·mL⁻¹ to prevent the dispersion from being too concentrated (above the critical overlap concentration c^*) and the subsequent build-up of intermolecular interactions. In a third experiment, the dissolution time was shortened from overnight to 3 h to prevent long-term rearrangement of molecules in solution. Further measurements with even shorter dissolution times of 25, 34, 43, 52 and 61 min were also conducted. All complementary experiments done on Gelose 80 yielded the same value of 86 % for the extent of dissolution $\%S_{\text{diss}}$, which is the same as in the initial experiment, within experimental error. Therefore the incomplete dissolution of Gelose 80 in anhydrous DMSO-d₆ (without LiBr) can not be explained by effects of concentration, temperature or dissolution time. Although incomplete, the dissolution of the high-amylose Gelose 80 reached an equilibrium within 25 min at 80 °C in DMSO, as it was the case for Sigma starch,^[38] which fully dissolved. It is reasonable to suppose that one effect contributing to the incomplete dissolution might be intra- or interchain hydrogen bonding interactions. It is known that lithium salts help solubilize polysaccharides in organic solvents by disrupting hydrogen bonds.^[45] Therefore, dissolution experiments with Gelose 80 and Gelose 50 in DMSO with addition of various amounts of LiBr were carried out. 0.5 wt.-% LiBr were first added; it corresponds to more than 3 moles of LiBr per mole of alcohol group on the starch and enables obtaining reproducible separations by SEC due to limited adsorption of dissolved starch onto the SEC stationary phase.^[8] This did not significantly improve the extent of starch dissolution (Table 1), likely due to interactions of LiBr with the DMSO, which “consumes” the LiBr. A study of chitin dissolution in dimethylacetamide with LiCl indeed suggests that very high Li salt concentrations may have to be used to achieve quantitative polysaccharide dissolution in organic solvents.^[58] Dissolution experiments with Gelose 80 and Gelose

50 in anhydrous DMSO-d₆ indeed show complete dissolution with 5 wt.-% LiBr (see Table 1 and supporting information). This confirms the observation from CP-MAS spectra that there is no significant amount of non-starch material in Gelose 50 and Gelose 80. It is also strongly supportive of the inference that high-amylose starches have stronger hydrogen-bonding interactions than regular starches, which must be overcome by addition of sufficient amounts of a hydrogen-bonding disrupter (such as LiBr) for complete dissolution.

As mentioned before, the only published method for the quantification of starch dissolution in an organic solvent^[41] consists of centrifuging the starch dispersion, isolating the supernatant, then precipitating the starch contained in the supernatant and quantifying it by gravimetry. This method makes the assumption that after centrifugation, all the starch contained in the clear supernatant is properly dissolved, and no aggregates or microgels are present. To test the validity of this assumption, 5 mg of Gelose 80 were dissolved overnight in 1 mL of DMSO in the thermomixer as described previously. We know from the NMR study above that only 85 % of the starch contained in the clear and transparent Gelose 80 dispersion is actually dissolved. The dispersion was then transferred into an Eppendorf vial and submitted to centrifugation in the same conditions as in reference ^[41]: at 2200 rpm for 15 min at room temperature. No precipitate could be observed. If the quantification method proposed in reference ^[41] was applied to this particular sample, after precipitation both dissolved and undissolved starch components of the supernatant would be measured as initially dissolved by gravimetry. This invalidates the quantification method proposed in reference ^[41].

To the best of our knowledge, the NMR method proposed here is thus the first method allowing the quantification of the extent of starch dissolution in an organic solvent. Furthermore, it is applicable to a dispersion of partially dissolved starch and does not require prior filtering or ultracentrifugation. This method and the discovered conditions for some starches allow obtaining true solutions of full starch with minimal degradation and open a pathway to separation and characterization. These starches are obtained from plants where they are present in various quantities together with proteins, lipids, etc. Conventional starch extraction processes use harsh conditions likely degrading the starch.^[59] To

characterize full starch as it is found in plants, extraction must be performed in mild conditions and with a high yield. The potential of the NMR method to monitor the dissolution of starch directly from plants is thus evaluated in the next part.

Dissolution of Starch from Waxy Rice Flours

The new NMR method was also applied to whole flours from waxy rice. Those samples strongly differ from the ones studied above, as they contain approximately 10 wt.-% of non-starch components (mainly proteins and lipids, dry-weight basis). ^1H NMR signals of proteins are expected mainly over the ranges from 5 to 9 ppm and from 1 to 4 ppm, while the lipid signals are expected between 0.5 and 2 ppm. Other minor components include inorganic materials (not detected by ^1H NMR) and non-starch polysaccharides, mainly cellulosic materials, whose chemical shifts are expected over the same ranges as the starch ones. Thus the lipids and inorganic materials do not interfere with the quantification of the extent of dissolution by ^1H NMR, while the proteins and the non-starch polysaccharides might interfere with the toluene and starch signals used for quantification respectively. Therefore a preliminary study of the components dissolved from the starch flours was conducted before the dissolution study itself.

A ^1H NMR spectrum of the flour from the variety Hom dissolved in DSMO-d_6 (with 0.05 wt.-% LiBr) was recorded without adding the toluene internal standard (Figure 3 (a)). The characteristic aromatic signals of proteins are observed between 6.5 and 7.5 ppm. However, their integral is negligible compared to the toluene signal used for quantification (see Figure 2 for the relative signals integrals of starch and toluene signals in the conditions used for quantification in this work). Therefore, even though some signals of the proteins overlap with the signal of toluene integrated for dissolution quantification, they are not interfering with the quantification itself. Characteristic protein methyl signals are observed between 1 and 3 ppm as well (Figure 3 (a)). The non-starch polysaccharide components of the flour are expected to exhibit signals for their OH groups and beta-linked anomeric protons between 4 and 5.5 ppm. This is the same range over which starch exhibits signals for its OH groups and alpha-linked anomeric protons.^[60] Therefore D_2O was added to the sample measured

previously to strongly attenuate the observed OH groups via proton-deuteron exchange and reveal the fine structure of the anomeric region (Figure 3 (b)). No beta-linked anomeric signal characteristic from non starch polysaccharides such as cellulose, arabinoxylan or β -glucans materials is visible between 4 and 4.5 ppm. Therefore, even though some signals of the non-starch polysaccharides would overlap with the signal of starch integrated for dissolution quantification, if detected, they are not interfering with the quantification itself. Note that some more protein signals are revealed after proton-deuteron exchange: weak broad signals between 5.3 and 5.5 ppm, as well as a sharp doublet at 5.25 ppm. However, their integral is negligible compared to the starch signal used for quantification, therefore they are not interfering with the quantification itself. Thus, in the particular case of the dissolution of waxy rice flours in DMSO, the other components do not interfere with the quantification of the extent of dissolution of starch by ^1H NMR with toluene as an internal standard.

The extent of dissolution of whole rice flours in anhydrous DMSO- d_6 (containing LiBr) at 80 °C overnight is shown in Table 1. Numerical values obtained for each repeat are shown in supporting information. For both flours, ca 90 % of the initially weighed samples (excluding moisture) dissolves as starch. As those samples contain approximately 10 wt.-% of components other than starch, it is concluded that the starch was fully dissolved.

It has been claimed that dissolution in DMSO can purify the starch, as non-starch components would not dissolve in this organic solvent.^[43, 61] However, it was clearly seen above (Figure 3) that some proteins do dissolve in DMSO under the conditions used here. It was observed that some lipids also dissolve, as indicated by the presence of characteristic signals at 0.8 and 1.2 ppm (Figure 3 (a)). Quantification of the proteins or lipid levels will require a precise knowledge of their chemical structure, which is beyond the scope of this work. It is however concluded that dissolution of starch in DMSO may restrict but does not completely prevent the co-dissolution of proteins and lipids. Direct extraction of starch from grains or flour by a simple dissolution in DMSO may thus not be suitable for further separation and characterization by DLS, SEC, FFF etc.

Conclusion

Solution-state ^1H NMR can be used to quantify dissolution of starch molecules in a solvent. To the best of our knowledge, it is the first method allowing the quantification of the extent of dissolution of starch in an organic solvent. The method was first validated on a model commercial rice starch in anhydrous DMSO-d_6 , then applied to maize starches with varying amylose content and to waxy rice flours. The dissolution of maize starches was shown by the new NMR method to be incomplete, although visual observation failed at detecting the presence of aggregates/microgels. Complete dissolution of high-amylose maize starches could however be attained when a high amount (5 wt.-%) of a hydrogen-bond disrupter, LiBr, was added. This strongly supports the inference that high-amylose starches have stronger hydrogen-bonding interactions than regular starches. The only method proposed previously for the quantification of the extent of starch dissolution in an organic solvent^[41] was tested on this partially dissolved sample and shown to be inadequate. In the case of flours, it was checked that the non-starch components did not interfere with the quantification of extent of starch dissolution. The starch did dissolve quantitatively from those flours, but contrary to previous claims,^[43, 61] some lipids and proteins did co-dissolve in anhydrous DMSO-d_6 .

The method presented here for the quantification of the extent of starch dissolution in DMSO opens the way to further work on the separation, characterization and functionalization of starch molecules. Ensuring complete dissolution is indeed a prerequisite for meaningful characterization of whole starch molecules (with techniques such as light scattering, liquid chromatography, field-flow fractionation) and their homogeneous chemical modification. The size distribution of starch molecules is known to be broad, and the partial dissolution is likely to introduce a bias in the distribution of molecules actually present in solution compared to the initial sample (toward amylose or toward the largest molecules for example). Identifying the non-dissolved components is a highly difficult task and is prone to error. We thus rather recommend using the NMR method validated here. This is a prerequisite to be able to characterize starch, e.g. determining the size of the largest soluble starch

molecules. Furthermore, the method is applicable on a routine solution-state NMR spectrometer to a wide range of samples of solvents, provided an appropriate internal standard can be found; its application to aqueous solvents would require further method development due to the large ^1H NMR signal of water.^[62] The method could be invaluable for non-starch polysaccharides with known solubility issues.^[63, 64] Moreover, complementary investigation by high-resolution magic-angle spinning (HR-MAS) NMR of the aggregates of microgels present in incompletely dissolved high-amylose starch samples would bring interesting insight into the mechanism of dissolution.

There may be an interesting correlation between the present quantitative observation of factors affecting how starch granules dissolve in DMSO, and factors affecting starch digestibility. High-amylose starches are observed here to dissolve in DMSO with difficulty, and to require large amounts of a hydrogen-bond disrupter (LiBr) for complete dissolution. Starches with a high amylose content are enzymatically digested *in vivo* slowly to lead to a lower glycemic index (a reduced rate of glucose uptake in the bloodstream) and to a higher fraction of resistant starch (that portion of starch that escapes digestion in the small intestine and enters the colon).^[19, 57] While the connection here is purely a correlation, and the differences on dissolution of starch granules are in DMSO and in aqueous media, there is the possibility of a common origin of the incomplete dissolution in DMSO and slow digestion.

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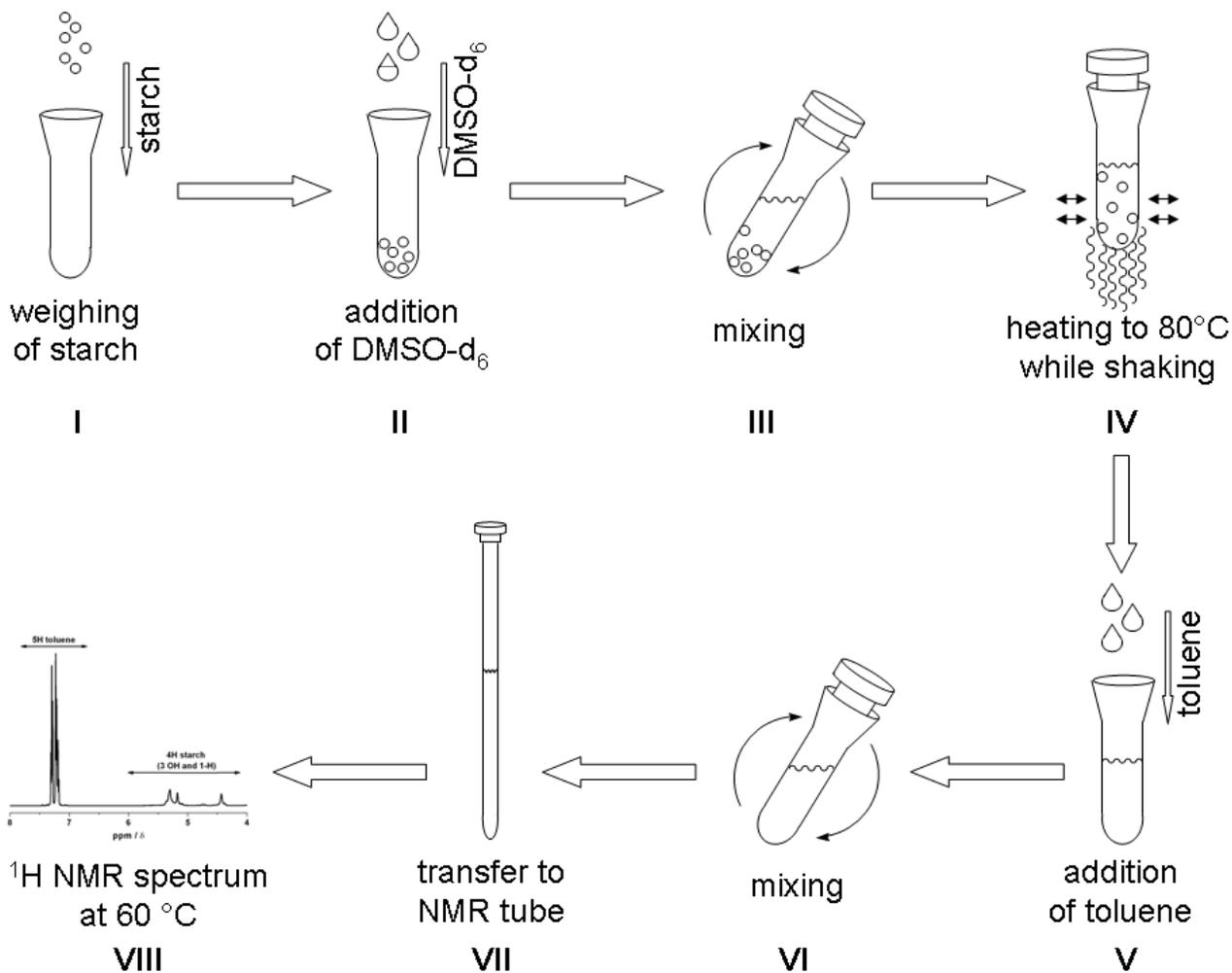


Figure 1. Sample preparation for the determination of the extent of starch dissolution $\%S_{\text{diss}}$ in DMSO-d₆ via ¹H NMR measurements, using toluene as an internal standard.

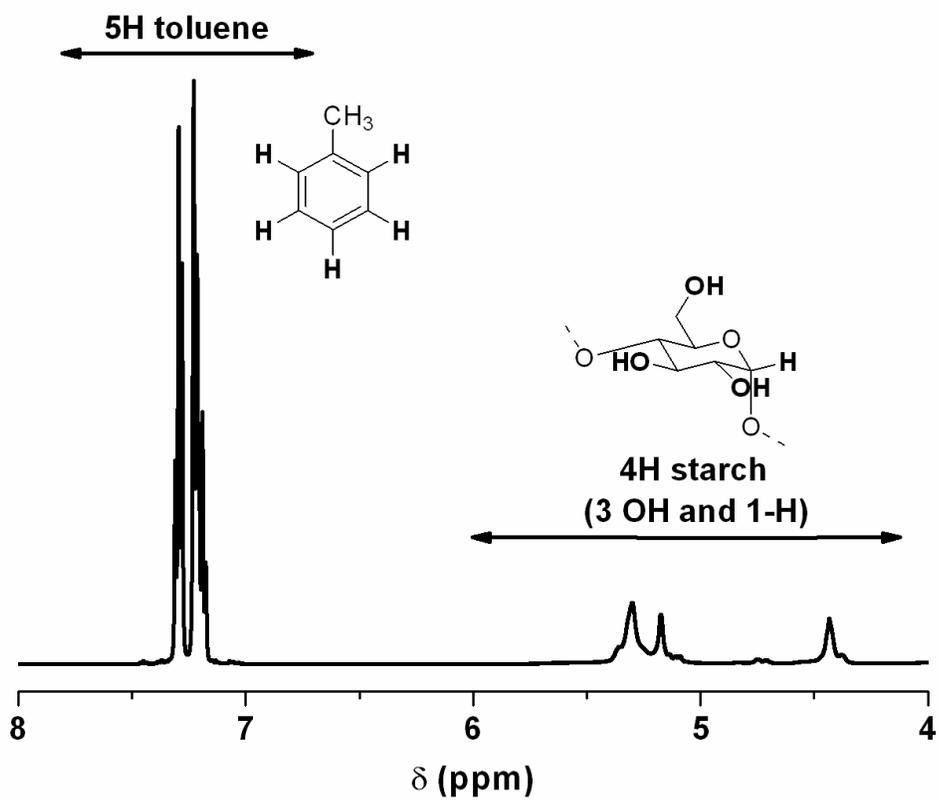


Figure 2. Partial ^1H NMR spectrum of sigma rice starch and toluene standard measured in DMSO-d_6 at 60°C with the corresponding integration ranges. The integrated protons are indicated in bold.

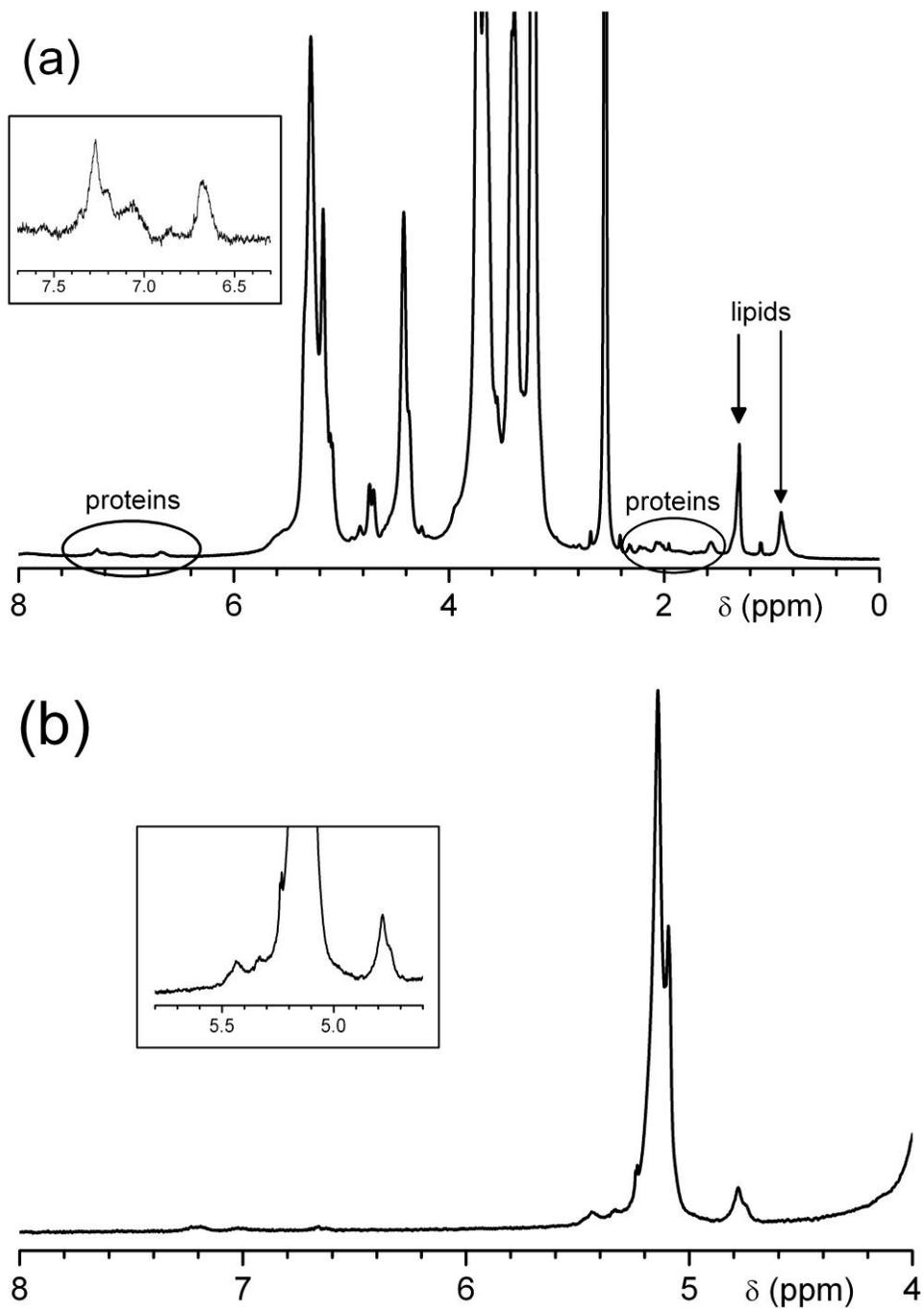


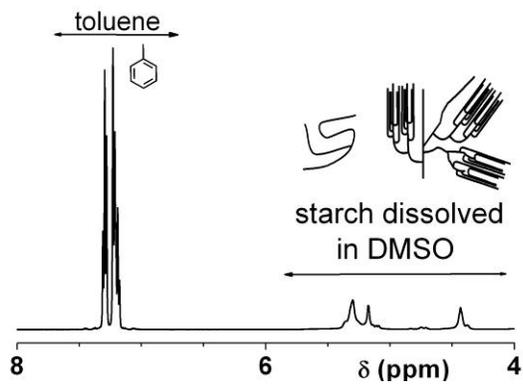
Figure 3. ^1H NMR spectra of Hom waxy rice flour at 60 °C (a) in DMSO-d_6 (+ 0.05 wt.-% LiBr) and (b) partial spectrum in DMSO-d_6 (+ 0.05 wt.-% LiBr) / D_2O 2/1. Inserts show zooms in selected regions.

Table 1. Percentage %S_{diss} of starch dissolved in anhydrous DMSO-d₆ in 12 h at 80 °C under gentle shaking (100 % would be 10 mg·mL⁻¹), with standard deviation. Note that some measured values are higher than 100 %; however, their difference to 100 % is always smaller than the experimental error, thus this does not mean that the actual value is higher than 100 %. Amylose contents are indicated for information.^[49, 50] wt.-% LiBr values are relative to DMSO-d₆.

starch/flour type	rice starch		maize starch		waxy rice flour	
starch/flour name	Sigma	Avon	Gelose 50	Gelose 80	Hom	Makfay
amylose (%)	36	24	56	83	0 - 2	0 - 2
%S_{diss}, no LiBr	96.5 ± 7	91 ± 6	91 ± 3	85 ± 3	-	-
%S_{diss}, 0.05 wt.-% LiBr	-	-	-	-	91 ± 5	90 ± 3
%S_{diss}, 0.5 wt.-% LiBr	-	-	-	85 ± 2	-	-
%S_{diss}, 5 wt.-% LiBr	-	-	101 ± 5	104 ± 4	-	-

Table of Content

Starch is a major component in human diets and industrial products, but its characterization is plagued by solubility issues. We present the first method to quantify the extent of starch dissolution in dimethylsulfoxide, by ^1H NMR with a toluene standard. Visual observation fails at detecting aggregates/microgels of starch in DMSO. Lipids and proteins co-dissolved from flours are detected.



Supporting Information for

“Assessment of the Extent of Starch Dissolution in Dimethylsulfoxide by

¹H NMR spectroscopy”

by Sarah Schmitz, Anthony C. Dona, Patrice Castignolles, Robert G. Gilbert, Marianne Gaborieau

Percentage of Starch Dissolution for all Investigated Samples

Sample	%S _{diss} values (%)	Average (%)	remarks
Sigma rice starch	93.2, 96.6, 96.8, 109.0	96.5 ± 7.0	usual sample preparation
Avon maize starch	102.7, 90.4, 87.4, 92.1	91.1 ± 6.6	usual sample preparation
Gelose 50	94.0, 96.0, 93.5, 89.4	91.1 ± 1.2	usual sample preparation
Gelose 80	86.9, 88.5, 88.1, 82.3	84.5 ± 2.9	usual sample preparation
Gelose 80	88.4*, 83.4, 88.0, 85.3	84.4 ± 2.4	no cooling before toluene addition
Gelose 80	88.8	88.8	a new measurement of sample * from the line above, after cooling down
Gelose 80	86.5, 87.4, 83.4, 78.8	82.2 ± 3.9	[starch] = 5 mg·mL ⁻¹ instead of 10
Gelose 80	87.4, 88.2, 86.8, 85.3	85.0 ± 1.2	t (dissolution) = 3 h
Gelose 80	85.7, 85.8, 85.6, 85.8, 86.0		t (dissolution) = 25, 34, 43, 52 and 61 min
Gelose 80	83.6, 84.2, 86.9	85 ± 2	0.5 wt.-% LiBr in DMSO-d ₆
Gelose 80	107.2, 99.6, 100.2, 107.5	103.6 ± 4	5 wt.-% LiBr in DMSO-d ₆
Gelose 50	99.9, 106.5, 104.0, 94.6	101.2 ± 5	5 wt.-% LiBr in DMSO-d ₆
Hom flour	83.3, 90.5, 96.3, 92.4, 90.5	90.6 ± 4.7	usual sample preparation, 0.05 wt.-% LiBr in DMSO-d ₆
Makfay flour	91.5, 87.7	89.6 ± 2.7	usual sample preparation, 0.05 wt.-% LiBr in DMSO-d ₆

Derivation of the Equation Used to Calculate the Extent of Dissolution from NMR

Integrals

The NMR signal is proportional to the number of nuclei of the dissolved species. Thus the fraction of initial starch actually dissolved can be determined as follows. This fraction of initial starch actually dissolved, $\%S_{\text{diss}}$, is defined in Equation 1, where $\int S_{\text{diss}}$ is the actual integral of the starch signal in the spectrum, and $\int S_{\text{in}}$ is the starch integral expected if all the initial starch had actually dissolved:

$$\%S_{\text{diss}} = \frac{100 \cdot \int S_{\text{diss}}}{\int S_{\text{in}}} \quad (3)$$

The expected value of the starch integral $\int S_{\text{in}}$ can be calculated according to Equation 2 from the integral of the toluene signal $\int T$, taking into account the molar amounts of introduced toluene and starch (excluding moisture) n_{T} and n_{Sdry} and the number of protons corresponding to the integrated toluene and starch signal H_{T} and H_{S} :

$$\int S_{\text{in}} = \int T \cdot \frac{n_{\text{Sdry}} \cdot H_{\text{S}}}{n_{\text{T}} \cdot H_{\text{T}}} \quad (4)$$

$$\text{where } n_{\text{T}} = \frac{V_{\text{T}} \cdot d_{\text{T}}}{M_{\text{T}}} \text{ and } n_{\text{Sdry}} = \left(1 - \frac{mc}{100}\right) \frac{m_{\text{Swet}}}{M_{\text{S}}} \quad (5)$$

with V_{T} the introduced toluene volume, d_{T} the relative toluene density at room temperature, m_{Swet} the mass of (wet) starch initially weighed, mc its moisture content in wt.-%, and M_{T} and M_{S} the molar masses of toluene and of a single anhydroglucose unit of starch respectively.

Combining Equation 3 to 5, the fraction of initial starch actually dissolved, $\%S_{\text{diss}}$, is calculated according to Equation 4:

$$\%S_{\text{diss}} = \frac{100 \cdot \int S_{\text{diss}} \cdot V_{\text{T}} \cdot d_{\text{T}} \cdot H_{\text{T}} \cdot M_{\text{S}}}{\int T \cdot \left(1 - \frac{mc}{100}\right) \cdot m_{\text{Swet}} \cdot H_{\text{S}} \cdot M_{\text{T}}} \quad (1)$$

The molar masses of toluene and the anhydroglucose units are 92.14 and 162.14 g·mol⁻¹ respectively, the relative density of toluene at room temperature is 0.865. Furthermore, the maize starches

investigated have a moisture content of 10 wt.-%, and the integrated signals correspond to 4 and 5 protons of the anhydroglucose unit and toluene respectively. Thus the numerical Equation (2) was used for quantification:

$$\% S_{\text{diss}} = 211.4 \times \frac{\int S_{\text{diss}}}{\int T} \cdot \frac{V_{\text{T}}}{m_{\text{Swet}}} \quad (2)$$

where $\int S_{\text{diss}}$ and $\int T$ are in the same arbitrary unit, V_{T} (introduced toluene volume) is in μL and m_{Swet} (mass of (wet) starch initially weighed) is in mg.

Examples of Sample Preparation Protocols Yielding Inaccurate Results

Protocols:

- a) The starch was weighed in a glass vial. Then DMSO- d_6 and toluene were poured in the vial using a precision pipette. The vial was heated in an oil bath at 80 °C overnight. The mixture was then transferred with a Pasteur pipette into a NMR tube.
- b) The starch was weighed in a glass vial. DMSO- d_6 and toluene were poured in a NMR tube using a precision pipette. The tube was shaken then the mixture was transferred with a Pasteur pipette to the vial containing the starch. After dissolution in an oil bath at 80 °C overnight, the starch dispersion was transferred into a NMR tube with a Pasteur pipette.
- c) The ingredients were weighed: the starch was weighed in a glass vial; the DMSO- d_6 was added dropwise into the vial using a Pasteur pipette until the required weight of DMSO- d_6 was achieved. The vial was set in an oil bath at 80 °C overnight. Toluene was then added with a Pasteur pipette until the required weight of toluene was achieved. The mixture was transferred with a Pasteur pipette into a NMR tube.
- d) The ingredients were weighed: starch was weighed into a glass vial; first DMSO- d_6 and then toluene were added dropwise into the vial using a Pasteur pipette until the required weight of each solvent was

achieved. The mixture was dissolved in an oil bath at 80 °C overnight, and then transferred into a NMR tube with a Pasteur pipette.

e) Starch was weighed in a glass vial. DMSO-d₆ was added with a precision pipette. Toluene was added with a Hamilton syringe. The vial was sealed and set in an oil bath at 80 °C overnight under stirring at 100 rpm. The mixture was then transferred into a NMR tube with a Pasteur pipette.

NB: starch, DMSO and toluene quantities are listed in the sample preparation section of the main text.

The dissolution step was done without shaking or stirring unless specified otherwise.

Results:

All the experiments were done with Sigma rice starch.

Protocol	%S _{diss} values (%)	Average (%)
a)	82.9, 96.8	89.9 ± 10
a)	74.4, 80.7, 86.3	80.5 ± 6
b)	96.8, 104.8	100.8 ± 6
b)	96.3, 93.6, 87.9	92.6 ± 4
c)	104.8, 98.2, 93.6	98.9 ± 6
d)	98.0, 97.2, 100.4	98.5 ± 2
e)	96.0, 120.9, 102.6, 106.1	106.4 ± 11