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Weak self-association in a carbohydrate system

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Key words: Heteroxylans, Immunostimulatory, self-association, analytical ultracentrifugation, SEC-MALLs
Abstract

The physiological importance of weak interactions between biological macromolecules (molar dissociation constants >10μM) is now well recognised, particularly with regard to cell adhesion and immunological phenomena, and many weak interactions have been measured for proteins. The concomitant importance of carbohydrate-carbohydrate interactions has also been identified, although no weak interaction between pure carbohydrate systems has ever been measured. We now demonstrate for the first time using a powerful probe for weak interactions - sedimentation velocity in the analytical ultracentrifuge - that at least some carbohydrates (from the class of polysaccharides known as heteroxylans and demonstrated here to be biologically active) can show well-defined weak self-interactions of the 'monomer-dimer' type frequently found in protein systems. The weak interaction between the heteroxylans is shown from a temperature dependence study to be likely to be hydrophobic in nature.
Introduction

Weak interactions between macromolecules underpin many cellular processes, and are particularly important where transient assembly processes and reversible cell adhesion and immunological recognition phenomena are involved (1-4). For example transient interactions between leucocytes have long been known to be critical for the normal function of the immune system (5, 6). Cell-cell adhesion involves the formation of multiple cell adhesion molecule complexes at the cell surface and if cell de-adhesion is to occur, these complexes need to be disrupted. The affinities of cell adhesion molecular interactions must be weak to allow this reversible de-adhesion of cell-cell interactions (2, 3) and this is precisely what has been observed (7-20). Much of the attention of researchers has been devoted to protein-protein interactions (PPIs) although the significance of carbohydrate-protein interactions (CPIs) and carbohydrate-carbohydrate interactions (CCIIs) has also been identified (21).

Molecular interaction strengths are conveniently represented in terms of equilibrium dissociation constants $K_d$, whose units are conventionally M, $\mu$M or nM: the larger the $K_d$ the weaker the interaction. The CCIIs measured so far have been found to be generally in the ‘tighter’ end of the binding range as compared to PPI/CPIs, being in the “moderate strength” region 10 $\mu$M down to 100 nM or “strong” (1) (<100nM) and nothing has been observed in the important “weak” region (10’s and 100’s of $\mu$M level) associated with – and reported for - cell adhesion and immunological phenomena (Table 1). One of the reasons given for the absence of data in this important physiological region has been the difficulty in measuring such interaction strengths in this range (4) although the greater availability of hydrodynamic methods – preferred tools for looking at weak interactions (22, 23) - now renders this region accessible for study. Using this technology we can now demonstrate for the first time that carbohydrates from a class of polysaccharide known as the heteroxylans can show well-defined weak self-interaction at the level frequently found in protein systems. This observation is particularly interesting in that many of these hemicellulose polysaccharides exhibit immunostimulatory and complement activation properties (24-30). The demonstration that weak interactions are possible in carbohydrates may help towards a proper understanding of the mechanisms involved in such behaviour.

Heteroxylans

Hemicelluloses are the second most abundant group of biopolymers on this planet and of these the heteroxylans are one of most important polysaccharides of this group. They consist of $\beta(1\rightarrow4)$ linked D-xylopyranosyl residues backbone with side chains of $\alpha(1\rightarrow3)$ linked L-arabinofuranose residues (31) (Figure 1) and are often conjugated with phenolic acids such as ferulic acid and p-coumaric acid (32-35). In the extraction and purification of these polysaccharides alkali treatment followed by ultrasound-assisted water extraction is normally required to remove starch and proteins (36, 37), a process which also reduces the phenolic acid content. Although heteroxylan is mainly
used as a dietary fibre, it has recently been reported that heteroxylans can act as immunostimulators and activate human macrophages, and that they can inhibit microbial adhesion (25, 28-30, 38-40).

<Figure 1 near here>

Three heteroxylans (PO2, PO5, PO6) were chosen with different bioactivities (Figure 2) and different severities of extraction (PO6>PO5>PO2). The particular approach we have adopted to investigate possible self-association phenomena in these substances is sedimentation velocity analytical ultracentrifugation (41).

Polysaccharides are more difficult molecules to study compared to proteins because of their polydispersity (i.e. they are characterised by sedimentation coefficient distributions rather than by single values). But provided that the average sedimentation coefficient $s_w$ can be reasonably defined, this enables us to study the effect of variation in solute concentration on $s_w$ and thus help us assess associative behaviour in terms of $K_a$ – the molar association constant, or more conventionally, $K_a$. Such a computation requires that non-specific or “non-ideality” interaction effects (42), need to be taken into account. We take advantage of newly written software to achieve optimal fitting via a non-linear least squares (Levenberg-Marquadt (43)) approach, improving on a ‘trial and error’ fitting algorithm approach used earlier to study the weak interaction between the cell adhesion molecules CD2 and CD48 (15).

Materials and Methods

*Heteroxylans.* Samples PO2, PO5 and PO6 were prepared from wheat bran by the multi-step extraction procedure described by Hromádková and co-workers (44). After separation of mechanically non-removed starch particles from wheat bran by cold water decantation, the de-starched bran (PO) was treated in succession with 1% NH$_4$OH and 0.5% NaOH at room temperature for 2 hours yielding the fraction known as PO2 and after dialysis used to separate salts and low-molecular products. The de-starched bran was refluxed with isopropanol and the residue was successively treated with pectinase in acetate buffer of pH 6.1 at 25 °C for 52 hrs and then finally heated at 100 °C for 15 min to solubilise pectic substances. A part of the solid residue was extracted using 0.5 M NaOH at room temperature for 2 hrs and from the extract fraction PO5 was recovered by dialysis of the neutralized extract and then freeze-dried. From the second part of the residue, fraction PO6 was prepared by the same procedure but with saturated Ca(OH)$_2$ as the extracting agent.

Prior to all experiments, samples were dissolved in a phosphate-chloride (pH 7.0, I = 0.1M) buffered solution (45) in screw-capped tubes with constant stirring at low speed. During this period the temperature was raised to 80.0 °C for 10 minutes to obtain maximum solubility. Stirring continued at room temperature (20.0 °C) overnight at low speed. Samples were then subjected to preparative centrifugation at 40,000 rpm for 15 min (Beckman L8- 55 M Ultracentrifuge, Beckman Instruments, Palo Alto, USA) to
remove any insoluble particles or aggregates. Solution concentrations were estimated using a differential refractometer (Atago DD5, Jencons Scientific, Leighton Buzzard, UK) and a refractive index increment, \( dn/dc \) of 0.151 mL/g (46).

**Composition analysis.** Residual protein content was calculated as \% Nitrogen x 6.25 using an Elemental Analyser (Perkin-Elmer, Model 240, Wellesley, USA). Samples were hydrolysed in 2M TFA (2,2,2 trifluoroacetic acid) for 2 hrs to measure the monosaccharide composition (47) using paper chromatography and GLC of alditol trifluoroacetates (Hewlett-Packard, Model HP 5890, Palo Alto, USA). The amount of feruloyl groups (mg FA /g sample) was determined using a spectrophotometer (48) by direct absorbance measurement at 375 nm of freshly prepared solutions of hemicelluloses (0.5 mg/mL) in 0.07 M glycine-NaOH buffer (pH 10.0) with the SPECORD M-20 UV-VIS spectrophotometer (Zeiss, Jena, Germany). A calibration curve was constructed using ferulic acid as standard.

**Lymphocyte transformation test.** The method described by Iribe and Koga (49) was followed to study the level of bioactivity (29, 50). Rat thymocytes (strain Wistar, males weighing about 200 g) in RPMI-1640 medium supplemented with 5% fetal calf serum were cultivated at \( 1.5 \times 10^5 \) cells in 0.2 mL per well with or without 25 \( \mu \)g/mL of the T-mitogen phytohaemagglutinin (PHA). Test compounds were added at final concentrations of 3, 10, 30, 100, 300, and 1000 \( \mu \)g/mL. After 72 hrs of cultivation, thymocyte proliferation was measured by incorporation of \(^{3}\)H-thymidine expressed in **counts per minute** (cpm). In each of 3 independent experiments, mean cpm for each set of 4 replicas was used to calculate the stimulation indices (SI).

**Complement fixation activity.** The procedure of Michaelsen et al. (51) and Nergard et al. (52) was followed and is based on the inhibition of hemolysis of antibody-sensitized sheep erythrocytes by human sera. The % inhibition of lysis = 100 x \( (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \).

**Sedimentation velocity experiments.** An Optima XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, USA) was employed equipped with a Rayleigh interference optical system. Solutions (380 \( \mu \)L)/ reference solvent (400 \( \mu \)L) were injected into double sector, carbon filled 12mm path length centrepieces, and then loaded into an 8-hole titanium rotor. Solutions of PO2, PO5 and PO6 were run at a rotor speed of 40,000 rpm and a temperature of 20.0 °C. PO5 solutions were also measured at temperatures of 5.0 °C and at 30.0 °C. Changes in the concentration distribution in the ultracentrifuge cell as a function of time were analysed using the so-called least squares methods incorporated into the finite-difference algorithm SEDFIT (53, 54). The weight average \( s \) for a particular component was then corrected to standard solvent conditions (density and viscosity of water at 20°C) to yield \( s_{20,w} \) (S) (55). The \( s_{20,w} \) was measured at a range of concentration, \( c \), for all samples. The \( s_{20,w} \) vs \( c \) dependence was then analysed using the routine MONOMER-DIMER to yield estimates for the zero concentration or “ideal” value \( s'_{20,w} \) and the dissociation constant \( k_d \) (g/mL). MONOMER-DIMER is a locally written algorithm defined within the software proFit™ (Quantum Soft, Zürich). It derives from the original work of Gilbert and Gilbert
(56), adjusted to take into account the hydrodynamic dependence (non-specific or “non-ideality” effects) of the s value of the sedimenting species (57). kₚ for the monomer species was computed independently, knowing the molecular weight and s²₀,ₐₚ values, and the partial specific volume. The kₚ for the dimer was assumed to be identical, and both were fixed in the fitting (58).

**Molecular weight determination.** A value for the (weight average) molecular weight $M_w$ for each of PO2, PO5 and PO6 was required to enable conversion of $k_p$ values to molar dissociation constants, $K_d$. Size exclusion chromatography (SEC) coupled with multi-angle laser light scattering (MALLs) was used. The chromatographic assembly consisted of an HPLC pump (Model PU-1580, Jasco Corporation, Tokyo, Japan), a Rheodyne injection valve (Model 7125, Rheodyne, St Louis, USA) fitted with a 100 µL loop, a Phenomenex guard column (Phenomenex, Macclesfield, UK), TSK (Tosoh Bioscience, Tokyo, Japan) Gel G4000 PW connected in series with TSK Gel G3000 PW. The angular scattering envelope was recorded using a Dawn DSP multi-angle laser light scattering photometer and concentration was determined using an Optilab 903 interferometric refractometer (both instruments from Wyatt Technology, Santa Barbara, USA) with PBS as mobile phase. The SEC-MALLs system was equilibrated overnight with the phosphate-chloride buffer at flow rate of 0.8 mL/min and room temperature. Samples (100 µL) with accurately known concentration and filtered through 0.45 µm filters (Whatman, Maidstone, UK) were injected at the same flow rate. The primary data obtained form the light scattering photometer and the refractometer were captured and analyzed on a PC using the ASTRA™ (for Windows 98) software supplied by the manufacturer. Because of the low loading concentrations used followed by further dilution on the columns, thermodynamic non-ideality effects were not taken into consideration.

**Results and Discussion**

**Integrity and bioactivity**

The monosaccharide composition of the PO2, PO5 and PO6 fractions is summarized in **Table 2**. Using the data from **Table 2** the ratio of arabinose/xylose was calculated to give values of 0.73, 0.89, and 0.86 for PO2, PO5 and PO6 respectively. In addition to arabinose and xylose, all the fractions contain considerable amount of glucose and very small amount of galactose and rhamnose. The amount of ferulic acid was also measured using spectrophotometer with the highest levels detected in the PO5 fraction. Only small amounts of protein impurity were detected as manifested by the low levels of nitrogen.

<Table 2, Figure 2 near here>

Both mitogenic-comitogenic and complement fixation experiments (**Figure 2a**) showed that all three preparations of heteroxylan were bioactive but to different extents. The direct mitogenic effect was expressed as $S_{int} = \text{mean counts per minute (cpm)}$ for test compound / mean cpm for the control without stimulant. The comitogenic effect was expressed as $S_{comit} = \text{mean cpm (test compound + PHA)} / \text{mean cpm for PHA}$. The mean cpm for control cultures without any addition was 924 (704 – 1143). For cultures
incubated with PHA, the mean cpm was 1341 (1081 – 1601). The eventual contamination of the samples by endotoxin was checked in a parallel test performed in the presence of polymyxin B, which inhibits the biological effects of endotoxin including its mitogenic activity (59): this was negative for all our samples. These results are reinforced by the complement fixation activity experiments for the heteroxylans. In Figure 2b dose-dependent response curves are used to represent the concentration of test sample able to give 50% inhibition of lysis (ICH50), where low ICH50 means high complement fixing activity. Here, low ICH50 means high complement fixing activity. It is evident from Figure 2b that all three samples show clear activity.

Sedimentation velocity and molar dissociation constants
The sedimentation distribution profiles for PO2, PO5 and PO6 revealed a nearly homogenous distribution confirming the integrity of the sample preparation procedure (Figure 3). The sedimentation coefficient of all three samples at 20.0 °C and of PO5 at 5.0 °C as well as 30.0 °C, were corrected to the sedimentation coefficient under the standard conditions of the density and viscosity of water at 20.0 °C, s_{20,w}. It was observed from plots for PO2 and PO5 that the sedimentation coefficient (s_{20,w}) values tended to increase as the concentration is elevated (Figure 4a,b), with this tendency in the order PO2>PO5. As this is an effect of the opposite sign to what is predicted on the basis of non-specific interaction (above), we can be confident that a specific interaction must be present, at least for PO2 and PO5. PO6 by contrast appeared to show more normal behaviour (Figure 4c). Since a self-association is evident the data of Figure 4 were then fitted using the algorithm MONOMER-DIMER to yield estimates for s_0^{20,w} and k_d, the dissociation constant (g/mL), and the data is presented in Table 3. In order to convert k_d values to molar dissociation constants, K_d, we use the weight average M_w from SEC-MALLs (~32,700 g/mol) for all samples. PO2 and PO5 are classical “weak” interactions (K_d’s ~ 340 and 660 µM respectively), with PO6 showing little or no interaction (K_d > 3000 µM)

<Figures 3, 4 and Table 3 near here>

Effect of temperature
In Figure 5 we have explored the effect of temperature on the self-association. To facilitate the comparison (performed on PO5) plots were of s_{20,w}/s_0^{20,w} vs. concentration. The ’monomer’ and ‘dimer’ lines refer to the 30.0 °C sample, which shows the highest degree of s-c dependence as a consequence of its elevated frictional ratio. The corresponding ’monomer’ and ‘dimer’ lines for the 5.0 °C and 20.0 °C data are omitted in the interests of clarity: they are much less steep than the 30.0 °C lines. Interestingly the highest degree of self-association occurred at 30.0 °C with a K_d of ~ 140 µM whereas, at 5.0 °C, there was no significant self-interaction (K_d > 3000 µM).

<Figures 5 and 6 near here>
Concluding remarks

It is clear that the heteroxylan samples exhibit weak but clear self-association phenomena in the order PO2>PO5>PO6, an order corresponding to different severities of treatment. Temperature dependence studies indicate that the interaction is hydrophobic in nature, with increasing temperature increasing the strength of the interaction (60): there may be hydrophobic or sticky patches on the polysaccharide backbone causing neighbouring molecules to associate in a weak or transient fashion (Figure 6). Hydrophobic interactions are not unexpected in polysaccharides since they can be amphiphilic in nature, possessing both hydrophobic (carbon/hydrogen atoms) and hydrophilic (oxygen possessing) faces. It has been suggested that “carbohydrate-carbohydrate interactions rely on this amphiphilicity as the driving force for association, largely by removing the hydrophobic faces from bulk aqueous solution” (61, 62). What is unexpected is the weakness of the interaction – polysaccharides generally interact either strongly or not at all (63).

Several workers have reported interactions between carbohydrate moieties of glycoprotein/glycolipids, but only in the presence of divalent cations (64-76) and some also have reported effects independent of the presence of salt (61, 70, 77-80). Bucior and Burger (76) studied the effect of Ca$^{2+}$ concentration and suggested that adhesion force increases (from 310 pN to 375 pN) with increase in Ca$^{2+}$ concentration from 10 mM to 100 mM.

Our present finding is, to the best of our knowledge, the first report of a reversible weak self-association both in a polysaccharide and a carbohydrate system not conjugated to a protein. Although this particular weak interaction is not directly important for the bioactive behaviour of these substances nonetheless the fact that we have demonstrated that carbohydrate groups are capable of weak hydrophilic self-interactions - precisely in the range that others have shown for proteins to be crucial for molecular recognition - makes a significant step forward in our understanding of carbohydrates in both bioactivity and other recognition processes: they may be capable of weak interactions with receptor molecules, whatever they may be.

Acknowledgement

We thank Professor A. van der Merwe of the Sir William Dunn School of Pathology, Oxford for helpful discussions and Professor Terje Michaelsen of the Institute of Pharmacy, Oslo for assistance with the complement activation studies. This work was supported by the SAS-COST D28 action programme of the European Community and the Slovak Grant Agency VEGA, grant No. 2/6131/06.
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Table 1: Summary of the range of molecular interactions in biological systems. For other interactions see ref (3).

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Strength, $K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme inhibitor: enzyme</td>
<td>~0.01nM</td>
</tr>
<tr>
<td>Cytokine:receptor</td>
<td>~1nM</td>
</tr>
<tr>
<td>Antibody:antigen</td>
<td>~10nM</td>
</tr>
<tr>
<td>Cell-cell recognition molecules</td>
<td>10-200μM</td>
</tr>
<tr>
<td>CD2:CD58</td>
<td>~10μM (14)</td>
</tr>
<tr>
<td>2B4:CD48</td>
<td>10μM (16)</td>
</tr>
<tr>
<td>KIR:MHC I</td>
<td>10μM (18, 19)</td>
</tr>
<tr>
<td>CD28:CD86</td>
<td>20μM (17)</td>
</tr>
<tr>
<td>CD2:CD48</td>
<td>50μM (15)</td>
</tr>
<tr>
<td>CD8:MHC class I</td>
<td>50-200μM (7-11)</td>
</tr>
<tr>
<td>CD4:MHC class II</td>
<td>&gt;200μM (12, 13)</td>
</tr>
</tbody>
</table>

Table 2. Composition analysis of heteroxylans PO2, PO5, and PO6a.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PH (%)</th>
<th>FA mg/g</th>
<th>Rha (%)</th>
<th>Fuc (%)</th>
<th>Ara (%)</th>
<th>Xyl (%)</th>
<th>Man (%)</th>
<th>Glc (%)</th>
<th>Gal (%)</th>
<th>Ara/Xyl molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO2</td>
<td>9.8</td>
<td>19.9</td>
<td>-</td>
<td>-</td>
<td>38.8</td>
<td>53.1</td>
<td>0.9</td>
<td>3.4</td>
<td>3.8</td>
<td>0.73</td>
</tr>
<tr>
<td>PO5</td>
<td>7.4</td>
<td>32.3</td>
<td>1.1</td>
<td>1.9</td>
<td>44.6</td>
<td>50.3</td>
<td>-</td>
<td>2.1</td>
<td>-</td>
<td>0.89</td>
</tr>
<tr>
<td>PO6</td>
<td>8.4</td>
<td>27.1</td>
<td>0.8</td>
<td>-</td>
<td>44.1</td>
<td>51.3</td>
<td>0.7</td>
<td>3.1</td>
<td>4.0</td>
<td>0.86</td>
</tr>
</tbody>
</table>

a The protein content (% N x 6.25) of the samples was < 0.5%.

PH: Phenolics determined as ethylacetate extract after alkaline hydrolysis (81).
FA: Ferulic acid, determined according to Izydorczyk & Biliaderis (48).
Table 3: Measured Dissociation Constants $K_d$

<table>
<thead>
<tr>
<th>Samples</th>
<th>Temp (° C)</th>
<th>$s_{20,w}^g$ (S)</th>
<th>$k_d$ (g/mL)</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO2</td>
<td>20.0</td>
<td>2.10 ± 0.05</td>
<td>0.011</td>
<td>340 ± 50</td>
</tr>
<tr>
<td>PO5</td>
<td>5.0</td>
<td>2.60 ± 0.02</td>
<td>&gt;0.100</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>PO5</td>
<td>20.0</td>
<td>2.20 ± 0.01</td>
<td>0.020</td>
<td>660 ± 20</td>
</tr>
<tr>
<td>PO5</td>
<td>30.0</td>
<td>1.80 ± 0.04</td>
<td>0.004</td>
<td>140 ± 40</td>
</tr>
<tr>
<td>PO6</td>
<td>20.0</td>
<td>2.20 ± 0.04</td>
<td>&gt;0.100</td>
<td>&gt;3000</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: Chemical structure of heteroxylan (from (31)). The linear backbone of the (cereal) heteroxylan chains consists of β-(1→4)-linked D-xylopyranosyl residues (Xylp) to which single α-L-arabinofuranosyl residues are attached at position 3 and/or both 2 and 3 positions of the Xylp residues as well as a small amount of 2-linked α-D-glucuronopyranosyl uronic acid units. Some galactose- and glucose-containing disaccharide side chains are found as well as phenolic acids (ferulic acid, p-coumaric acid, etc.), which esterify the arabinofuranosyl residues at position C-5.

Figure 2: Bioactivity of heteroxylans PO2, PO5 and PO6. (a) Lymphocyte activation test for heteroxylans (i) mitogenic activity and (ii) comitogenic activity. The test is essentially as described by Iribe and Koga (49) adapted for plant polysaccharides (29, 50). (b) complement fixation activity of heteroxylans. Based on the procedure of Michaelsen et al. (51) and Nergard et al. (52).

Figure 3: Plots of (a) g*(s) vs. s (S) and (b) g*(s) vs log(s) for PO2 (0.85 mg/mL), PO5 (0.75 mg/mL) and PO6 (0.85 mg/mL). All experiments performed at 20.0°C at a rotor speed of 40,000 rpm.

Figure 4: Change in apparent sedimentation coefficient, s_{20,w} with concentration. (a) PO2 (b) PO5 (c) PO6. The solid lines are fits given by the function MONOMER-DIMER. The broken line is the theoretical concentration dependence for no self-association.

Figure 5: (a) Change in apparent sedimentation coefficient, s_{20,w} with concentration for PO5 at 5.0 °C, 20.0 °C and 30.0 °C. The solid lines are fits given by the function MONOMER-DIMER. (b) Effect of temperature on the change in apparent sedimentation coefficient s with concentration for PO- 5 at 5.0 °C, 20.0 °C and 30.0 °C. The ‘monomer’ (black dashed line) and ‘dimer’ (black dotted line) lines refer to the 30.0° C sample.

Figure 6: Inter-chain interactions of PO2 and PO5 are of the order 10^2 μM (adapted from (82)).
Figures

Figure 1:
Figure 2(a):

![Graph showing Stimulation index, SI vs Dose, μg/ml for different PO concentrations.](image-url)
Figure 2(b):
Figure 4(a):

Figure 4(b):
Figure 4(c):
Figure 5(a)

Figure 5(b):
Figure 6: