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# Sugar–lectin interactions investigated through affinity capillary electrophoresis

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**Abstract**

The affinity interactions of *Concanavalin* A (Con A) with various saccharide oligomers (dextrins, dextrans, and selected N-linked glycans from various glycoproteins) have been investigated through a capillary electrophoresis approach. Con A has shown a notable binding discrimination between the α-1,6-linked dextran and α-1,4-linked dextrin oligomers. Both the binding capacity and binding discrimination appear to decrease with an increase in sugar chainlength. While the core structure of N-linked glycans is deemed to be responsible for the overall binding of various glycans to Con A, the presence of mannose units at the non-reducing ends was found to be very beneficial to the affinity interaction with Con A. Finally, a connection between the glycan–lectin interaction and glycoprotein–lectin interaction has also been suggested. 

*Keywords:* Sugars; Lectins

## 1. Introduction

Lectins are proteins that bind carbohydrates with considerable specificity. They are normally found in a variety of organisms, being involved in numerous cellular processes. The most widely known lectin is *Concanavalin* A (Con A). Con A is a tetramer at pH above 7, and a dimer below pH 6, with each monomer (MW 26 500) possessing one saccharide binding site in addition to a transition metal-ion site (typically, $Mn^{2+}$) and a $Ca^{2+}$ binding site. The pioneering studies on Con A–sugar interactions of Goldstein et al. [1,2] has shown that the binding occurs with mono- and oligosaccharides with terminal non-reducing α-D-mannopyranosyl (α-D-man) and α-D-glucopyranosyl (α-D-glc) residues, and with certain internal glucose or mannose residues as well [3].

Con A's well-known property of binding specific sugars has made it very useful in a number of applications: Con A has been immobilized on Sepharose and routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids [4]. In a recent report [5], a glucose biosensor was proposed, based on the fact that glucose competes with the fluorescently-labeled dextran for the Con A binding sites. The fluorescence intensity was

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used to estimate the glucose level. Con A has also been immobilized on a sample plate for a MALDI (matrix-assisted laser desorption/ionization) mass-spectrometric characterization of certain sugar-containing molecules from microorganisms [6].

Capillary electrophoresis (CE) is eminently suitable for conducting affinity investigations. While working with the host and guest molecules in a free-buffer solution, it is often feasible to observe multicomponent interactions. For example, oligomer-specific information becomes available [7–9] in investigating oligosaccharide mixtures for their capacity to interact with various ligands. In studying various solution aspects of the binding conditions, it is relatively easy to make repeated runs in different CE buffers. Additionally, under a favorable set of experimental conditions, the so-called affinity capillary electrophoresis (ACE) becomes capable of measuring the values of binding constants.

Different modes of ACE have been utilized since the early 1990s. Among the most commonly pursued protein–ligand interactions, associations between various lectins and sugars were investigated in several reports [10–13]. Simultaneously, a simple ACE technique was developed in this laboratory for a case of weak sugar–ligand interactions [7], in which a mixture of fluorescently labeled oligomers was brought into equilibrium with a small ligand inside the CE capillary. The disturbances in the regular oligosaccharide migration pattern became highly indicative of the binding conditions. This simple methodology has now been applied successfully to observe the binding trends in dextrin-drug complexations [7], polyiodide association with amylose [8,14], sugar/metal-ion complexation [9] and sugar–sugar interactions [15]. In this report, we extend our ACE strategy to the investigations of several aspects of Con A–sugar interactions.

The monotonous oligosaccharides with different linkages, i.e., dextrans vs. dextrins, were evaluated first for their binding behavior vis-à-vis Con A. Subsequently, three types of glycoprotein N-glycans, featuring the same core structure but differing in their non-reducing-end sugar units, were investigated as the binding ligands. Finally, the results from glycoprotein–lectin interactions (using a fluorescently labeled Con A) were briefly correlated with those obtained with glycan–Con A interactions.

## 2. Experimental

### 2.1. Apparatus

All CE experiments were performed with a home-made capillary electrophoresis setup equipped with a laser-induced fluorescence (LIF) detection system, which was described previously [16]. Briefly, a high-voltage DC power supply (Spellman High Voltage Electronics Corporation, Plain View, NY), capable of delivering 0–40 kV, was used. The on-line fluorescence measurements employed a Model 543-AP argon ion laser (5 mW power at 488 nm) as the light source (Omnichrome, Chino, CA). The incident laser beam was aligned to its optimum position on the capillary flow-cell at a right angle, through adjusting the positioner-holding capillary. Fluorescence emission at 514 nm was collected through a microscopic lens. The emitted light was measured through a photomultiplier tube (Hamamatsu Photonics K.K., Shizuoka Prefecture, Japan), while the signal was further amplified by a lock-in amplifier (in phase with a mechanical chopper).

### 2.2. Materials and chemicals

Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50 μm, I.D. (350 μm, O.D.) were used as the separation columns for all CE/LIF experiments. Maltodextrin DE-10 and dextran (MW 1500) were both bought from Fluka Chemie (Buchs, Switzerland). Glucose, maltotriose, ribonuclease B (type III-B, from bovine pancreas), fetuin (from fetal calf serum), asialofetuin (type I, from fetal calf serum), a recombinant N-glycosidase F (EC 3.5.1.52) from Boehringer Mannheim Gmblt (Mannheim, Germany), TEMED buffer, sodium cyanoborohydride, γ-methacroyloxypropyl trimethoxysilane, Tris buffer, acetic acid, fluorescein isothiocyanate (FITC), *Concanavalin* A (Con A type VI), FITC-labeled Con A (from *Canavalia ensiformis*, type IV) were all purchased from Sigma Chemical Co. (St. Louis, MO). The fluorescence derivatization reagents, 1-aminopyrene-3,6,8-trisulfonic acid, trisodium salt (APTS), and 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS) were purchased from Molecular Probes, Inc. (Eugene, OR).

Finally, Sep-Pak Cartridges ($C_{18}$) were from Waters Associates (Milford, MA).

### 2.3. Capillary coating and sample derivatization

The inner surface of separation capillaries was coated with a layer of a linear polyacrylamide according to a slightly modified Hjertén's method [17]. Specifically, the new capillaries were first treated with 1 N sodium hydroxide for 1 h and rinsed with water and methanol for 10 min each. γ-Methacroyloxypropyl trimethoxysilane (30 μl dissolved in 1 ml $CH_2Cl_2$ containing 0.02 *M* acetic acid) was then reacted with the silica wall for 40 min (under nitrogen pressure). The capillary was then rinsed briefly with methanol and water, followed by 1 ml of 2.5% (w/w) acrylamide aqueous solution containing 2.5 μl of 10% (v/v) *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED) and 2.5 μl of 10% (w/v) ammonium persulfate. The solution was passed through the capillary under nitrogen pressure for another 1 h. Finally, the capillary was rinsed with water and dried under a stream of nitrogen.

The sample derivatization [18] of various oligosaccharides was accomplished through mixing a 1.5 mg sample with 30 μl of 20 m*M* APTS aqueous solution (containing 3% (v/v) acetic acid in a glass vial). Subsequently, 2 μl of 1 *M* $NaBH_3CN$ aqueous solution was added to the above mixture and the glass vial was heated at 60°C in a metal block for 2 h. After cooling, the solution was diluted to a 200 μl volume with distilled water and stored at −20°C. Freshly diluted samples were prepared daily.

### 2.4. Enzymatic cleavage of N-linked oligosaccharides from glycoproteins

A total of 3 mg glycoprotein samples were reconstituted in 300 μl of 10 m*M* sodium phosphate buffer solution (pH 7.5), followed by addition of 2 μl (2 units of activity) of PNGase F solution. The solution was incubated at 37°C for 24 h, during which time an additional 2 μl of the enzyme solution was added after 12 h of incubation. The mixture was then taken out from the water bath and passed through a $C_{18}$ cartridge to remove proteins. The cartridge was conditioned with methanol, acetonitrile, and 5% methanol aqueous solution before applying a sample solution to it. The applied mixture was washed with 5% methanol aqueous solution and a 2-ml elute was collected. The collected glycan solutions were lyophilized.

## 3. Results and discussion

During different modes of ACE, various specific interactions occurring between a biomolecule and its ligand can be evaluated from the corresponding shift in the electrophoretic mobility. Additionally, band-broadening and peak distortion may manifest important trends in binding behavior. Besides the many advantages of CE as a separation tool, the ACE techniques can benefit various biomolecular binding studies, in that (a) only minute amounts of biological materials are needed for CE runs; (b) a CE buffer can easily be adjusted to mimic biologically relevant environment (e.g., in preserving the natural conformation of biomolecules); and (c) if impurities in the sample matrix occur, they are often readily separated during the CE procedure. However, after each run, a brief rinse with fresh buffer solution is recommended so that any extraneous, potentially interfering, materials become effectively removed. Good baseline stability is a suitable criterion for this latter aspect.

### 3.1. Binding between linear saccharides and Con A

Since Con A is known for its specific binding to either mannose or glucose residues [1–3,5], we initiated our studies with two sets of readily available oligomers featuring glucose as their only building unit: α-1,6-linked dextrans and α-1,4-linked dextrins. Fig. 1 demonstrates a typical electromigration behavior of both fluorescently-labeled oligomeric series when Con A was added to the background electrolyte at 0.5 mg/ml concentration.

While comparing in Fig. 1 the electropherograms A vs. B and C vs. D, it is obvious that the migration times and peak shapes for the derivatization reagent, APTS (indicated by arrows) are identical, suggesting that there is no specific interaction between APTS

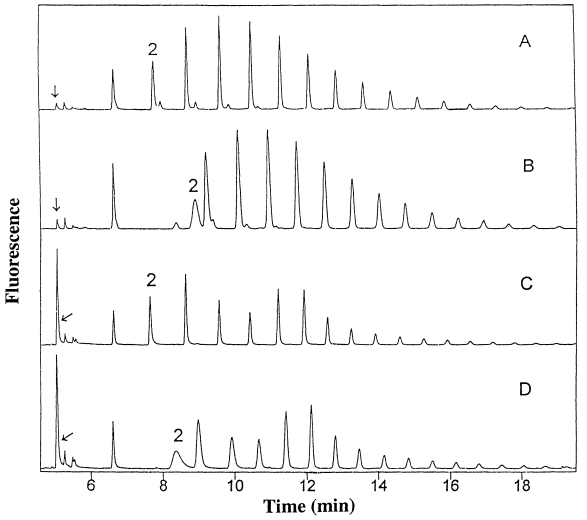


Fig. 1. Electrophoretic profiles of dextran (A, B)/dextrin(C, D) oligomers in the absence (A, C) and presence (B, D) of Con A in the buffer solution. CE conditions: 45+15 cm coated capillary, 20 m*M* Tris–HCl; Con A 0.5 mg/ml; pH 7.4; applied voltage, 18 kV; current, 8 μA. The arrows indicate the peaks for the derivatization reagent APTS; number ''2'' represents a degree of polymerization number (DP) of 2.

and Con A, and that the addition of a small amount of the lectin to buffer has not changed the solution viscosity appreciably.

Glucose (DP1), which is known to interact strongly with Con A [1–3] did not show a shift in migration either. Apparently, derivatization with APTS prevented glucose from forming the ring structure necessary for recognition. Additionally, the three sulfated groups on the reagent molecule can also be too close to the hydroxyl groups participating in the recognition process. However, an intact glucose molecule was still capable of a very strong interaction with Con A, in our buffer system, as shown through a competitive binding experiment (see below).

Starting with DP2, the presence of Con A has delayed the electromigration for all oligomers in both the dextran and dextrin series, albeit to a different degree. The data from Fig. 1 were plotted (Fig. 2) to determine the effect of molecular size on a shift in migration time. The relative mobility shift $\Delta\mu_r$ (caused by addition of Con A) is defined as:

$$\Delta\mu_r = \mu_{r1} - \mu_{r0}$$

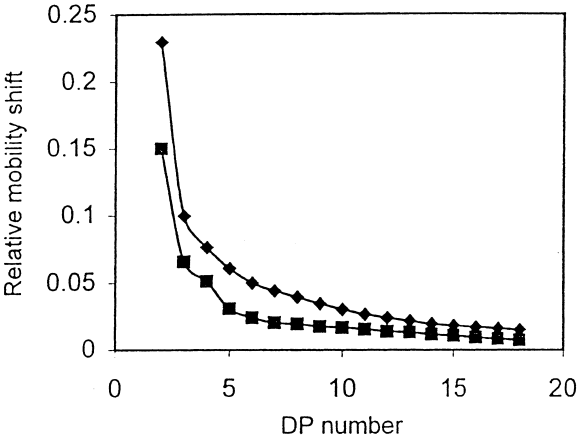


Fig. 2. Plot of relative mobility shift against oligosaccharide DP number. (♦) dextrans; (■) dextrins. Data were extracted from Fig. 1.

where $\mu_{r1} = \mu_x^*/\mu_i$, and $\mu_{r0} = \mu_x/\mu_i$, while $\mu_x^*$ is the mobility of $DP_{(x)}$ in the presence of Con A, $\mu_x$ is the mobility of $DP_{(x)}$ in the absence of Con A, and $\mu_i$ is the mobility of an internal standard (APTS-derivatized glucose has been chosen).

Fig. 2 shows clearly that the saccharides with DP2, for both linkage forms, exhibit the strongest binding to Con A, as evidenced by the migration shifts and altered peak shapes. Additionally, for the oligomers smaller than DP=10, the α-1,6-linked dextran series peaks have about twice as large mobility shifts as compared with the corresponding α-1,4-linked (dextrin) series. For the larger oligomers (DP>10), the differences became much less pronounced. Apparently, while the above measurements draw attention to the importance of linkage in the binding process, molecular modeling at the Con A binding site and spectroscopic measurements are likely to be more indicative of the molecular mechanisms involved in recognition. While the lesser binding of higher oligomers could be justified for both kinetic and steric reasons, the results obtained here appear at variance with those reported recently by Taga et al. [13]. There may be legitimate reasons for this discrepancy. The lectins studied [12,13] in their work possess a net positive charge at the indicated pH, while the labeled oligosaccharides are all negative. Under our set of conditions, Con A, which is a different lectin from those separated [12,13], has a net negative charge, while the oligo-

saccharides are also negative. The observed mobility shifts should depend on the mobility of the formed complex as well as the strength of binding. Under a different experimental arrangement (a difference between the ''classical'' affinity CE and pre-equilibration CE [19,20]), the magnitude of the electromigration shift does not necessarily imply larger binding constants. Clarification of this is needed in future research.

The previous references [1–3] have stressed the importance of metal ions ($Ca^{2+}$ and $Mn^{2+}$) in the process. In our CE experiments, it has not been feasible to employ the usual concentrations of these cations due to their adverse effects of increasing the electrophoretic current and the problems associated with electroplating of the electrodes [21]. Only small additions of these ions (0.5 and 1 m*M*) were tested in this work. Surprisingly, the results showed no difference from the zero addition of these cations (results not shown), suggesting that enough $Ca^{2+}$ and $Mn^{2+}$ may already have been incorporated in the Con A preparations to facilitate the process.

### 3.2. Effects of buffer pH

Since Con A is a protein with a variable charge in different environments (p*I*-values ranging from 4.5 to 5.5), it has become important to consider the effect of pH on the outcome of our experiments. When the buffer pH was decreased to 4.6, the migration shifts were observed not only for sugar oligomers, but also for the derivatization agents (data not shown). Undoubtedly, at this low pH, a positively charged Con A can interact, electrostatically, with APTS. To avoid misinterpretations, all following experiments were run at pH 7.4.

### 3.3. Binding constant measurements

As evidenced in Figs. 1 and 2, the migration times and peak shapes are perturbed in the presence of Con A. The migration shifts for different Con A concentrations can conveniently be used to construct a Scatchard plot, estimating the binding constants for the sugar–Con A interactions. As an example, Fig. 3 illustrates variations of the relative mobility of dextran oligomers at different Con A concentrations.

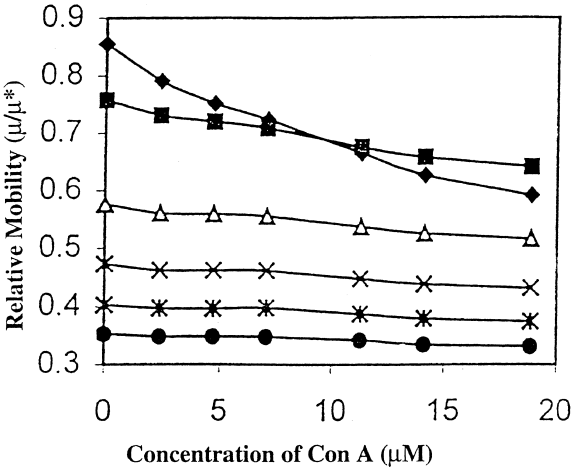


Fig. 3. Effect of Con A's concentration on the dextran oligomer migration. (♦) DP 2; (■) DP 3; (△) DP 6; (×) DP 9; (*) DP 12; (●) DP 15. Other CE conditions were the same as in Fig. 1.

Apparently, only smaller sugars (DP 2 and 3) provide meaningful migration shifts, while the larger oligomers become more difficult to evaluate due to their apparent weak binding. Very similar trends were also experienced for the dextrin series (data not shown). As an example for the binding constant measurement, the disaccharides from both series were evaluated. Several methods have been previously proposed [22–24] for the determination of binding constants through ACE. In a simplified process, the Scatchard analysis can be performed as based on a slightly modified dimensionless number [25,26], with the shifting coefficient R [$R=(M_0-M)/M_0$], and $M_0$ representing the mobility of free sugar, and M being the observed sugar mobility during the interaction with Con A. A plot of R/[Con A concentration] versus R should yield a straight line with a slope of $-1/K_d$ for determination of the binding constant.

Graphical analysis of the dextran DP 2 and dextrin DP 2 migration profiles at different Con A concentrations is shown in Fig. 4. In both cases, straight lines were obtained; the dissociation constants, $K_d$, were found as 17 μ*M* and 34 μ*M*, respectively. At the used pH, Con A is known to exist as a tetramer. Since each protein monomer has one binding site for a sugar molecule, a multiple binding process is expected. The linear plot (therefore, one binding

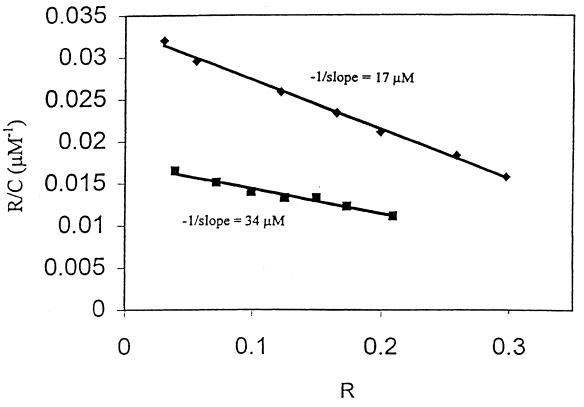


Fig. 4. Scatchard plot of Con A–sugar interaction derived from the binding profile data using ACE. (♦) dextran DP 2; (■) dextrin DP 2. R is defined in the discussion section.

constant) in a multiple binding suggests that the association of the sugar to each of Con A tetramer's four binding sites is an independent event.

We have also measured the binding constant between dextran DP 2 and Con A after derivatizing the sugar with another fluorescent reagent, aminonaphthalene trisulfonic acid (ANTS), which has a smaller molecular size than APTS. The $K_d$ value in this case was 16 $\mu M$, which agreed very well with that from the APTS-derivatized sugar (17 $\mu M$). This indicates that the end-label does not affect the binding between a sugar and the Con A molecule.

### *3.4. Competitive binding experiment*

To verify that underivatized glucose still can bind to Con A in our studied system, a competitive experiment was designed. First, it was ascertained that the peak corresponding to the APTS-derivatized maltotriose became broader and migrated considerably slower than before Con A (0.88 mg/ml) was added to the buffer. However, when glucose was also added to the buffer, the maltotriose peak reverted to its original condition (data not shown). Thus, glucose successfully competes with maltotriose for the binding sites of Con A. At a certain glucose concentration (3.6 mg/ml), apparently all binding sites were occupied by glucose, while the triose peak behaved as if Con A was not present at all. Coincidentally, a recent report [5] describes monitoring of blood glucose levels as based on competition of glucose with a fluorescently labeled dextran for the binding sites on Con A. Apparently, a relative fluorescent signal due to the displaced dextran is proportional to glucose concentration.

### *3.5. Interactions between glycoprotein glycans and Con A*

Lectins have been used extensively in the isolation of glycoproteins from various biological materials. The selectivity of various lectins for carbohydrate moieties is known to vary [27], while choosing a particular lectin for isolation tasks appears largely empirical. Con A-based chromatographic packings appear most universally employed, and their use in the microscale glycoprotein analysis appears feasible [28,29]. In developing the analytical potential of lectins as carbohydrate selectors, more information is clearly needed as to which glycan moieties are involved in the sugar–lectin interactions. The classical methodologies in the area appear tedious and time-consuming, so that ACE could provide rapid information in differentiation of glycans. Moreover, the inherent sensitivity of LIF detection is a distinct advantage in dealing with small amounts of biological materials.

In order to assess the capability of ACE for investigating glycan–lectin interactions, three model systems were chosen: (a) high-mannose glycans from ribonuclease B; (b) sialic acid-containing oligosaccharides isolated from bovine fetuin; and (c) enzymatically desialylated glycans from (b), i.e., asialofetuin. They all represent previously identified N-glycan structures [30–32], all featuring the usual N-linked consensus sequence, but differing in the presence of sugars at the non-reducing end of the molecules (see Fig. 5). They all are easy to cleave enzymatically, with an N-glycanase, from their respective glycoproteins.

#### *3.5.1. Glycans from ribonuclease B*

After the enzymatic cleavage with PNGase F and derivatization with APTS, a number of peaks appear in the electropherogram (Fig. 6A). To facilitate the identification of each peaks, we used a CE buffer similar to that reported previously [33], while omitting the 0.4% polyethyleneoxide additive, which

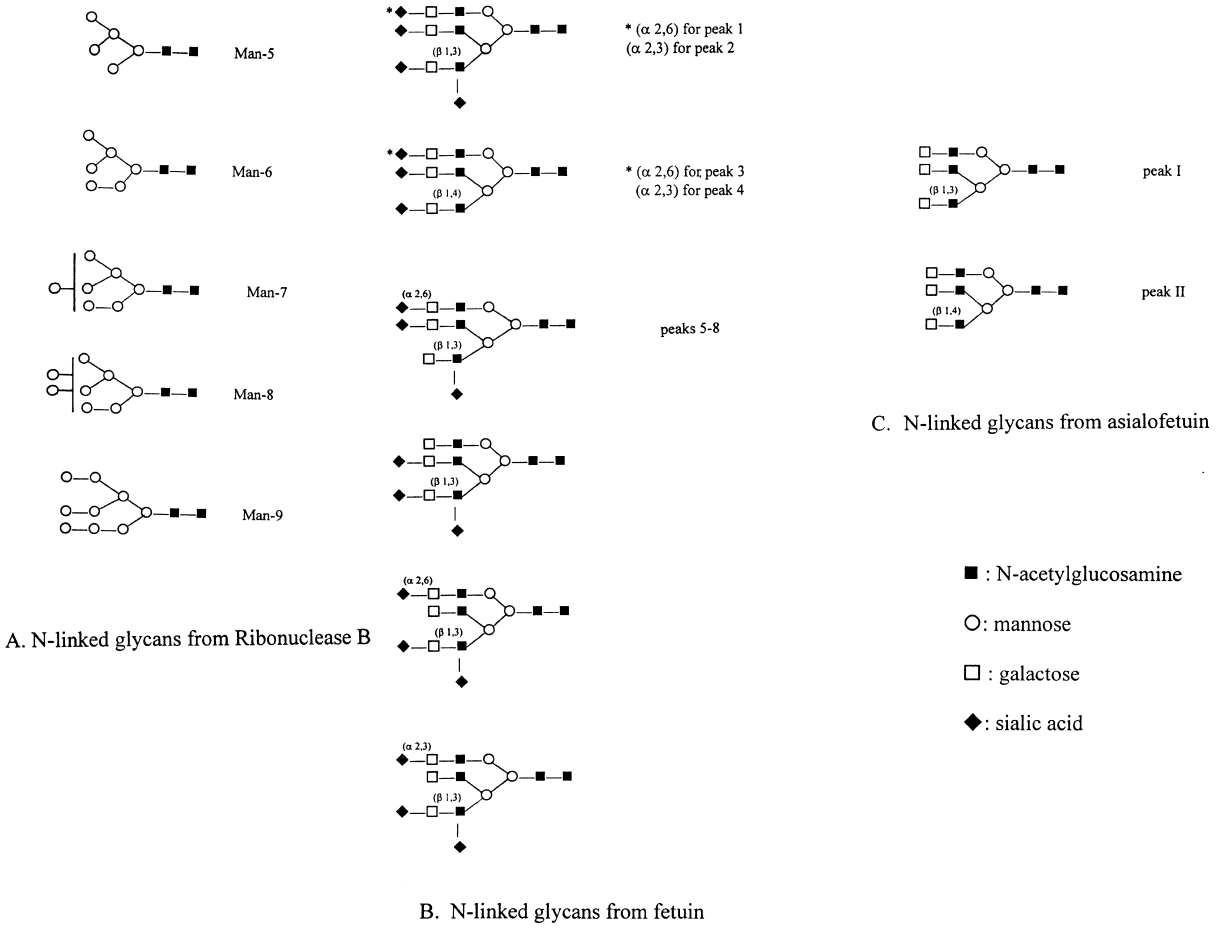


Fig. 5. Structures of N-linked glycans used in this work.

might interfere with the expected binding process. Through comparing the peak positions with those of the reported data [33], dextrin oligomers (data not shown) and all peaks could be positively identified. After adding a trace amount of Con A (2.7 μg/ml) into the buffer, all zones began to migrate more slowly, while Man-5 and Man-6 peaks became broader (Fig. 6B). The high-mannose type glycans, especially smaller ones, like the smaller dextrans and dextrins, appear very sensitive to the presence of Con A. Already when the concentration of Con A was increased to 18 μg/ml, all glycans experienced much slower migration, with all peaks becoming much broader and even hard to recognize (Fig. 6C). Such strong interactions make it difficult to evaluate the binding constants.

### *3.5.2. Glycans from fetuin*

Fig. 7A shows the peak profile for the glycans isolated from fetuin. These are structurally distinct from the ribonuclease B glycans, as they contain the sialic acid and galactose residues at the non-reducing ends instead of mannose. Once again, peak identification was made by comparing the electrophoretic mobilities with those of dextrin oligomers and the literature results [34]. Three peak clusters were readily observed: (1) Peaks 1 and 2 represent the molecules with four sialic acids at their non-reducing ends, differing only with respect to one of the sialic acid linkages; (2) Peaks 3 and 4 represent the molecules with three sialic acids at their non-reducing ends, differing again with respect to one of the sialic acid linkages; and (3) As for peaks 5–8, they

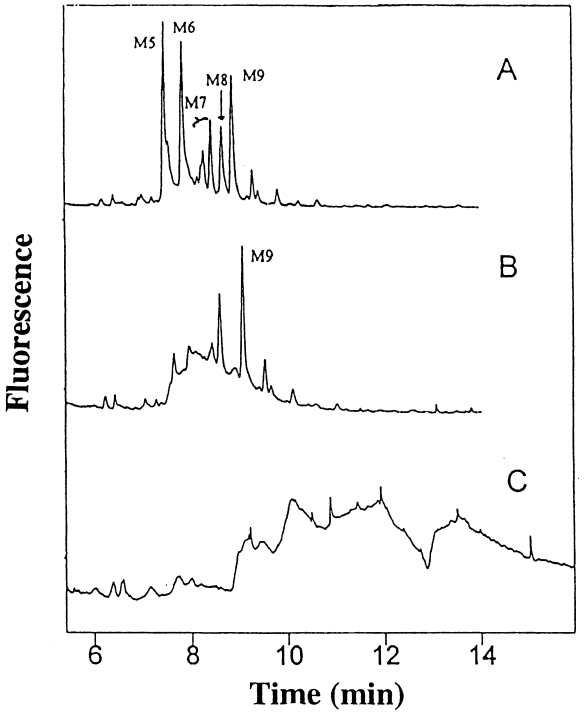


Fig. 6. Peak profiles of high-mannose type glycans in the presence of Con A. (A) 0; (B) 2.7 μg/ml; (C)18 μg/ml. CE conditions were the same as in Fig. 1, except that the capillary was 36+13 cm, and the current was 10 μA.

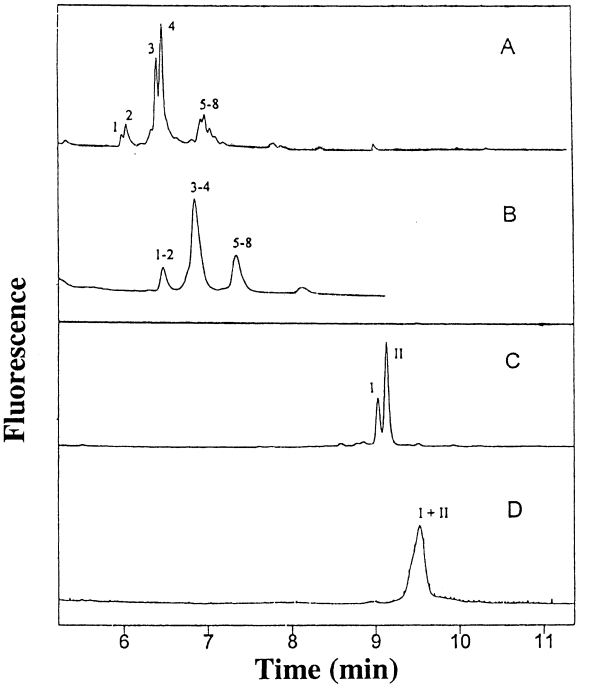


Fig. 7. Peak profiles of glycans from fetuin: without Con A (A); with Con A (1.48 mg/ml) (B); and glycans from asialofetuin, without Con A (C); and with Con A (1.48 mg/ml) (D). CE conditions were the same as in Fig. 6.

all feature three sialic acids at the non-reducing ends, but their structures are more distinct from those representing peaks 3 and 4. Fig. 7A demonstrates that these complex glycans can be resolved in the absence of Con A.

When Con A was added to the buffer, each peak cluster became one major peak, while the extent of delay in migration of each of the three major peaks was about the same (Fig. 7B). Apparently, the presence of sialic acid and galactose at the non-reducing ends does not seem to contribute to a recognition by Con A. The migration shifts of those peaks are mainly attributable to the glycans' core structure (which contains three mannose units).

### *3.5.3. Glycans from asialofetuin*

As deduced from Fig. 5B, only two isomeric glycans can be generated after desialylation of fetuin glycans: (1) these from the sialylated glycans 1 and 2 and 5–8; and (2) those originated from 3 and 4. The two resulting glycans differ only in their galactose linkage positions (β–1, 3 vs. β–1, 4). Fig. 7C shows these two glycans well resolved from each other. It can be seen from Fig. 7A that peaks 1, 2 and 5–8 together are less intense than are the peaks 3 and 4. Therefore, in Fig. 7C, the higher peak (II) is likely to represent the desialylated glycan from 3 and 4.

After addition of Con A, peaks 1 and 2 merged and migrated more slowly (Fig. 7D). Interestingly, the fetuin and asialofetuin glycans (at the same concentration of Con A) had very similar migration shifts, indicating that the sialylation of glycans does not affect their binding to Con A. Once again, the core structure, alone seems to determine the binding capacity.

Comparing high-mannose type glycans with those containing mannose only in their core structures yields another interesting observation: high-mannose type glycans are more sensitive to the presence of Con A. For high-mannose type sugars, to exhibit the migration shifts (binding capacities) of a magnitude

similar to that of the other types of glycans, *two orders* lower concentrations of Con A were needed.

We have also briefly investigated the interactions between Con A and the corresponding glycoproteins. While a FITC-derivatized Con A was used as the sample, different glycoproteins were added into the buffer. While fetuin and asialofetuin (ranging in concentration from 0.05 to 0.5 mg/ml for both cases) did not seem to have any effects on Con A's migration time and peak shape, ribonuclease B did induce a significant migration shift, and affected the peak shape for Con A (Fig. 8). Using the same Scatchard plot strategy as above, the binding constant between ribonuclease B and Con A was estimated to be $K_d = 74\ \mu M$. This behavior is consistent with the results from glycan–Con A interaction studies, suggesting that the lectin–glycoprotein interactions closely parallel the lectin–glycan interactions.

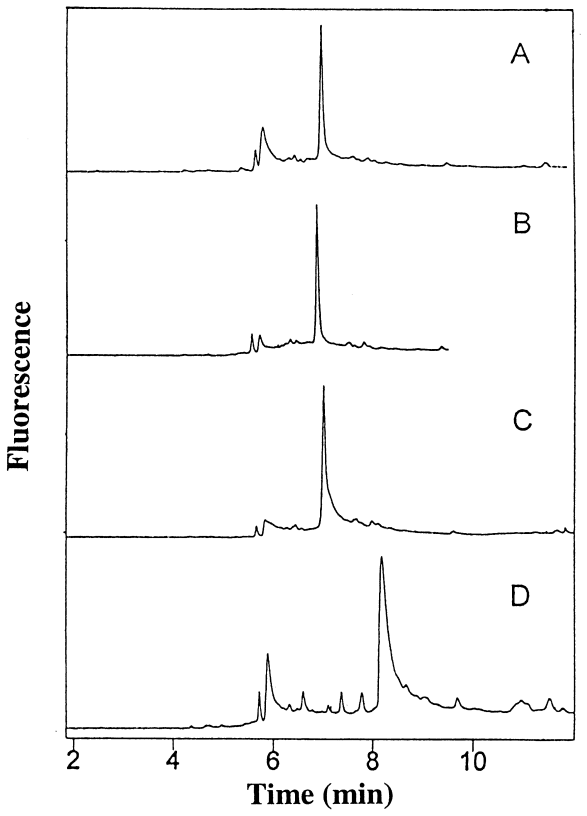


Fig. 8. Demonstration of a lectin–glycoprotein interaction. FTIC-labeled Con A in (A) buffer only; (B) buffer with 0.20 mg/ml fetuin; (C) buffer with 0.20 mg/ml asialofetuin; (D) buffer with 0.14 mg/ml Ribonuclease B. CE conditions were the same as in Fig. 6.

## 4. Conclusions

Our previously reported version of ACE [7–9], using the oligosaccharide probes labeled fluorescently at the reducing end, appears suitable for studying the glycan–lectin complexation phenomena. A different binding behavior of the oligosaccharides with different linkage forms warrants further investigation through spectroscopic techniques. The core structure of the N-linked oligosaccharides appears to be a major cause of the Con A–sugar binding behavior, while the sugar residues at the non-reducing ends also participate strongly. The fluorescently-labeled lectins appear suitable probes for the glycoprotein–lectin binding behavior in future studies.

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