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Capillary electrophoretic determination of the association constant of a protein and a neutral carbohydrate by introducing mercaptoethanesulfonate tags to the carbohydrate

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Abstract

The association constant between a protein and a carbohydrate can be determined based on the relationship between the delay of migration time of a protein as sample and the concentration of a ligand as additive in capillary zone electrophoresis. In this determination the carbohydrate as ligand must have an electric charge to enable accurate estimation of the migration delay caused by the ligand. This paper proposes a convenient method for conversion of neutral carbohydrates having no electric charge to derivatives having a strongly negative charge. It is based on dithioacetalation with 2-mercaptoethanesulfonate in trifluoroacetic acid. The derivatization is rapid and almost quantitative at room temperature, and does not cause cleavage of interglycosidic linkages between hexose residues nor removal of the sialic acid residue. This paper demonstrates the usefulness of the proposed method for the determination of the association constants of neutral carbohydrates to proteins, using simple oligosaccharides and lectins as models.

Keywords: Association constants; Proteins; Carbohydrates; Mercaptoethanesulfonate; Oligosaccharides; Lectins

1. Introduction

Capillary electrophoresis (CE) has been applied to various fields, since its high capabilities have been well recognized. Among various advantages of free solution CE, that based on its single phase property is notable. It allows direct observation of chemical as well as physical reactions occurring in capillaries.

We have published a few papers on chemical derivatization by various techniques [1–3]. We also presented the first paper on the determination, by CE, of the association constant between a protein as sample and a carbohydrate as ligand in an electrophoretic solution [4], based on the relationship between the delay of the migration time of the sample and the ligand concentration. This method is noteworthy because an interaction under circumstances similar to physiological conditions can be observed without immobilizing a ligand to a supporting material as in affinity chromatography or a chip as in surface plasmon resonance. It is a convenient method capable of estimating with high reproducibility the association constant with small amounts of a protein and a ligand. The protein sample may be a lectin [5,6], an enzyme [7–10] an antibody [11], or other functional protein [12,13]. The ligand can be

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replaced by a drug [7–9], or a metal [12]. Because of these advantages this method has become widely used in many fields such as biochemistry and molecular biology, and there is an extensive review by Shimura and Kasai [14]. Such studies are highly important, because many biological phenomena are considered to be triggered by such specific interactions between substances.

In our first paper of association constant determination [4] the association of a protein (RCA_{60}, a lectin from *Ricinus communis*) and a carbohydrate (lactobionic acid, an acidic carbohydrate having the carboxyl group) was studied as a model system. The electric charge is essential for this method, since it is based on the change of mobility, and hence migration velocity or migration time; without migration no information will be available on the molecular interaction. Often however, in the study of protein–carbohydrate interactions, the protein has only a low mobility, because the interaction is observed at around its isoelectric point, where it exists as a zwitterion. In addition the carbohydrate as ligand usually does not have an electric charge. Therefore, we should develop a method to apply this method to neutral carbohydrates. Though there has been a number of methods for pre-column labeling of reducing carbohydrates, which give ionic derivatives (e.g. [15–20]), all of them absorb the ultraviolet or visible light, and therefore will give high background level.

In this paper we propose a chemical method introducing the sulfonate group via the $-S-CH_2-CH_2-$ group, which is almost transparent in the ultraviolet and visible regions.

2. Materials and methods

2.1. Chemicals

An extra pure grade sample of sodium salt of 2-mercaptoethanesulfonic acid (MerES) was obtained from Nakalai Tesque (Nijo-karasuma, Sakyo-ku, Kyoto, Japan). Trifluoroacetic acid (TFA) of reagent grade was purchased from Wako (Dosho-machi, Higashi-ku, Osaka, Japan). These reagents were used without further purification. Other chemicals were of the highest grade commercially available. The sources of the carbohydrate samples were as follows: gentiobiose from Nakalai Tesque; isomaltose, lactose, maltose, melibiose, maltotriose and maltopentose from Wako; neuraminyllaltose from Sigma (St. Louis, MO, USA). The lectins from *Ricinus communis* (RCA_{60}), *Concanavalia ensifolis* (Con A), and *Lens culinaris* (LCA) were from Wako. Deionized and glassware distilled water was used for the preparation of running buffers for CE.

2.2. Derivatization with MerES

In a large scale procedure a sample of a reducing oligosaccharide (40 μmol) was dissolved in TFA (6 ml) in a small Erlenmeyer flask, and sodium salt of MerES (250 mg) was added. The solution was allowed to stand for 5 min at 25°C and evaporated to dryness by placing the vessel in a desiccator containing pellets of sodium hydroxide. The residue was cooled and dissolved in a small volume of iced water and applied to a Sephadex G-10 column. The phenol–sulfuric acid-positive [21] fractions were collected and lyophilized. The authentic specimens of the MerES derivatives of oligosaccharides could be prepared by repeating the chromatography process on the same Sephadex G-10 column three more times.

The reaction scale could be reduced. Since the elution volume was almost unchanged even with this scale down, the clean-up of reaction product could be carried out by collecting the fractions at the same elution volume using the same column.

2.3. Capillary electrophoresis

CE was performed using a Perkin-Elmer 270A high-performance capillary electrophoretic system equipped with a vacuum injector, a multiple wavelength UV detector, and a data processor. A fused-silica capillary (70 cm×50 μm I.D.) obtained from Polymicro Technologies (Phoenix, AZ, USA) was installed in the CE apparatus and analysis was performed at 20 kV (for MerES derivatives) or 15 kV [for 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives]. Before each analysis the capillary was thoroughly rinsed with 1 *M* sodium hydroxide, then

equilibrated with the running buffer. The running buffer was 200 m*M* borate buffer, pH 10.5 (for MerES derivatives) or 100 m*M* borate buffer, pH 10.0, containing sodium dodecyl sulfate to a concentration of 50 m*M* [for 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives]. Sample solutions were introduced by suction to the anodic end for 1.5 s. The length of the introduced plug was ≈4 mm under these conditions. Detection was carried out at 200 nm or 245 nm, respectively.

2.4. Estimation of the yields of the products

The final residue obtained by lyophilization of the eluate from the Sephadex G-10 column was dissolved in distilled water (2 ml) and a 100 μl-aliquot of the solution was transferred to a micro-tube containing 40 nmol of cinnamic acid (internal standard, I.S.) . After the I.S. was well dissolved, the solution was analyzed by CE. The MerES derivative of an oligosaccharide (major product) and accompanying MerES derivative(s) of the constituent monosaccharide(s) as minor product(s) were determined based on the relative responses to I.S. against the calibration curves prepared from the authentic specimens of MerES derivatives.

Another 500 μl-aliquot was passed through a small column (14 cm×8 mm I.D.) containing Amberlite CG-120 (H^+ form, 1 ml) and Amberlite CG-400 (OAc^- form, 1 ml), and the column washed with distilled water (20 ml). The combined eluate and the washings were evaporated to dryness and the residue was subjected to CE analysis as PMP derivatives [17] using rhamnose as I.S.

The procedures used for the estimation of the main

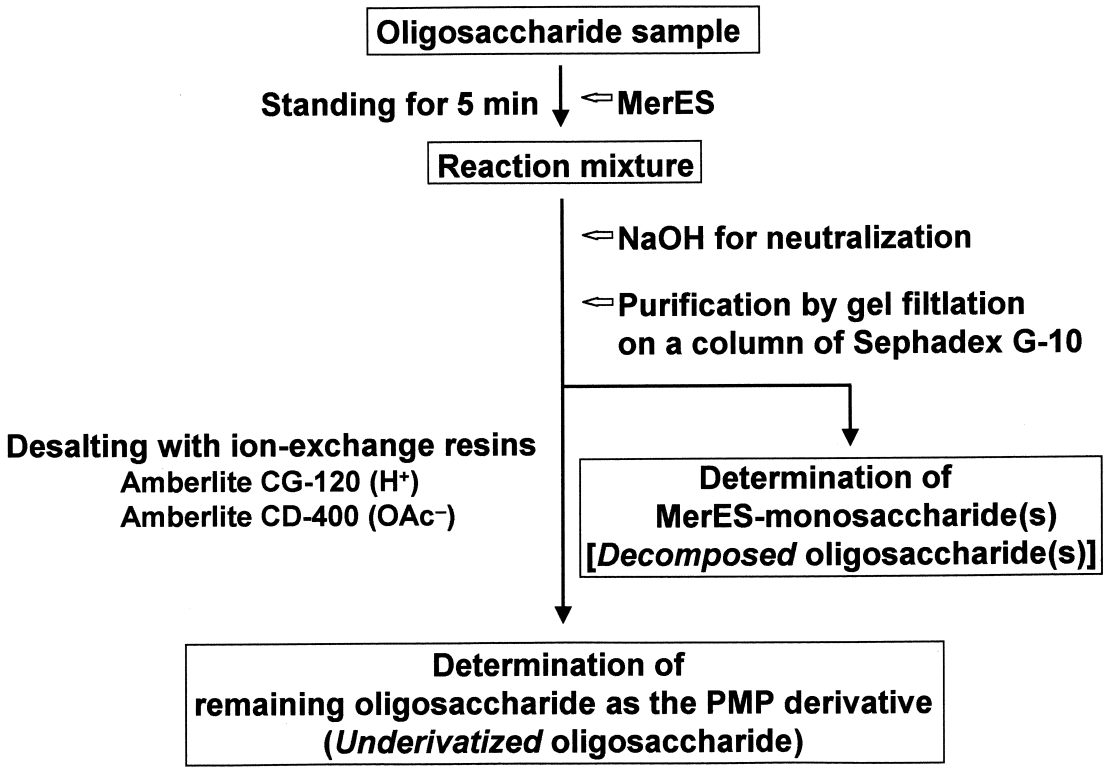

Fig. 1. Schematic of the procedure for the determination of the yields of the main product, by-product(s) and unreacted material in the derivatization of an oligosaccharide with MerES. The reaction solvent was TFA. An evaporation step for the reaction solution is omitted in this schematic.

products, by-products and the remaining materials are schematically summarized in Fig. 1.

2.5. Mass spectrometry

The mass spectrum of the final product of dithioacetalation of lactose with MerES was obtained using a Jeol JMS-SX102 mass spectrometer equipped with a fast atom bombardment (FAB) interface. The sample was ionized in a glycerol matrix under a Xe gas atmosphere and the spectrum was obtained by the negative ion mode at an acceleration potential of −8 eV. An intense peak (56%) at m/z 629 and a moderate peak (19%) at m/z 607 were assingnable to $(M-H+Na)^-$ and $(M-H)^-$, respectively, where the numbers in the parentheses are the percentage relative abundances to the base peak at m/z 141 ($HS-CH_2-CH_2-SO_3^-$).

2.6. Determination of association constants

This was done as described in our previous paper [4]. Briefly, the MerES derivative of an oligosaccharide obtained as mentioned above was dissolved in the 50 m*M* phosphate buffer, pH 6.8, and the solution was diluted with the same buffer to various concentrations. Each of the resultant solutions was filled in the capillary thermostated at 23 or 30°C, and a lectin solution in the same buffer (1 mg/ml) was introduced by suction to the capillary for 1.5 s. The migration time was measured accurately at the peak top position using the data processor installed in the apparatus and corrected for slight variation of electroosmotic flow (EOF), as described in Ref. [4]. The plot of the reciprocal migration time difference against reciprocal ligand concentration was constructed using the software, CRICKET GRAPH of Cricket Software (Malvern, PA, USA).

3. Results and discussion

3.1. Selection of the method for derivatization

In order to ensure the accuracy of association constant determination, the derivatization must meet the requirements that the reaction is quantitative and does not cause decomposition of the oligosaccharide chain structure. In addition it is desirable that the reaction is rapid and the clean-up of the product is easy. There have been a number of methods for the pre-column/pre-capillary derivatization of reducing carbohydrates based on reductive amination [15,16,18–20]. However, all of them used acid as a catalyst, which may cause degradation of part of the carbohydrate structure. The derivatization with PMP [17,22] has also been used, but it introduces only weakly acidic groups. In our previous paper on gas chromatography of carbohydrates we successfully utilized dithioacetalation and established the ethanethiol method as a general method for the analysis of component monosaccharides [23]. It can also be applied to simultaneous analysis of the products of periodate oxidation [24] and the hydrolysis products of permethylated polysaccharides [25]. We attempted modification of the ethanethiol molecule by sulfonation as shown in Fig. 2.

In the presence of an excess amount of MerES, two MerES groups are introduced to the reducing end as in the reaction with ethanethiol [24], as evidenced by the presence of the $(M-H+Na)^-$ and $(M-H)^-$ peaks in the FAB-mass spectrum. Fortunately the reducing ends of oligosaccharides reacted with MerES as smoothly as with ethanethiol and the oligosaccharides were rapidly converted to the bis-MerES derivatives in high yields even at room temperature.

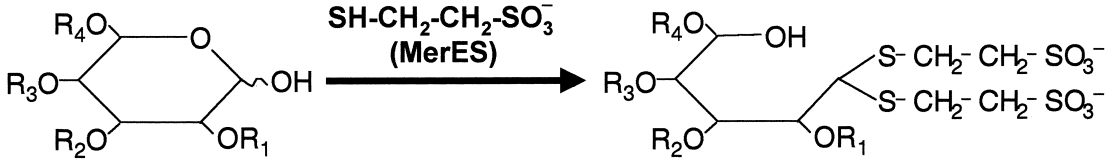

Fig. 2. Conversion of an oligosaccharide to the MerES derivative having strongly negative charge.

3.2. Yields of MerES derivatives

Fig. 3a shows an example of the analysis of MerES derivatives from gentiobiose (β1→6 linked glucobiose) by CE as borate complexes. The derivatization was performed at 25°C, as in the dithioacetalation with ethanethiol for gas chromatography [23–25].

The main peak at ≈11 min in Fig. 3a came from MerES-gentiobiose and that at ≈9 min arose from cinnamic acid (I.S.). The direct estimation of the yield of MerES-gentiobiose was not easy, though a high value was obtained using the calibration curve. This is because a completely pure authentic specimen was not available. The excess MerES could not be completely removed even by repeated gel filtration on the Sephadex G-10 column. There are practically no other peaks at least within 20 min except for a small peak at ≈17 min, which was assigned to MerES-glucose. The appearance of the MerES-glucose peak can be attributed to a partial cleavage of the interglycosidic linkage of the gentiobiose as starting material and/or MerES-gentiobiose as product, during the reaction and/or the cleanup process. The yield of MerES-glucose was as low as 0.46 μmol from 40 μmol of gentiobiose (Table 1).

It may have come from the glucose moieties at both the reducing and the non-reducing ends. Therefore, the loss of gentiobiose by this mechanism

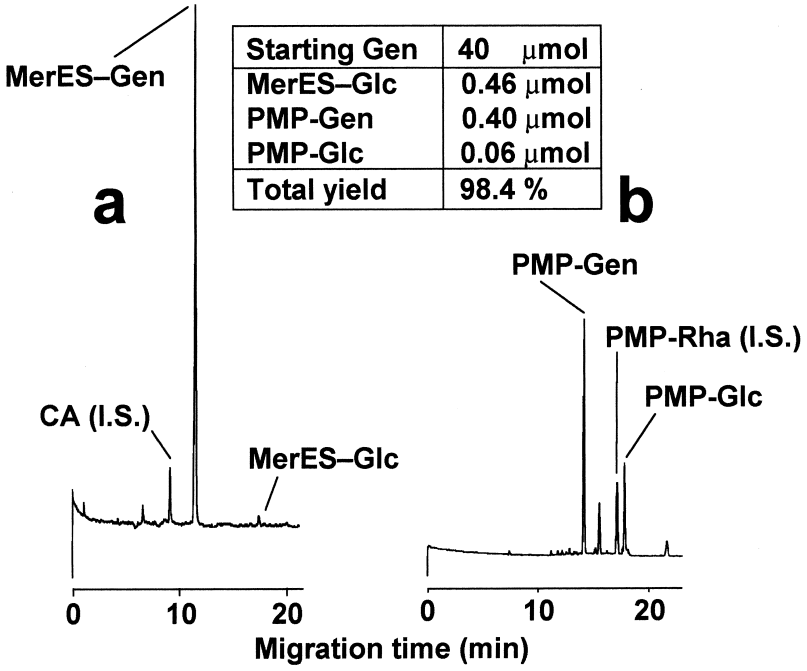

Fig. 3. Analysis of the main product and a by-product formed by the dithioacetalation of gentiobiose with MerES, together with the remaining starting material. (a) Analysis of the MerES derivatives in the purified product. Capillary, uncoated fused-silica (70 cm×50 μm I.D.); capillary oven temperature, 23°C; running buffer, 100 m*M* borate buffer (pH 10.5); sample introduction, vacuum injection (1.5 s); applied voltage, 20 kV; detection, UV absorption at 200 nm. MerES-Gen=MerES derivative of gentiobiose, MerES-Glc=MerES derivative of glucose, CA (I.S.)=cinnamic acid. (b) Analysis of the carbohydrates in the deionized fraction of the reaction mixture of dithioacetalation of gentiobiose with MerES as PMP derivatives. Capillary, uncoated fused-silica (70 cm×50 μm I.D.); capillary oven temperature, 23°C; running buffer, 200 m*M* borate buffer (pH 10.0) containing sodium dodecyl sulfate (50 m*M*); sample introduction, vacuum injection (1.5 s); applied voltage, 15 kV; detection, UV absorption at 245 nm. PMP-Gen=PMP derivative of gentiobiose, PMP-Glc=PMP derivative of glucose, PMP-Rha=PMP derivative of rhamnose (I.S.). The inset shows the estimated value of each derivative in μmoles from gentiobiose (40 μmol). Gen=gentiobiose.

Table 1
Yields of the main products, by-products, and the remaining materials in the dithioacetalation of oligosaccharides with MerES[a]

Oligosaccharide (O)		MerES-Glc (μmol)	PMP-O (μmol)	PMP-Glc (μmol)	Total yield (%)
Gentiobiose	Glcβ1→6Glc	0.46	0.40	0.06	98.4
Isomaltose	Glcα1→6Glc	0.40	1.54	0.01	95.7
Melibiose	Galα1→6Glc	0.94	0.74	0.07	97.0
Maltose	Glcα1→4Glc	1.02	1.60	0.07	94.7
Lactose	Galβ1→4Glc	1.37	2.29	0.36	92.6
Maltotriose	Repeat Glcα1→4Glc	0.32	1.41	0.21	>96.2
Maltopentaose	Repeat Glcα1→4Glc	0.24	1.52	0.20	>96.0

[a] Starting oligosaccharide (40 μmol) → dithioacetaled oligosaccharide (as MerES–O); hydrolysis products (as MerES–Glc/-Gal + PMP-Glc/-Gal); underivatized oligosaccharide (as PMP-O)

Dithioacetaled oligosaccharide = starting oligosaccharide − (total hydrolysis products + underivatized oligosaccharide) =

$$40 - \left(\frac{\text{MerES-Glc}/\text{MerES-Gal}}{\text{DP}} + \text{PMP-O} \right)$$

$$\text{Total yield (\%)} = \frac{\text{dithioacetalated oligosaccharide}}{\text{starting oligosaccharide}} \times 100$$

during derivatization can be estimated to be 0.46×1/2÷40×100=0.58%. On the other hand the neutral fraction obtained after deionization of the reaction mixture was analyzed by CE as PMP derivatives according to our established procedure [17] using rhamnose as I.S (Fig. 3b). The obtained values for gentiobiose and glucose were 0.40 and 0.06 μmol, respectively (Table 1). The estimated value of gentiobiose obviously represents the amount of unreacted gentiobiose, which is equivalent to 0.40÷40×100=1.0%. The PMP-glucose is considered to be due to partial release of the MerES group from MerES-glucose but its value was low as compared to that of MerES-glucose (0.46 μmol). From these data the overall yield of MerES-gentiobiose was estimated to be 100−(0.58+1.0)=98.4% (Table 1). Namely the total amounts of the unreacted gentiobiose and the loss of the product by partial decomposition were less than 2%. Thus, the total yield of the MerES derivative of an oligosaccharide can be calculated by subtracting the sum of the total amounts of the hydrolysis products, MerES-monosaccharide(s), divided by degree of polymerization (DP), and the oligosaccharide left underivatized (estimated as the PMP-oligosaccharide) from the amount of the oligosaccharide subjected to derivatization.

The total yields of the MerES derivatives of other oligosaccharides could be estimated by similar experiments. Table 1 summarizes the data obtained. It is indicated that the 1→6 linked disaccharides gave higher values of total yield than 1→4 linked disaccharides. The total yield seems to have increased with increasing DPs in the maltoligosaccharide series. Although there was slight variation with oligosaccharide species, the total yield was more than 92% for all oligosaccharides examined. These high yields ensure reliable estimation of association constants of neutral carbohydrates to proteins.

3.3. Reactivity of oligosaccharides to MerES and stability of interglycosidic linkages

Inspection of the data in Table 1 gives information on the reactivity of these oligosaccharides to MerES and the stability of the interglycosidic linkages during derivatization, since the yield of the PMP derivative of the oligosaccharide indicates the extent of incompleteness of the reaction, and the total yields of the component monosaccharides are indicative of the ease of cleavage of the interglycosidic linkages. Isomaltose (α1→6 linked glucobiose) gave a higher value (1.54 μmol) of the PMP–oligosaccharide (unreacted oligosaccharide) than gentiobiose (0.40

μmol), implying lower reactivity. On the other hand the MerES-glucose value of isomaltose (0.40 μmol) was slightly lower than that of gentiobiose (0.46 μmol) due to slightly higher stability of the interglycosidic linkage. Substitution of the glucose residue at the non-reducing end by the galactose residue (melibiose) gave a lower value of the PMP-oligosaccharide (0.74μmol) than isomaltose (1.54 μmol), while it gave a larger total value of MerES-monosaccharides (0.94 μmol), indicating lower stability. In this heteroglycan the MerES-glucose and MerES-galactose were not well resolved under the conditions employed, giving a fused peak, hence both were estimated together. The 1→4 linked disaccharides (maltose and lactose) gave much higher values of MerES-glucose/MerES-galactose. In these disaccharides both low reactivity (PMP-oligosaccharide values of 1.60 and 2.29 μmol, respectively) and ready cleavage (MerES-glucose/MerES-galactose values of 1.02 μmol and 1.37 μmol, respectively) caused the reduction of the total yield. In the maltooligosaccharide series the reactivity to MerES gradually decreased but the total yield became rather high, as DP increased.

Thus, the data in Table 1 not only compares the total yields of MerES derivatives but also gives useful information on the reactivity of oligosaccharides to MerES and the stability of the interglycosidic linkages during derivatization.

3.4. Stability of the sialic acid residue

It is well known that the sialic acid residues in N- and O-glycans are specially labile to acid. In order to confirm the stability of the sialic acid residue to the proposed method, neuraminyllactose was subjected to dithioacetalation with MerES. Analysis of the reaction products, after purification by gel filtration, indicated the presence of a large peak of MerES-neuraminyllactose at 10.8 min together with a minor peak corresponding to MerES-lactose at 12.2 min. However, the peak response of this minor peak was less than 2% of that of the main peak of MerES-neuraminyllactose. Since the neuraminyllactose sample also contained a small amount of lactose, the yield of MerES-lactose could be considered to be of negligible extent. Thus, the obtained result provides an evidence for high stability of the sialic acid residue during dithioacetalation with MerES.

3.5. Association constants of the MerES derivatives of oligosaccharides to lectins

The theory of the association constant determination by CE has already been described in our previous paper [4]. It can be briefly summarized as shown in the Appendix.

When a protein (a lectin in this case) sample (P) is present in an appropriate medium containing a carbohydrate (C) as ligand, the protein sample binds to the ligand to yield a complex (P–C), and there is an equilibrium between the unbound (P) and bound (P–C) protein. Thus, there exists the basic equation of equilibrium, $K_a=[P]^{-1}[C]^{-1}[P–C]$, provided the binding occurs in the monovalent mode, where the brackets mean the concentrations of the indicated solutes. Introduction of the molar fraction α (which is equivalent to the ratio of [P–C] to the sum of [P] and [P–C]) to the equilibrium equation results in Eq. (A.1). On the other hand, when a sample solution of a protein in the running buffer (50 m*M* phosphate buffer, pH 6.8., in this case) is introduced from the anodic end of a capillary filled with the running buffer and a high voltage is applied between both ends, the protein sample migrates towards the cathode by the combined effects of electroosmosis and electrostatic attraction. The velocity of the sample (V_p) is the algebraic sum of the velocity of EOF (v_{eo}) and the velocity of electrophoretic migration of the protein (v_p). Thus, a velocity equation, $V_P = v_{eo} - v_P$, is obtained. Here, the positive sign is given to the movement from the anode to the cathode. If a carbohydrate (the MerES derivative of an oligosaccharide in this case) is added to the running buffer, the movement of the protein sample is retarded in such a manner that the molar fractions of the unbound and bound proteins ($1-\alpha$ and α, respectively) are incorporated to their velocities of electrophoretic migration. Therefore, the velocity of the protein (V_{P-C}) can be expressed as $v_{eo} - [\alpha v_{P-C} + (1-\alpha)v_P]$. Subtraction of the 1st velocity equation from the 2nd one gives an important equation, Eq. (A.2). Substitution of α in Eq. (A.2) by that in Eq. (A.1) and conversion of the velocity terms by migration times result in Eq. (A.3), which expres-

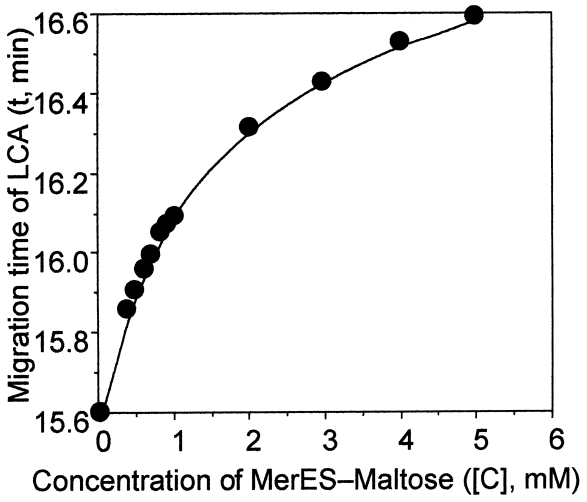

Fig. 4. Plot of the migration time, *t*, of LCA vs. MerES-maltose concentration, [C], in the running buffer.

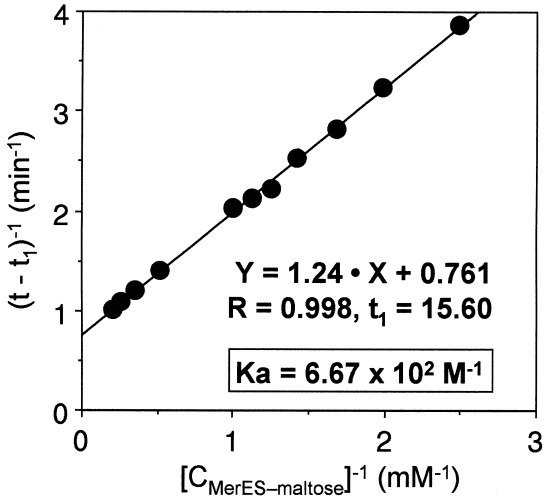

Fig. 5. Plot of reciprocal migration time difference, $(t-t_1)^{-1}$, vs. reciprocal MerES-maltose concentration, $[C_{MerES\text{-}maltose}]^{-1}$. Symbols as in Fig. 4.

ses the 1st order relationship between reciprocal migration time difference $(t-t_1)^{-1}$ and reciprocal ligand concentration $[C]^{-1}$, where t and t_1 are the migration times in the presence and the absence, respectively, of the ligand. The association constant (K_a) between P and C can be obtained by Eq. (A.4) using the slope (A) and the y-intercept (B) of the straight line prepared from Eq. (A.3).

Fig. 4 shows the t–[C] curve for the LCA–MerES-maltose system. It shows gradual increase of t with increasing [C]. Conversion of this direct plot to the reciprocal plot resulted in Fig. 5. Good linearity (coefficient of correlation, 0.998) was observed between $(t-t_1)^{-1}$ and $[C]^{-1}$, demonstrating the monovalent mode binding of MerES-maltose to LCA. The K_a value of MerES-maltose to LCA obtained by Eq. (A.4) using the estimated A and B values was $6.67\cdot10^2\ M^{-1}$. The K_a values of MerES-maltose to Con A and MerES-lactose to RCA_{60} were obtained in a similar manner at 30°C. They were $5.03\cdot10^2\ M^{-1}$ and $2.47\cdot10^3\ M^{-1}$, respectively. The obtained value for MerES-lactose to RCA_{60} was close to that for lactobionic acid to RCA_{60} ($3.3\cdot10^3\ M^{-1}$ at 30°C [4]).

The association constants of LCA to the same series of oligosaccharides (maltooligosaccharides) obtained in a similar manner (Table 2) indicate gradual increase of affinity with DPs, well consistent with our earlier findings obtained by high-performance affinity chromatography [26].

Correction of the [C] values by the estimated yields of individual MerES derivatives involves the changes of A and B values to A' and B', respectively. The corrected values of K_a ($K_{a'}$) for maltooligosaccharides (Table 2) obtained by redrawing the $(t-t_1)^{-1}$ vs. $[C]^{-1}$ curves are also given in Table 2. The difference of $K_{a'}-K_a$ for the oligomers with DPs 2, 3 and 5 were 5.7, 3.9 and 4.2%, respectively. With the correction for the yield mentioned above the proposed method is quite reliable

Table 2
Association constants of LCA to maltooligosaccharides obtained by the proposed method

Oligosaccharide	A	B	K_a (M^{-1})	A'	B'	$K_{a'}$ (corrected) (M^{-1})	Error (%)
Maltose	1.24	0.761	$6.67\cdot10^2$	1.17	0.761	$7.05\cdot10^2$	5.7
Maltotriose	1.45	1.33	$9.64\cdot10^2$	1.39	1.33	$<10.02\cdot10^2$	<3.9
Maltopentaose	1.67	2.22	$13.66\cdot10^2$	1.60	2.22	$<14.23\cdot10^2$	<4.2

for the determination of the association constant between a neutral oligosaccharide and a protein and will be widely used for the studies of protein–carbohydrate interaction.

4. Conclusions

The proposed method for the conversion of oligosaccharides to the dithioacetal derivatives with MerES was proved almost quantitative. In addition the cleavage of the interglycosidic linkages between hexose residues and the removal of the sialic acid residue during derivatization was minimized. The introduced MerES group did not have remarkable absorption at around 214 nm, where the protein samples were to be detected. This is advantageous for base line stabilization of the electropherogram. Small amounts of by-products were formed due to the cleavage of the interglycosidic linkage(s), but the extent of this scission was almost negligible.

Modification of the oligosaccharide structure by MerES may affect affinity to a protein by blocking the binding site or changing electron density of the atom(s) involved in the binding. If the binding occurs at the exterior portion of an oligosaccharide chain as in the exoglycosidase digestion, modification of the non-reducing end will give a crucial damage to the binding. On the contrary modification at the reducing end will give only slight influence. Although there is only a little knowledge on the binding of lectins to oligosaccharides, the fact that N-glycans released from a glycoprotein having affinity to a lectin still retain its affinity to the lectin [27] suggests that the exterior portion of the oligosaccharide chain is much more important for the binding to the lectin. Thus, the proposed method in which the reducing end is blocked by the MerES group will give only small influence on the binding to lectins, unless the chain length of the oligosaccharides is too short. Although reliable values of association constant have not been available in the literature for unprotected maltose to LCA as well as Con A, a value was reported for lactose to RCA_{60} [28] based on the equilibrium dialysis method. The value obtained for MerES-lactose to RCA_{60} ($5.03\cdot10^{2}\ M^{-1}$) was considerably lower than the reported value of lactose to RCA_{60} ($1.5\cdot10^{4}\ M^{-1}$), probably because this short chain (DP 2) oligosaccharide was modified by MerES. The obtained value was, however, comparable to that for lactobionic acid to RCA_{60} ($3.3\cdot10^{3}\ M^{-1}$) in our previous paper [4].

Thus, the usefulness of the dithioacetal method was demonstrated by CE using a few model systems. An interesting phenomenon was observed for isomaltooligosaccharides having varying DP values. It was also demonstrated that this method can clearly differentiate such small difference of association constant, and the obtained result was consistent with our earlier study by high-performance affinity chromatography [26]. Though the lectin examined in the earlier study (Con A) was different from that used in the present work, there seems to exist a similar mechanism without regard to lectin species, and the result obtained in the present work suggests participation of the interior portion of the oligosaccharide chain, though not to so much extent as compared to that of the outermost monosaccharide residue, to the binding to the lectin.

The proposed method is considered to have wide applicability to oligosaccharides. Although simple oligosaccharides composed solely of aldohexoses were adopted as the model systems, the proposed method can also be applied to N- as well as O-glycans in various glycoconjugates. Application to sialylated glycans is also ensured, though it will be not necessary to derivatize sialylated glycans with MerES in most cases, since they have weakly negative charge even in intact state. Therefore, the most highly expected objects will be asialoglycans. Application to such kinds of glycans are on-going, and the results will appear elsewhere.

Appendix A. Theory of the determination of association constant from migration times

Equilibrium: P + C⇌P–C (P: protein, C: carbohydrate)

$$K_a = \frac{[\text{P–C}]}{[\text{P}][\text{C}]} \quad \alpha = \frac{[\text{P–C}]}{[\text{C}] + [\text{P–C}]} \quad \therefore \frac{1}{K_a[\text{C}]} + 1 = \frac{1}{\alpha} \tag{A.1}$$

Regarding the velocities of the protein and the complex:

$$V_{P-C} = v_{eo} - \alpha v_{P-C} - (1-\alpha)v_P,$$
$$V_P = v_{eo} - v_P$$
$$\therefore V_P - V_{P-C} = \alpha(v_{P-C} - v_P) = \alpha\Delta v \quad \text{(A.2)}$$

From Eqs. (A.1) and (A.2),

$$\frac{1}{K_a[C]} + 1 = \frac{\Delta v}{V_P - V_{P-C}},$$

where

$$V_{P-C} = \frac{\ell}{t}, V_P = \frac{\ell}{t_1}$$

Then,

$$\frac{1}{t - t_1} = \frac{\ell}{\Delta v t_1^2} \cdot \frac{1}{K_a} \cdot \frac{1}{[C]} + \frac{\ell}{\Delta v t_1^2} - \frac{1}{t_1}$$

where

$$\Delta v = v_{P-C} - v_P = \ell\left\{\left(\frac{1}{t_0} - \frac{1}{t_2}\right) - \left(\frac{1}{t_0} - \frac{1}{t_1}\right)\right\}$$
$$= \frac{\ell(t_2 - t_1)}{t_1 t_2}$$

Therefore,

$$\frac{1}{t - t_1} = \frac{t_2}{t_1} \cdot \frac{1}{t_2 - t_1} \cdot \frac{1}{K_a} \cdot \frac{1}{[C]} + \frac{1}{t_2 - t_1} \quad \text{(A.3)}$$

$1/(t - t_1)$ vs. $1/[C]$ plot gives a straight line having its slope (A) and y-intercept (B)

$$A = \frac{t_2}{t_1} \cdot \frac{1}{t_2 - t_1} \cdot \frac{1}{K_a} \quad B = \frac{1}{t_2 - t_1}$$

K_a can be expressed as follows

$$K_a = \frac{Bt_1 + 1}{At_1} \quad \text{(A.4)}$$

6. Symbols

[C]:	concentration of C in running buffer
t_1:	migration time of P
t_2:	migration time of P–C complex
t:	migration time of P at [C]
v_{eo}:	velocity of electroosmotic flow
v_{P-C}:	velocity of electrophoretic migration of P–C complex
v_P:	velocity of electrophoretic migration of P
V_{P-C}:	velocity of P in the presence of C
V_P:	velocity of P in the absence of C

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