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Two-Dimensional ^{1}H and ^{13}C NMR Spectroscopy and the Structural Aspects of Amylose and Amylopectin

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Summary. A complete assignment of the ^{13}C and ^{1}H NMR signals of amylose dissolved in dimethylsulfoxide was achieved using two-dimensional H–H and C–H correlated spectroscopy and deuterium exchange. The same methods together with an INEPT experiment provided the assignments of the ^{1}H and ^{13}C NMR signals of the amylose type glucan units, the branch end glucan fragments, and a few assignments of nuclei within the branching glucan units of amylopectin dissolved in dimethylsulfoxide. From these assignments and from the integration of pertinent proton signals the branching degrees of amylose and amylopectin were derived to amount to 3.9 and 8–9%.

Keywords. Amylose; Amylopectin; Branching; Two-dimensional NMR.

Zweidimensionale ^{1}H und ^{13}C-NMR-Spektroskopie und die strukturellen Aspekte von Amylose und Amylopektin

Zusammenfassung. Die vollständige Zuordnung der ^{13}C- und ^{1}H-NMR-Signale von in Dimethylsulfoxid gelöster Amylose konnte mit Hilfe zweidimensionaler H–H- und C–H-Korrelationsspektroskopie und Deuteriumaustausch erzielt werden. Die gleichen Methoden sowie ein INEPT-Experiment ermöglichten auch die Zuordnung der ^{13}C- und ^{1}H-NMR-Signale der amylosischen Glucaneinheiten, der Verzweigungsendfragmente und einzelner Kerne im Bereich der Verzweigungsstellen von in Dimethylsulfoxid gelöstem Amylopektin. Aus diesen Zuordnungen und der Integration entsprechender Protonensignale konnte ein Verzweigungsgrad von Amylose und Amylopektin von 3.9 und 8–9% abgeleitet werden.

Introduction

Starches are one of the most important renewable sources of materials for nutritional and industrial purposes [1]. Their two main constituents, amylose and amylopectin, are of fundamental interest for a wide variety of applications either as pure materials, as mixtures which are naturally produced in form of the various starches, or as application tailored derivatives [2].

Despite their importance and considerable efforts to investigate their structural aspects, a fast and easily applicable tool to analyze these aspects is still lacking. Recent efforts to use ^{13}C NMR spectroscopy have provided a valuable means to

clarify structural problems [3, 4]. However, due to the low natural abundance of the carbon-13 isotope, this method demands rather long spectroscopic acquisition times. Moreover, quantitative estimates, although possible in principle, are rather limited.

^{1}H NMR spectroscopy, characterized by a higly predominant natural abundance of the proton and a proper integrability of signals, would have allowed a much better solution of this problem in principle. However, due to severe signal overlap this approach has so far not been applied successfully to our knowledge. With the advent of extremely sensitive high field NMR instruments, the application of ^{1}H NMR spectroscopy to structural problems of starches and starch derivatives seemed to be within reach. Therefore, we set out to explore this possibility and to advance at least partial signal assignments of the ^{1}H NMR spectra of the main starch constituents amylose and amylopectin by means of two-dimensional H–H and H–C NMR correlation spectroscopy.

Results and Discussion

The linearly built amylose displays an extended series of 1–4 linked glucan moieties (Fig. 1a). Although these units are slightly different from each other in principle, depending on their position within the chain, they will behave indistinguishable viewed from the standpoint of chemical shift differences of their various carbon and hydrogen atoms. The linear chain is capped by a terminal residue with a free 4-hydroxyl group (4e′) at one end and a glucan moiety with a free anomeric position (1e) at the other one.

The branching units of amylopectin which are characterized by 1–6 linkages (b) are surrounded by essentially the same units as encountered in amylose (Fig. 1). Again, all these units 4e and b which are slightly different fram each other depending on their position within the molecular frames will be more or less indistinguishable with respect to their chemical shifts. Of course, in all cases the small structural differences will result in *distributions of chemical shifts* which will be reflected mainly in the line shapes of the various signals.

It should be noted that – with respect to the rather high molecular masses of these two biopolymers – the amount of 1e and 4e′ fragments in amylose and of the 1e moiety as well as of the 4e′ residue of amylopectin are negligible (i.e., they will constitute less than 0.1% of all the glucan moieties and thus contribute signals in NMR spectra which are below the detection limits). Due to the cluster structure of amylopectin [1], which to our feeling should be more properly be interpreted as a natural dendritic molecule (for such molecules see Ref. [5]), the amylose type terminal group becomes indistinguishable from the terminal groups that result from branching and, consequently of course, the number of branchings becomes equivalent to the number of the terminal groups labeled 4e.

Since the NMR spectra of amylose are, of course, much simpler that those of amylopectin, the former substance was chosen as the starting point for signal assignments. As could be derived from the C–H correlation experiment shown in Fig. 2, these signals correlated nicely with several distinct signals of the ^{1}H NMR spectrum. Deuterium exchange corroborated the nature of the remaining signals as those pertaining to hydroxyl protons. This result was nicely complemented by the

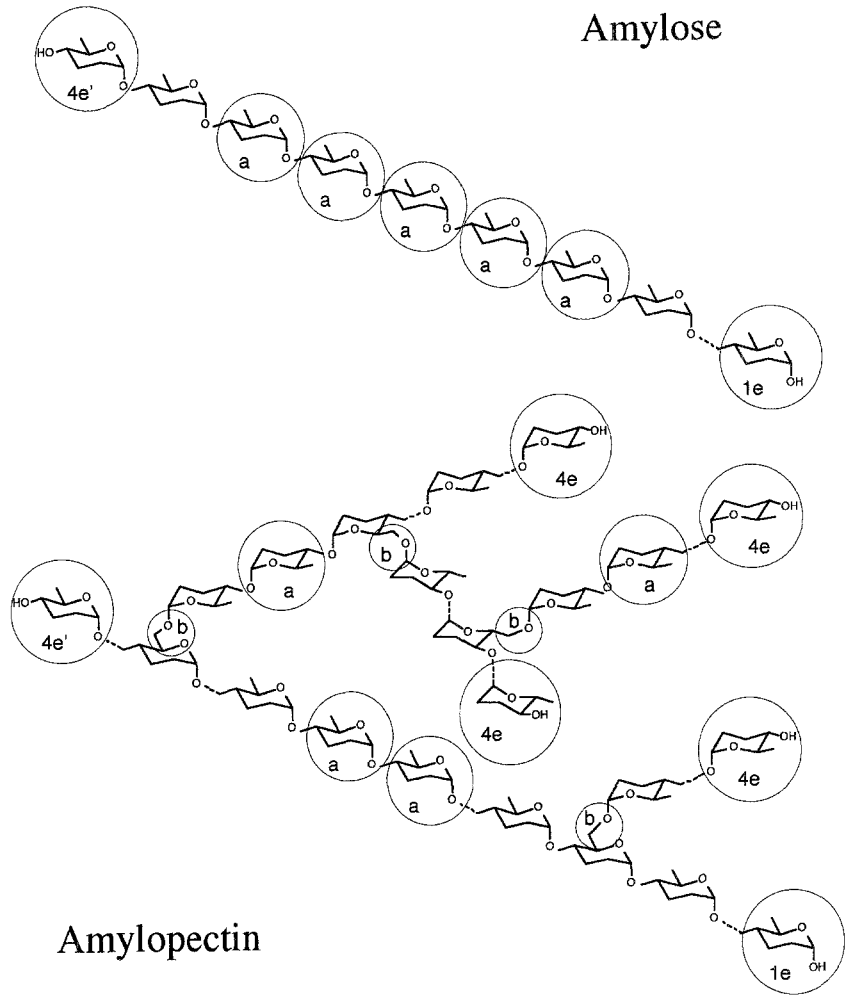

Fig. 1. Schematic structures of amylose and amylopectin; amylose type glucan units within the chains, branching units, terminal glucans with free 4-OH group, and terminal glucans with non bonded anomeric carbon atom were denoted by a, b, 4e, 4e′, and 1e

H–H COSY experiment displayed in Fig. 3 which, in addition, allowed correlations of the hydroxylic protons. Eventually, these experiments permitted a complete assignment of the ^{13}C and ^{1}H NMR signals of the amylose type glucan units (a) of amylose as given in Fig. 4. However, it should be stressed that the assignments of the C-2 and C-3 atoms had to be interchanged with the assignments given previously for *DMSO* solutions [3]. These assignments have been reached mainly from shift arguments and analogy with the assignments given for water solutions [6]. The new assignments are based upon the fact that in the H–H COSY experiment the signal of H-1 (correlated before – Fig. 2 – unequivocally with the signal of C-1) at 5.11 ppm was found to correlate with the signal at 3.33 ppm (Fig. 3), which therefore had to be assigned to H-2. The latter signal was correlated in the C–H COSY experiment to the carbon signal at 71.84, which therefore had to be assigned to C-2. Of course, the signal of H-2 was then found in the H–H COSY

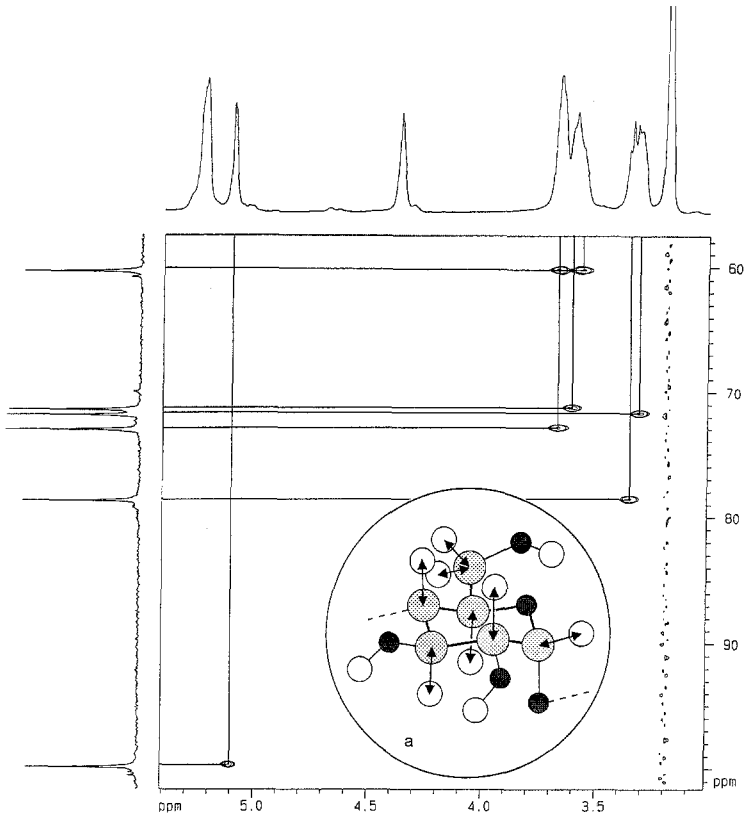

Fig. 2. 2D H–C COSY spectrum of amylose dissolved in *DMSO*-d_6 (straight arrows in the insert indicate the correlations derived from the cross peaks indicated in the spectrum; for the assigned shift values, cf. Fig. 4)

experiment to be correlated with the signal at 3.68 ppm; therefore it had to be assigned to H-3.

As also had been derived recently [3], the ^{13}C NMR signals of predominant intensity of amylopectin can easily be assigned just by relating them with the corresponding signals of the amylose spectra. The H–C COSY experiment shown in Fig. 5 nicely displayed these correlations between the amylose type ^{13}C and ^{1}H signals pertaining to the interchain glucan units a, yielding also the same correlation features as in the case of amylose (cf. Fig. 4). In addition to these main features, the experiment also allowed to correlate and thereby assign the distinct minor signals stemming from the branching, and it turned out that the prominent residues which could be singled out in this way were those of the branch-end fragments 4e (cf. Fig. 1). Again, the H–H correlation experiment corroborated the assignments (Fig. 6) and, moreover, allowed also the assignment of the corresponding hydroxyl protons. According to theses experiments, the ^{13}C and ^{1}H signal assignments for amylopectin are the ones given in Fig. 4 for the a glucan moieties and those of Fig. 7 for the 4e glucan moieties.

It should be mentioned that the small ^{13}C NMR signal at about 70 ppm has been assigned previously to the 6-CH_2 group of branching units b [3]. However, a

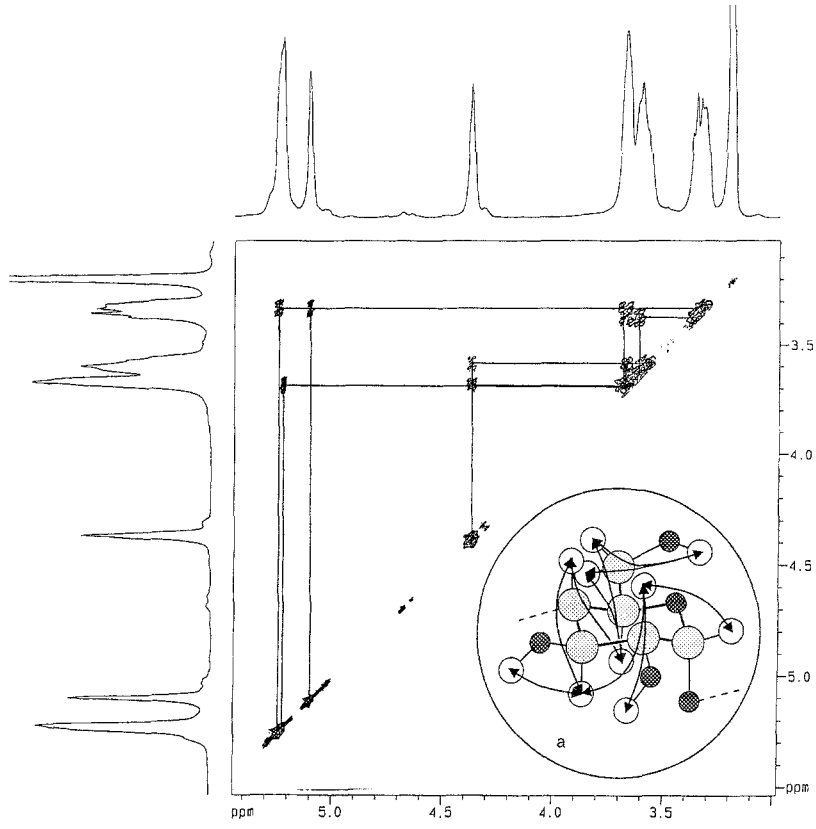

Fig. 3. 2D H–H DQF COSY spectrum of amylose dissolved in *DMSO*-d_6 (bent arrows in the insert indicate the correlations derived from the cross peaks indicated in the spectrum; for the assigned shift values, cf. Fig. 4)

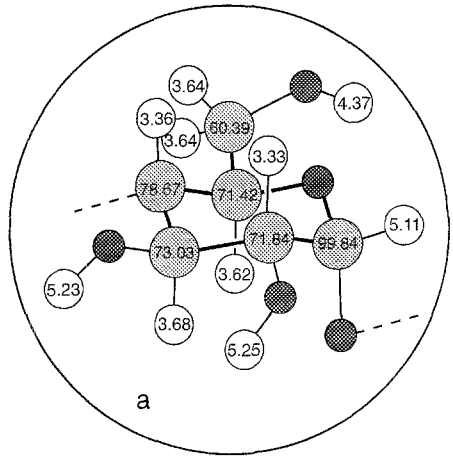

Fig. 4. ^{13}C and ^{1}H signal assignments of the glucan units a (*DMSO*-D_6, cf. Fig. 1)

^{13}C DEPT experiment, which pinpointed it to be bonded to only one proton instead of two, together with the correlation experiments described above, unequivocally showed that it had to be assigned to the non bonded position 4 of the 4e moiety. Most signals pertaining to the branching units b (cf. Fig. 1) could not be identified and assigned with any certainty with the exception of C-1 and H-1 at 100.29 and

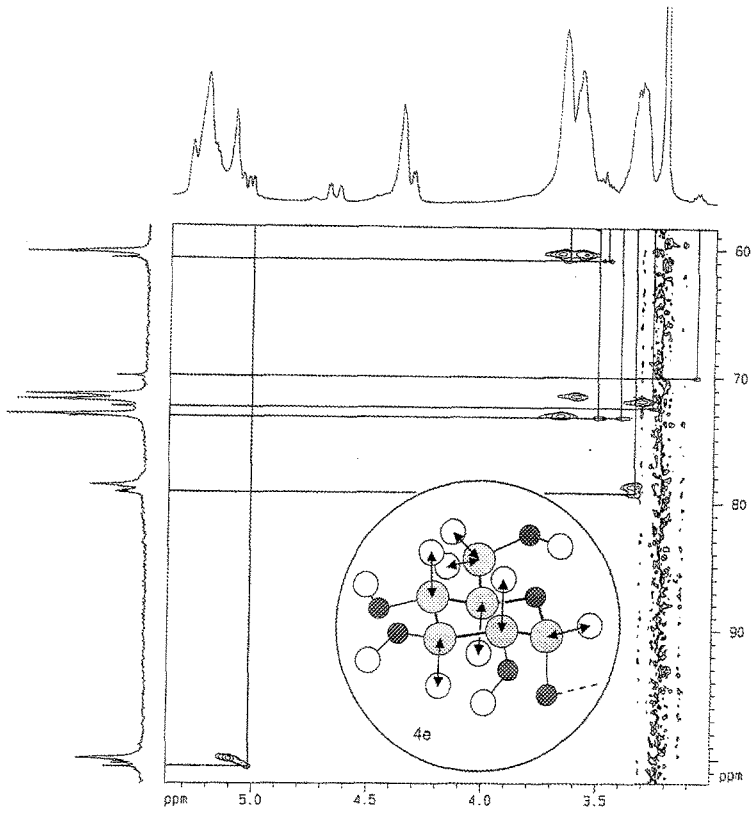

Fig. 5. 2D H–C COSY spectrum of amylopectin dissolved in *DMSO*-d_6 (straight arrows in the insert indicate the correlations derived from the cross peaks indicated in the spectrum; only those lines pertaining to the fragment 4e were drawn; for the assigned shift values, cf. Fig. 7)

5.03 ppm. Moreover, the signals of C-1 and H-1 of the glucan moieties giving rise to the branching in their position 6 could be assigned at 100.14 and 5.07 ppm.

Having achieved the ^{13}C and ^{1}H NMR signal assignments of the amylose and amylopectin a and 4e fragments and of certain signals belonging to the branching fragment themselves, the question of the degree of branching of amylose and amylopectin could be addressed from the standpoint of proton NMR spectroscopy. The integration and comparison of intensities in ^{1}H NMR spectra is superior to the corresponding procedure in ^{13}C spectroscopy. Due to severe overlaps (cf. Figs. 3 and 6) of the various OH protons, one was forced to integrate over the signals of 6-OH(a)+6-OH(4e)+3-OH(4e) or, alternatively, 3-OH(a)+2-OH(a)+1-CH(a)+3-OH(4e), and to compare these sum intensity with those of the resolved signal of 4-OH(4e). These two methods resulted in branching degrees of 3.9 and 3.9% for amylose and 8 and 9% for amylopectin. These values are systematically somewhat higher than those reported recently (1.4 and 6% [3]). This could be attributed to the inherent problems of integrating carbon-13 signals, since relative signal intensities in proton NMR spectra can be derived with high precision and without intrinsic shortcomings. Thus, the new branching values had to be taken with higher

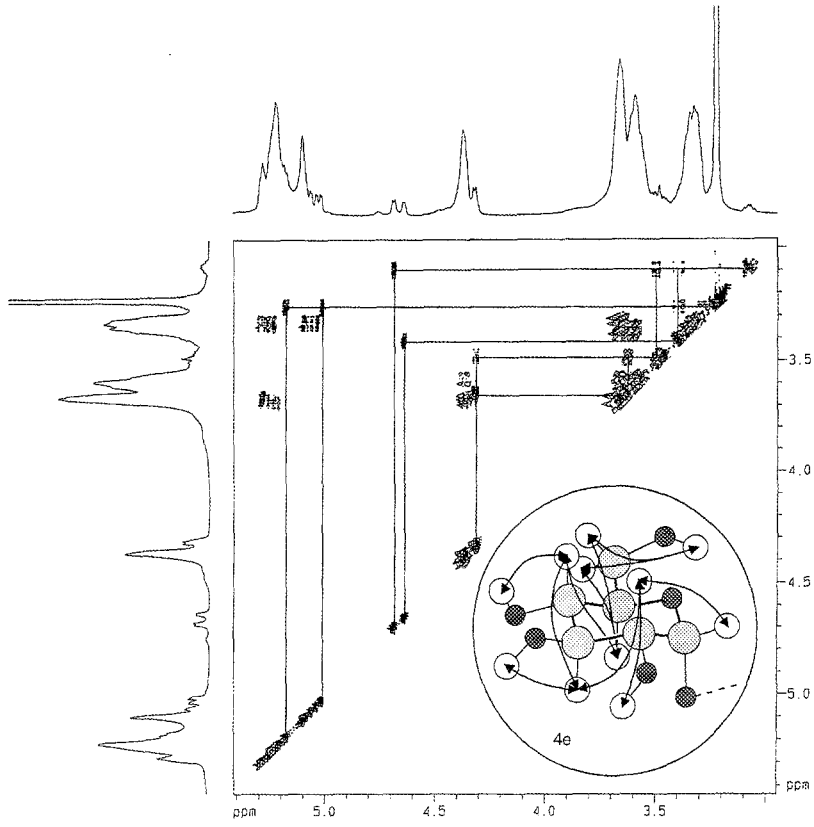

Fig. 6. 2D H–H DQF COSY spectrum of amylopectin dissolved in *DMSO*-d_6 (bent arrows in the insert indicate the correlations derived from the cross peaks indicated in the spectrum; only those lines pertaining to the fragment 4e were drawn; for the assigned shift values, cf. Fig. 7)

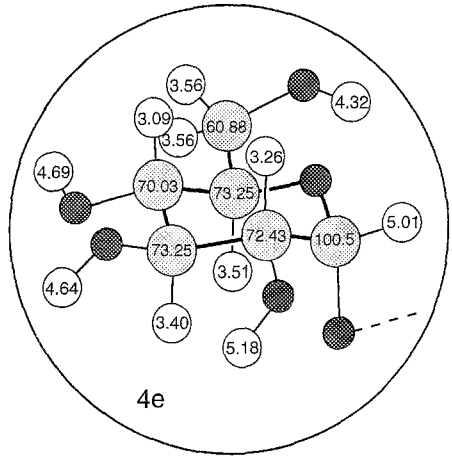

Fig. 7. ^{13}C and ^{1}H signal assignments of the glucan units 4e (*DMSO*-D_6, cf. Fig. 1)

confidence. Since the branching degrees found for the natural products using enzymatic methods [7] are also systematically lower (2 and 5%), it was suspected that the enzymatic method somewhat underestimates the true branching degree. Accordingly, integration of the proton signals as indicated above should provide a

valuable means for the simple and reliable derivation of branching degrees for starches and starch derivatives in general.

Experimental

Native amylopectin from waxy maize and amylose were of commercial origin ("Wachsmaisstärke, alkaligewaschen"; AGRANA Stärke GmbH, Gmünd, Austria, and "Lösliche Stärke"; SERVA, Germany). The NMR samples were prepared by dissolving 30.0 mg of the respective material in 0.5 ml dimethylsulfoxide-d_6 (99.95% D; Uetikon) under an argon atmosphere and heating at 60°C for 45 min in 5 mm quartz sample tubes. The spectra were recorded at 332 ± 1 K on a Bruker DRX 500 instrument at a proton frequency of 500.13 MHz and a carbon frequency of 125 MHz under non spinning conditions. 2D (HH and HC COSY) experiments were performed using the gradient enhanced phase sensitive HH DQF-COSY technique [8] (4096 data points in F2, 1024 data points in F1; 4 scans; 16 dummy scans; 2976.2 Hz spectral width in both dimensions; phase sensitive (TPPI); apodization function: sinebell squared) and the inverse HC-COSY strategy (*ge*-HMQC [9]; 4096 data points in F2, 512 data points in F1; 8 scans; 32 dummy scans; 2976.2 Hz spectral width in F2, 11340 Hz spectral width in F1; phase sensitive (TPPI); apodization function: sinebell). Deuterium exchange was accomplished by adding a drop of D_2O to the sample dissolved in *DMSO*-d_6. ^{13}C DEPT spectra were recorded with a pulse angle of $\theta = 3\pi/4$, 40000 scans, 4 dummy scans 25062 Hz spectral width, and transformed after exponential multiplication.

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