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^{13}C CP/MAS NMR study of the interaction of bile acids with barley β-D-glucan

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Dietary β-D-glucan is associated with low blood cholesterol levels due to increased excretion of bile acids, but there is little evidence for the mechanism involved in enhanced bile acid clearance. Solid-state ^{13}C NMR spectroscopy has now been used to investigate the possibility of specific binding between a representative bile acid salt, glycocholic acid, and barley β-glucan. From the similarity in chemical shift values for β-D-glucan in the solid and solution states, it is concluded that the β-D-glucan adopts the same conformation in both states. The dye Congo red is known to form a complex with β-D-glucan that can precipitate from aqueous solution. In this case, the formation of a complex involving specific binding was evidenced by the appearance in the solid-state spectrum of resonances characteristic of the dye molecule, that otherwise would not be present. In contrast, no resonances characteristic of the bile acid were observed in the solid-state NMR spectrum of the mixture of β-D-glucan and the bile acid salt. These results suggest that the hypocholesterolemic property of β-D-glucan does not involve a simple binding of bile acid salt molecules to specific sites on the β-D-glucan polymer. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

β-D-Glucan samples extracted from barley are composed of linear polymers containing a mixture of (1→3)- and (1→4)-linkages in a ratio of about 1:2.5 (Dais & Perlin, 1982; Tvaroska *et al.*, 1983). They have high molecular weights and form viscous solutions. The main repeating units of β-D-glucan from barley are three and four β-(1→4) linked glucopyranosyl units, that is, cellotriosyl and cellotetraosyl units, connected by single β-(1→3)-linkages.

Inclusion of cereal β-D-glucan in the human diet appears to have a positive effect in lowering blood cholesterol levels. It is generally supposed that this hypocholesterolemic property of β-D-glucan is due to the formation, on dissolution, of a highly viscous solution that adsorbs bile acids secreted into the duodenum, and impedes the readsorption of bile acids in the small intestines (Bengtsson *et al.*, 1990). As no enzymes are present in the human small intestine for the digestion of β-D-glucan, this polymer reaches the large intestine essentially unmodified. The decreased readsorption of bile acids could be due to slow diffusion from a viscous layer, but could also involve specific binding of the hydrophilic surfaces of bile acid micelles, or bile acids themselves, to the β-D-glucan. Bile acids at concentrations as low as 0.3 mM and at physiological pH of about 6.5, associate with fatty acids to form micelles which dissolve fats and oils (Davenport, 1977).

A number of dyes, including Calcofluor and Congo red are known to bind strongly to β-D-glucan (Wood & Fulcher, 1978; Wood *et al.*, 1983). These dye molecules are stains for cellulose, so it assumed that they interact with the cellotriosyl and cellotetrosyl units of the β-D-glucan. The interaction is strong enough that it can cause the β-D-glucan to precipitate from solution as a β-D-glucan and dye molecule complex (Wood, 1982). These observations prompted us to consider whether bile acid molecules may be binding strongly to specific sites on the β-D-glucan in the same way. Such binding could be mediated by both hydrogen bonding and hydrophobic interactions.

We have used ^{13}C CP/MAS NMR to examine bile acid and Congo red absorption to barley β-D-glucan. CP/MAS NMR is an ideal technique for investigating static interactions, since these interactions could cause additional peaks to appear in the solid-state NMR spectrum as a result of the changes to the conformation of carbon atoms in both the complexed molecules and the surrounding host matrix. Conformational changes of a variety of dry, hydrated and gelled polysaccharides have been studied in the solid-state by NMR techniques including ^{13}C CP/MAS NMR. Several papers have

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investigated the correlation of the inter-residual torsional angles to the chemical shift of the C1 and C4 carbons of α-D-glucopyranosyl residues in starches, glycogen and cyclodextrins (Veregin *et al.*, 1987; Heyes *et al.*, 1992), and of β-D-glycopyranosyl residues in cellulose and cellulose oligomers (Dudley *et al.*, 1983).

There are few examples, however, of solid-state NMR studies on the interaction of small molecules with polysaccharides and none using β-D-glucan. Starch has been extensively studied since it forms inclusion complexes with a variety of hydrophobic molecules that are incorporated inside a hydrophobic cylinder formed from a single helical chain of the starch polymer (Saito *et al.*, 1982; Veregin *et al.*, 1987; Gidley & Bociek, 1988). The resulting structural changes in the starch on the formation of such inclusion complexes are noticeable as chemical shift differences in the ^{13}C CP/MAS NMR. Changes in the chemical shift values of ^{13}C atoms of the included molecule can also occur. For included fatty acids, changes in the chemical shift of the carbons in the alkyl chain can be as much as a few ppm.

Using ^{13}C CP/MAS NMR techniques, we have examined the solid-state conformation of barley β-D-glucan. We have also sought evidence for the binding of bile acids and dye molecules with a β-D-glucan matrix, by looking for solid-like behaviour of the incorporated molecules and changes in the solid-like components of the β-D-glucan.

MATERIALS AND METHODS

Preparation of the β-D-glucan

Bran (40 g) from a non-commercial hull-less barley line, Crop and Food Research Limited strain 917/6, was washed three times with 80% (w/v) ethanol (200 ml) to remove oligosaccharides. It was then suspended in 0.1 M HCl (200 ml) which contained 55 mM NaCl. These conditions serve to inactivate endogenous β-glucanase. This preparation was incubated at 37°C, with regular stirring for 2 h, after which the HCl was neutralised by addition of 8.0 ml of 10% NaOH. Incubation at 37°C was continued for a further 3.5 h, after which the extraction mixture was centrifuged at 5000 ***g*** for 10 min and the supernatant retained.

An equal volume of acetone was added to the supernatant and the precipitate sedimented at 5000 ***g*** for 5 min. The supernatant was discarded, and the precipitate resuspended in 100 ml of distilled water. A few drops of a bacteriostat were added and the sample incubated at 37°C for 2 h. Despite this treatment only about half of the precipitate redissolved. The sample was centrifuged at 5000 ***g*** for 5 min, and the supernatant decanted. Both supernatant and precipitate were frozen and lyophilised. The lyophilised supernatant was found to be contaminated with polysaccharides other than β-D-glucan and was not used in this study.

NMR spectroscopy

^{13}C NMR solution state spectra of 5% w/w β-D-glucan were obtained at 90°C in D_2O on a Varian Unity 500 at 125 MHz. DMSO (39.4 ppm) was used as an internal reference. For the solid-state ^{13}C NMR spectra, moistened samples (33% w/w water) were packed into 5 mm rotors equipped with close fitting end caps, sealed with Halogen grease, and spun at speeds of 4 kHz in a probe from Doty Scientific. Spectra were recorded at room temperature on a Varian Unity 500 MHz spectrometer using CP/MAS, and proton r.f. fields of about 60 kHz during decoupling and cross-polarisation. Liquid-like components in the samples were examined with a single pulse Bloch decay sequence and magic angle spinning, SP/MAS (Gidley, 1992). Spectra were referenced to an external secondary standard of hexamethylbenzene (17.4 ppm for the methyl groups). Resolution enhanced spectra were obtained by applying a Lorentzian to Gaussian transformation to the FID.

For solid-state NMR analysis of moistened samples of glycocholic acid with β-D-glucan, the glycocholic acid (20 mg) was dissolved in water (10 ml), and neutralised to pH 7 with dilute sodium hydroxide. To the solution, 300 mg of the lysophilised β-D-glucan was added and the solution was heated in a water bath to dissolve the β-D-glucan. Water was removed from the solution by rotary evaporation. The dry material was moistened with water (33% w/w) until a viscous mixture was formed, which was then placed into 7 mm rotors and spun at speeds of 3 kHz in a probe from Doty scientific. Spectra were recorded on a modified Varian XL200 spectrometer.

The β-D-glucan complex with Congo red was prepared by dissolving the precipitated barley β-D-glucan (400 mg) in boiling water (50ml), and adding to the cooled solution Congo red (40 mg) in water (1 ml). The red precipitate that formed was washed with two 5 ml portions of water, before being lyophilised. For NMR analysis the lyophilised material was moistened with water (33% w/w) and spectra recorded as for the sodium glycocholate/β-D-glucan sample, above.

RESULTS AND DISCUSSION

Solution state ^{13}C NMR spectroscopy

The ^{13}C NMR spectra of β-D-glucan samples extracted from oats, barley and lichen, in deuterated dimethylsulfoxide solution at 95°C, show similar features (Dais & Perlin, 1982). A peak at 103.36 ppm was assigned to C1 of the (1→3)-linkage and the two peaks at 102.45 and 102.33 ppm, to C1 of different residues involved in (1→4)-linkages. The assignments were based on the spectra of several oligosaccharides obtained by enzymatic hydrolysis of the β-D-glucan. However, ^{13}C NMR spectra of barley β-D-glucan solution in D_2O (Bock *et*

al., 1991) show only two peaks in the anomeric region at 103.8 and 103.6 ppm. It seems likely that differences in solvation of β-D-glucan by deuterated dimethylsulfoxide and D_2O are responsible for these changes in the chemical shift. The β-D-glucan isolated for this study gave spectra in D_2O solutions at 95°C essentially the same as those reported by Bock *et al.* (Fig. 1a).

Solid-state ^{13}C NMR spectra of barley β-D-glucan

The solid-state conformation of β-D-glucan from lichen and barley has been determined by a combination of X-ray fibre diffraction and conformational modelling studies (Tvaroska *et al.*, 1983). It was concluded that the diffraction pattern is consistent with an antiparallel arrangement of double helical chains of $P3_1$ symmetry. It was noted that for different barley β-D-glucan samples, an increase in the proportion of (1→4)-linkages corresponds to a decrease in the degree of crystallinity.

We report here the first examination of barley β-D-glucan by ^{13}C CP/MAS NMR spectroscopy. Both dry and moistened samples of the precipitated barley β-D-glucan were examined. For the dry β-D-glucan sample, the spectrum (not shown) contained a number of broad peaks of which only those assignable to C1 and C6 carbons were differentiated clearly. The spectrum of the moistened β-D-glucan sample, however, had increased resolution. This could be due to increases in crystallinity or mobility, both of which tend to average out chemical shift differences. With the application of resolution enhancement, a series of relatively sharp peaks were obtained (Fig. 1b). Due to differences in referencing, the chemical shifts were uniformly moved to lower field by one or two ppm relative to those observed for solution spectra in D_2O, although a shoulder at 107 ppm in the region of the anomeric carbons and a broad peak at 94 ppm appeared uniquely in the solid-state NMR spectrum. If the moistened sample is first gelled, by heating briefly at 95°C, and the spectrum is acquired again, at room temperature, then the shoulder at 107 ppm is greatly reduced in intensity and the spectrum is similar to that of the solution-state spectrum at 95°C, albeit broader. These results indicate that the solid-state conformation is not too different to that in solution. The presence of resonances at 107 and 94 ppm suggested that some of the precipitated β-D-glucan has regions that are in a different conformation, perhaps due to chain entanglement. Such chain entanglement may be the explanation for why the native β-D-glucan, though soluble in water at 37°C, will not redissolve in water at 37°C once precipitated. It requires temperatures as high as 100°C to cause the precipitated β-D-glucan to redissolve completely.

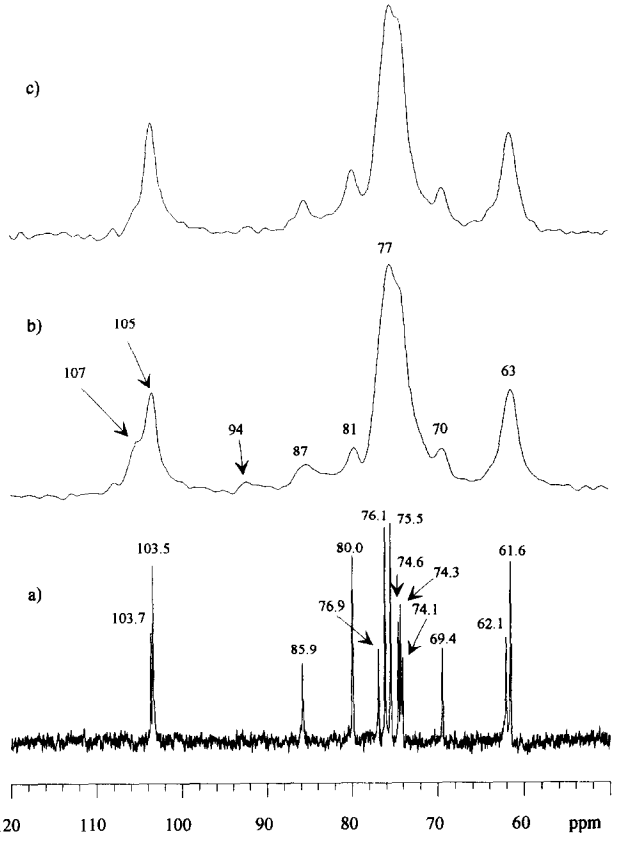

Fig. 1. ^{13}C NMR spectra of barley ß-D-glucan: (a) solution NMR spectrum at 90°C in D_2O; (b) CP/MAS NMR resolution-enhanced spectrum with 33% w/w water at 25°C; and (c) CP/MAS NMR resolution-enhanced spectrum with 33% w/w water at 25°C after gelation for 15 min.

Although the CP/MAS NMR spectrum confirms that there are solid-like components in the moistened β-D-glucan, since only solid-like components will cross-polarise, some parts of the β-D-glucan may have liquid-like behaviour. It is possible to investigate this by acquiring spectra with the SP/MAS sequence (Gidley, 1992). Results show that the SP/MAS spectrum of the moistened β-D-glucan at 25°C is similar to the solution spectrum of the β-D-glucan at 90°C, but again peaks are broader. This confirms that at room temperature there is still considerable mobility of the β-D-glucan in moistened samples.

Interaction of small molecules with β-D-glucan

The ^{13}C CP/MAS spectra of the moistened precipitate of barley β-D-glucan with Congo red is shown in Fig. 2a. Extra peaks in the aromatic region indicated that the Congo red was immobilised onto the barley β-D-glucan. Congo red would normally dissolve in water under these conditions, and as such, would have given no resonances in the CP/MAS spectrum. The peaks assignable to the β-D-glucan in the complex are similar, but broader, to those of β-D-glucan alone. Evidently the interaction is not causing a major change in the conformation of the β-D-glucan.

The interaction of β-D-glucan with bile acids was modelled by a glycocholic acid/β-D-glucan mixture at pH of 7.0 and a water content of 33% w/w. A pH of 7.0 was chosen as glycocholic acid itself is only sparingly soluble in water at low pH, and a pH of 7.0 is close to the physiolo-

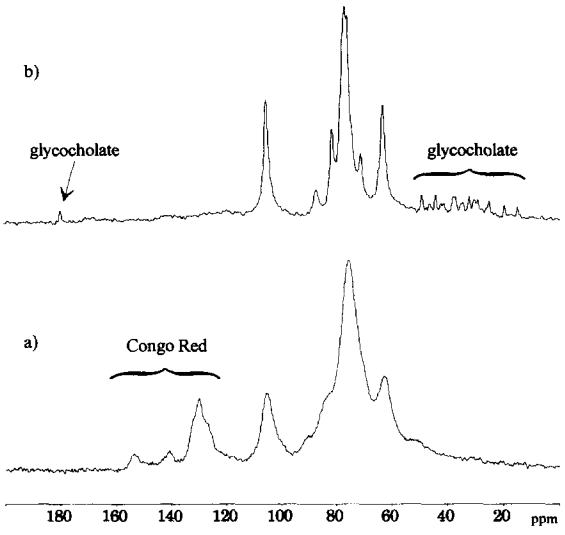

Fig. 2. (a) ^{13}C CP/MAS NMR resolution-enhanced spectrum of the barley ß-D-glucan/Congo red precipitate moistened with 33% w/w water; (b) SP/MAS NMR spectrum of ß-D-glucan, glycocholic acid and 33% w/w water at a pH of 7.0.

gical pH in the small intenstines. The CP/MAS NMR spectrum at 25°C of the gelled β-D-glucan with glycocholic acid was identical to that shown in Fig. 1c for the β-D-glucan alone, and there were no peaks assignable to the glycocholic acid. This indicated that glycocholic acid is in solution, because dissolved molecules do not cross-polarise. This was confirmed by application of the SP/MAS sequence, which gave a spectrum (Fig. 2b) virtually identical to glycocholic acid dissolved in D_2O and the liquid-like components of the β-D-glucan.

These results confirm that glycocholic acid is not bound to the β-D-glucan, but rather the glycocholic acid is mobile and in solution, which contrasts with the strong interaction found for Congo red with β-D-glucan. This supports the proposition that the ability of β-D-glucan to inhibit readsorption of bile acids is a function of its high viscosity in aqueous solution, rather than any specific binding or complexation. It is possible, however, that binding might occur between β-D-glucan and micelles formed from bile and fatty acids rather than the isolated bile acid salts alone, as modelled above. This aspect remains to be investigated.

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