We developed capillary affinity electrophoresis (CAE) to analyze the molecular interaction between carbohydrate chains and proteins in solution state (Nakajima et al. [2003] J. Proteome Res., 2, 81–88). A mixture of oligosaccharides derived from a glycoprotein was labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS), and used as glycan library without isolation. Interaction of a carbohydrate-binding protein with each oligosaccharide in the mixture could be simultaneously observed, and relative affinities of oligosaccharides toward the protein were accurately determined. In this study, we applied CAE to detect the presence of lectins in some plants (Japanese elderberry bark and tulip bulb). In the crude extract of the elderberry bark, binding activity toward sialo–carbohydrate chains could be easily detected. We also examined the presence of lectins in the crude extract of tulip bulbs and determined the detailed carbohydrate-binding specificity of *Tulipa gesneriana* agglutinin (TGA), one of the lectins from tulip bulbs. Kinetic studies demonstrated that TGA showed novel carbohydrate-binding specificity and preferentially recognized triantennary oligosaccharides with Gal residues at nonreducing termini and a Fuc residue linked through α(1-6) linkage at chitobiose portion of the reducing termini but not tetraantennary carbohydrates. The results described here indicate that CAE will be a valuable method for both screening of lectins in natural sources and determination of their detailed carbohydrate-binding specificities.

**Key words:** capillary affinity electrophoresis/carbohydrate-binding specificity/8-aminopyrene-1,3,6-trisulfonate/lectin

**Introduction**

Glycosylation is one of the most important events of posttranslational modification of proteins, and it is involved in various processes, such as protein folding, molecular recognition, and protein stabilization (Hakomori, 2000; Lasky, 1995). To understand the function of carbohydrates in glycoproteins, it is important to elucidate their structures and distributions. Lectins are a versatile tool for categorizing carbohydrates because they specifically recognize carbohydrate structures. When a lectin is used for recognition of carbohydrates, carbohydrate-binding specificity of the lectin should be strictly defined. Although a large number of lectins have been reported, only a limited number of reports are available for carbohydrate-binding studies. This is because most of the binding specificities have been determined based on semiquantitative methods, such as an agglutination assay, which is still one of the most popular methods.

Most of the assay methods for carbohydrate-binding proteins, such as surface plasmon resonance (Shinohara et al., 1997), fluorescence polarization (Oda et al., 1998), and time-resolved fluorometry (Lee et al., 1998; Nakajima et al., 2002), essentially require carbohydrates in a pure state. However, it is often too laborious to obtain pure carbohydrates in amounts necessary for kinetic studies by either chemical synthesis or isolation from natural sources.

We have developed capillary affinity electrophoresis (CAE) to analyze the molecular interaction between carbohydrates and proteins in solution state (Nakajima et al., 2003). A mixture of oligosaccharides derived from a glycoprotein was labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS) and used without isolation of each oligosaccharide. Interaction between a lectin and each carbohydrate chain in a mixture was determined simultaneously based on their change of electrophoretic mobilities or peak intensities. Furthermore, we found that CAE allowed calculation of affinity constants without determination of the accurate concentrations of carbohydrates (i.e., ligands) that are often difficult to measure. We showed that CAE was applied to classify carbohydrate chains in biological samples using a few lectins (Nakajima et al., 2003).

In the present study, we show that CAE can examine the presence of a lectin in a crude biological extract using an appropriate set of oligosaccharides. At the initial step, a mixture of oligosaccharides of known compositions is analyzed in the absence of oligosaccharides. Then the same mixture is analyzed in the same buffer containing the extract. If we observe changes of migration of oligosaccharides, such changes indicate that a substance (typically lectins) that interacts with the carbohydrates is present in the extract. Another set of a mixture of different oligosaccharides is analyzed in the same manner as described. By repeating these procedures, we can confirm the presence of a lectin and determine the detailed binding specificity of the lectin.
It is important to select an appropriate set of carbohydrates from various glycoproteins. In the previous study, we showed that human α1-acid glycoprotein (AGP), bovine IgGs, porcine thyroglobulin, bovine ribonuclease B, and bovine fetuin were appropriate as sources of N-linked oligosaccharides, because oligosaccharide compositions of these glycoproteins have been well characterized by a number of studies. The N-linked oligosaccharides or their desialylated oligosaccharides derived from these glycoproteins is shown in Table I. These oligosaccharides were previously labeled with APTS before use.

N-linked oligosaccharides of AGP, porcine thyroglobulin, and fetuin are available for detection of both sialic acid–binding lectins and lectins recognizing complex-type oligosaccharides (Kakehi et al., 1999; Nakajima et al., 2003; Yamamoto et al., 1981). Thyroglobulin is also available for finding mannose-specific lectins because it contains high-mannose type oligosaccharides. One of the asialo-triantennary oligosaccharides derived from AGP and fetuin contains one di- and two triantennary complex-saccharides with α1(3) and α(1-6) linked fucose residues, respectively, and they are used for detection of fucose-binding lectins. Asialo-fetuin contains one di- and two triantennary complex-type oligosaccharides. One of the asialo-triantennary oligosaccharides has three Galβ(1-4)GlcNAc residues, and another oligosaccharide has one Galβ(1-3)GlcNAc branch and two Galβ(1-4)GlcNAc residues (Green et al., 1988). Bovine IgG has diantennary complex-type oligosaccharides with α(1-6) linked fucose residues (Raju et al., 2000). Oligosaccharides of ribonuclease B are high-mannose type (Man5–Man9) (Fu et al., 1994). By combination of these sets as glycan libraries, we can easily obtain information on the carbohydrate-binding specificity of a novel lectin.

Results

Detection of lectin activity in a crude extract of the bark of Sambucus sieboldiana

Two lectins with different carbohydrate-binding specificity, sialic acid–specific Sambucus sieboldiana agglutinin (SSA) and galactose-specific ribosome-inactivating protein (RIP) were reported in Japanese elderberry (S. sieboldiana) bark (Kaku et al., 1996; Rojo et al., 1997). We examined the presence of these lectins by observing the change of migrations using a mixture of oligosaccharides derived from AGP in the electrolyte containing the crude extract from S. sieboldiana bark. As shown in Figure 1A, addition of crude protein fraction (250 μg/ml) in the electrolyte obviously caused change of migrations of sialo-oligosaccharide peaks.

At a concentration of 500 μg/ml crude protein fraction, the peaks almost disappeared. In contrast, migrations of asialo-oligosaccharides were not obviously affected (Figure 1B), although nonspecific retardation of migration times was observed due to the presence of proteins in the crude extract. These data clearly indicated that the crude extract contained a sialic acid–binding protein but did not show clear affinity toward asialo-oligosaccharides derived from AGP.

Migrations of the oligosaccharides in the presence of purified SSA and RIP are shown in Figure 2. The purified SSA obviously lowered the peak height of sialo-oligosaccharides and the peaks disappeared at the concentration of 0.8 μM (Figure 2A) as observed for the crude extract, but SSA did not affect the migration of asialo-oligosaccharides (Figure 2B). In contrast, RIP did not cause change of migrations of sialo-oligosaccharides (Figure 2A). However, asialo-oligosaccharides (2 and 4) showed higher affinities to RIP than those having a fucose residue (3 and 5) and were observed gradually later with increase of the concentrations of the lectin. The peaks of these carbohydrates were overlapped at 8 μM RIP (Figure 2B). It should be noted that SSA showed clear binding at 0.8 μM, but RIP required 8 μM concentration for specific binding to asialo-oligosaccharides.

Detection of lectin activity in crude extract of tulip bulbs

Two carbohydrate-binding proteins have been reported in tulip bulbs. One is a mannose-specific lectin, Tulipa gesneriana lectin (TGL), which preferentially binds α(1-6) linked manno-oligosaccharides derived from mannan (yeast cells) (Oda and Minami, 1986). The other is T. gesneriana agglutinin (TGA), which agglutinates animal blood cells. Asialo-thyroglobulin showed the most potent inhibition toward binding of 125I-labeled TGA to mouse erythrocytes among the examined glycoproteins, and asialo-AGP also showed inhibitory effect (Oda et al., 1987). However, precise binding specificity of TGA has not been determined.

Interactions between crude protein fraction in tulip bulbs and the carbohydrates derived from a few glycoproteins are shown in Figure 3. In the electrolyte-containing crude extract (5 mg/ml), migrations of asialo-oligosaccharides including (10) derived from porcine thyroglobulin showed dramatic changes, and the major peaks became broad (Figure 3AII). Asialo-oligosaccharides derived from AGP showed interesting binding to the crude extract, and peaks derived from triantennary oligosaccharides (2 and 3) obviously became broader and appeared later on addition of the crude extract in the buffer (Figure 3BII). In contrast, presence of the crude extract of tulip bulbs did not cause obvious changes of migrations of any oligosaccharides derived from ribonuclease B (Figure 3CII). In the previous article, Oda and Minami (1986) reported that TGL showed specific affinity to mannan derived from yeast. Because the structures of mannan from yeast and high-mannose oligosaccharides are different, TGL did not show affinity toward high-mannose oligosaccharides derived from ribonuclease B.

Detailed studies on carbohydrate-binding specificity of TGA

We used four sets of oligosaccharide mixture derived from AGP, fetuin, porcine thyroglobulin, and bovine IgG as glycan libraries to determine the detailed carbohydrate-binding specificity of TGA using CAE.

AGP contains di- (1), tri- (2 and 3), and tetra- (4 and 5) antennary oligosaccharides. Some of the tri- and tetraantennary oligosaccharides are substituted with a fucose residue at one of the lactosamine branch to form sia1y Lewis x structure (3 and 5).

Figure 4 shows the interactions between asialo-oligosaccharides of AGP and TGA at various concentrations. Addition of TGA in the electrolyte caused specific retardation
Table 1. List of oligosaccharides

<table>
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<tr>
<th>Peak number</th>
<th>Structure<em>1</em>2</th>
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<tbody>
<tr>
<td>1</td>
<td>Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manο1-3</td>
</tr>
<tr>
<td>2</td>
<td>Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manο1-3 Galβ1-4GlcNAcβ1-4</td>
</tr>
<tr>
<td>3</td>
<td>Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manο1-3 Galβ1-4GlcNAcβ1-4 Fucα1-3</td>
</tr>
<tr>
<td>4</td>
<td>Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manο1-3 Galβ1-4GlcNAcβ1-4</td>
</tr>
<tr>
<td>5</td>
<td>Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manο1-3 Galβ1-4GlcNAcβ1-4 Fucα1-3</td>
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<tr>
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<td>Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-3GlcNAcβ1-2Manο1-3 Galβ1-4GlcNAcβ1-4</td>
</tr>
<tr>
<td>7</td>
<td>GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS GlcNAcβ1-2Manο1-3 Fucα(1-6)</td>
</tr>
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<td>8</td>
<td>Galβ1-4GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS GlcNAcβ1-2Manο1-3 Fucα(1-6)</td>
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<tr>
<td>9</td>
<td>GlcNAcβ1-2Manο1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manο1-3 Fucα(1-6)</td>
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<tr>
<td>20</td>
<td>Manα1-6 Manα1-3Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Manα1-2Manα1-3</td>
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of the migration times of triantennary carbohydrates (2 and 3), but did not affect the migrations of tetraantennary carbohydrates (4 and 5). Peaks of tri- (2 and 3) and tetra- (4 and 5) antennary carbohydrate chains were overlapped at 4.5 μM TGA, and finally, the migration order of them were reversed and the peaks of 2 and 3 were fused to a broad peak at 12.0 μM TGA and observed at 7.7 min.

TGA showed a small effect on migration of diantennary oligosaccharide (1) compared to those of triantennary carbohydrates. At 12.0 μM TGA, the migration time of (1) was observed slightly later (5.8 min) than in the absence of the lectin (5.2 min).

Table I. continued

| Peak number | Structure††‡
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<td>Manα1-6&lt;br&gt;Manα1-3Manα1-6&lt;br&gt;Manβ1-4GlcNAcβ1-4GlcNAc-APTS&lt;br&gt;Manα1-2Manα1-2Manα1-3</td>
</tr>
<tr>
<td>22</td>
<td>Manα1-2Manα1-6&lt;br&gt;Manα1-3Manα1-6&lt;br&gt;Manβ1-4GlcNAcβ1-4GlcNAc-APTS&lt;br&gt;Manα1-2Manα1-3</td>
</tr>
<tr>
<td>23</td>
<td>Manα1-6&lt;br&gt;Manα1-2Manα1-3Manα1-6&lt;br&gt;Manβ1-4GlcNAcβ1-4GlcNAc-APTS&lt;br&gt;Manα1-2Manα1-3</td>
</tr>
<tr>
<td>24</td>
<td>Manα1-6&lt;br&gt;Manα1-2Manα1-3Manα1-6&lt;br&gt;Manβ1-4GlcNAcβ1-4GlcNAc-APTS&lt;br&gt;Manα1-2Manα1-3</td>
</tr>
<tr>
<td>25</td>
<td>Manα1-2Manα1-6&lt;br&gt;Manα1-2Manα1-3Manα1-6&lt;br&gt;Manβ1-4GlcNAcβ1-4GlcNAc-APTS&lt;br&gt;Manα1-2Manα1-2Manα1-3</td>
</tr>
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</table>

† The abbreviations used for the structures are: Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose; NeuAc, N-acetyleneuraminic acid.
‡ All oligosaccharides were used after labeling with 8-aminopyrene-1,3,6-trisulfonate APTS by reductive amination.

Fetuin contains two triantennary oligosaccharides (2 and 6) and a diantennary oligosaccharide (1). One (2) of the triantennary oligosaccharides has three Galβ(1-4)GlcNAc branches, and the other (6) contains one Galβ(1-3)GlcNAc as well as two Galβ(1-4)GlcNAc branches (Table I). TGA clearly distinguished these triantennary oligosaccharides, as shown in Figure 5.

The mobility of triantennary oligosaccharide (6), which has a Galβ(1-3)GlcNAc branch, became obviously smaller than that of (2) in the presence of TGA and was observed later at higher concentrations of TGA. The migration order of triantennary oligosaccharides (2 and 6) was reversed at 6.0 μM TGA. These data indicate that oligosaccharide (6) has higher affinity toward TGA.

Bovine IgG contains four major diantennary oligosaccharides (7–10) that have α(1-6) linked fucose residues at chitobiose portion of the reducing end. Interestingly, TGA showed obvious retardation effect on the migration times of these oligosaccharides (7–10), as shown in Figure 6. The complete form (10) of diantennary oligosaccharide was observed latest, and the one lacking both Gal residues (7) showed the weakest interaction with TGA (Figure 6A).

Although GlcNAc residue in the reducing end of these oligosaccharides is an open-chain form (i.e., N-acetylglucosaminitol), these oligosaccharides show distinct affinities toward TGA. In contrast, diantennary oligosaccharides that do not have a fucose residue in the reducing end did not show affinity toward TGA as mentioned in the binding to the oligosaccharides derived from fetuin and AGP. To
confirm these observations, we digested the oligosaccharides with fucosidase and examined the binding of defucosylated oligosaccharides (11–13, and 1). We found that these defucosylated oligosaccharides did not show obvious interaction with TGA (Figure 6B).

Porcine thyroglobulin contains complex type oligosaccharides (10 and 14) and high-mannose type oligosaccharides (HM in Figure 7) as minor oligosaccharides (Kakehi et al., 1999). Addition of TGA decreased the peak intensity of triantennary oligosaccharide (14) even at 0.2 μM TGA in the electrolyte and also retarded diantennary oligosaccharide (10) at higher concentrations than 0.8 μM TGA, as observed for the interactions with oligosaccharides derived from IgG. Migration times of high-mannose oligosaccharides were not changed even in the presence of 12.0 μM TGA.

Finally, we examined the interactions between TGA and sialic acid–containing oligosaccharides derived from fetuin (Figure 8A) and porcine thyroglobulin (Figure 8B). A group of sialo triantennary oligosaccharides observed at 3.9 min were resolved into three groups around 4.5 min and 5.2 min at 12.0 μM TGA (Figure 8A). In the analogy of the analysis of asialo-oligosaccharides, we speculated that sialo-triantennary oligosaccharides having Galβ(1-3)GlcNAc branch showed different affinity to TGA, although further studies are required. In Figure 8B, results on the interactions between TGA and sialo-oligosaccharides from thyroglobulin are shown. High-mannose type oligosaccharides showed no interactions (see small peaks at 4.5–5.5 min). In contrast, 18 showed obvious retardation of migration times in the presence of TGA.

Determination of binding affinity constants

Binding constants for interactions between TGA and various carbohydrates were determined according to the method reported previously (Nakajima et al., 2003). The values of association constant (K_a) to oligosaccharides are summarized in Table II.

The method using CAE does not require the data concerning the concentrations of ligand (i.e., oligosaccharide), therefore is quite useful for the binding studies using oligosaccharide mixture as ligands. The oligosaccharide (14) that has a triantennary structure with an α(1-6) linked fucose branch showed the highest affinity (K_a = 1.7 x 10^6 M^-1), followed by 6 (K_a = 6.4 x 10^5 M^-1) and 10 (K_a = 5.8 x 10^7 M^-1). Oligosaccharides (2 and 3) showed almost the same affinities, indicating that fucose residue with α(1-3) linkage in the outer chain was not involved in the binding. Comparison of 10 with 7 and 1 indicates that Gal residues at nonreducing termini and α(1-6) linked fucose residue at the reducing terminal are important for the binding. The K_a values for sialo-carbohydrates are smaller than those of corresponding asialo-carbohydrates: 1.5 and 0.6 x 10^5 M^-1 for 1 and 15, 4.4 and 2.0 x 10^5 M^-1 for 2 and 16, 17 and 5.8 and 3.4 x 10^5 M^-1 for 10 and 18, respectively. These indicate that masking of Gal residues at nonreducing termini with sialic acids considerably lowers the affinity.

Discussion

We observed the interactions between the crude extract from Japanese elderberry bark or tulip bulbs and some sets of oligosaccharides derived from a few glycoproteins using CAE. Addition of crude extract from Japanese elderberry bark to the electrolyte obviously changed the mobility of sialo-oligosaccharides of AGP. These results were clearly due to the presence of SSA in the extract. However, another lectin, RIP, in the crude extract was not detected. The

Fig. 1. Capillary affinity electrophoresis of oligosaccharides derived from AGP in the presence of crude protein fractions from S. sieboldiana bark. An aqueous solution (10 μl) of the mixture of (A) sialo- and (B) asialo-oligosaccharides derived from AGP was assayed in the presence of crude protein fraction from S. sieboldiana bark at the concentrations of 0, 250, and 500 μg/ml as protein, respectively. Running buffer: 100 mM Tris-acetate buffer (pH 7.4) containing 0.5% polyethylene glycol (PEG 70000) and crude protein extract. Capillary: ECAP N-CHO coated capillary, 30 cm length (effective length, 20 cm, 50 μm I.D.). Applied potential: 18 kV. Injection: pressure method (0.5 psi for 5 s). Fluorescent detection at 520 nm excited with an argon laser with a 488 nm filter. The structures of the oligosaccharides are shown in Table I.

Fig. 2. Capillary affinity electrophoresis of sialo- and asialo-oligosaccharides derived from AGP in the presence of purified lectins from S. sieboldiana. (A) Sialo- and (B) asialo-oligosaccharides. The analytical conditions were the same as those described in Figure 1.
The analytical conditions were the same as in Figure 1. T. gesneriana. Asialo-oligosaccharides derived from (A) porcine thyroglobulin, (B) AGP, and (C) bovine ribonuclease B. These oligosaccharide mixtures were assayed in the presence of (I) buffer only, and (II) crude protein extract (5 mg/ml) from bulbs of T. gesneriana. The minimum concentrations of the purified SSA and RIP to show obvious interactions with carbohydrate chains of AGP were estimated to be 0.8 μM and 8.0 μM, respectively (Figure 2). The reason we could not detect RIP in the crude extract may be due to the low amount of it and low affinity to asialo-carbohydrates of AGP.

In the crude extract of tulip bulbs, lectin activity of TGA was also easily detected by CAE. However, it was difficult to detect the activity of another lectin in tulip bulbs, TGL. TGL showed only small effect on migration times of high-mannose oligosaccharides derived from ribonuclease B, because TGL preferentially binds manno-oligosaccharides derived from yeast cells.

We also showed that CAE is a powerful method for studies on the detailed carbohydrate-binding specificity using TGA. Because the method allows observation of the interactions of oligosaccharides simultaneously, relative affinities of each oligosaccharide can be easily calculated. TGA showed higher affinity to triantennary oligosaccharides (2 and 3) than diantennary carbohydrate (1), whereas TGA showed no significant interactions with tetraantennary carbohydrates (4 and 5). Among triantennary oligosaccharides, 2 and 3 showed almost the same affinities, but affinity of 6 was higher than that of 2. These indicate that Fuc residue linked through α(1-3) linkage to GlcNAc in the outer chain does not interfere the binding with TGA, and the triantennary oligosaccharide with Galβ(1-3)GlcNAc at the nonreducing terminus binds more tightly than that having Galβ(1-4)GlcNAc. Fucosidase digestion of oligosaccharides derived from bovine IgG reduced their affinities toward TGA, indicating that Fuc residues linked through α(1-6) linkage to the innermost GlcNAc are involved in the lectin binding.

In conclusion, TGA shows the highest affinity to triantennary carbohydrates with three Gal residues, especially, Gal with β(1-3) linkage at nonreducing termini and diantennary oligosaccharide with a Fuc residue linked through α(1-6) linkage to the innermost GlcNAc. These characteristic affinities are well described in Table II. Galactose-binding lectin in the seeds of Tetrarcarpidium conophorum agglutinin (TCA) has been reported to show similar affinities to those of TGA (Sato et al., 1991). This lectin shows high affinity to triantennary oligosaccharides but only weak binding to tetraantennary glycans. However, TCA binds more tightly to Galβ(1-4)GlcNAc than to Galβ(1-3)GlcNAc residue. Furthermore, fucose residue linked through α(1-6) linkage to GlcNAc in diantennary oligosaccharide does not affect the binding for TCA, Datura stramonium seed lectin and Phaseolus vulgaris leucoagglutinin (PHA) also show high affinities to N-glycans of complex type carrying Gal residues at the nonreducing termini (Kaneda et al., 2002; Yamashita et al., 1987). However, these lectins bind tetraantennary glycans as well as triantennary glycans. It should be noted that TGA does not recognize tetraantennary oligosaccharides. Thus we could determine the detailed carbohydrate-binding specificity of TGA using four sets of oligosaccharide mixture derived from fetuin, IgG, thyroglobulin, and AGP as glycan libraries.

With the present method, we found that the binding between carbohydrates and a lectin is observed in two different manners. Substantial peak broadening and peak retardation are observed in many of the figures, and in some cases, peak disappearance suggested that ligand exchange rates are comparable or slow compared with electrophoretic migration.

We are collecting the data for carbohydrate–protein interactions and found that ligand exchange rates largely depend on the lectins. For example, lectins such as Concanavalin A, Ricinus communis agglutinin (RCA), and SSA show peak disappearing effect, but those such as TGA, wheat germ...
agglutinin, and PHA show peak retardation effect. Although precise mechanism showing such difference is not clear, these characteristics are useful for analysis of complex mixture of oligosaccharides. Some lectins are known to recognize N-linked carbohydrates as well as O-linked carbohydrates. Matsumoto et al. (2001) proposed a dot-blot method for screening lectins on a membrane using glycoproteins coupled with enzyme or
Biotinyl albumin. This method does not require multivalent binding between protein and ligand, unlike hemagglutination assay, and allows screening of lectins from 16 cultivable mushrooms. In CAE, interactions of a lectin with each carbohydrate chain in a mixture of carbohydrates (glycan library) can be observed at the same time. Therefore when a lectin activity is detected in screening assay, we can know which of carbohydrate chains in glycan libraries are.

Fig. 6. Capillary affinity electrophoresis of oligosaccharides derived from bovine IgG in the presence of TGA. (A) Native and (B) fucosidase-digested oligosaccharides. The analytical conditions were the same as in Figure 1.

Fig. 7. Capillary affinity electrophoresis of asialo-oligosaccharides derived from porcine thyroglobulin in the presence of TGA. Peaks of high-mannose type oligosaccharides (HM) are enclosed by rectangle in dotted line. The analytical conditions were the same as in Figure 1.
responsible for the binding with the lectin. Furthermore, it should be emphasized that the binding affinity for the interaction can be easily estimated. Thus the present method using CAE may be a powerful tool for finding a new lectin.

Materials and methods

Materials

Human AGP, bovine fetuin, bovine IgG, bovine ribonuclease B (pancreas), and porcine thyroglobulin were obtained from Sigma-Aldrich Japan (Minato-ku, Tokyo). Peptide-$N^\alpha$-(acetyl-$\beta$-$D$-glucosaminyl)asparagine amidase ($N$-glycoamidase F) and fucosidase (bovine kidney) were purchased from Roche Molecular Biochemicals (Minato-ku, Tokyo) and Sigma-Aldrich, respectively. APTS was a product of Beckman-Coulter (Fullerton, CA). All other reagents were of the highest grade commercially available or of high-performance liquid chromatography grade. All aqueous solutions were prepared using water purified with a Milli-Q purification system (Millipore, Bedford, MA).

Preparation of a mixture of fluorescent-labeled oligosaccharides from glycoprotein sample

We prepared the fluorescent-labeled oligosaccharides (glycan library) from AGP, fetuin, porcine thyroglobulin, bovine IgG, and ribonuclease B (bovine pancreas) (Kakehi and Honda, 1996; Ma and Nashabeh, 1999).

Briefly, a sample of glycoprotein (1 mg) was dissolved in 20 mM phosphate buffer (pH 7.0, 50 µl), and $N$-glycoamidase F (5 µM, 5 µl) was added. After the mixture was incubated at 37°C for 24 h, the solution was kept in a boiling water bath for 5 min and centrifuged at 10,000 × g for 10 min. The supernatant containing the oligosaccharides was evaporated to dryness by a centrifugal vacuum evaporator (Speed Vac, Savant, Farmingdale, NY).
When asialo-oligosaccharides were prepared, the residue was dissolved in 2 M aqueous acetic acid (50 μl), and the mixture was kept at 80°C for 3 h to remove sialic acids (Morimoto et al., 2001). After evaporation of the mixture, the residue was dissolved in 15% aqueous acetic acid (5 μl) containing APTS at the concentration of 100 mM. A freshly prepared solution of 1 M NaBH₄CN in tetrahydrofuran (5 μl) was added to the mixture. The mixture was overlaid with mineral oil (100 μl, nD 1.4670, d 0.838; Aldrich) to prevent evaporation of the reaction solvent (Ma and Nashabeh, 1999; Sei et al., 2002). The mixture was kept at 55°C for 90 min. Water (200 μl) was added to the mixture, and the fluorescent yellowish aqueous phase (lower layer) was collected. The aqueous layer was applied on a column of Sephadex G-25 (1 cm, 50 cm length) equilibrated with water. The fluorescent fractions eluted earlier were pooled and evaporated to dryness. The dried fluorescent oligosaccharides were stable at least for several months at −25°C. The residue was dissolved in water (100 μl), and a portion (10 μl) was used for CAE.

Preparation of defucosylated oligosaccharides

Defucosylated oligosaccharides were obtained from the oligosaccharide mixture derived from bovine IgG (1 mg) after digestion with α-L-fucosidase (200 mU) in 60 μl 50 mM sodium citrate buffer (pH 5.0) at 37°C for 24 h, and then were labeled with APTS as described.

Preparation of crude protein fractions and purified lectins from Japanese elderberry and tulip bulbs

Pulverized bark (50 mg) was suspended in 1 ml of 100 mM Tris–acetate buffer (pH 7.4) using a vortex mixer, and the supernatant obtained by centrifugation (3000 g, 10 min) was used as the crude protein fractions. Crude protein fractions from tulip bulbs were prepared as previously reported (Oda and Minami, 1986). Briefly, two tulip bulbs (about 70 g) were homogenized with a Waring blender with water (300 ml). After centrifugation of the mixture, solid ammonium sulfate was added to the supernatant to the concentration of 60% saturation. The precipitate collected by centrifugation was dissolved in 100 ml Tris±acetate buffer (pH 7.4) using a vortex mixer, and the fluorescent yellowish aqueous phase (lower layer) was collected. The aqueous layer was applied on a column of Sephadex G-25 (1 cm, 50 cm length) equilibrated with water. The fluorescent fractions eluted earlier were pooled and evaporated to dryness. The dried fluorescent oligosaccharides were stable at least for several months at −25°C. The residue was dissolved in water (100 μl), and a portion (10 μl) was used for CAE.

Protein assay

Protein concentrations were determined based on Lowry method using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) (Schaffner and Weissmann, 1973).

CAE

CAE was performed using a P/ACE MDQ glycoprotein system (Beckman Coulter) equipped with an eCAP N-CHO capillary (20 cm effective length, 30 cm total length, 50 μm ID, Beckman Coulter) using an argon laser-induced fluorescence detector as described previously (Nakajima et al., 2003). Detection was performed by installing a 520-nm filter for emission with a 488-nm argon laser for excitation. Tris–acetate buffer (100 mM, pH 7.4) was used as the electrolyte throughout the work. The sample solution was introduced to the capillary by pressure method (0.5 psi, 5 s). Separation was performed at 25°C at the applied potential of 18 kV. Data were collected and analyzed with a standard 32 Karat software (version 4.0, Beckman Coulter) on Microsoft Windows 2000. The procedures are briefly as follows.

Prior to CAE, a mixture of fluorescent-labeled oligosaccharides was analyzed in the absence of lectin. Then the same electrolyte containing a lectin at the specified concentration was filled in the capillary, and the same mixture of fluorescent oligosaccharides was analyzed. After observing the migrations at different concentrations of the lectin, association constants for the interactions were calculated according to the method as reported previously (Nakajima et al., 2003).

Abbreviations

AGP, α1-acid glycoprotein; APTS, 8-aminopyrene-1,3,6-trisulfonate; CAE, capillary affinity electrophoresis; N-glycoamidase F, peptide-N-α-[acetyl-β-D-glucosaminyl] asparagine amidase; RIP, ribosome-inactivating protein; SSA, Sambucus sieboldiana agglutinin; TCA, Tetracarpidium conophorum agglutinin; TGA, Tulipa gesneriana agglutinin; TGL, Tulipa gesneriana lectin.

References


