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Simultaneous determination of the association constants of oligosaccharides to a lectin by capillary electrophoresis

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Abstract

The association constants (K_a values) of oligosaccharides as 8-amino-1,3,6-naphthalenetrisulfonate (ANTS) or 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives to lectins were determined in the reverse (an oligosaccharide derivative as sample and a lectin as additive) system by electrophoresis in a capillary coated with linear polyacrylamide. The determination was as reliable as in the direct (a lectin as sample and an oligosaccharide derivative as additive) system by electrophoresis in a capillary base fused silica. The reverse system had the advantage that the K_a values of multiple oligosaccharides could be determined simultaneously. Thus, the K_a values of lactose and melibiose (as PMP derivatives) to M_r 60 000 *Ricinus communis* agglutinin could simultaneously be determined by this method. The K_a values of a number of isomaltooligosaccharides (as ANTS derivatives) having various degrees of polymerization (DPs) to M_r 46 000 *Lens culinaris* agglutinin could also be simultaneously determined. The K_a values gradually increased with DP, implying the participation of not only the non-reducing end but also the interior portion of the oligosaccharide chain. The K_a value of an oligosaccharide obtained in the multiple solute reverse system was consistent with that obtained by the single solute reverse system, demonstrating the reliability of this simultaneous determination.

Keywords: Association constants; Carbohydrate–protein interactions; Derivatization, electrophoresis; Oligosaccharides; Lectins

1. Introduction

Recent progress in life sciences has permitted understanding many of biological phenomena in relation to interactions between biological substances. With this advancement a keen demand has arisen for a rapid and reliable micro method for the estimation of the magnitude of interaction. Although it is also significant to elucidate the mechanism of molecular interaction in detail by various instrumental methods, the determination of the association constant is of primary importance in such binding studies. For this reason we have been engaged in method development for association constant determination using modernized techniques such as affinity chromatography [1–3] and surface plasmon resonance [4]. These methods allow determination of association constant using minute amounts of substances, but require immobilization of either of the involved substances to the supporting material (silica gel, agarose gel, etc.) or a metal-vaporized chip,

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respectively. Immobilization to such solid phases, however, poses a problem of steric hindrance.

Capillary electrophoresis (CE) has high capabilities in separation and detection. Free solution CE has an additional advantage based on its single phase property. It allows observation of a physical interaction taking place between substances in any places in a capillary. We already published a paper proposing a CE method for the determination of the association constant (K_a) between a carbohydrate and a lectin [5] utilizing this advantage. This report played a role as a trigger for binding studies and a number of papers (e.g., [6–9]) as well as a review [10] based on this principle have been published in these five years.

In our first paper [5] a sample of a protein (a lectin) was introduced to an electrophoretic solution containing an acidic disaccharide as ligand, and the dependence of the migration time delay of the sample protein on ligand concentration was analyzed. This method is also considered to be applicable to the reverse system composed of a ligand as sample and a protein as additive. However, our early studies indicated that adhesion of proteins on the capillary wall was so serious that the velocity of electroosmotic flow (EOF) fluctuated throughout analysis. Detailed studies of capillary coating, however, have solved this problem. Careful chemical coating of a capillary with linear polyacrylamide has enabled reproducible determination of association constant even at high protein concentrations.

The reverse system has the advantage that a number of solutes in a sample bind to a protein in a buffer while they are being separated by CE, and thus permits simultaneous determination of their association constants to the protein.

This paper demonstrates the reliability of the association constant determination in the reverse system and its advantage of simultaneous determination of association constants.

2. Methods and materials

2.1. Chemicals

Reagent grade samples of chemicals were obtained from the following sources: acrylamide from Seikagaku Kogyo (Tokyo, Japan), ammonium peroxodisulfate from Wako (Osaka, Japan), methacryloxypropyltrimethoxysilane (MAPTMS) from Shinetsu Kagaku (Tokyo, Japan), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) from Nakalai Tesque (Kyoto, Japan), tri(hydroxymethyl)-aminomethane (Tris) from Sigma (St. Louis, MO, USA), 8-amino-1,3,6-naphthalentrisulfonate (ANTS) from Tokyo Kasei Kogyo (Tokyo, Japan), 1-phenyl-3-methyl-5-pyrazolone (PMP) from Kishida (Osaka, Japan). All other chemicals were also of the highest grade commercially available. The sources of the carbohydrate samples were as follows: cellobiose from Wako, gentiobiose from Nakalai, lactose from Kishida, maltose from Wako, melibiose form Wako, rhamnose from Nakalai, an isomaltooligosaccharide mixture from Seikagaku Kogyo. The samples of the M_r 60 000 lectin from *Ricinus communis* (RCA_{60}) and the M_r 46,000 lectin from *Lens culinaris* (LCA) were obtained from Wako. All these chemicals as well as carbohydrate and lectin samples were used as obtained.

Acetonitrile of liquid chromatography grade was purchased from Nakalai. Deionized and glassware-distilled water was used for the preparation of running buffers.

2.2. Labeling of oligosaccharides

Derivatizations of oligosaccharides with ANTS [12] and PMP [13] were performed according to the literature. For derivatization with ANTS an oligosaccharide sample (5 mg) was dissolved in a mixture of a 15% (v/v) acetic acid solution (500 μl) of ANTS (0.2 *M*) and a 15% (v/v) acetic acid solution (500 μl) of dimethylamine borane complex (1 *M*). The mixed solution was incubated for 15 h at 37°C. By this treatment the oligosaccharide was reductively aminated to give a glycamine derivative whose amino group is substituted by naphthalenetrisulfonate. The reaction solution was applied onto a Sephadex G-25 column (50 cm×10 mm I.D.). The phenol–sulfuric acid-positive fractions were collected and evaporated to dryness by a Speed Vak. The residue was dissolved in 50 m*M* phosphate buffer, pH 6.8, to make a $1.0 \cdot 10^{-4}$ *M* solution, and an aliquot of the solution was subjected to association constant determination. The sample scale could

be reduced, once the elution position of the product was ascertained. For derivatization with PMP a disaccharide sample (50 nmol) or an oligosaccharide mixture (100 μg) was dissolved in a 0.5 *M* methanolic solution of PMP (50 μl) and 0.3 *M* sodium hydroxide (50 μl) was added to convert PMP to its sodium salt. The solution was heated for 30 min at 70°C to give a glycosylated 1-deoxyalditol derivative in which each hydrogen atom at C-1 is replaced by a PMP group. The reaction solution was neutralized with 0.3 *M* hydrochloric acid (50 μl). The neutralized solution was evaporated to dryness by a Speed Vak, the residue dissolved in water (200 μl), and the solution extracted three times with chloroform (200 μl). The aqueous layer was evaporated to dryness, the residue dissolved in 50 m*M* phosphate buffer, pH 6.8 to make a $1.0 \cdot 10^{-4}$ *M* solution, which was subjected to association constant determination.

2.3. Coating of capillaries

This was carried out basically according to the procedure developed by Baba et al. [11], briefly as follows. A piece of a 50 μm I.D. capillary of fused silica from Polymicro Technologies (Phoenix, AZ, USA) was rinsed with 1 *M* sodium hydroxide, water and acetonitrile in this order for 15 min each. An acetonitrile solution containing MAPTMS and acetic acid to a common concentration of 0.4% (v/v) was allowed to flow through the capillary for 1 h, then the capillary was rinsed with water for 15 min, followed by acetonitrile for 15 min. Finally an acrylamide solution, prepared by adding 16 μl of 10% (v/v) TEMED and 4 μl of 10% (w/v) ammonium peroxodisulfate to 1 ml of a 3% (w/v) acrylamide solution containing Tris (50 m*M*), boric acid (50 m*M*) and EDTA (2.5 m*M*), was allowed to flow through the capillary for 10 min, and the capillary was stood for further 30 min for polymerization. All processes of flowing the reagent solutions and rinsing the capillary were carried out by the flushing mode of the injection system of the apparatus used.

2.4. CE

This was performed using an Applied Biosystems 270A high-performance CE system equipped with a vacuum sample injection system, a continuous-wavelength UV detector and a data processor. A portion of the capillary coated with linear polyacrylamide (see Section 2.2) was installed onto the apparatus and analysis was done by applying a specified voltage indicated in each figure caption between both ends. The capillary was thermostated at 30±0.1°C by circulating the air. Before each run the capillary was equilibrated with the running buffer. Detection of the ANTS and PMP derivatives of oligosaccharides was carried out by monitoring the absorption at 280 nm and 245 nm, respectively, using the UV detector installed in the apparatus.

2.5. Determination of association constant

This was done as described previously [5]. Briefly, 1 mg of a lectin (ca. $2 \cdot 10^{-5}$ *M*) was dissolved in 50 m*M* phosphate buffer, pH 6.8 (200 μl), and lectin solutions of various concentrations were prepared by diluting this original solution with the same buffer. After rinsing the capillary with this buffer, each lectin solution was filled in the capillary by suction. Sample solutions were prepared by dissolving a final product of derivatization in 50 m*M* phosphate buffer, pH 6.8 to a concentration of $1.0 \cdot 10^{-4}$ *M*. Introduction of each solution was carried out by suction for 1.5 s using the sample injection system installed in the apparatus. Suction for 1.5 s introduced a ca. 4-mm plug to the capillary. Under these conditions ca. 800 fmol of the sample was introduced to the capillary. The peaks of the derivatives of oligosaccharides as well as the internal standard were recorded on the data processor and the migration time observed for each lectin concentration was corrected for the migration time of the internal standard in the first run.

3. Results and discussion

3.1. Problems in the reverse system

It is well known that in CE analysis positively charged substances especially cationic macromolecules are liable to be adsorbed on the inner wall of a fused silica capillary which has the silanol group. The magnitude of adsorption depends on the pH

values of the running buffer. When a proteinaceous substance is added to the buffer having a lower pH value than its isoelectric point (p*I*), the protein is adsorbed on the capillary inner wall by ionic association, because the protein has a positive charge, whereas the silanol group is dissociated to give a negative charge under such conditions. Excessively low pH is unfavorable for this effect, because the silanol group is not dissociated. Due to this effect the peak of a protein is retarded and sometimes it gives a flattened peak or an increased baseline level. In addition such phenomena become more and more pronounced with repetition of analytical runs. Thus, pH values excessively lower than the p*I* are crucial for protein analysis when an intact fused silica capillary is used.

Binding studies of ligands to proteins are generally done at pH values near p*I*, because specific interaction occurs at such pH values. RCA_{60} used in the present study had a p*I* value of 7.1 [15], which is very close to the pH value of the buffer employed for the determination of association constant. The p*I* value of LCA is reported to be 7~8 by the manufacturer, rather higher than the pH value of the buffer employed. Although the magnitude of adsorption is not so drastic under such conditions, as compared to that at lower pH values, the retardation of a ligand peak from the previous run in repeated analyses is generally so marked that reliable data are hardly obtained even with correction for EOF. For this reason the reverse system, which utilizes buffers containing high concentrations of proteins, poses a difficult problem to solve unless the capillary surface is modified.

We tried modification of the capillary inner wall by physical coating based on ionic association with various compounds but the result was not satisfactory. Even though a suitable cationic compound such as cadaverine bound to the capillary inner wall by rinsing it with a buffer containing this amine, the bound amine came off from the inner wall, if the capillary was rinsed with the running buffer not containing the amine. The magnitude of the amine release was not reproducible; it depended on the time elapsed after switching the buffer (data not shown). It is a fundamental problem that binding constant determination cannot be easily performed in the presence of such an amine, because the amine also takes part in the binding of the ligand to the protein. Use of a quaternary ammonium salt such as Polybrene, suppressed its release on buffer change, but re-coating was necessary before each run.

Based on this experience we focused on chemical coating based on covalent bonding. Among various materials for chemical coating we found linear polyacrylamide the best. Coating should be as completely as possible; it should not leave any silanol groups. The procedure by Baba et al. [11] met this requirement and we could use a capillary coated by this procedure at least throughout a series of analysis using five different concentrations of a protein.

3.2. Labeling of oligosacharides

As mentioned above binding studies are generally performed at around the pH values near the p*I* of the proteins involved. Under such conditions (pH values 7~8 in the present case), however, intact oligosaccharides have no electric charge unless they are carboxylated, sulfated, phosphorylated, or sialylated. Therefore, a suitable ionic group should be introduced when they have no such groups. Among various methods for pre-column/pre-capillary derivatization we selected reductive amination with ANTS [12] and condensation with PMP [13], because the derivatization procedures are simple and the yields of the derivatives are high in both methods. The ANTS and PMP derivatives have strong and weak negativity due to the presence of the sulfonate and the enol (formed by the keto enol tautomerism) groups, respectively, and they are attracted to the anode by electrostatic force. Since EOF was almost null in the present work due to the blocking of the silanol group by linear polyacrylamide coating, these derivatives moved fast and slow, respectively, to the anode and were sensitively detected by UV absorption.

3.3. Simultaneous determination of the association constants of oligosaccharides to a lectin

Fig. 1a and b show examples of the analysis of ANTS-lactose by the single solute reverse system in the absence and the presence, respectively, of RCA_{60}.

It is clearly shown that the ANTS-lactose peak

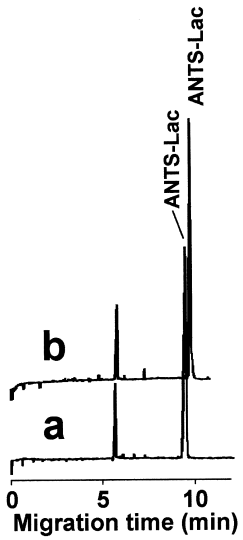

Fig. 1. CE of ANTS-lactose in the absence (a) and presence (b) of RCA_{60}. Capillary, fused silica coated with linear polyacrylamide (72 cm×50 μm I.D.); temperature of the capillary oven, 30°C; running buffer, 50 m*M* phosphate buffer, pH 6.8 (a) or (a) containing RCA_{60} (2 mg/ml) (b); applied voltage, 20 kV; detection, UV absorption at 280 nm. A sample solution of ANTS-lactose in 50 m*M* phosphate buffer, pH 6.8 was injected for 1.5 s to the capillary from the cathodic end. ANTS-Lac = ANTS-lactose.

was retarded in the presence of RCA_{60}. The peaks at ca. 6 min are those of the remaining ANTS, which can be used as reference. Thus, under these non-EOF conditions both ANTS and ANTS-lactose migrated to the anode and were detected by UV absorption. Since ANTS-lactose interacted with RCA_{60} which was almost in the zwitterionic state and almost in no motion, it migrated at lower velocity than that in the absence of RCA_{60}.

The association constant can be determined by the same principle as reported in our previous paper [5]. Since the theory was described in this and other [10] papers in detail, only the final conclusion is shown in Eq. 1, where t is the migration time of the ligand (a derivative of an oligosaccharide in this case) in the presence of the protein (RCA_{60} in this case).

$$\frac{1}{(t-t_1)} = \frac{t_2}{t_1} \cdot \frac{1}{(t_2-t_1)} \cdot \frac{1}{K_a} \cdot \frac{1}{[P]} + \frac{1}{(t_2-t_1)} \qquad (1)$$

The terms t_1 and t_2 are the migration times of the ligand and the complex, respectively. The term t_1 can be obtained as the migration time of a ligand in the absence of the protein and t_2 can be approximated as the migration time of the ligand at the concentration of RCA_{60} at the onset of a plateau in the t–$[P]$ curve. K_a is the association constant and $[P]$ is the concentration of the protein in the buffer. In this theory the binding is assumed to occur in the monovalent mode for simplicity. Eq. 1 means that the plot of $(t-t_1)^{-1}$ vs. $[P]^{-1}$ gives a straight line. From the slope $[A,\ t_1^{-1}\ t_2\ (t_2-t_1)^{-1}\ K_a^{-1}]$ and the y-intercept $[B,\ (t_2-t_1)^{-1}]$, K_a can be calculated as follows.

$$K_a = \frac{Bt_1 + 1}{At_1} \qquad (2)$$

Fig. 2 shows the plot of $(t-t_1)^{-1}$ vs. $[P]^{-1}$ for the interaction of ANTS-lactose with RCA_{60}.

The obtained values of A and B were $6.7 \cdot 10^{-5}$ M min^{-1} and 5.8 min^{-1}, respectively, and the K_a calculated from these values was $8.9 \cdot 10^4$ M^{-1} at 30°C. The reproducibility of the determination was high with a relative standard deviation (RSD) ($n=5$) of ca. 4%. The obtained K_a value for the interaction of ANTS-lactose with RCA_{60} was considerably higher, though in the same order, than the reported value for the interaction of unprotected lactose with RCA_{60} obtained by the equilibrium dialysis method ($1.5 \cdot 10^4$ M^{-1}, room temperature, pH 7.1) [14]. In

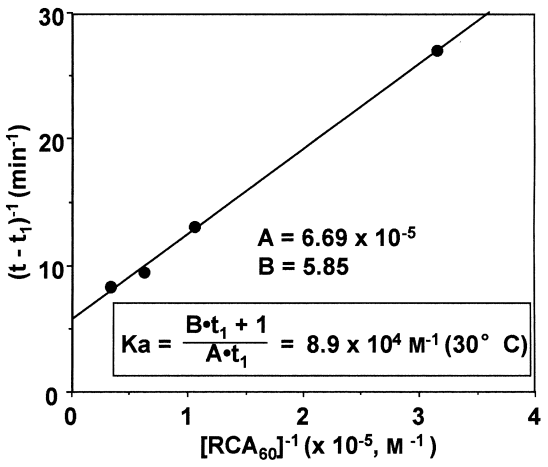

Fig. 2. Plot of $(t-t_1)^{-1}$ vs. $[P]^{-1}$ for the ANTS-lactose–RCA_{60} system.

contrast, a K_a value of $1.1 \cdot 10^4\ M^{-1}$ was obtained for the interaction of PMP-lactose with RCA_{60} by a similar procedure by CE. This is mentioned later in more detail. Though there might be slight temperature and pH variations between the present CE conditions (30°C, pH 6.8) and the equilibrium dialysis conditions in the literature (room temperature, pH 7.1), the difference of K_a values observed between ANTS-derivatized lactose and intact lactose was considered to be much larger than expected from these temperature and pH variations. The higher value in the ANTS derivative is obviously due to the presence of ANTS group, but further studies are necessary to explain this overestimation.

One of the advantages of the reverse system is in its capability of simultaneous determination of the association constants for a plural number of ligands to a protein. Fig. 3b shows an example of the analysis of such system composed of several PMP derivatives of disaccharides (cellobiose, gentiobiose maltose, melibiose and lactose) and RCA_{60}.

The electropherogram in the presence of RCA_{60} (Fig. 3b) is compared to that in the absence of this lectin (Fig. 3a). RCA_{60} is known to interact with the galactose moiety in oligosaccharides, and this specificity could be confirmed by the strong retardation of the PMP-lactose peak. The PMP-melibiose peak was also retarded to a considerable extent. A plot of $(t-t_1)^{-1}$ values for PMP-lactose vs. $[P]^{-1}$ for RCA_{60} gave A and B values of $9.24 \cdot 10^{-6}\ M\ \text{min}^{-1}$ and $3.58 \cdot 10^{-2}\ \text{min}^{-1}$, respectively. From these data K_a could be calculated as $1.1 \cdot 10^4\ M^{-1}$. Similarly the A and B values for the PMP-melibiose–RCA_{60} interaction were $3.02 \cdot 10^{-5}\ M\ \text{min}^{-1}$ and $2.02 \cdot 10^{-1}\ \text{min}^{-1}$, and the calculated value of K_a was $8.9 \cdot 10^3\ M^{-1}$. Unfortunately no literature values are available for the interaction of RCA_{60} with intact melibiose to compare with.

In this multiple solute reverse system the PMP-melibiose migrated faster than PMP-lactose, i.e., PMP-lactose interacted with RCA_{60} after PMP-melibiose. The RCA_{60} concentration along the capillary, after the PMP-melibiose passed through the RCA_{60}-containing buffer, will rapidly decrease as it parts from the inlet, because PMP-melibiose consumes RCA_{60} in the second order function with

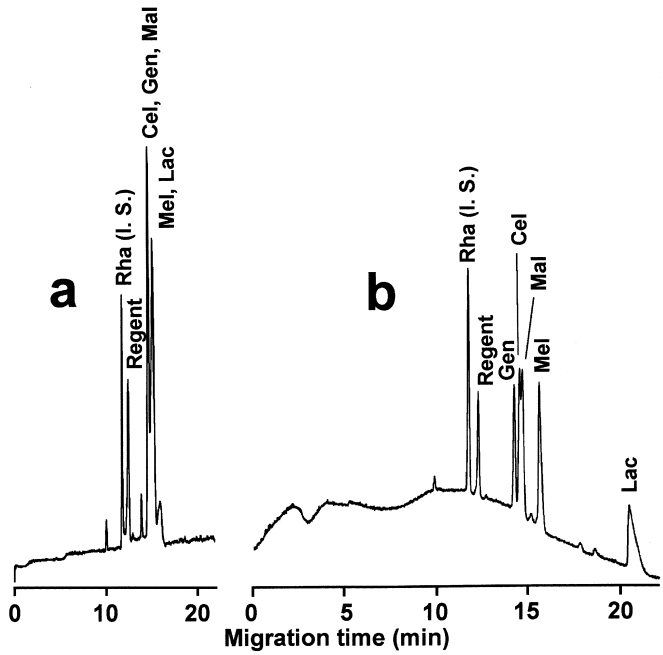

Fig. 3. CE of a mixture of the PMP derivatives of various disaccharides in the absence (a) and presence (b) of RCA_{60}. Analytical conditions as in Fig. 1, except that the applied voltage in (b) was 18 kV and detection wavelength was 245 nm. Cel = Cellobiose, Gen = gentiobiose, Mal = maltose, Mel = melibiose, Lac = lactose, Rha = rhamnose (internal standard).

respect to reaction time, accordingly the distance from the inlet. Therefore, PMP-lactose, following PMP-melibiose, will travel through the capillary, facing this concentration change. This situation is different from the competition of two ligands to a protein in the same medium as seen in equilibrium dialysis. Thus, the faster moving ligand (PMP-melibiose) will continue to interact with RCA_{60} of a constant concentration, whereas the slower moving ligand (PMP-lactose) will experience the concentration decrease mentioned above. If the reaction rates of both ligands were slow demanding several minutes or more for completion, their migration times, and accordingly the K_a value would be affected by this RCA_{60} concentration variation. In reality, however, the association of these reactants is considered to have proceeded much more rapidly in the present case, considering from our surface plasmon resonance studies [4]. Since the time required for the association of the half amount of a carbohydrate immobilized to a sensor chip (τ) can be calculated as $k_{ass}^{-1}[C]^{-1}$, where k_{ass} and $[C]$ are the association rate constant and the concentration of the lectin in a solution in contact with the chip, respectively, the rapidity of the association can be evaluated from the τ value. For example, the association rate constant (k_{ass}) of the interaction of maltose (as maltamine) immobilized to a sensor chip with LCA in a solution was estimated to be $9.57 \cdot 10^3\ M^{-1}\ s^{-1}$. Therefore, the τ value for this immobilized maltose–LCA system at $[C] = 2.1 \cdot 10^{-5}\ M$, which is similar to the lectin concentration used in the CE method, was as short as 4.8 s. The k_{ass} value for the immobilized maltopentaose (as the glycamine derivative)–LCA system was $2.79 \cdot 10^4\ M^{-1}\ s^{-1}$ and the τ value at $[C] = 2.1 \cdot 10^{-5}\ M$ was much shorter (1.7 s). From these data obtained for similar systems the concentration change of RCA_{60} along the capillary can be neglected, and the use of the multiple solute reverse system in CE ensures reliable simultaneous determination of the association constants of multiple ligands to a protein.

Based on this result and discussion a multiple solute system containing a larger number of ligands were examined. Fig. 4a and b show the electropherograms of a mixture of ANTS derivatives of

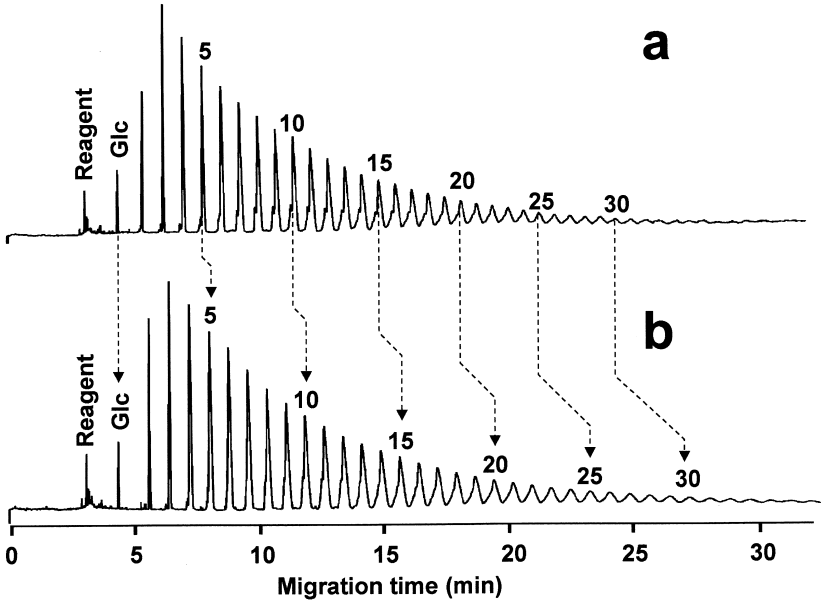

Fig. 4. CE of a mixture of the ANTS derivatives of isomaltooligosaccharides having various DPs in the absence (a) and presence (b) of LCA. Capillary, fused silica coated with linear polyacrylamide (50 cm×50 μm I.D.); running buffer, 50 m*M* phosphate buffer, pH 6.8 (a) or (a) containing LCA (0.6 mg/ml) (b); applied voltage, 15 kV. Other analytical conditions as in Fig. 1. The peak numbers correspond to DPs. Reagent = ANTS.

isomaltooligosaccharides having various degrees of polymerization (DPs) in the absence and presence, respectively, of LCA.

It is indicated that the ANTS derivatives having any DP values were retarded in the presence of LCA, indicating interaction of these ligands with this lectin. Fig. 5 gives the K_a values obtained for individual derivatives of isomaltooligosaccharides. Reference values for the interaction of LCA with individual intact isomaltooligosaccharides are not available in the literature.

It is notable that the small difference of K_a values was well differentiated and the increasing tendency of K_a with DP in these homologous series of oligosaccharides was clearly indicated. We already pointed out the gradual increase of K_a with DP, using a different method (high-performance affinity chromatography) and a different system (isomaltooligosaccharides-Con A) [3]. The present result confirms this previously reported tendency in a straight chain oligosaccharide–lectin interaction. It is obvious from the present and the earlier findings that a homologous oligosaccharide binds to an lectin through not only the hydroxyl groups in the non-reducing monosaccharide residue but also the hydroxyl groups in the interior portion of the oligosaccharide chain. The contribution of the interior portion is not so great as compared to that of the non-reducing end; it plays only an auxiliary role.

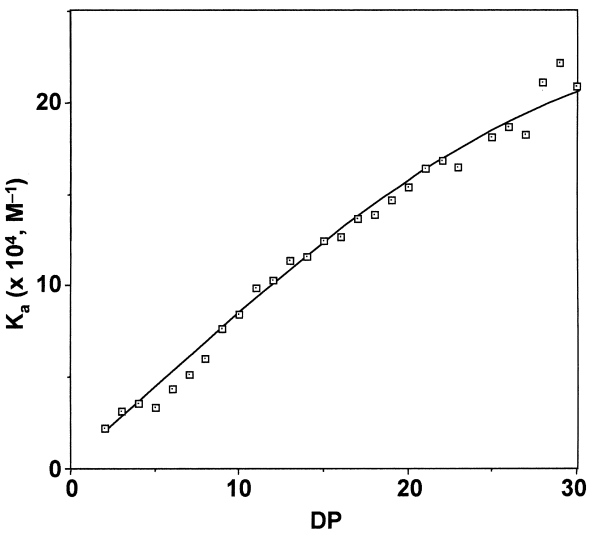

Fig. 5. Plot of the association constants obtained for ANTS derivatives of isomaltooligosaccharides vs. DPs.

3.4. Comparison of a single component system with a multiple component system

In order to confirm the accuracy of the determination of association constant in the multiple system, the K_a value of ANTS-isomaltose to LCA determined by using an isomaltooligosaccharide mixture ($2.19 \cdot 10^4\ M^{-1}$) was compared with that obtained by using isomaltose alone ($2.21 \cdot 10^4\ M^{-1}$). Similarly, the K_a value of ANTS-maltopentaose to LCA ($7.4 \cdot 10^4\ M^{-1}$) obtained by using an maltooligosaccharide mixture was compared with its K_a value obtained by using maltopentaose alone ($7.3 \cdot 10^4\ M^{-1}$). In both instances the corresponding values were in good agreement. Thus, the reliability of the multiple reverse system was confirmed.

4. Conclusions

The interaction between two substances in the reverse system resembles the throughout-capillary technique of in-capillary derivatization [16], but the reaction is reversible in the present case. The association constant of a ligand to a protein can be determined accurately with high reproducibility using small amounts of the ligand and the protein by the direct system (composed of a protein as sample and a ligand as additive) in a bare fused silica capillary, based on averaging the velocities of electrophoretic migration of unbound and bound proteins by their molar fractions (Eq. (1)) [5]. The determination of the association constant in the reverse system (a ligand as sample and a protein as additive) has a problem of protein adhesion on the capillary inner wall. This problem could be solved by efficient coating of the capillary with linear polyacrylamide by the procedure of Baba et al. [11]. The association constant could be determined with high reproducibility by use of a thus-coated capillary, in a similar manner as in the direct system. The obtained values were approximately the same as those obtained by the direct system.

The biggest advantage of the reverse system is the possibility of simultaneous determination of multiple

ligands to a protein. The effect of non-uniformity of the protein concentration in the running buffer should be discussed in multiple solute system, but this effect was not so great in such rapid associations as observed in the present study. An association rate constant (k_{ass}) of a protein to a ligand can be determined by surface prasmon resonance. From the association rate constants, required times for 87.5% binding of LCA to maltose and maltopentaose were estimated (ca. 15 s and 5 s, respectively). Therefore, the equilibrium between LCA and maltooligosaccharides might have been reached within scores of seconds.

This paper only presents a model experiment using simple oligosaccharides of homologous series. Further application to complex carbohydrates in glycoconjugates is now going on.

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