

Determination of The Composition of a Tetrasaccharide Isolated from Glycosylated Barley β -Amylase Isozyme Using Nuclear Magnetic Resonance

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Abstract : In order to determine the composition of a tetrasaccharide obtained from glycosylated barley β -amylase isozyme we applied two-dimensional (2-D) nmr (nuclear magnetic resonance) techniques. COSY (CORrelated Spectroscopy) and TOCSY (TOTally CORrelated Spectroscopy) pulse sequences enabled us to achieve an unambiguous assignment of four monosaccharide units by allowing us to follow the shift-correlation map in each monomeric ring. After careful comparison to proton chemical shifts as well as the resonance patterns in literature we have found that there are α -D-GlcNAc, β -D-GlcNAc, α -D-Man and β -D-Man in the tetrasaccharide.

Proteins, nucleic acids and carbohydrates are the three main building blocks of biological systems. Characterization of the precise three-dimensional structures of these molecules is crucial in understanding their biological roles (Anthony *et al.*, 1992; Poppe *et al.*, 1993). For determining structures of proteins and nucleic acids with molecular weights of 15,000 daltons or less, nmr spectroscopic methods have been well established over the last two decades (Wuthrich, 1986).

In the case of carbohydrates which play an important role in molecular recognition, the immune response, and cell metabolism (Clark *et al.*, 1987; Ziska and Henderson, 1988; Sairam and Bhargavi, 1985; Calvo and Ryan, 1985; Dennis *et al.*, 1987), application of nmr methods has not been so successful. One main difficulty in using nmr techniques for carbohydrates stems from the fact that most of the proton resonances of carbohydrates appear within a narrow spectral

region of 3~4 ppm creating a severe resonance overlap (Vliegthart *et al.*, 1983). The situation becomes worse when one has to deal with many different forms of isomers that are possible due to a variety of glycosidic linkages. One saving feature is that anomeric protons in carbohydrates are usually well separated from the rest of the proton resonances. Consequently, nmr structural studies regarding carbohydrates has been limited to comparing different carbohydrates only by looking at two or more 1-D (one-dimensional) spectra obtained under identical conditions, especially near the anomeric proton region (Vliegthart *et al.*, 1983).

Introduction of 2-D nmr methods in the mid-1970s (Jeener, 1971; Aue *et al.*, 1976), together with the availability of high-field nmr instruments, has revolutionized the nmr application for structural determination of biomolecules by providing a way of overcoming such resonance overlap problems. By carefully tracing down the through-bond correlations from the anomeric protons to H2, H3, H4, H5 and H6 protons in

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a shift-correlated spectrum such as COSY (Homans *et al.*, 1983) and TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) one should be able to assign all the resonances for relatively small carbohydrates with a few glycosidic linkages.

Here, we report an unambiguous resonance assignment of all the proton resonances in a tetrasaccharide using 2-D nmr methods. We also demonstrate the importance of nmr sample preparation for successful nmr data collection and analysis.

Materials and Methods

A tetrasaccharide (TS1) attached to barley (*Hordeum vulgare*, Lemend LAMARK) β -amylase isozyme was released by enzymatic cleavage using endo- β -N-acetyl-glucosaminidase (Endo H) purchased from Boehringer Mannheim GMBH. The sugar moiety was dissolved in D_2O , and was subject to nmr studies without further purification. Details regarding the preparation of TS1 will be published elsewhere (Kim and Lee, 1993). The concentration of the nmr sample was approximately 2 mM. In an effort to reduce the intense residual HOD peak which appears in the anomeric region, the nmr sample was lyophilized a few times in a speed-vac evaporator (Savant Instrument Inc. Model AS160) and finally dissolved in D_2O in an air-free environment.

The nmr experiments were performed using a UNITY 500 spectrometer equipped with a linear amplifier in the observe transmitter channel. The residual HOD which resonates at 4.76 ppm at 298 K is the reference for proton chemical shifts. Proton 1-D as well as homonuclear COSY (Homans *et al.*, 1983) and TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) methods were used. Experiments were carried out at 298 K.

Results and Discussion

Fig. 1 is a 1-D proton nmr spectrum of TS1 obtained at 298 K. Notice the size of the small residual HOD resonance which appears at 4.76 ppm. Before the sample was prepared in an air-free environment the size of this resonance was too big and potentially obscured the resonances at 4.87 and 4.69 ppm. Fig. 1

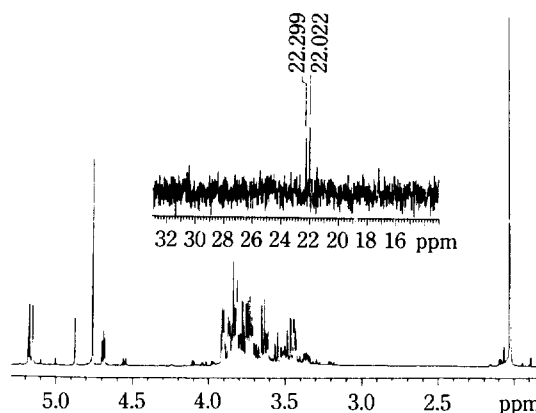


Fig. 1. One-dimensional proton nmr spectrum of TS1 obtained in D_2O at 298 K. Chemical shifts are referenced to the residual HOD which at 298 K resonates at 4.76 ppm. Pulse width for 90 degree pulse is 11.3 μ s and pulse repetition delay is 10 s. The insert shows a region of C-13 spectrum with two methyl resonances from N-acetyl groups.

Table 1. Summary of proton chemical shifts in ppm for TS1 in D_2O at 298 K. Chemical shifts are referenced to the residual HOD which resonates at 4.76 ppm at 298 K

Monomeric unit	Ano-meric	H2	H3	H4	H5	H6
α -D-GlcNAc	5.18	3.85	3.73	3.46	3.83	3.78, 3.89
α -D-Man	5.15	3.91	3.82	3.63	3.79	3.74, 3.86
β -D-Man	4.87	3.91	3.62	3.54	3.35	3.70, 3.88
β -D-GlcNAc	4.69	3.66	3.52	3.43	3.43	3.72, 3.89

shows that there are 4 major anomeric protons, therefore, 4 monosaccharide units. Two-dimensional spectra shown below confirm that there are 4 monosaccharide units in TS1. The resonance at 4.55 ppm seems to belong to an anomeric proton of the β isomer of the D-GlcNAc whose α isomeric anomeric proton resonates at 5.18 ppm (see Table 1). It should be pointed out here that our study concentrates on the major component in the nmr sample. The nmr sample contains minor components other than TS1 as is evidenced by small peaks in Fig. 1. They are present in the nmr sample since no efforts have been made to isolate them from the main tetrasaccharide TS1 for which resonance assignments were carried out in the present study.

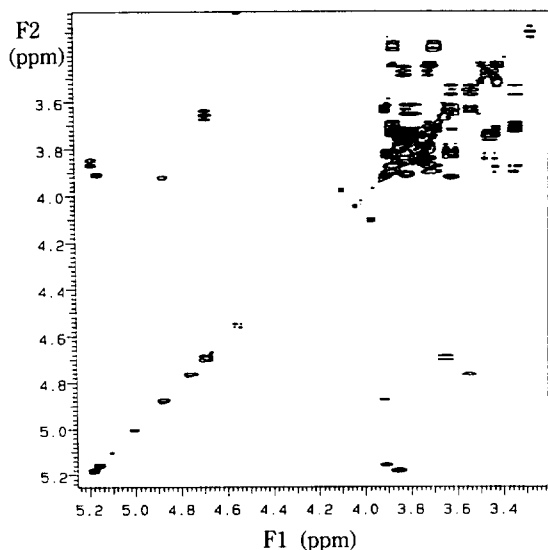


Fig. 2. Proton COSY spectrum of TS1 in D₂O at 298 K obtained in a phase-sensitive mode. Zero-filling was done once in F2 dimension and thrice in F1 dimension. Final data matrix is composed of 4096×4096 data points. Pulse repetition delay is 1.5 s. Four anomeric-to-H2 cross peaks are found on the bottom right of the spectrum, indicating the presence of four monosaccharide units. No symmetrization has been applied. The peak at 4.76 ppm along the F2 axis is an artifact due to the residual solvent. Note that corresponding is absent on the left top of the spectrum.

There are two N-acetyl groups in TS1. Methyl proton resonances from these N-acetyls overlap exactly at 2.03 ppm. Integration near 2.03 ppm indicates that there are 6 protons, pointing to the fact that there should be two methyl groups. Presence of two N-acetyl groups in TS1 has also been confirmed by observing two carbon-13 resonances at 22.299 and 22.022 ppm, corresponding to methyl carbons from the two N-acetyl groups (see the insert in Fig. 1). N-acetylation is quite common among carbohydrates (Vliegthart *et al.*, 1983; Agrawal, 1992) and our data suggest that there are two N-acetylation points in TS1.

Fig. 2 is a homonuclear COSY spectrum of TS1 and an expanded plot is shown in Fig. 3. Even though TOCSY is very powerful in showing the overall shift-correlation (Fig. 4), simple COSY proves to be more useful in picking out the H2 protons. Fig. 2 clearly indicates that there are four major anomeric protons.

Fig. 4 is a TOCSY spectrum of TS1. With known

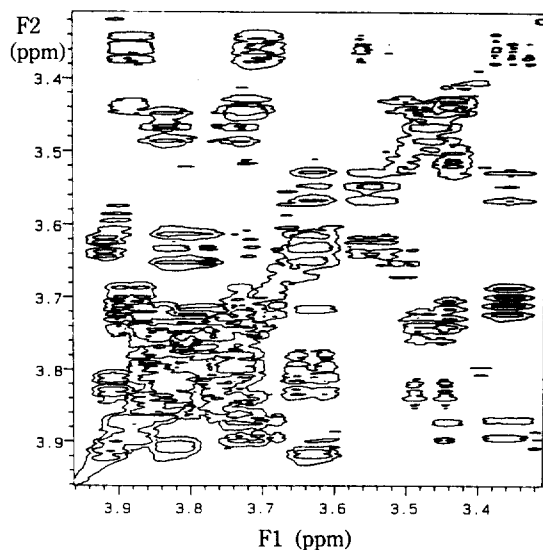


Fig. 3. Expanded region of the COSY spectrum shown in Fig. 2. This region shows only the through-bond connectivities among H2, H3, H4, H5 and H6 protons. Experimental conditions were the same as those for Fig. 2.

H2 proton locations from the COSY spectrum one can now estimate the chemical shifts of the H3, H4, H5 and H6 protons for a particular monosaccharide ring from the TOCSY spectrum. This illustrates the power of TOCSY over COSY. However, it is important to realize that the TOCSY spectrum does not tell us if a resonance belongs to H3, H4, H5 or H6. We only learn which 6 resonances form a particular monosaccharide unit. In order to complete the resonance assignment one therefore has to resort to the COSY data such as the one shown in Fig. 3.

For example, the α -D-Man whose anomeric proton resonates at 5.15 ppm has 5 cross peaks in the TOCSY spectrum. The COSY spectrum shown in Fig. 2 showed that the H2 proton of this monosaccharide unit resonates at 3.91 ppm. If we look at the COSY spectrum in Fig. 3 the diagonal peak at 3.91 ppm has a COSY cross peak at 3.82 ppm and this is the chemical shift of the H3 proton of the α -D-Man. The diagonal peak at 3.82 ppm has a cross peak at 3.63 ppm, which belongs to the H4 proton. This H4 proton is again connected to the H5 proton that resonates at 3.79 ppm. Finally, the H5 proton is correlated with the two H6 protons at 3.74 and 3.86 ppm. Similar procedures have been used for the other residues. It

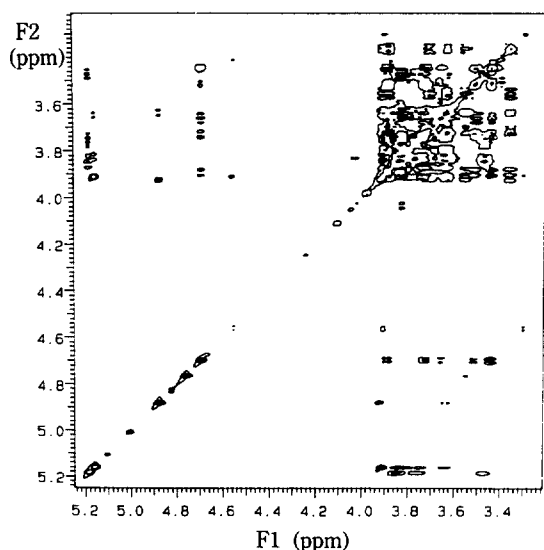


Fig. 4. TOCSY spectrum of TS1 in D₂O at 298 K. Mixing time of 152 ms was used, including 2 ms for a trim pulse. Pulse width for 90 degree pulse during the spin-locking period was set to 24 μ s. Other experimental conditions were the same as those for the COSY spectrum shown in Fig. 2.

is important to cross check the chemical shift of each proton obtained from COSY with that indicated by a TOCSY spectrum. In other words, if one comes up with a chemical shift for a particular proton from a COSY spectrum which is not supported by a TOCSY spectrum it is advisable to look for a mistake in picking the COSY cross peak.

In Fig. 5 the expanded regions of the TOCSY spectrum shown in Fig. 4 are shown along with traces in order to clearly indicate the transfer of correlations from the four anomeric protons to the rest of the protons within each monosaccharide unit.

Consistency with the literature (Vliegthart *et al.*, 1983; Agrawal, 1992) has been checked as well. For glucose and mannose anomeric protons of alpha isomers were usually downfield compared to beta; in TS1 assigned chemical shifts are 5.18 ppm for α -D-GlcNAc, 4.69 ppm for β -D-GlcNAc and 5.15 ppm for α -D-Man, and 4.87 ppm for β -D-Man. Excluding monosaccharides which have H3 resonances at a higher field than H2 resonances, for the anomeric proton at 5.18 ppm we can find 4 candidates from literature; α -D-Man, α -D-GlcNAc, α -D-GalNAc and α -D-ManNAc.

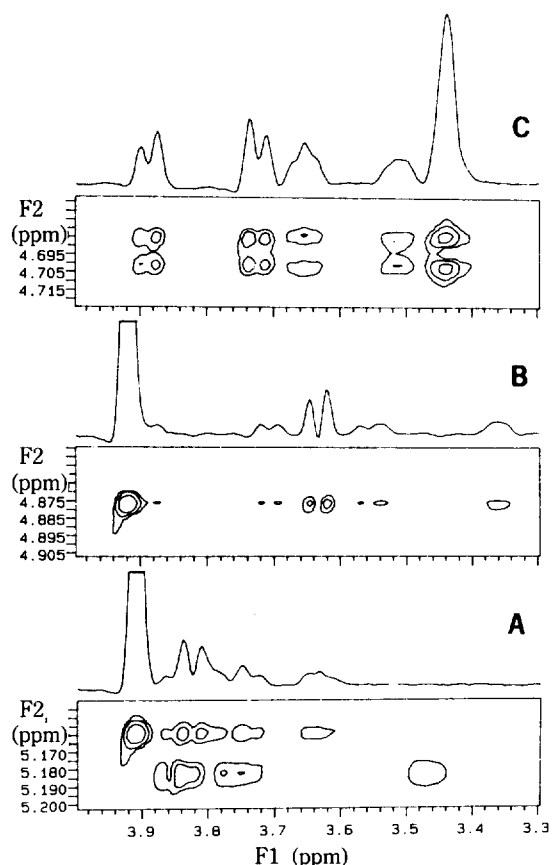
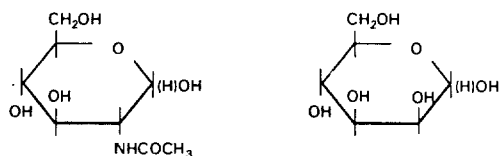


Fig. 5. Expanded regions of the TOCSY spectrum shown in Fig. 4 with traces on the top demonstrating clear coherence transfer within (A) α -D-GlcNAc and α -D-Man (B) β -D-Man and (C) β -D-GlcNAc. In (A) only the trace along the α -D-Man is shown to avoid complication.

Among them, H2 proton resonances of α -D-GalNAc and α -D-ManNAc typically resonate near 4.19 ppm and 4.32 ppm, respectively. For α -D-Man, the H4 proton usually resonates near 3.68 ppm. Therefore, α -D-GlcNAc is the only one that satisfies the observed proton chemical shifts. The small coupling constant between the anomeric proton and the H2 proton (<3.5 Hz) in α -D-GlcNAc also supports this conclusion. For the anomeric proton at 5.15 ppm, α -D-Man and α -D-ManNAc are the only two that can show this resonance. Since the H2 resonance of the latter is known to appear at 4.31 ppm while the observed chemical shift is 3.91 ppm, the 5.15 ppm anomeric proton is assigned to that of α -D-Man. Following similar arguments, we have assigned the resonances at 4.87



N-Acetyl Glucosamine

Mannose

Fig. 6. Structures of N-acetyl glucosamine and mannose.

ppm and 4.69 ppm to anomeric protons of α -D-Man and β -D-GlcNAc, respectively.

Structures of N-acetyl glucosamine and mannose are shown in Fig. 6.

In this report, we have only concentrated in determining the composition of TS1. More work is on the way in order to determine the sequence and three dimensional structure of TS1.

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References

- Anthony, J., Near, R., Wong, S., Iida, E., Ernst, E., Wittekind, M., Haber, E. and Ng, S-C. (1992) *Mol. Immunol.* **29**, 1237.
- Agrawal, P. (1992) *Phytochemistry*, **31**, 3307.
- Aue, W. P., Bartholdi, E. and Ernst, R. R. (1976) *J. Chem. Phys.* **64**, 2229.
- Bax, A. and Davis, D. G. (1985) *J. Mag. Res.*, **65**, 355.
- Braunschweiler, L. and Ernst, R. R. (1983) *J. Mag. Res.* **53**, 512.
- Calvo, F. O. and Ryan, R. J. (1985) *Biochemistry*, **24**, 1953.
- Clark, B. L., Oka, J. A. and Weigel, P. H. (1987) *J. Biol. Chem.* **262**, 17384.
- Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L. and Kerbel, R. S. (1987) *Science* **236**, 582.
- Homans, S. W., Dwek, R. A., Fernandes, D. L. and Rademacher, T. W. (1983) *Biochim. Biophys. Acta.* **760**, 256.
- Jeener, J. (1971) Ampere International Summer School, Basko Polje, Yugoslavia.
- Kim, C-H. and Lee, D-S. (1993) (manuscript in preparation).
- Poppe, L., York, W. S. and van Halbeek, H. (1993) *J. Bio. NMR* **3**, 81.
- Sairam, M. and Bhargavi, G. N. (1985) *Science*, **229**, 65.
- Vliegthart, J. F. G., Dorland, L. and van Halbeek, H. (1983) *Adv. Carbohydrate. Chem. Biochem.* **41**, 209.
- Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley New York, N. Y.
- Ziska, S. E. and Henderson, E. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 817.