Identification of carbohydrates binding to lectins by using surface plasmon resonance in combination with HPLC profiling

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Introduction

Carbohydrates, present as free oligosaccharides or as glycoconjugates, play an important role in many biological events (Dwek, 1996; Varki, 1993). By interacting with carbohydrate-binding proteins, for example, lectins or antibodies, they either promote or prevent cellular adhesion processes. Insight in these carbohydrate-mediated interactions requires detailed knowledge about the carbohydrate structures involved and the precise mechanisms underlying the interactions. Significant advances have been made with the development and application of techniques like chemically induced dynamic nuclear polarization nuclear magnetic resonance (CIDNP-NMR) (Siebert et al., 1997), isothermal titration calorimetry (Doyle, 1997), glycoaffinity chromatography (Caron et al., 1998), affinity capillary electrophoresis (Heegaard et al., 1998), surface plasmon resonance (SPR) (Haseley et al., 1999), and frontal affinity chromatography (Hirabayashi et al., 2000). Most of these techniques allow the evaluation of kinetic parameters of the interaction, but all require the use of purified, well-defined carbohydrate structures. Biologically interesting glycoconjugates are often available in limited amounts only or in mixtures of compounds. This has prompted the search for highly sensitive techniques for the analysis of carbohydrate structures, either obtained directly from complex matrices or after partial purification. Improvements in the fields of chromatography and capillary electrophoresis have made it possible to develop these techniques as predictive, ultra-sensitive profiling methods, especially when combined with fluorescent tagging (Anumula and Dhume, 1998; Liu et al., 1997, 1999). Many of the available fluorescent probes (reviewed in Rice, 2000; Anumula, 2000) allow low fmol detection levels, quantification, and, importantly, do not significantly alter the physical properties of the carbohydrates. In combination with external (Guile et al., 1996) or internal (Charlwood et al., 1999) standards and sequential exoglycosidase digestions, carbohydrate chains can be analyzed in detail (Royle et al., 2002; Rudd et al., 1997, 1999).

In this study the potential of SPR to monitor interactions taking place in solution under dynamic conditions at lectin-coated surfaces was combined with the resolving power of high-performance liquid chromatography (HPLC) for the detection of fluorescently labeled (2-aminobenzamide; 2AB) high-affinity carbohydrate epitopes from complex mixtures. In the development of the method, oligomannose-type N-glycans binding to concanavalin A (Con A) were used. With the authentic mixture of glycans, derived from RNase B, and in a single experiment the...
preferential binding of Man7GlcNAc2 (Man7), Man8GlcNAc2 (Man8), and Man9GlcNAc2 (Man9) could be demonstrated. These findings were corroborated employing well-defined structures and are in agreement with earlier observations (Mega et al., 1992), thus validating this approach. Subsequently, using the fucose-binding lectin from Lotus tetragonolobus purpureus (LTA) and a mixture of fucosylated milk oligosaccharides, the selectivity and sensitivity of the combination method was demonstrated. Finally, the experimental setup was exploited to identify fucose-containing oligosaccharides in complex O-glycan mixtures derived from bovine submaxillary gland mucin.

Results

The combination of SPR and HPLC for carbohydrate ligand fishing

The HPLC profile of 2AB-labeled oligomannose-type N-glycans (not shown) as released from RNase B revealed the presence of Man5GlcNAc2 (Man5), Man6GlcNAc2 (Man6), Man7GlcNAc2 (Man7/70, Man70GlcNAc2 (Man7), and Man9GlcNAc2 (Man9) (Figure 1) in a molar ratio of 22.0:6.13:2.4:0.7 (Table I).

Con A was immobilized at the surface (~8000 response units [RU]) at pH 4.5, at which the lectin associates as a dimer (Gupta et al., 1997) to avoid cooperative binding of the ligands with Con A tetramer. However, to avoid any effects of low pH, the binding experiments were performed at pH 7.4 (physiological pH). Furthermore, the average density of Con A was calculated to correspond to Con A dimers occurring every 100 Å (BIATechnology Handbook, 1994) on the surface, minimizing the possibilities of ligand bridging between two adjacent Con A dimers. A sample (50 µl) of 2AB-labeled oligomannose-type N-glycans, containing approximately 1.5 pmol carbohydrate in total, was injected across the Con A surface at a flow rate of 5 µl/min (Figure 2A), collecting the flow-through fraction (injection; fraction B). Subsequently buffer was flowed across for 18 min, during which three fractions (30 µl each) were collected (wash 1–3; data not shown). Finally, the surface was regenerated using 2, 5, and 25 mM methyl α-D-mannopyranoside (15 µl each), collecting the four effluents (regeneration 1–4; fractions C–F). After filtration and lyophilization, collected fractions B–F were profiled on HPLC using solvent gradient 1. The chromatogram of fraction B (Figure 2B) was nearly identical to that of the starting mixture, as reflected by the relative peak intensities (Table I).

It turned out that during the washing procedure most bound material was retained on the surface. The intensities of the individual peaks in the profiles of the regeneration

Table I. Oligomannose-type structures as present on RNase B

<table>
<thead>
<tr>
<th>Structure</th>
<th>GU value</th>
<th>N</th>
<th>I</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
</thead>
<tbody>
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<td>6.12</td>
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<td>10.3</td>
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<td>22.0</td>
<td>23.6</td>
<td>15.7</td>
<td>16.7</td>
<td>17.4</td>
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<td>7.79</td>
<td>6.6</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>5.9</td>
<td>12.7</td>
<td>16.7</td>
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</tr>
<tr>
<td>Man8</td>
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<td>12.0</td>
<td>26.6</td>
<td>39.2</td>
<td>42.4</td>
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<tr>
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<td>4.8</td>
<td>9.8</td>
<td>17.2</td>
<td>20.4</td>
</tr>
</tbody>
</table>

Symbols: 2AB, 2AB labelled GlcNAc; β, β-GlcNAc; α, α- or β-Man.

Mol percentages are given for the compounds in the original mixture (N), injection (I), and four regenerations (R1-R4) after the SPR experiment. The values of Man7, Man70, and Man7/70 for N, I, and R1 represent the sum of the three isoforms, whereas those for R2 to R4 represent that of Man7 only (isoform retained by Con A).
(fractions C–F in Figure 2C–F) represented between 20 and 100 fmol per peak. The relative intensities of the individual peaks (Figure 2C–2F) reflected the affinity of the corresponding oligomannose-type structure for Con A. In Figure 3 and Table I, the relative amounts of each structure in the individual fractions are shown with respect to the initial composition (N). The relative amounts of Man5 and Man6 remain unaltered or show a slight increase in

Fig. 2. SPR combined with HPLC analysis of 2AB-labeled oligomannose-type structures interacting with Con A. (A) Sensorgram; (B) injection (representing only 20% of the amount of material recovered during injection); (C) regeneration 1 (2 mM methyl α-D-mannopyranoside); (D) regeneration 2 (5 mM methyl α-D-mannopyranoside); (E, F) regeneration 3 and 4 (10 mM methyl α-D-mannopyranoside). Dots with 2AB, 2AB-labeled GlcNAc; plain dots, β-GlcNAc; diamonds, α- or β-Man (see Figure 1). It should be noted that because the regeneration steps are performed using a large molar excess with respect to the amount of 2AB-labeled material injected, the sensorgram readouts in these steps are not directly related to the corresponding HPLC profiles. This holds for Figures 4, 7, and 8 as well.
the flow-through fraction (I), whereas those of Man7/7′/7″, Man8, and Man9 appear to remain unaltered or decrease slightly. Even though only ~10% of the injected material had interacted with the Con A surface, the trend indicated that Man5 and Man6, the major constituents in the mixture, have a lower affinity for Con A than the other components. This observation is clearly corroborated in the four regeneration fractions (R1 to R4) with the incessant increase of the relative amounts of Man7, Man8, and Man9 and the decrease to baseline level of Man5, Man6, and Man7/7′/7″.

The distinction between the binding of the three Man-βGlcNAc2 structures to Con A was concluded after using isolated structures. These results clearly reveal the usefulness of this approach for the identification of high-affinity ligands directly from a mixture. Moreover, by selecting the appropriate regeneration conditions, an affinity ranking can be established. Although Man5 was present in a 10-fold excess over Man9 in the mixture, the higher affinity excess of a structure or structures, that only display non-specific interactions, does not interfere with the ligand binding.

Evaluation of the interaction kinetics of Man5-βGlcNAc2-Con A binding

The kinetics of the interaction between the isolated oligomannose-type glycans Man5 to Man9 (using free and 2AB-labeled compounds) and Con A were performed at a surface containing a low amount of immobilized dimeric lectin (~150 RU, bound at pH 4.5; less than 1 Con A molecule every 1000 Å). The phenomenon of mass transport was assessed by using a standard protocol (Myszka, 1999). By saturating the Con A surface with each of the isolated oligosaccharides in separate experiments and recording the level of response in each sensorgram, cooperative binding effects could be completely ruled out. The gradual increase in SPR response from Man5 (10 RU) to Man9 (20 RU) is indicative of comparable surface coverage, the response being linearly dependent on the molecular mass of the glycan, demonstrating a similar concentration of bound material in each case. Divalent binding would reduce the number of bound molecules by two, and for example Man9 would have produced a response of ~10 RU.

The sensorgrams for the binding of each nonlabeled sample are shown in Figure 5. Differences between the binding characteristics of Man5, Man6, and Man7/7′/7″ on the one hand, and Man7, Man8, and Man9 on the other, are the slower association and dissociation (shallower slope of the curves) of the latter structures. Similar sensorgrams and kinetics were also obtained for the corresponding 2AB-labeled oligomannose-type structures (Figure 6). A comparison of both labeled and unlabeled Man6 and Man9 at identical concentrations proved that the 2AB-label does not influence the interaction between the oligomannose-type structures and Con A. Calculation of the kinetics by fitting the sensorgram to a 1:1 Langmuir binding profile produced χ2 values with a good fit for the binding of Man5, Man6, and Man7/7′/7″ and KX values of between 1–3 × 105 M⁻¹ (Table II). The other sensorgrams, that is, those of Man7, Man8, and Man9, proved more difficult to fit to the usual binding models as generated by the BIAS evaluation software, however it was clear from the sensorgrams that a more stable complex was formed. The typical structural difference between the group of Man7/Man8/Man9 structures as compared to the group of Man5/Man6/Man7/7′/7″ is the presence of a Man-D₃ unit at the B(A)₄ fragment. An explanation for the poor fit to usual binding models
could include a distortion of the glycosidic bond between Man-D_3 and Man-B. An analogous suggestion has been made for Man_a^-6(Man_a^-3)Man extended with a GlcNAc residue (Moothero and Naismith, 1998). An approximation of the kinetics indicated Man_7, Man_8, and Man_9 to have a 10-fold higher affinity ($K_A = 1 \times 10^5 M^{-1}$) than Man_5, Man_6, and Man_7^-7' ( $K_A = 1 \times 10^4 M^{-1}$), with the increase in affinity most likely originating from the lower dissociation rates recorded (Table II). As expected, the Man_7^-7' mixture has similar binding characteristics to Man_5 and Man_6, whereas Man_7 has a similar profile to Man_8 and Man_9. In addition to the care taken to avoid possible cooperative binding effects at the Con A surface, the higher-affinity binding of Man_7 cannot have been caused by this phenomenon because only the high-affinity oligomannose binding site epitope (Mandal and Brewer, 1993; Mandal et al., 1994) is present in this molecule (Figure 1).

**Milk oligosaccharides interacting with LTA lectin**

Using the fucose-binding LTA lectin and a mixture of 2AB-labeled oligosaccharides (Figure 7B), isolated from human
milk, a similar SPR experiment as for the Con A system was set up. The components in the mixture have been analyzed by monosaccharide analysis, NMR, mass spectrometry, and HPLC profiling in combination with exoglycosidase digestions (Gutiérrez Gallego, 2001; Haseley et al., 1998).

For the SPR experiment, a solution of the 2AB-labeled milk oligosaccharides (~2 pmol) was injected across the surface at a flow rate of 5 μl/min for 10 min, during which the flow-through fraction was collected (injection; fraction C in Figure 7A). Subsequently, the system was washed with buffer for 24 min, collecting two fractions (wash 1 and wash 2; fractions D and E in Figure 7A). Finally, regeneration was performed with 2 mM methyl α-L-fucopyranoside (40 μl), followed by 10 mM methyl α-L-fucopyranoside (40 μl) to yield regenerations 1 and 2, respectively (fractions F and G in Figure 7A). All fractions collected were filtered, lyophilized, and profiled on HPLC using solvent gradient 3.

The HPLC profiles of the isolated milk oligosaccharides prior to SPR experiments (Figure 7B) and of the injection (Figure 7C) showed subtle differences, as demonstrated on integration of the peaks (Table III).

A comparison of the relative peak areas, representing the absolute amounts of different oligosaccharides, indicated that the structures lacking fucose or carrying fucose α-1, 3/4-linked to N-acetylglucosamine were more prominent in the injection. This implied that these oligosaccharides had no or weaker interaction with the lectin than the oligosaccharides containing fucose α-1,2-linked to galactose. During the washing procedure, most bound material was retained on the surface (Figure 7D, E). The HPLC profile of the first regeneration fraction (Figure 7F) showed the increase in relative peak areas (Table III) of the structures containing fucose α-1,2-linked to galactose and the absence of structures lacking fucose. These findings are consistent with the specificity of the LTA lectin, exhibiting an increased affinity for the blood group H determinant Fu(α1-2)Gal and an especially high affinity for the Fu(α1-2)Gal(β1-4)GlcNAc fragment (Pereira and Kabat, 1974).

In the HPLC profile of regeneration 2, using 10 mM methyl α-L-fucopyranoside, only traces of the most abundant peak (2-fucosyl-lacto-n-hexaose) were evident.
(Figure 7G), indicating almost complete regeneration of the surface with 2 mM methyl α-L-fucopyranoside. Repetitive experiments yielded identical results, validating the sensitivity, accuracy, and potential of the combination of SPR and HPLC profiling. In addition, these experiments show that even if no appropriate regeneration conditions are available, a comparison of the HPLC profiles from the native mixture and the injection would allow a prediction of the interacting structure.

**Bovine submaxillary gland mucin type I O-glycans interacting with LTA lectin**

Using the same fucose-binding lectin, the SPR behavior of a mixture of 2AB-labeled O-glycans from bovine submaxillary gland mucin type I (BSM-I) was studied. The O-glycans have been previously isolated and characterized by NMR spectroscopy as their corresponding alditols (Chai et al., 1992). Using the NMR data and published glucose unit (GU) values for 2AB-labeled O-linked glycans (Matti et al., 1998; Royle et al., 2002; Rudd et al., 1999), several peaks in the HPLC profile could be assigned. The HPLC profile (solvent gradient 3) of the original mixture of O-glycans and the injection fraction (~2 pmol of carbohydrate were injected) were identical (Figure 8B, C). In the two subsequent washing steps, residual material was washed away (Figure 8D, E). The HPLC profile of the first regeneration step, employing 2 mM methyl α-L-fucopyranoside (40 μl), showed four major peaks (a–d in Figure 8F) with an intensity order of d > b > a > c. The second regeneration step, using 10 mM methyl α-L-fucopyranoside (40 μl), contained the same four peaks but with an intensity order of a > b > d > c (Figure 8G). The sum of the peaks from both regeneration steps represented ~1% (50–100 fmol) of the total amount of material used for the SPR experiment, and the elution positions of a–d corresponded to elution positions of minor components in the original profile (Figure 8B). Based on the elution positions (GU values) of peaks a–d, they could not belong to difucosylated core 2 type {GlcNAc(β1-6)[Gal(β1-3)]GalNAc} or core 3 type [GlcNAc(β1-3)GalNAC] structures.

Using this restriction, the NMR data (Chai et al., 1992) and the observed differences in affinity for peaks a–d, it is evident that peaks a–d correspond to relatively short glycans containing fucose residues and that the linkage type is most likely different in peaks a and b (α-1,2-) when compared to d (α-1,3-). This approach not only allowed the identification of fucose-containing structures that might have been ignored if conventional techniques had been used but also permitted direct quantification of glycans in a mixture. The combination of the SPR and HPLC approach may prove particularly valuable for identifying trace amounts of epitopes responsible for specific interactions as, for example, the inhibition of *Escherichia coli* adhesion by a fuco-oligosaccharide present in milk at a concentration of 20 pmol/L (Cravioto et al., 1991).

**Discussion**

The combination of an SPR biosensor and HPLC with fluorescent detection provides a powerful profiling technique capable of ligand fishing (oligosaccharides) and ordering the binding of oligosaccharides to complementary molecules according to affinities. In the development...
of the presented approach, different oligosaccharide-lectin combinations were successfully examined. The system is very sensitive (fmol) and suitable for selecting particular structures from a mixture, even when present as minor constituents. In addition, the method can be used for the detection and characterization of unknown carbohydrate-binding molecules, present at the surface of intact microorganisms (Gutiérrez Gallego, 2001).
Table III. GU values for the 2AB-labeled milk oligosaccharides from HB1

<table>
<thead>
<tr>
<th>Structure</th>
<th>GU value</th>
<th>% (mol)</th>
<th>Native</th>
<th>Recovery</th>
<th>Regen 1</th>
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<tr>
<td>6-4-3-2</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
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Symbols: ■, 2AB-labeled Glc; □, β-Gal; ◻, β-GlcNAc; ●, α-Fuc.
In addition, mol percentages are given of each identified structure present in the HPLC profile of the native mixture, the injection, and regeneration 1.

In the oligosaccharide–Con A study, oligomannose-type glycans were allowed to compete for the binding sites on Con A in a dynamic situation, and the course of interaction was quantitatively monitored. We observed that the monovalent interaction of oligomannose-type structures containing the Man3 unit D3 connected to Man-B of the B(A)4' fragment has a higher affinity for the binding site of dimeric Con A than that of the B(A)4' structure alone, and therefore the optimum binding epitope is actually this tetrascarhide unit (Figure 1); a preliminary communication about the ranking of Man3GlcNAc2 to Man3GlcNAc2 has appeared (Haseley et al., 2001). Over the past 20 years, the specificity and the thermodynamics of interaction of Con A have been studied extensively (e.g., Clegg et al., 1981; Dam et al., 2000; Derewenda et al., 1989; Goldstein et al., 1974; Goldstein and Poretz, 1986; Gupta et al., 1997; Mandal and Brewer, 1993; Mandal et al., 1994; Mega et al., 1992). These studies have disclosed the different affinities that mono- (\(K_A = 8.2 \times 10^3 \text{ M}^{-1}\)), di- (\(K_A = 4 \times 10^4 \text{ M}^{-1}\)) and trisaccharides (\(K_A = 1.5 \times 10^5 \text{ M}^{-1}\)) built up from mannose display toward Con A. Crystallographic studies of the binding of well-defined oligosaccharides to Con A (Bouckaert et al., 1999; Moothoo and Naismith, 1998; Moothoo et al., 1999; Naismith and Field, 1996) revealed that the binding site is an elongated cleft on the surface of the protein. This site is sufficiently large to accommodate a pentascarhide [GlcNAc2Man2]6[GlcNAc2Man2]Man: \(K_A = 1 \times 10^6 \text{ M}^{-1}\), flexible enough to allow the binding of a Man2Man2OMe unit in two different ways, and requires at least one monosaccharide in the appropriate orientation to allow interactions with Asn-14, Leu-99, Tyr-100, Asp-208, and Arg-228 of Con A. It is generally assumed that the high-affinity binding epitope for Con A consists of a triscarhide [B(A)4' or 4(4')]3.

Using pyridylamino-derivatized oligosaccharides Mega et al. (1992) conducted a study employing microequilibrium dialysis at pH 7.0 followed by HPLC. Our observations concur with their findings in the identification of the contribution of Man-D3 to the increased affinity and of the order of magnitude for the difference in affinity between oligomannose-type structures containing the D3B(A)4' epitope and those without. However, it is very likely that the reported \(K_A\) values reported in that study are overestimated by a factor of 3–5, judging from other literature data and our own results; also, they could not completely rule out cooperative binding as the experiments were performed with tetramer Con A.

In view of differences between our observations, in agreement with one other report (Mega et al., 1992) and other literature, it was decided to examine the interaction between Con A and different oligomannose-type structures in more detail. The first and most important item to rule out was that of cooperative binding events at the surface of the SPR biosensor. To avoid the appearance of cooperative binding, Con A was immobilized to the SPR sensor chip at pH 4.5, at which the lectin associates as a dimer. In this way divalent binding, as has been reported for the interaction to the tetramer, could be avoided. However, to exclude any effects of a lower pH on the interaction, the glycan–lectin binding experiments were performed at a physiological pH. The lectin was immobilized with a low level (150 RU) of Con A, representing less than one Con A molecule every 1000 Å, so that the possibility of a glycan bridging two Con A dimers could be completely ruled out. Moreover, it was shown that saturation of the surface with Man5 to Man9 brought about a gradual increase of the maximum response level, a result in agreement with the absence of cooperative binding events. Finally, the higher-affinity binding of Man7 but not of Man7' confirmed that the difference in affinity was a result of binding at one binding site.

The conformation of the lectin has not been changed by immobilization to the surface because the calculated affinities are in agreement with those reported by using other methods (Dam et al., 1998, 2000; Gupta et al., 1997; Mandal and Brewer, 1993; Mandal et al., 1994). Finally, the possible effect of the 2AB label in the initial experiments...
was completely ruled out by observing identical kinetics of labeled and nonlabeled glycans.

In conclusion, we suggest here that the Man$_\alpha$2Man element in D$_3$B(A)$^4\gamma$, present in Man7 to Man9, binds in a fashion similar to the GlcNAc$_\beta$2Man$_\alpha$6 epitope in the pentasaccharide GlcNAc$_\beta$2Man$_\alpha$6(GlcNAc$_\beta$2Man$_\alpha$3)Man (Moothoo and Naismith, 1998), thereby contributing significantly to the higher affinity. We propose the optimum

**Fig. 8.** SPR combined with HPLC analysis of 2AB-labeled BSM-I O-glycans interacting with the LTA lectin. (A) Sensorgram; (B) native mixture (~5 pmol); (C) injection (representing only 20% of the amount of material recovered during injection); (D) wash 1; (E) wash 2; (F) regeneration 1 (2 mM methyl $\alpha$-l-fucopyranoside); (G) regeneration 2 (10 mM methyl $\alpha$-l-fucopyranoside). Half-shaded diamonds with 2AB, 2AB-labeled GalNAc; dots, $\beta$-GlcNAc; closed squares, $\beta$-Gal; open squares, $\alpha$-Fuc; triangles, $\alpha$-Neu5Ac. For graphical linkage types information, see Table III.
binding carbohydrate epitope of Con A to be Man\(_2\) Man\(_6\) (Man\(_3\)) Man \[D_3B(A)4\]^0, Figure 1\], rather than the Man\(_6\) (Man\(_3\)) Man structure \[B(A)4\]^0. The tetrasaccharide does not appear to bind via a simple 1:1 interaction model but is more likely to undergo a conformational change to incorporate the mannose D\(_3\) residue.

Interestingly, Bachhawat \textit{et al} (2001), using SPR, noted a comparable binding phenomenon between oligomannose-type structures (free oligosaccharides and glycopeptides) and the garlic lectin from \textit{Allium sativum}, although a discrimination between D\(_2\) B(A)4 and B(D\(_2\) A)4' was not made.

Improvements of the profiling system could involve the development of an SPR surface capable of binding more molecules, which at present is in the picomole to femtomole range. In addition, the implementation of already commercially available micro flow cells in the fluorescence detector could enhance the sensitivity even further. The combination of SPR and HPLC could be interfaced in the future without too many difficulties. The collected SPR fractions never exceeded the maximum injectable volume for the analytical HPLC column; the buffer systems of both techniques are compatible, and no major purification is needed after SPR. Finally, the volatile buffer system applied for the HPLC profiling permits amplification of the system with an in-line coupled mass spectrometer. This would facilitate detailed structural information and further broaden the scope of the technique.

### Materials and methods

**Chemicals**

RNase B (EC 3.1.27.5), Con A, LTA, and BSM-I were purchased from Sigma (St. Louis, MO); and recombinant peptide-\(N^\alpha-(N\text{-acetyl-}\beta\text{-glucosaminyl})\)asparagine amidase F (PNGase F; EC 3.5.1.52) from Roche Diagnostics GmbH, Mannheim, Germany. All other chemicals were of highest purity commercially available.

**Preparation of oligosaccharides**

N-glycans of RNase B (2 mg) were released by PNGase F digestion as described elsewhere (Van Rooijen \textit{et al}., 1998). The mixture of liberated oligosaccharides was separated from detergent, protein, and salts in a single step, on graphitized carbon columns (Packer \textit{et al}., 1998). The O-glycans of BSM-I (25 mg) were released by manual hydrazinolysis (4 h, 65°C) and further purified as described (Patel \textit{et al}., 1993). Human milk oligosaccharides were a gift from Prof. H. H. Baer (University of Ottawa, Canada).

**Labeling of the glycans**

Oligosaccharides were fluorescently labeled with 2AB essentially as described. Briefly, to a solution of 23.6 mg 2AB in 500 \(\mu\)l dimethylsulfoxide/acetic acid (70:30, v/v) was added 35.4 mg NaCNBH\(_3\), and the mixture was heated for 2 min at 65°C to yield a clear solution. An aliquot (5 \(\mu\)l) of the solution was added to dried oligosaccharide (P\(_2\)O\(_5\)), and the mixture was incubated twice for 1 h at 65°C with intermediate mixing. After cooling to room temperature, the mixture was transferred onto an acid-preconditioned QMA strip (3 \(\times\) 10 cm), and the residual reagents were eluted from the labeled glycan mixture by ascending chromatography using acetonitrile. The labeled glycan mixture (remaining at the baseline) was excised from the strip, placed in an ultrafree MC centrifugal Eppendorf filter (5000 nmwl), and recovered by centrifugation with water (3 \(\times\) 200 \(\mu\)l, 8000 \(\times\) g, 15 min). The resulting solution was lyophilized and redissolved in 100 \(\mu\)l water; an aliquot was used for SPR and/or HPLC analysis. Quantifications of the 2AB-labeled oligosaccharides are based on 2AB calibration curves.

**Isolation, purification, and characterization of oligomannose-type glycans**

Oligomannose-type N-glycans were enzymatically released from RNase B (20 mg) using PNGase F and fractionated by high-performance anion exchange chromatography on a Dionex LC system, using a CarboPac PA-1 pellicular anion-exchange column (0.9 \(\times\) 25 cm, Dionex, Sunnyvale, CA) and a gradient buffer consisting of 0.1 M NaOH/0.5 M sodium acetate (Van Rooijen \textit{et al}., 1998). Collected fractions were neutralized with diluted acetic acid, desalted by gelfiltration on HiTrap columns (5 \(\times\) 5 ml bed volume), then lyophilized. Man\(_5\), Man\(_6\), Man\(_8\), and Man\(_9\) were obtained as pure compounds. The Man\(_7\) fraction contained 5–10 mol% Man\(_5\) and Man\(_6\). Man\(_7^\alpha\) and Man\(_7^\beta\) (molar

### Table IV. Proton chemical shifts (referenced to internal acetone, \(\delta\) 2.225) of the oligomannose-type N-glycans, isolated from RNase B, recorded at 300 K in D\(_2\)O

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Man(_5)</th>
<th>Man(_6)</th>
<th>Man(_7)</th>
<th>Man(_7^\alpha)</th>
<th>Man(_7^\beta)</th>
<th>Man(_8)</th>
<th>Man(_9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc-1(\alpha)</td>
<td>5.189</td>
<td>5.187</td>
<td>5.187</td>
<td>5.188</td>
<td>5.188</td>
<td>5.187</td>
<td>5.189</td>
</tr>
<tr>
<td>Man-4</td>
<td>5.095</td>
<td>5.346</td>
<td>5.347</td>
<td>5.340</td>
<td>5.340</td>
<td>5.338</td>
<td>5.330</td>
</tr>
<tr>
<td>Man-A</td>
<td>5.093</td>
<td>5.092</td>
<td>5.089</td>
<td>5.040</td>
<td>5.092</td>
<td>5.087</td>
<td>5.400</td>
</tr>
<tr>
<td>Man-B</td>
<td>4.906</td>
<td>4.907</td>
<td>5.145</td>
<td>4.907</td>
<td>4.907</td>
<td>5.145</td>
<td>5.139</td>
</tr>
<tr>
<td>Man-C</td>
<td>—</td>
<td>5.051</td>
<td>5.053</td>
<td>5.055</td>
<td>5.303</td>
<td>5.303</td>
<td>5.304</td>
</tr>
<tr>
<td>Man-D1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5.042</td>
<td>5.042</td>
<td>5.047</td>
</tr>
<tr>
<td>Man-D2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5.056</td>
<td>—</td>
<td>—</td>
<td>5.058</td>
</tr>
<tr>
<td>Man-D3</td>
<td>—</td>
<td>—</td>
<td>5.042</td>
<td>—</td>
<td>—</td>
<td>5.042</td>
<td>5.047</td>
</tr>
</tbody>
</table>
ratio, 2:3) coeluted, and the Man7/7" fraction contained ~5 mol% Man8. The structures of the oligosaccharides in each fraction were identified by 1H-NMR spectroscopy (Table IV) (Hård et al., 1991; Priem et al., 1993; Tseneklidou-Stoeter et al., 1995). Aliquots of the oligosaccharides were fluorescently labeled with 2AB as reported previously (Stroop et al., 2000).

NMR spectroscopy

Prior to analysis, oligosaccharides were repeatedly exchanged in D2O (99.9 atom % D, Cambridge Isotope Laboratories, Andover, MA) with intermediate lyophilization and finally dissolved in 450 µl D2O (99.96 atom % D, Isotec). Resolution-enhanced 1H 1D and 2D NMR spectra were recorded on a Bruker DRX-500 instrument, equipped with a 5 mm TXI-probe, at probe temperatures of 300 K (Department of NMR Spectroscopy, Utrecht University). Chemical shifts (δ) are expressed in ppm relative to internal acetate (δ 1.908, acetone δ 2.225). HOD signal suppression was achieved by applying a WEFT pulse sequence (Hård et al., 1992) in 1D 1H experiments and by presaturation for 1 s in 2D experiments. 2D total correlation spectroscopy spectra were recorded by using MLEV-17 mixing sequences with effective spin-lock times between 20 and 100 ms. 1H 1D and 2D spectra were processed on Silicon Graphics IRIS work stations (Indigo 2 and O2) using TRITON software (van Kuik et al., Bijvoet Center, Departments of Bio-Organic Chemistry and NMR Spectroscopy).

SPR

All SPR experiments were performed on a BIACore 2000 system, using a running buffer (pH 7.4) consisting of 10 mM Tris, 150 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2. For the experiments, carboxymethylated dextran-coated sensorchips (CM5, Pharmacia, Uppsala, Sweden) were activated (Haseley et al., 1999), and different lectins immobilized. For the preparation of a Con A lectin surface, dimeric Con A (in 10 mM NaOAc, pH 4.5) was attached to the filters were washed with 3 × 100 µl double distilled water and centrifuged. The pooled effluents were lyophilized, redissolved in 100 µl starting HPLC buffer, and profiled. The HPLC system used for the profiling consisted of a Waters (Milford, CT) 2690 XE module equipped with an in-line degasser, a temperature control unit (maintained at 30°C throughout the experiments) and a 474 scanning fluorescence detector. The system was controlled via a LAC/E interface using Waters Millennium 32 software. The GlycosepN, normal phase HPLC column (4.6 × 100 mm) was obtained from Oxford Glycosciences. The column was calibrated in GU with a standard mixture of glucose oligomers.

HPLC profiling

Samples collected during the SPR experiments were directly applied to a ultrafree MC centrifugal Eppendorf filter (5000 nMw), and centrifuged at 8000 × g for 7 min. Subsequently, the filters were washed with 3 × 100 µl double distilled water and centrifuged. The resulting response was ~10,000 RU for each of the four surfaces. Regeneration was accomplished by using 2 mM (40 µl) and 10 mM (40 µl) methyl α-L-fucopyranoside.

Oligosaccharide fractions, at concentrations between 6.25 and 100 µmol/L, were injected for 3 min and left to dissociate for a further 3 min. Saturation of the surface was achieved by injecting 1 mM of each isolated oligosaccharide fraction across the surface.

Association and dissociation rate constants (kₐ and k₅, respectively), and the equilibrium association constant (Kₐ) were calculated by nonlinear fitting of the primary sensorgram (BIACore evaluation program version 3.0, 1997) data using the BIACore evaluation 3.0 software (Pharmacia).

For the preparation of the LTA lectin surface, a similar protocol was used, injecting the lectin solution across the surface for 4 min (20 µl). The resulting response was ~10,000 RU for each of the four surfaces. Regeneration was accomplished by using 2 mM (40 µl) and 10 mM (40 µl) methyl α-L-fucopyranoside.
Acknowledgments

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Abbreviations

2AB, 2-aminobenzamide; BSM-I, bovine submaxillary gland mucin type I; Con A, concanavalin A; GU, glucose units; HPLC, high-performance liquid chromatography; LTA, Lotus tetragonolobus purpureus agglutinin; NMR, nuclear magnetic resonance; PNGase F, peptide-N\(^\alpha\)- (N-acetyl-\(\beta\)-glucosaminyl) asparagine amidase; RU, response unit; SPR, surface plasmon resonance.

References


