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Review

Analysis of carbohydrates as 1-phenyl-3-methyl-5-pyrazolone derivatives by capillary/microchip electrophoresis and capillary electrochromatography

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

The 1-phenyl-3-methyl-5-pyrazolone (PMP) method has many advantages over hitherto reported methods based on reductive amination and hydrazone formation. This short review summarizes the various aspects of the PMP method, including the principle of derivatization, the simplicity of derivatization procedure, the high sensitivities to UV monitoring and ESI-MS, and the diversity of separation modes in capillary electrophoresis, and presents a number of application data for carbohydrate analysis in biological samples by this method. It also describes successful automation of carbohydrate analysis by in-capillary derivatization with PMP and miniaturization to microchip electrophoresis with whole channel UV detection allowing rapid (within 1 min) analysis of small amounts of PMP derivatives of carbohydrates. Furthermore, it discusses the possibility of capillary electrochromatography in carbohydrate analysis as PMP derivatives, and proposes an in-capillary modification strategy for improving column efficiency and elution time reproducibility.

Keywords: Carbohydrate analysis; Capillary electrophoresis; Microchip electrophoresis; Capillary electrochromatography; 1-Phenyl-3-methyl-5-pyrazolone derivatives; PMP method; Protein component monosaccharides; Milk oligosaccharides; Raffinose-family oligosaccharides; Glycoprotein glycans; Proteoglycan-derived oligosaccharides; Glycolipid glycans; In-capillary derivatization; Multi-mode analysis; Electrospray ionization mass spectrometry

1. Introduction

In early days before 1950, the major interests in carbohydrate function was in energy storage (as in starch from plant seeds and roots) and structure supporting living bodies (as in cellulose in plant barks, chitosan in sea animal shells and cell wall materials of bacteria). Recent progress of life

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science has, however, revealed the importance of other types of oligo- and polysaccharides in animal bodies and given rise to much interest in the biological functions of these special types of glycans. Such glycans exist in not only free state but also conjugated state as glycoproteins, proteoglycans, and glycolipids. The *N*- as well as *O*-glycans in glycoproteins are considered to have a function to specifically recognize certain kinds of proteins, and this function is believed to be related to important events of animal [1, Chapter 5]. These glycoconjugates are biosynthesized in the cells by glycosylation of polypeptide cores formed in the Golgi bodies and the thus-synthesized glycoproteins can now be regarded as the most important group of compounds in the post-genome and proteome era. Recent glycoproteinaceous drugs produced by biotechnology pose a problem in that they are different in glycosylation pattern from native glycoproteins, which may cause change of specificity in recognition of receptors [1, Chapter 3]. On the other hand, advance in immunology, pathology and related fields has necessitated the insight into fine structures of various kinds of polysaccharides in bacteria and plants. With these trends in glycobiology and glycotechnology, demand for development of intelligent methods for micro–ultra micro analysis of carbohydrates has become more and more strong.

Meanwhile, carbohydrate analysis has a special feature that it must be capable of high-resolution separation, because samples are almost always accompanied by a number of structurally resembling substances such as isomers and homologues. In addition the structural diversity due to the variation in sequence, linkage type and attaching position of the monosaccharide residues must be taken into account. Therefore, the history of carbohydrate analysis has been how to effectively adapt to the separation methods of individual times. Thus, carbohydrate analysis owed its progress to paper and thin-layer chromatography before 1970, gas chromatography and paper/membrane electrophoresis in the 1970–80s, and high-performance liquid chromatography and gel electrophoresis from the 1980s on. Currently capillary electrophoresis (CE), microchip electrophoresis (ME) and capillary electrochromatography (CEC) are also devoted to the progress of carbohydrate analysis. The authors' group has engaged in method development for carbohydrate analysis throughout this history, and published books and/or reviews on gas chromatography (GC) [2], high-performance liquid chromatography (HPLC) [3], CE [4], and CEC [5] of carbohydrate analysis. Although many other excellent books and reviews (e.g. [6–11]) have appeared on the same subject from different views, our coherent strategy has been the most appropriate adaptation to each of these separation methods, considered from both separation and detection sides.

The latest technique of CE has no doubt the highest capabilities in both separation and detection. However, there is a problem that most carbohydrates have no electric charge. This is a fetal problem of CE, which can normally separate only ions. A recent method of micellar electrokinetic chromatography has made it possible to analyze also neutral compounds, but carbohydrate samples must be moderately hydrophobic, though unfortunately most carbohydrates are strongly hydrophilic. Another problem is the inherent lack of sensitively detectable functional groups in carbohydrate molecules. One of the methods that overcomes these difficulties is to analyze carbohydrates in strongly alkaline solutions. Most carbohydrates are dissociated to give negatively charged species under such conditions, and they can be separated by zone electrophoresis and detected by electrolysis on a copper electrode [12]. Repeated monitoring by the pulse amperometric method using a gold electrode with integration allows much more sensitive detection at the *p*mol level [13]. However, this method permits analysis of only limited species of carbohydrates, because only a single separation mode of zone electrophoresis is allowed. In addition, skilful operation is required for detection. The most promising solution of these problems is chemical derivatization; it can solve both difficulties coming from the lack in charge/hydrophobicity and detectable functional groups.

Numerous methods have been developed for pre-capillary derivatization of carbohydrates as summarized in Table 1 [5]. They can be classified into four categories involving reductive amination

Table 1
Various methods for the derivatization of carbohydrates

Reductive amination
4-Aminobenzoic acid ethyl ester (ABEE) [52], 4-aminobenzoic acid hexyl ester (BHE) [53], trifuloroacetamiodoaniline (TFAN) [54], 4-aminobenzonitrile (ABN) [55], 2-AP [21], 2-amino-6-cyanoethylpyridine (ACP) [56], biotinylated diaminopyridine (BDP) [57], 6-aminoquinoline (6AQ) [58], 2-aminobenzoic acid (2ABA) [59], 4-aminobenzoic acid (4ABA) [60], 2-aminobenzamide (2AB) [61], 2-aminoacridone (AMAC) [62], 2-amino-biphenyl (2ABP) [63], 8-aminonaphthalene-1,3,6-trisulfoante (ANTS) [64], 7-aminonaphthalene-1,3-disulfonate (ANDS) [65], 8-aminopyrene-1,3,6-trisulfonate (APTS) [66], 3-acetyamino-6-aminoacridone (AAMC) [67], 7-amino-4-methylcoumarin (AMC) [68]
Amine coupling via. glycamine
Phenylisothiocyanate (PITC) [69], 4-carboxytetramethylrhodamine succinyl ester (TRSE) [70], 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [71]
Hydrazone formation
Dansylhydrazine (DNS-hydrazine) [72], fluorenylmethoxycarbonylhydrazone (FMOC-hydrazine) [73]
Condensation with the active methylene group
1-Phenyl-3-methyl-5-pyrazolone(PMP) [14], 1-(4-methoxy)phenyl-3-methyl-5-pyrazoline (PMPMP) [17]

with various kinds of amines, conversion to glycamines followed by coupling to an amino group-reactive compound, hydrazone formation, and condensation with the active methylene group.

The methods involving reductive amination [21,51–68] are based on Schiff base formation between the amino group in an amine reagent and the reducing end of a reducing carbohydrate, followed by reduction to a glycamine derivative. The Schiff base formation requires acid catalyst normally at elevated temperatures, which may cause partial degradation of the acid-labile groups such as the *N*- as well as *O*-sulfate groups and the sialic acid residues in carbohydrate samples. Each derivative has its own characteristic features with regards to electric charge, hydrophobicity, UV absorption, fluorescence generation, etc., depending on the kind of amine reagent used. The second type is a modification of the reductive amination method, and it is performed in two steps [69–71]. In the first step a reducing carbohydrate is transformed to a glycamine by either reductive amination with an ammonium salt [69,71] or a multi-step procedure involving hydrogenation of a benzylated glycamine derivative [70]. Thus-obtained glycamine is coupled to an amino group-reactive compound. This type of derivatization is tedious involving at least two steps, and the FITC [69] and CBQCA [71] methods also need acid catalyst. Hydrazone formation [72,73] also suffers from the same problem of the necessity of acid catalyst, and in addition it has the theoretical problem of *syn–anti* isomerism of the products. For these reasons this type of reaction has not become popular. As we have already described details of these methods in our reviews [4,5], we omit here the argument for individual methods.

Condensation with an active methylene group is a quite different type of reaction, requiring no acid catalyst. The condensation with 1-phenyl-3-methyl-5-pyrazolone (PMP) is a typical example of this type of reaction. It was first developed in 1989 [14] by the authors' group for HPLC of reducing carbohydrates, and the utility for CE has also been reported from 1992 [15] in a number of papers. The basal part of this method was already included in our above-mentioned review [4] published in 1998, but great development has been made thereafter and applicability has been extended by using ME and CEC. This type of reaction proceeds in quantitative yields under mild conditions, and free from the disadvantages of the above types of reactions. The products have strong absorption in the UV region, are readily oxidizable on a glassy carbon electrode, and can be detected sensitively by ESI-MS. It is also notable that the derivatives can be separated by multiple modes of CE. For these reasons the PMP method has become a robust, convenient means for carbohydrate analysis. We summarize here various aspects of the PMP method, placing emphasis on the recent advancement during the last several years.

2. Principle of derivatization with PMP

A description of the reaction of the aldehyde group with the active methylene group in the pyrazolone ring was documented in an early book [16], but further studies on this type of

reaction have not been found in the literature until an example of the condensation of glucose with PMP was reported by the authors' group [14]. At that time the authors' group was undertaking method development studies for quantitative *O*-glycan release from mucous glycoproteins. Since *O*-glycans were known to be released with alkali but the products were rapidly decomposed after release under such drastic conditions, a suitable reagent was explored, which can block the reducing end of the released *O*-glycans to prevent decomposition and at the same time introduce a chromophore to the released *O*-glycans. PMP was one of the candidates tested for this purpose, and its efficacy for this purpose was demonstrated (unpublished result). Since it was found that the condensation of *O*-glycans with PMP proceeded in not only alkaline media but also almost neutral media, an attempt was made to use this reagent for derivatization of reducing carbohydrates. The first paper for the derivatization of reducing carbohydrates [14] was thus published in 1989 after detailed optimization studies, though it aimed at the use in HPLC. The authors have used the name of 1-phenyl-3-methyl-5-pyrazolone throughout their publications, because many chemical companies such as ICN, Tokyo Kasei, and Wako have used this name in their lists, but another name, 3-methyl-1-phenyl-2-pyrazolin-5-one, has also been used by a chemical company (Aldrich). It is documented as "2,4-dihydro-5-methyl-2-phenyl or 1,2-dihydro-5-methyl-2-phenyl under a term 2H-pyrazol 3-one" in Chemical Abstract.

The proton NMR spectrum of the product from glucose indicated the presence of two PMP groups and one glucose unit in a molecule of the derivative. The negative FAB-MS spectrum also supported this composition by giving an intense M-1 ion peak at *m*/*z* 509. Based on the assignment of the major ^{1}H signals, the structure of PMP-glucose was determined as shown in Fig. 1.

Use of insufficient amounts of PMP resulted in a mono-PMP derivative, and the reaction of the mono-PMP derivative with an additional amount of PMP gave bis-PMP. This experimental evidence indicated that the PMP group was introduced one by one to the reducing end of a reducing carbohydrate. The reaction can be conducted by adding an aqueous methanol solution of PMP to an aqueous solution of a carbohydrate sample. PMP is a kind of acid in the sense that the keto group in the pyrazolone ring can be converted to the weakly acidic enol group by tautomerism. Since the sodium salt was more reactive, the reagent solution was neutralized with sodium hydroxide prior to mixing with the sample solution. Under the optimized conditions derivatization was complete in 30 min at 70 °C. It is beneficial for carbohydrate analysis that the reaction proceeds in aqueous methanol solutions, because almost all carbohydrate samples are readily dissolved in such solvents.

The PMP method is widely applicable to all reducing carbohydrates. Ketoses are less reactive to PMP, resulting in incomplete derivatization (unpublished result). Free amino sugars and oligosaccharides having the *N*-sulfate group at the C-2 position (for example the oligosaccharides from heparin) resist condensation with PMP, though *N*-acetylation or desulfation followed by re-*N*-acetylation allowed quantitative derivatization with PMP (unpublished result). The sialic acid residues, either in conjugation with or released from oligosaccharides, cannot be derivatized by the PMP method, due to the presence of the electron-withdrawing carboxyl group in the neighboring position (unpublished result).

An analogue of PMP in which the phenyl group is substituted by the *p*-methoxy group (PMPMP) [17] was reported to react with reducing carbohydrates with approximately the same reactivity as that of PMP.

3. Various properties of PMP derivatives

3.1. Physical properties for detection

From the analytical point of view the most characteristic feature of PMP derivatives is the strong absorption in the UV region. The absorption maximum of PMP-glucose is at 245 nm and the molar absorptivity is 29 400 in ethanol [14]. This is an excellent property for detection. Another attractive property is the high electrochemical reactivity. PMP derivatives are readily

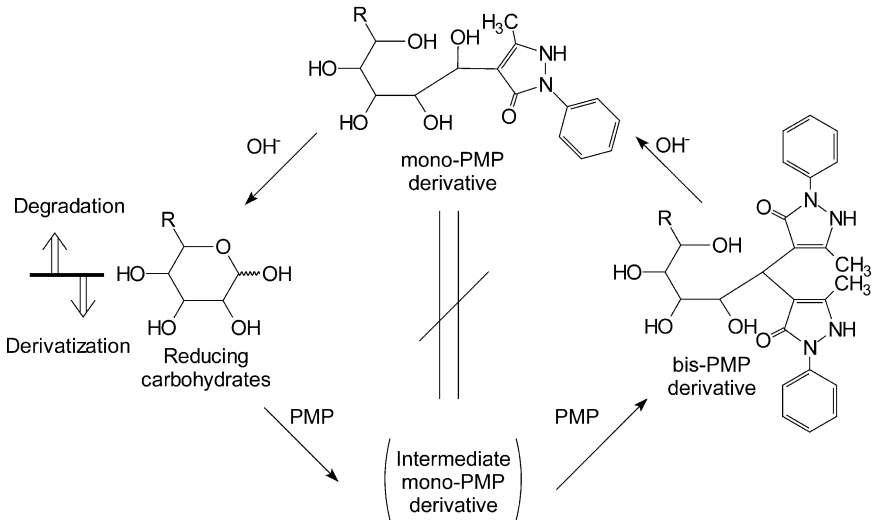

Fig. 1. Derivatization of a reducing carbohydrate with PMP and decomposition of the PMP derivative in weakly alkaline solutions.

oxidizable on a glassy carbon electrode. The electric current generated at 600 mV allowed detection at a concentration of one order of magnitude lower than that in UV detection [14]. Although electrochemical detection is not easy in CE at the present stage, because no commercial detectors are available and it requires a special technique when a home-made device is used, this is a useful property for highly sensitive detection.

3.2. Chemical properties allowing parent sugar recovery

The glycamine derivatives formed in reductive amination are generally stable in acidic and alkaline solutions. This property is useful for carbohydrate analysis, because the derivatives can be stored for a long time. PMP derivatives have a different feature from that of the glycamine derivatives.

When a weakly alkaline solution of PMP-glucose (the bis-PMP derivative) was heated, one of the PMP groups was removed, giving the mono-PMP derivative, but this mono-PMP derivative was not identical with the intermediate mono-PMP derivative formed in the course of derivatization. Further conversion to the parent sugar (glucose) by the removal of one more PMP group was not easy, requiring much more drastic conditions, but it was possible. These chemical properties of the bis- and mono-PMP derivatives are common to other carbohydrates, as generalized in Fig. 1 (unpublished result).

This series of reactions is useful to recover the original reducing carbohydrates from the PMP derivatives for further use, such as structure elucidation, enzyme inhibition assay, etc.

Notwithstanding these chemical properties under weakly alkaline conditions at high temperatures, the PMP derivatives can be stored for a long time when stored in a refrigerator. It is not recommended, however, to store PMP derivatives in a PMP-free state after solvent extraction, if analyses must be done in a long duration. Evaporation of the PMP-free aqueous solutions to dryness and storage of the dried products in a refrigerator is also recommendable.

3.3. Physicochemical properties for versatile separation modes

Introduction of two PMP groups into each carbohydrate molecule endows the derivative physicochemical properties based on the introduction of this tag. The PMP group has high hydrophobicity due to the presence of the pyrazolone ring as the basal structure. The presence of the phenyl and methyl groups at N-1 and C-3 in the pyrazolone

ring enhances its hydrophobicity. This property is useful for micellar electrokinetic chromatography as described later. On the other hand the keto groups in the pyrazolone ring can be tautomerized to the enol group, which gives negative charge by ionization. Since this tautomerism is affected by pH, pH change gives influence on the negativity of the derivatives, accordingly charge-based separation. The separation modes based on charge difference is also described later.

4. Mass spectrometry

Mass spectrometry generally provides versatile information on the structures and amounts of carbohydrates in samples. The quality of structural information is dependent on the mode of ionization. Electron impact (EI) is the most general mode giving a number of fragment ions, and the total ion monitoring based on this ionization mode allows sensitive detection of sample components separated by various separation method including GC, HPLC, CE, ME and CEC. However, this method is not suitable for obtaining the information on molecular mass, which is basically important for structural analysis of carbohydrates. Fast atom bombardment (FAB) is generally a convenient mode to obtain intense peaks related to molecular mass. Unfortunately, however, intact carbohydrates are quite susceptible to the accelerated atom in FAB to give further degradation products. Therefore, sensitivities of intact carbohydrates are normally low. Electrospray ionization (ESI) is also a useful mode for the analysis of molecular ions for certain kinds of compounds such as proteins and related substances, but it is not favorable for intact carbohydrates, either, giving poor ionization. Under these circumstances, we compared the sensitivities of various derivatives, including aminopyridine (AP), *p*-amionobenzoic acid ethyl ester (ABEE), aminoethanethiol (AET), aminobenzenethiol (ABZ), PMP, and *p*-methoxy-PMP (PMPMP) derivatives of maltopentaose, together with maltopentaitol, its reduced form [18], using various sample matrices. The PMP derivative gave the lowest detection limit in the triethanolamine matrix in the negative mode, but did not give the highest sensitivity in other matrices in both positive and negative ion formats. In contrast, the ESI interface in the positive ion format gave the lowest detection limit among these derivatives. It gave exclusively an intense peak of m/z 1159, which is assignable to $(M+H)^+$. PMPMP, the *p*-methoxy derivative of PMP, also gave an intense peak of $(M+H)^+$. Since both the ESI interface and CE are well compatible to each other, both of which use high electric fields, the high sensitivity of PMP as well as PMPMP greatly benefits this coupling. The ESI-MS as PMP derivatives will be a promising technique for carbohydrate analysis.

Thus, the above results also indicate the priority of the PMP method in mass spectrometry.

5. Standard procedure for derivatization

Optimization studies indicated that derivatization was complete in 30 min at 70 °C, when 250 mM PMP solution in a 50 v/v% aqueous methanol was used and it was neutralized with 150 mM sodium hydroxide prior to the addition to a sample [14]. Reaction conditions could easily be optimized, and the removal of the excess reagent was a key step to establish an efficient procedure. Fortunately the excess amount of PMP could easily be removed from reaction solution by extraction with ethyl acetate, unlike in most other methods for pre-capillary derivatization, in which solvent extraction is not efficient due to low hydrophobicity of reagents. For analyses of monosaccharides ethyl acetate should be replaced by chloroform in order to minimize the loss of the derivatives due to slightly lower hydrophobicity. Other solvents including benzene, carbon tetrachloride, and *n*-hexane are not useful for this purpose, giving poorer extraction efficiencies. In most other methods, cleanup by columns is employed, but this procedure has drawbacks of tedious operation and low reproducibility. In addition errors due to loss of derivatives may occur, especially when different batches of columns are used.

The established standard procedure for derivatization is as follows. Add a reagent solution (100

μl) to an aqueous sample solution (50 μl) of either a reducing carbohydrate or a mixture of reducing carbohydrates (~ 10 nmol each) contained in a 1.5-ml Eppendorf tube. Cover the tube with a stopper and keep the tube for 30 min at 70 °C. Open the cover, add 300 M (50 μl) hydrochloric acid, and dry up the mixture in a centrifugal concentrator. Dissolve the residue in water (100 μl) and extract the solution with ethyl acetate (100 μl) three times. Evaporate the aqueous layer by placing the tube in a vacuum desiccator containing pellets of sodium hydroxide. Dissolve the final residue in an appropriate volume of water and analyze an aliquot of the solution by CE.

The reagent solution can be prepared by dissolving PMP in methanol to a concentration of 500 mM and adding 300 mM sodium hydroxide (1:1, by volume). The concentrations of the sodium hydroxide and hydrochloric acid should be exactly normalized and the indicated volumes of the solutions should be exactly taken by whole pipettes. When needed, a known amount of an internal standard, preferably a reducing carbohydrate different from the sample components (for example, L-rhamnose) in cases where it is not the analytes should be added to the sample solution.

In ordinary analyses this procedure ensures accurate determination of reducing carbohydrates, but sometimes the accompanying salt (sodium chloride) of high concentration due to neutralization of the reaction solution may interfere with the separation of sample components. Deionization by micro scale electrodialysis by using an Acylizer (Showa Denko, Shiba-Daimon, Minato-ku, Tokyo) is quite effective in such cases.

6. Stability of the acid-labile groups

As pointed out in Section 1, one of the problems of the existing methods based on reductive amination and hydrazone formation is the drastic reaction conditions using acid catalysts at high temperatures. Treatment of glycans under such conditions causes partial release of acid-labile groups such as *N*- as well as *O*-sulfate groups and the sialic acid residues. In contrast, the PMP method is free from this problem.

Fig. 2a shows an electropherogram of the reaction mixture of a ΔDi-4S and ΔDi-6S mixture, which are the isomeric disaccharides composed of a Δ4,5-unsaturated uronic acid residue and an *N*-acetygalactosamine residue having the *O*-sulfate group at the 4- and 6-positions, respectively (obtainable from chondroitin sulfates A and C, respectively), subjected to derivatization with PMP (unpublished result). If desulfation occurred, the PMP derivative of ΔDi-0S should have been formed. In reality there was no peak of PMP-ΔDi-0S, indicating that this was not the case.

Similarly a mixture of 3′-sialyllactose and 6′-sialyllactose was subjected to derivatization with PMP by the standard procedure. If desialylation occurred, a peak of PMP-lactose in Fig. 2b should have appeared, but only a small peak was observed at the position of PMP-lactose, which was considered to be due to an impurity in the sample (unpublished result).

The results shown above demonstrate that neither de-*O*-sulfation nor desialylation occurred during derivatization. Since the sulfate groups in proteoglycans and the sialic acid residues in glycoproteins are considered to have important roles in the biological functions of these glycoconjugates, and any other existing methods do not allow derivatization without all/partial release of these functional groups, the PMP method can be highly evaluated as a reliable method for glycan analysis.

7. Diversity of separation modes in CE of PMP derivatives

PMP derivatives of carbohydrates can be separated by various modes of CE. This is based on the excellent physicochemical properties mentioned in Section 7.3.

7.1. Zone electrophoresis mode

PMP derivatives of carbohydrates can be separated from each other by this basal mode of CE based on the difference of charge to mass ratio. Since the negative charge is resulting from the enolization of the keto group in the pyrazolone

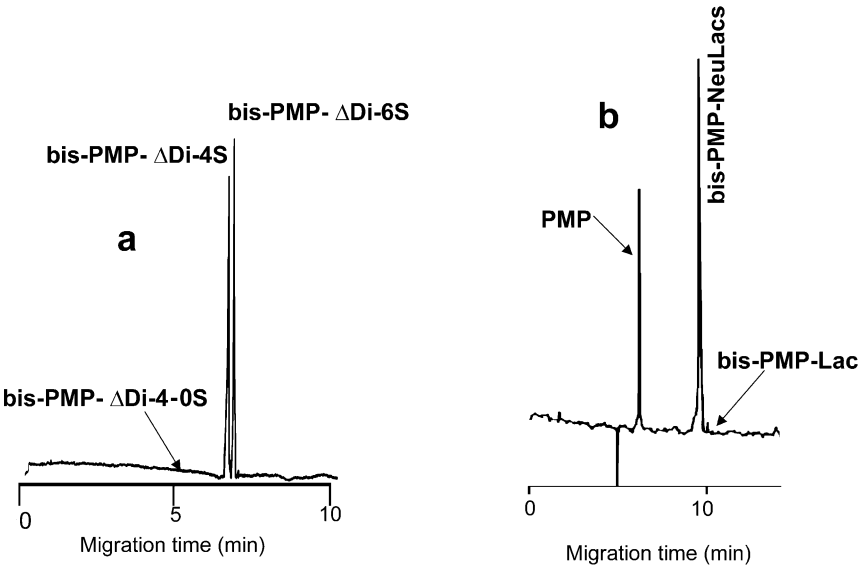

Fig. 2. (a) Stability of the sulfate group in Δdi4S and Δdi6S during derivatization with PMP. A mixture of Δdi4S and Δdi6S was derivatized with PMP by the standard procedure. Analytical conditions: capillary, fused silica (75 μm i.d., 55.7 cm); running buffer, 100 mM borate buffer (pH 9.5); applied voltage, 20 kV; detection, UV absorption at 245 nm. (b) Stability of the sialic acid residue in 3′-NeuAcLac and 6′-NeuAcLac during derivatization with PMP. A commercial sample of a mixture of 3′-NeuAcLac and 6′-NeuAcLac was derivatized with PMP by the standard procedure. Analytical conditions: capillary, fused silica (50 μm i.d., 70 cm); running buffer, 50 mM phosphate buffer (pH 6.5) containing SDS (20 mM); applied voltage, 25 kV; detection, UV absorption at 245 nm.

ring followed by dissociation of the resultant enol group, all reducing carbohydrates are considered to have the same charge, hence carbohydrate species having the same mass, as in the aldopentose and aldohexose sets are not expected to be separated by this mode. Surprisingly, however, good separation occurred among the epimers of either set. An example for aldopentose epimers is shown in Fig. 3a [19]. We postulated intramolecular ring formation through hydrogen bonding between the keto group and one of the hydroxyl groups in the aldoses, in order to explain this phenomenon (Fig. 3b).

The aldoses having more favorable steric disposition will more easily form a ring through hydrogen bonding, and the easier bonding will leave small proportions of the keto group intact, which is enolizable and in turn dissociable to the enolate ion. As a result negative charge will decrease and accordingly velocity in CE will slow down. When a running buffer with pH around 7 was filled in a capillary and a mixture of the PMP derivatives of aldopentoses were introduced from the anodic end, EOF was toward the cathode and the PMP derivatives having weakly negative charge were held back to the anode. Separation occurred by the difference of negativity, i.e. the difference in the magnitude of ring formation. In the aldopentose set retardation of ribose was the greatest, while arabinose showed the weakest retardation. The best separation was achieved at around pH 8, and the migration times were in the order of arabinose, xylose, lyxose, ribose. Comparison of the configurations of the hydroxyl groups indicated the importance of the hydroxyl groups at C-2 and C-3, and the assumption of intramolecular ring formation was verified by thermodynamic analysis by Yamamoto [20]. In aldohexose sets the migration time order was altrose, glucose, mannose, allose for the D-conformation of the hydroxyl group at C-4 in the D-series, and galactose, idose, gulose, talose for the same hydroxyl group in the L-series. The pH range permitting separation of aldoses was narrow and careful pH control was necessary, but it should be noticed that these basal monosaccharides could

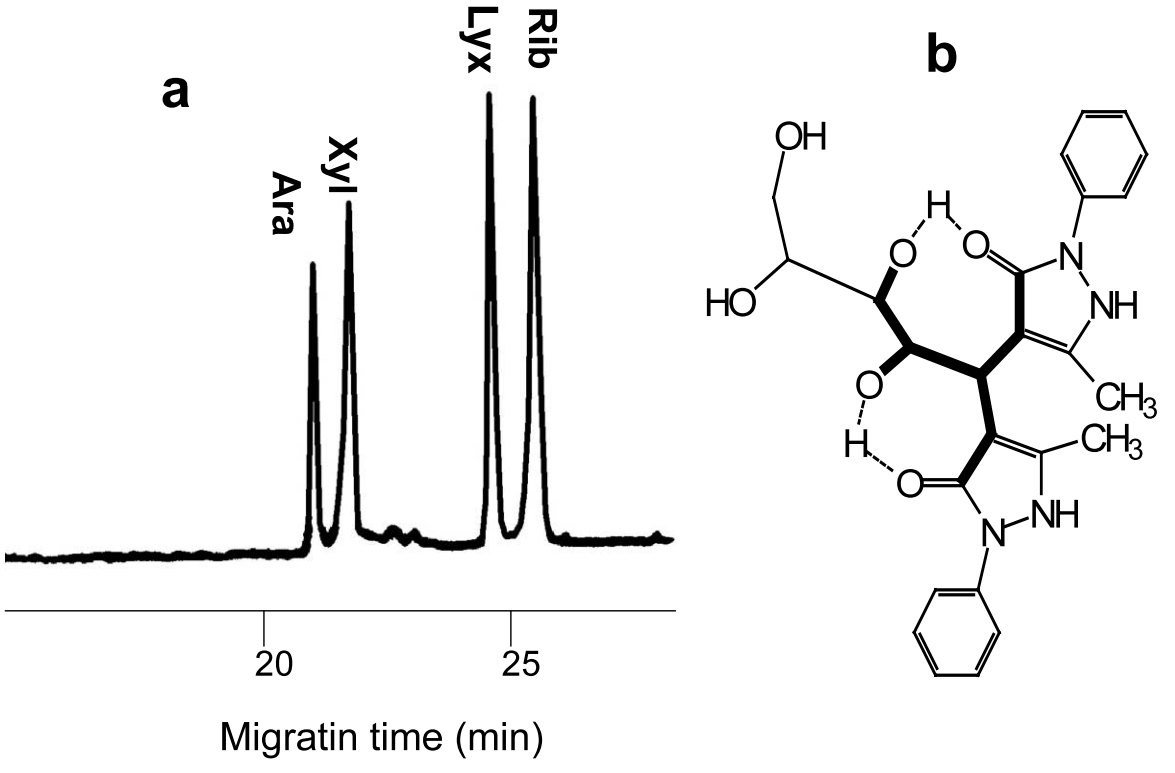

Fig. 3. (a) Separation of the PMP derivatives of aldopentoses by zone electrophoresis in a slightly alkaline phosphate buffer. Capillary, fused silica (50 μm i.d., 69 cm); running buffer, 50 mM phosphate buffer (pH 8.0); sample introduction, from the anodic end; applied voltage, 10 kV; detection, UV absorption at 245 nm. (b) Postulation of intramolecular ring formation through hydrogen bonding to explain the varying mobilities due to varying negative charges.

easily be separated only by pH control. This phenomenon is inherent to PMP derivatives, not observed for any other derivatives.

Separation of oligosaccharides showed a different feature. For example, it was shown that isomaltooligosaccharides having different degrees of polymerization (DPs) gave peaks in the order of increasing DPs, fairly well separated from each other by zone electrophoresis (unpublished result). Since the configuration of the C-2 through C-4 hydroxyl groups in the glucose residue at the reducing end was common for all these oligosaccharides, the negative charge should have been at the same extent. Therefore, the separation was considered to be due to the difference of molecular mass.

7.2. Zone electrophoresis as borate complexes

The complexation of the hydroxyl groups with the borate ion to give anionic complexes has been well known and this phenomenon has long been utilized for carbohydrate separation in the anion-exchange mode of HPLC. The first example of carbohydrate separation by CE was also based on this mode, though not with PMP derivatives but with AP derivatives [21]. Since hydroxyl groups having different configurations react with the borate ion with different magnitudes, the total charge is varied among carbohydrate species, resulting in mutual separation even though there is no difference in molecular size. Complexation is enhanced as borate concentration increases. It is also enhanced by increasing pH, because the borate ion concentration increases. At a borate concentration of 200 mM at pH 10.5 all naturally occurring monosaccharides were completely separated from each other (figure not shown). The excess amount of the AP reagent did not interfere with separation, because it migrated the fastest, well isolated from the peaks of monosaccharide derivatives. The PMP derivatives of monosaccharides were also well separated by this mode. Fig. 4 shows typical examples of separation of the PMP derivatives of aldopentoses (a) and aldohexoses (b) by this mode [15].

It is striking that all sets of aldopentoses and aldohexoses were completely separated from each other in a running buffer having a simple composition of 200 mM borate buffer, pH 9.5. This was the most efficient separation of monosaccharides, hitherto reported for any kinds of separation methods.

7.3. Zone electrophoresis as metal complexes

The hydroxyl groups in carbohydrates can also react with some metal ions to form cationic complexes. This type of complexation is also useful for separation of carbohydrates. Among various kinds of metal ions investigated, alkaline earth metal ions proved to be best suited for this purpose. Fig. 5 shows an example of separation of the PMP derivatives of aldopentoses based on this mode [22].

The PMP derivatives of aldopentoses were easily separated from each other simply by introducing a mixture to an aqueous solution of the acetate salt of an alkaline earth metal. Complexation was rapid but there was a problem of the gradual reversal of EOF, because the metal ions were adsorbed on the surface of the capillary inner wall. The adsorbed metal ions could be removed from the capillary inner wall, but the removal rate was very slow. It should be noticed that the PMP portion was negatively charged but the positive charge of the complex is not large, and as a result the total charge of a derivative was still negative. Therefore, the derivatives introduced from the cathodic end migrated toward the anode by EOF and the movement to the anode was weakly accelerated by the net negative charge. Separation occurred due to the difference of the total negativity among aldoses. The difference of migration time among the kinds of metal ions is due to the different charge of the derivatives.

7.4. SDS-micellar electrokinetic chromatography

This mode, based on different magnitudes of solubilization of hydrophobic solutes to SDS micelles [23] is one of the promising modes for PMP derivatives of carbohydrates. Every member of the PMP derivatives of a homologous series has commonly two PMP groups but has a carbohydrate structure different from each other in degree

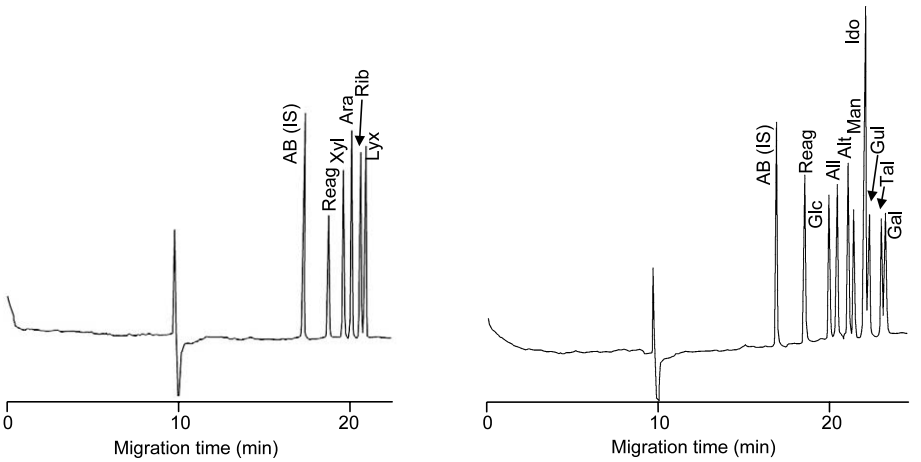

Fig. 4. Separation of the PMP derivatives of aldopentoses (a) and aldohexoses (b) by zone electrophoresis as borate complexes. Capillary, fused silica (50 μm i.d., 78 cm); running buffer, 200 mM borate buffer (pH 9.5); sample introduction, from the anodic end; applied voltage, 15 kV; detection, absorption at 245 nm.

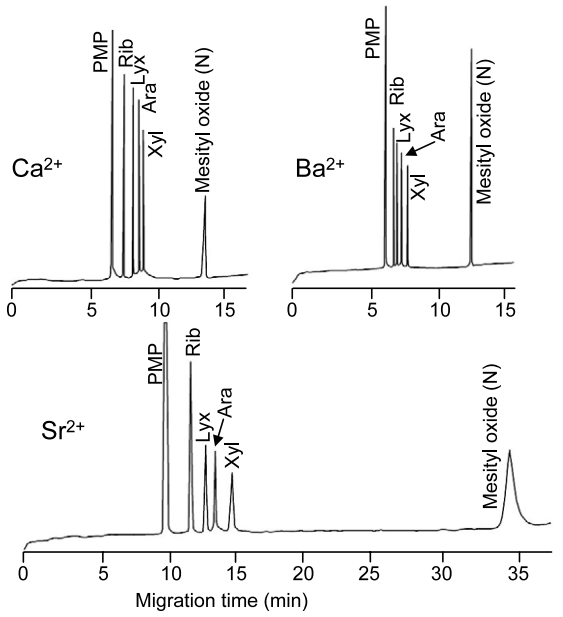

Fig. 5. Separation of the PMP derivatives of aldopentoses by zone electrophoresis as alkaline earth metal complexes. Capillary, fused silica (50 μm i.d., 53 cm); running buffer, 100 mM alkaline earth metal acetate; sample introduction, from the cathodic end; applied voltage, 10 kV; detection, UV absorption at 245 nm.

of polymerization. Therefore, the relative hydrophobicities are different from one another, and this difference causes variation of the magnitudes of solubilization, resulting in separation of homologues. Fig. 6 shows an excellent separation of the PMP derivatives of isomaltooligosaccharides (unpublished result).

The separation by this mode is not directly based on the configurational difference of hydroxyl groups as in the afore-mentioned direct zone electrophoresis mode and zone electrophoresis as ionic complexes, but this mode can separate even isomers, provided that there is a slight difference in relative hydrophobicity among derivatives. Separation of the PMP derivatives of ten monosaccharides and 16 mono- and oligosaccharides by Chiesa and coworkers [24] can be explained by this mechanism. Our result for the separation of *N*-glycans in a glycoprotein (fetuin), shown later in Section 9.4, is also one such example.

7.5. Ion-interaction electrokinetic chromatography

When an ionic polymer, anionic or cationic, is added to running buffer and solutes having counter charge are introduced, these solutes interact with the additive by ionic association. The magnitude of association is dependent on the electric charge, and therefore the separation will occur by the difference of the magnitude of association, though sample introduction should

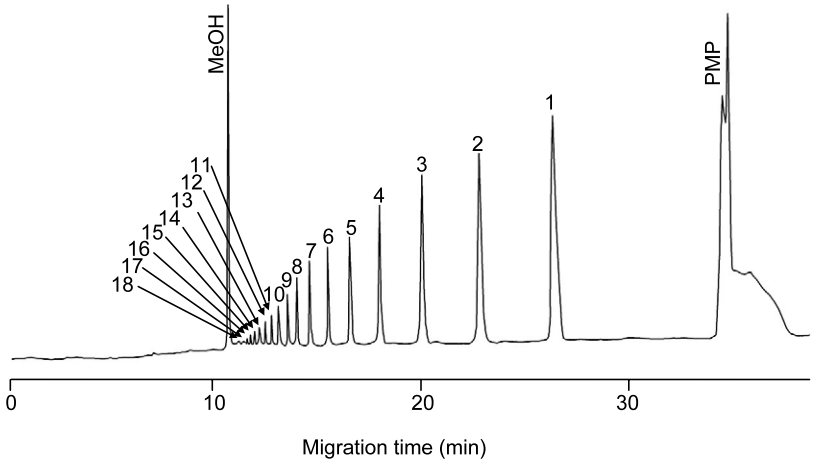

Fig. 6. Separation of the PMP derivatives of isomaltooligosaccharides having various d.p.s by electrokinetic chromatography mode in SDS micelles. Capillary, fused silica (50 μm i.d., 69 cm); running buffer, 50 mM phosphate buffer (pH 6.8) containing SDS (50 mM); applied voltage, sample introduction, from the anodic end; 20 kV; detection, UV absorption at 245 nm.

be done from the proper end depending on the direction of EOF.

In order to investigate the details of this ionic association mode we adopted hexadimethrine bromide (Polybrene) as a positively charged additive. Negatively charged samples should be selected for this additive, but most carbohydrates are almost neutral (even PMP derivatives have only weak negativity). Therefore, we adopted the in-situ formed borate complexes from PMP derivatives. We compared the separation of the PMP derivatives of aldopentoses between 50 mM borate buffer (pH 9.5) and this buffer containing Polybrene to a concentration of 1.0 w/v% [25]. Although the migration direction of these derivatives was reversed due to the reversal of EOF, the migration order was the same without regard to the presence and absence of Polybrene. However, resolution was greatly (one order of magnitude for some species) increased by the addition of Polybrene, presumably owing to the concerted effects of borate complexation and borate complex–Polybrene ionic interaction. In the analysis of PMP derivatives of aldohexoses a much more surprising phenomenon was observed. The migration order was greatly changed between the absence and presence of Polybrene, as follows; "glucose, allose, altrose, mannose, idose, gulose, talose, galactose" in the absence of Polybrene to "altrose, glucose, idose, allose, galactose, mannose, talose, gulose" in the presence of Polybrene (1.0 w/v%).

Thus, the binary system, made by coupling zone electrophoresis in the presence of a cationic polymer to zone electrophoresis as borate complexes, caused change of separation profile for PMP derivatives by altering migration order and resolution.

7.6. Size exclusion electrophoresis

Gel electrophoresis in a capillary gave excellent separation of the AP derivatives of oligosaccharides owing to the size exclusion mechanism. However, the PMP derivatives did not give a good result, due to difficulty in sample introduction (unpublished result). Since sample introduction must rely on the electromigration method, it hampered introduction of PMP derivatives having weakly negative charge, because the accompanying chloride ion having much stronger negativity was predominantly introduced. Desalting by an Acylizer still did not improve introduction of PMP derivative.

Use of entangled polymers of neutral polysaccharides was more practical for the separation of PMP derivatives (unpublished result). The separation of PMP derivatives of isomaltooligosacchar-

ides was improved by adding pullulan owing to molecular sieving effect, and greater improvement was obtained by increasing pullulan concentration

7.7. *Affinity electrophoresis*

The specific interaction of carbohydrates with other types of compounds, especially proteins, can be utilized for separation of PMP derivatives of carbohydrates. Fig. 7 shows a typical example of the separation of PMP-oligosaccharides by the affinity electrophoresis mode.

The PMP derivatives of some disaccharides (maltose, cellobiose, gentiobiose, melibiose and lactose) were not well separated by zone electrophoresis in a neutral phosphate buffer, giving only group separation into glucobiose (maltose, cellobiose and gentiobiose) and galactosylglucose (melibiose and lactose) peaks (Fig. 7a), but the galactosylglucose peaks, especially the lactose peak, were retarded by the addition of *Ricinus communis* agglutinin (RCA_{60}), a galactose-recognizing lectin [26]. The retardation became more prominent as RCA_{60} concentration increased, and at a RCA_{60} concentration of 5 mg/ml all the peaks of these oligosaccharides were almost completely resolved from each other (Fig. 7b). It was interesting that addition of two different kinds of lectins to a running buffer gave the combined effects of these lectins. Thus, addition of *Lens culinaris* agglutinin (LCA, 7 mg/ml), a glucose-recognizing lectin, at the RCA_{60} concentration of 3 mg/ml, which was not enough for complete separation of the above oligosaccharides, realized complete separation [27].

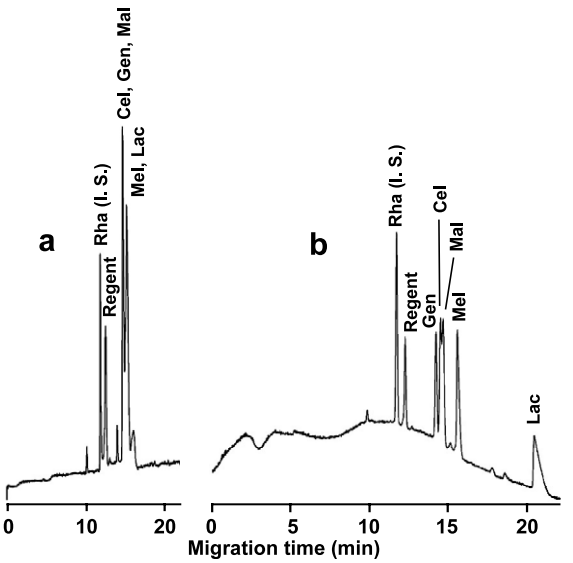

Fig. 7. Separation of the PMP derivatives of disaccharides by affinity CE. Capillary, linear polyacrylamide-coated fused silica (50 μm i.d., 50 cm); running buffer, 50 mM phosphate buffer (pH 6.8) (a) or 50 mM phosphate buffer (pH 6.8) containing RCA_{60} (5 mg/ml); sample introduction, from the cathodic end, applied voltage, 18 kV; detection, absorption at 245 nm.

7.8. *Hydrogen bonding formation electrophoresis*

Recently a number of chiral selectors have been developed for separation of drug enantiomers in HPLC and CE, but only a few selectors have been known for separation of carbohydrate enantiomers. We found that optically active dodecoxycarbonylvalines (*S*-/*R*-DCVs) were effective selectors for this purpose [28]. For example L- and D-glucoses were not separated at all in 50 mM phosphate buffer, pH 7.0 (Fig. 8a), whereas they were completely separated from each other in this order with separation factor and resolution of 1.036 and 2.11, respectively, when *R*-DCV was added at a *R*-DCV concentration of 50 mM (Fig. 8b).

Enantiomers of other aldoses including arabinose xylose and galactose could also be resolved completely. Since lauroylvaline did not give such chiral separation, the participation of the carbamoyl group in *R*-DCV is obvious, and since the ^{1}H-NMR spectrum of the reaction mixture indicated upfield shift of the sugar proton signals, the possible mechanism for chiral separation will be the difference in the magnitude of stable ring formation through hydrogen bonding (Fig. 8c). The stabilization of the ring should involve the hydroxyl group at/near the asymmetric carbon in the sugar ring.

Such easy separation of D-/L-enantiomers will be important for the analysis of the component monosaccharides in some plant polysaccharides which contain both enantiomers.

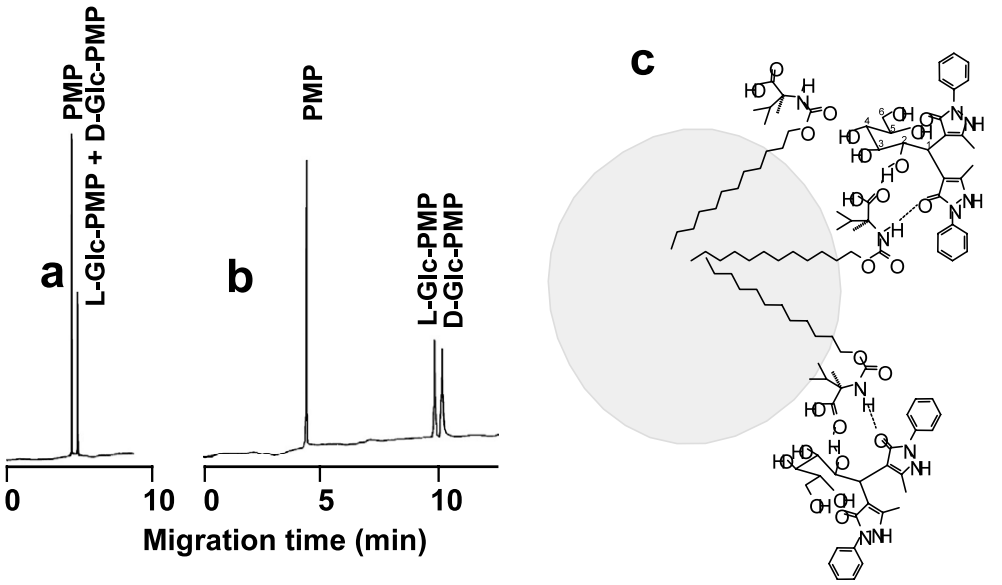

Fig. 8. Separation of the PMP derivatives of D- and L-glucoses in the absence (a) and presence (b) of *R*-dodecoxycarbonylvaline (*R*-DCV, 50 mM). Capillary, fused silica (50 μm i.d., 54 cm) running buffer, 50 mM phosphate buffer (pH 7.0) (a) or 50 mM phosphate buffer (pH 7.0) containing *R*-DCV (50 mM) (b); sample introduction, from the anodic end; applied voltage, 20 kV; detection, UV absorption at 245 nm. (c) Postulation of intermolecular hydrogen bonding between DCV and PMP-glucose, involving the hydroxyl groups in the sugar ring.

8. Quantification

Owing to the extraordinarily high absorptivity of PMP derivatives (ca. 30 000 at 245 nm), detection by UV absorption allowed their quantification at the μM level using ordinary UV detectors installed in commercial CE apparatus. This is corresponding to the 10 *f*mol level as injected amount, if the injected volume is assumed to be 10 nl, an average volume in CE when using a 50 μm i.d.-tube. We can further reduce the injected amount to one order of magnitude by using a bubble tube. Here we must take into account the fact that we need a much larger volume of sample solution, because the smallest sample volume we can handle with reasonable reliability is ca. 10 μl. Therefore, the smallest sample amount for CE analysis will be ca. 10 *p*mol, 1000 times larger than the injected amount. This is approximately the same level as in HPLC.

Calibration curves of sample components obtained by using an internal standard were linear over wide ranges, and allow reproducible determination of the components with relative standard deviations less than 3% in the middle of the linear ranges, though the determinable range varies slightly depending on the separation mode and analytical conditions. It is interesting that the slope of the calibration curve varies among carbohydrate species, though all derivatives commonly have two PMP groups. Since the yields of derivatives are almost the same and quantitative without regard to carbohydrate species, the variation of the slope is considered to have come from the variation of the keto-enol equilibrium due to intramolecular ring formation through hydrogen bonding. As the internal standard a carbohydrate species having a similar structure to the components is desirable. Rhamnose is recommended for the analysis of carbohydrates from biological sources.

9. Typical examples of application to carbohydrate analysis

The quantitative derivatization under mild conditions, the strong UV absorption, high sensitivity

in mass spectrometry of the products, and diverse separation modes permitted reliable analysis of mono- and oligosaccharides by CE as PMP derivatives. There have been a number of reports on the application of the PMP method. The following are representative examples from such accumulated data.

9.1. Component monosaccharides in glycoconjugates

The analysis of the component monosaccharides in glycoconjugates is primarily important to figure out the structural outline of the glycan part of the glycoconjugates. Especially the monosaccharide composition analysis of a glycoprotein gives basic information on the glycan. This kind of analysis has long been performed by GC [29] or HPLC [14,30,31]. CE as glycamine derivatives [21] also permits rapid analysis, but the preference of the use of PMP derivatives is as mentioned above. Fig. 9 shows an example performed by the zone electrophoresis mode as borate complexes (unpublished results).

The analytical procedure including the hydrolysis step is simple; hydrolysis of a glycoprotein sample in 2 M trifluoroacetic acid for 2 h at 100 °C, evaporation of the hydrolyzate, derivatization of the residue as mentioned above, introduction of the derivatized mixture to the anodic end of a capillary filled with 100 M borate buffer (pH 9.4), and application of a voltage; these all should be done. The released monosaccharides could accurately and reproducibly be determined by using a calibration curve using L-rhamnose as an internal standard.

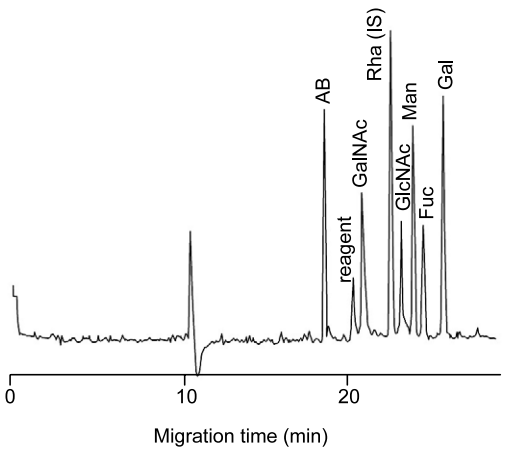

Fig. 9. Analysis of the component monosaccharides commonly found in glycoproteins as PMP derivatives. Capillary, fused silica (50 μm i.d., 78 cm); running buffer, 200 mM borate buffer (pH 9.5); sample introduction, from the anodic end; applied voltage, 15 kV; detection, UV absorption at 245 nm.

9.2. Naturally occurring oligosaccharides

Some kinds of oligosaccharides are present in not only plant but also animal bodies. Among the oligosaccharides of animal origin, milk oligosaccharides are especially interesting from the nutritional aspect for infancy. Most female mammals excrete high concentrations of lactose as a nutrient for feeding babies. It is interesting that colostrum excreted immediately after parturition contains relatively high concentrations of various kinds of oligosaccharides, which gradually decrease or disappear after a few weeks. These unique oligosaccharides are considered to be good carbon sources for the enterobacteria of newborn babies and recognized as *Bifidus* factors. Since such enterobacteria produce various immunogens in

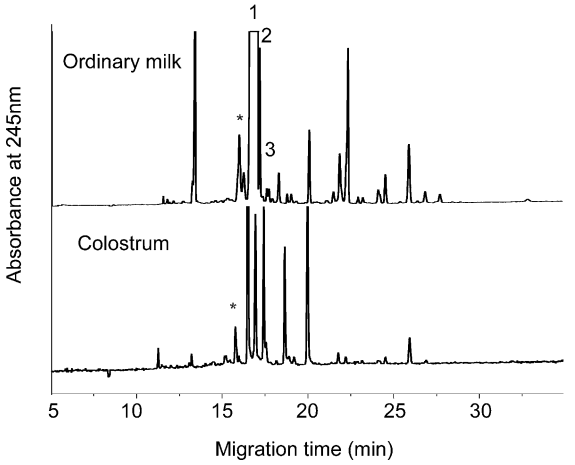

Fig. 10. Analysis of the oligosaccharides in the ordinary cow milk and cow colostrum as PMP derivatives. Capillary, fused silica (50 μm i.d., 60 cm); running buffer, 150 mM phosphate buffer (pH 9.7); sample introduction, from the anodic end; applied voltage, 20 kV; detection, UV absorption at 245 nm. Peaks: 1 = lactose, 2 = 3-NeuAcLac, 3 = 6-NeuAcLac. The asterisked peaks were from PMP.

digestive organs which are indispensable for antibody production, the excretion of such oligosaccharides in colostrums are considered to be important for not only pediatrics but also immunology. The biosynthesis of these oligosaccharides is not indifferent to the production of glycoproteins and glycolipids, which are closely related to immunological phenomena.

Fig. 10 shows examples of analysis of the oligosaccharides in cow milk, obtained by zone electrophoresis as borate complexes (unpublished results). The difference of the oligosaccharide profile between ordinary milk and colostrum was remarkable, and the relative peak intensities varied with individual samples. We should note that the volume of the ordinary milk that gave the upper chart was approximately 10 times as large as the volume that gave the lower chart (unpublished results). This means that the concentrations of the oligosaccharides in colostrum other than lactose were much higher than those in ordinary milk.

9.3. Glycans in glycoproteins

It is well known that most proteins are glycosylated and the glycoconjugates (glycoproteins) have heterogeneity with respect to glycan structure. There are two types of glycans, one bound to the asparagine residue of the pre-formed polypeptide core (*N*-glycans) and the other linked to either serine or threonine residue (*O*-glycans). These two types of glycans have structural features different from each other. *N*-Glycans have been relatively well studied and more than 300 species have been characterized. They can be classified into three categories, high-mannose type, complex type, and hybrid type. The high-mannose type *N*-glycans are first biosynthesized and the complex-type *N*-glycans were derived from them by processing with hydrolases. If the processing is not complete, leaving a part of the mannose chain intact, the resultant *N*-glycans are classified as the hybrid type. The systematic analysis of *N*-glycans has been studied using HPLC by a number of researchers. Especially the group of Takahashi has devoted to two [32] and three [33] dimensional mapping as AP derivatives, but this system requires direct comparison with authentic specimens due to not so high reproducibility. Since much higher reproducibility is ensured in CE provided relative mobility is taken as a migration index, the authors group attempted two-dimensional mapping of desialylated *N*-glycans as the same derivatives [34]. Hermentin and coworkers [35] also reported a similar attempt. This attempt by both groups was successful, and since then we have been engaging in multi-mode analysis of *N*-glycans as PMP derivatives. Although this program is still on the way, we can foresee successful multi-dimensional mapping that will permit reliable identification of *N*-glycans without direct comparison to authentic specimens. Fig. 11 shows one of the examples of the analysis of *N*-glycans by SDS micellar electrokinetic chromatography [36].

The glycan release was performed by the enzymatic digestion using recombinant PNGase F from *E. coli*. It is indicated that various species of complex type *N*-glycans were efficiently separated from each other.

9.4. Unsaturated oligosaccharides derived from proteoglycans

Proteoglycans are a group of glycosylated proteins, in which long-chain acidic polysacchar-

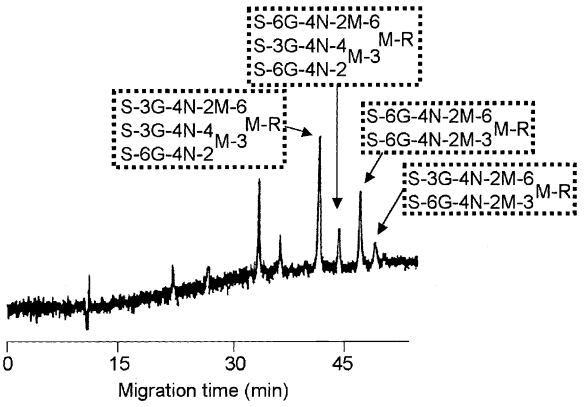

Fig. 11. Analysis of sialo-*N*-glycans from bovine fetuin as PMP derivatives. The sialo-*N*-glycans were released from bovine fetuin by digestion with PNGase F and derivatized with PMP by the standard procedures. Capillary, fused silica (50 μm i.d., 72.5 cm); running buffer, 50 mM phosphate buffer (pH 6.0) containing SDS (30 mM); sample introduction, from the anodic end; applied voltage, 20 kV; detection, UV absorption at 245 nm.

ides are attached. They are important not only as the constituents of connective tissues but also have many physiological functions including blood anticoagulation, lipemea clearing effect, cell proliferation inhibition, etc. Current interest is in the structural diversity near the glycosylation sites, because such diversity is considered to be related to many important biological phenomena.

The polysaccharide (glycosaminoglycan) portion of each proteoglycan has a basic structure composed of alternately linked hexosamine and uronic acid residues, but the hydroxyl group is variously modified by the sulfate group and the amino group is either acetylated or sulfated. On the other hand proteoglycans and the glycosaminoglycans therein are too large molecules to differentiate from each other by ordinary separation methods. However, the oligosaccharides derived from proteoglycans can be the objects of CE analysis. Therefore, simultaneous analysis of the oligosaccharides is the most important strategy to obtain useful information on the fine structures of proteoglycans, though it is an indirect method.

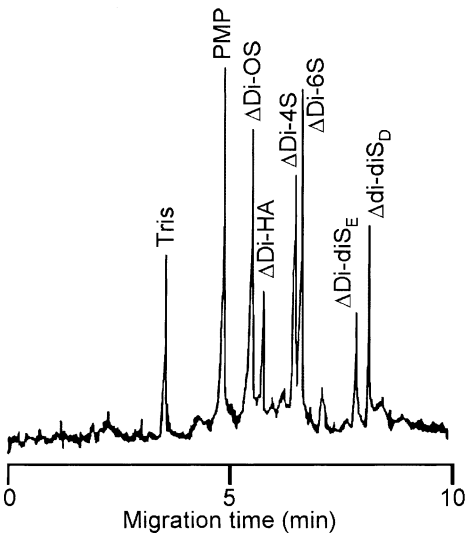

Fig. 12. Analysis of unsaturated disaccharides derived from proteoglycans by digestion with chondroitinase ABC, as PMP derivatives. Capillary, fused silica (75 μm i.d., 57 cm); running buffer; 100 mM borate buffer (pH 9.0); sample introduction, from the anodic end; applied voltage, 15 kV; detection, UV absorption at 214 nm.

The use of a series of eliminases facilitates release of oligosaccharides, yielding oligosaccharides containing uronic acid residue(s) having the double bond between C-4 and C-5. Fig. 12 shows the analysis of unsaturated disaccharides formed from various proteoglycans by digestion with chondroitinase ABC [37].

These oligosaccharides can be detected by the weak UV absorption due to the double bond, but derivatization with PMP enhanced sensitivity. Zone electrophoresis as borate complexes allowed simultaneous analysis of all possible unsaturated disaccharides formed with this most popular enzyme. Detection was not at 245 nm, where PMP derivatives gave the maximum absorption, but at 214 nm, simply because the apparatus used was equipped with only a discontinuous wavelength type detector with a zinc lamp and a corresponding interference filter. Heparin can be degraded to various unsaturated oligosaccharides with heparinases and heparitinase, but the oligosaccharide fragments cannot be quantitatively derivatized with PMP due to the presence of the electron-withdrawing *N*-sulfate group adjacent to the reducing end.

9.5. Oligosaccharides in glycolipids

Sphingoglycolipids exist generally on cell surface and play a role to recognize other cells and molecules in matrices. They have a tendency to make micelles by themselves in aqueous solutions, which hampers normal separation by CE. A certain kind of endoglycoseramidase can release oligosaccharides from the sphingosine moiety, and the released oligosaccharides can be analyzed by CE as PMP derivatives. Fig. 13 shows an example of analysis of the PMP derivatives of oligosaccharides released from bovine brain gangliosides with endoglycoceramidase [38]. Separation was done by the SDS micellar electrokinetic chromatography mode (Fig. 18a) and zone electrophoresis as borate complexes (Fig. 18b).

9.6. Other oligosaccharides

Plant oligo- and polysaccharides might be outside the scope of this journal, but we would like to

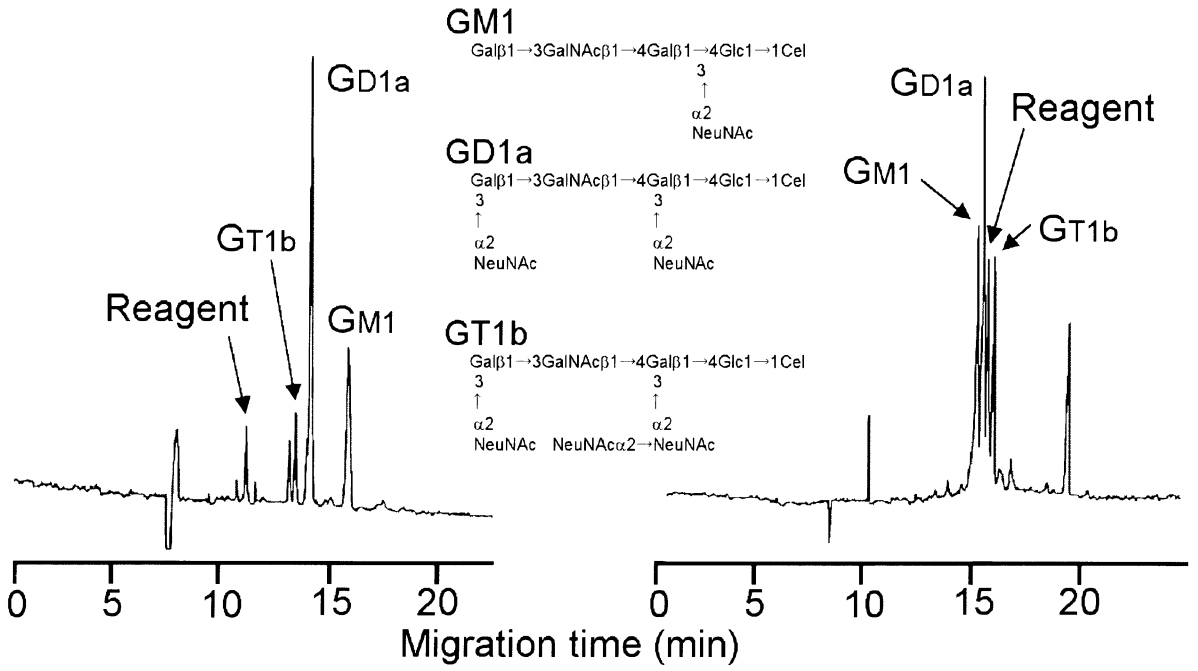

Fig. 13. Analysis of the oligosaccharides released from bovine brain gangliosides as PMP derivatives. The oligosaccharides were released by digestion with endoceramidase and derivatized with PMP. Capillary, fused silica (50 μm i.d., 70 cm); running buffer, 50 mM phosphate buffer (pH 6.0) containing SDS (30 mM) (a) or 60 mM sodium tetraborate (pH 9.3) (b); sample introduction from the anodic end; applied voltage, 15 kV; detection, UV absorption at 245 nm.

describe their analysis briefly, because they are widely and abundantly distributed in foods, and therefore important for human health. Among the plant oligosaccharides sucrose, an outstanding sweetener, is a unique one having a characteristic structure in which the hemiacetal and ketal groups in the two monosaccharide residues (glucose and fructose, respectively) are linked to each other. Application of the PMP method, therefore, requires prior conversion to the component monosaccharides, because it has no reducing end. This could easily be achieved by the use of intestinal invertase and the resultant glucose could be quantitatively derivatized with PMP. Galactosylation of the glucose residue in sucrose gives raffinose and further galactosylation results in stachyose. This series of oligosaccharides including sucrose, raffinose and stachyose, are widely distributed in plant roots and are recognized as potent *Bifidus* factors [39]. Raffinose and stachyose could also be hydrolyzed with invertase to melibiose and manninotriose, respectively. Since the latter have a reducing end, they could be derivatized with PMP. The authors' group demonstrated the usefulness of a series of procedures, based on invertase digestion and CE analysis as PMP derivatives, in the quality evaluation study of *Rehmaniae radix*, one of the popular crude drugs [40].

Fermentation products from starch, such as wine, beer and sake contain various kinds of glucooligosaccharides, and their contents contribute to the taste of these alcoholic beverages. Their analysis can also be achieved by CE as PMP derivatives (unpublished results).

10. Automated analysis by in-capillary derivatization

CE can be performed in free solution unlike other separation methods, and this single phase

property brings forth a number of advantages inherent to this method. So the authors' group has made systematic studies utilizing these advantages. The development of the in-capillary derivatization technique [41–43] is one of these systematic studies. We classified this technique into three types, including the at-inlet [41], zone-passing [42], and throughout-capillary [43] formats, and pointed out merits and demerits of each format. Although initially we used a combination of an amino acid mixture and *o*-phthalaldehyde as a model system, which completes almost instantaneously, later we extended this kind of research to systems involving slower reactions. In derivatization utilizing slow reactions a heating device is required and the modification of the at-inlet format was proved to be the most appropriate. Fig. 14a depicts an apparatus for this purpose, in which a heating oven is placed just after the inlet of a capillary. The distance between the capillary inlet and the oven was minimized.

Fig. 19b illustrates how the PMP reagent and a sample were introduced to and reacted with each other in a capillary. The reagent (PMP) solution, the running buffer, and a sample solution were introduced by pressure to the anodic end of an uncoated fused silica capillary in this order, and a high voltage was applied for a short period. The reagent and sample zones migrated toward the other end and were overlapped on each other at the entrance of the oven due to the difference of their velocities. The overlapped zones were allowed to stand for an appropriate period of time for completion of derivatization, and the derivatives were immediately analyzed by CE by applying a relevant voltage. The durations for the introduction of the reagent/sample solutions as well as the running buffer, and the voltage/time for transferring the reagent and sample could be calculated from the mobilities of the reagent and the sample, and an appropriate program could be constructed using these parameters. Therefore, this in-capillary derivatization system, based on a modified at-inlet format, permitted fully automated analysis of reducing carbohydrates, using small amounts of sample and reagent. Fig. 15 shows an example of the analysis of a maltooligosaccharide mixture by this system [44].

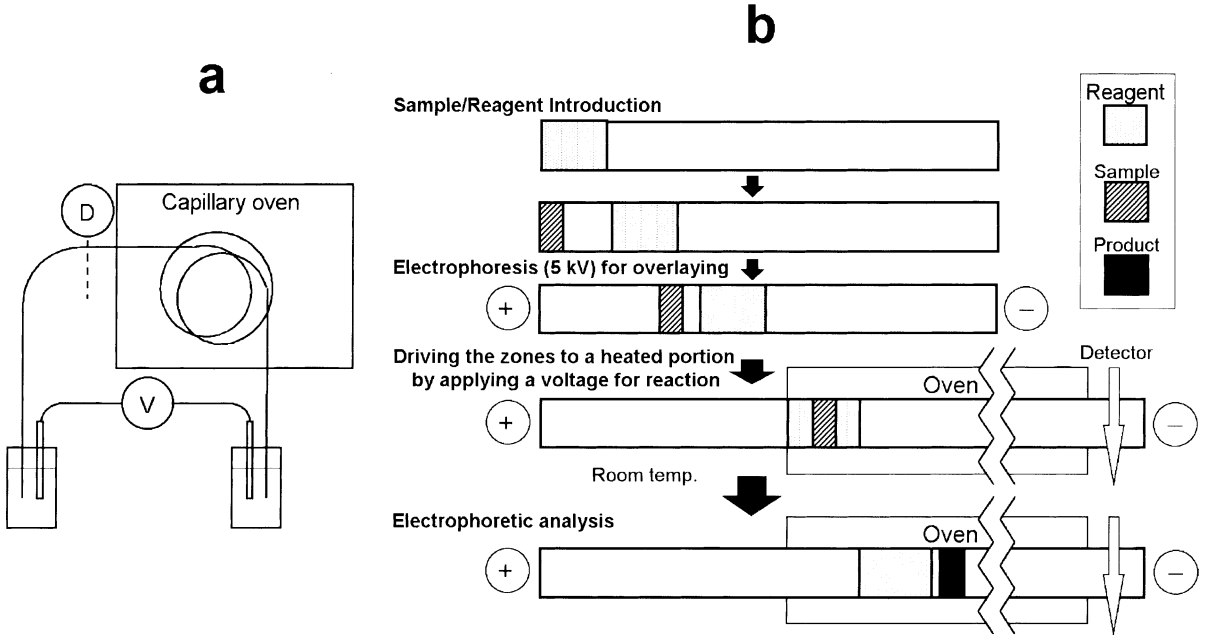

Fig. 14. An apparatus for in-capillary derivatization by the modified at-inlet format using a slow derivatization reaction (a) and illustration of sample/reagent introduction prior to derivatization reaction (b).

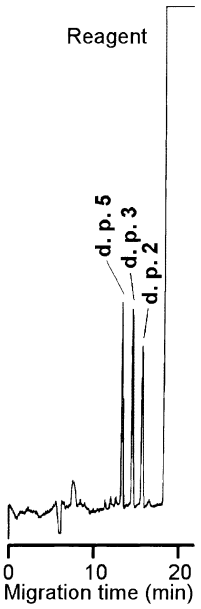

Fig. 15. Automated analysis of maltooligosaccharides by CE followed by in-capillary derivatization with PMP. Analytical conditions: capillary fused silica (50 μm i.d., 71 cm); running buffer, 200 mM borate buffer (pH 8.2) containing SDS (200 mM); applied voltage, 10 kV; detection, UV absorption at 245 nm). Sample solution, 200 μM each in the running buffer; reagent solution for the in-capillary derivatization, 25 mM PMP in the running buffer; reagent/sample introduction, from the anodic end by vacuum (10 and 1.5 s, respectively); buffer zone insertion, between the reagent and sample introductions 5 kV was applied for 4 min; reagent/sample plug transferring to the oven, 5 kV, 3 min; in-capillary derivatization, 35 min at 57 °C.

All the component oligosaccharides were well separated from each other with a column efficiency range of $1.3–1.5 \times 10^4$ and could automatically be quantified with reasonable reproducibility (RSD < 3%) in a sample concentration range of 10–500 μM.

11. Sample reduction by pre-concentration

Although PMP derivatives of carbohydrates can be detected at micromolar concentrations, much higher sensitivity is sometimes required for analysis of precious samples, especially those from biological sources. Use of laser-induced fluorescence is one of the promising methods used to answer this question, and it will be summarized in another review as a different topic. Here the author would like to point out a special technique of sample pre-concentration, which is inherent to CE as a different topic. Among several techniques of pre-concentration, the one based on counter voltage application was proved to be effective for carbohydrate analysis as PMP derivatives. Fig. 16a illustrates the principle of this technique.

When an analytical sample solution was introduced from the anodic end by pressure and a high voltage was applied for a relevant period of time to make a long plug, anionic components in the sample solution having high charge/mass ratios, such as the chloride ion coming from neutralization of the derivatization mixture and SDS added as a denaturant in the process of *N*-glycan release, migrated to the anode and moved into the reservoir. The PMP derivatives of oligosaccharides were concentrated around the anodic end, because they moved slowly. After a reasonably programmed time the concentrated PMP derivatives were analyzed in reverse polarity. Fig. 16b shows an example of analysis of the *N*-glycans from human transferrin. Although migration time was not so sensitively detected in normal technique, lower limit of detection decreased to approximately two orders of magnitude and the *N*-glycans from a glycoprotein sample of as small as 2 ng could be detected. This technique was useful for routine analysis of samples containing a small number of components. For samples containing a large number components, complete separation of sample components was not ensured.

12. Oligosaccharide sequencing by exoglycosidases

Step by step elimination of the outermost monosaccharide residues by digestion with exoglycosidases is the most reliable and useful method for oligosaccharide sequencing. Application of this method to PMP derivatives of oligosaccharides indicated that each exoglycosidase could release the corresponding outermost monosaccharide almost quantitatively. The presence of the PMP groups did not give any significant influence on enzyme reactions, and the stepwise elimination of

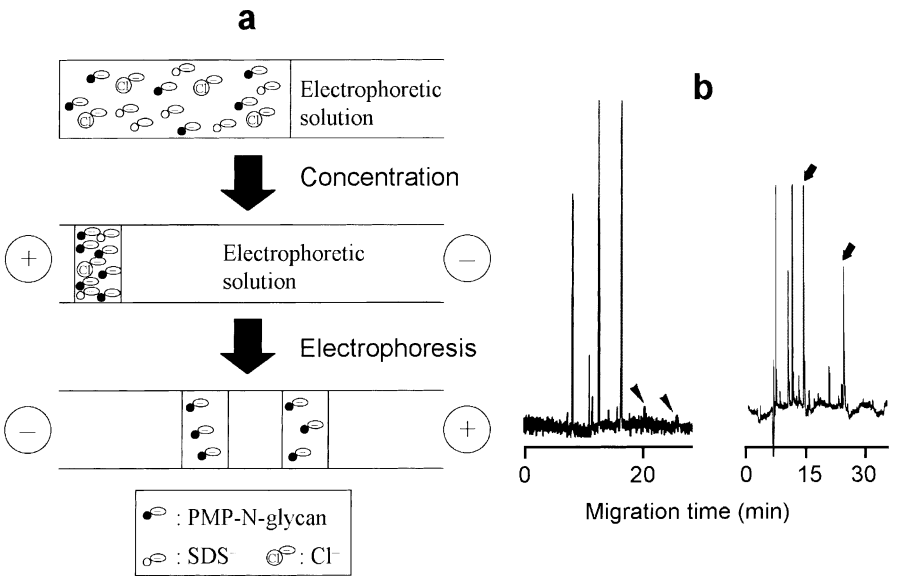

Fig. 16. (a) Pre-concentration of the analytical solution by counter voltage application. The derivatives of sialo-*N*-glycans from human transferrin (20 ng) was introduced from the anodic end for 30 s and a high voltage (5 kV) was applied for 10 min to drive out the strongly negative ions such as the chloride and SDS ions to the anodic reservoir. Then, the polarity was switched and the concentrated PMP derivatives were analyzed toward the cathode (electropherogram on the right hand side). (b) Reference obtained from the analytical solution from the 2 μg-amount of transferrin.

the terminal monosaccharides provided important information on oligosaccharide sequencing. Fig. 17 shows the result of sequencing of the major *N*-glycan from transferrin released with PNGase F. Sequential digestion with neuraminidase, β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase resulted in the elimination of the corresponding monosaccharide residue to yield smaller glycans.

No release of the PMP group occurred. Derivatization of the digestion product with PMP after each step gave a peak of the PMP derivative of the released monosaccharide, together with the peak of the newly formed shorter chain oligosaccharide (result not shown).

13. Microchip electrophoresis

Recent advancement of miniaturization of analytical instruments is overwhelming. Miniaturization of CE has been investigated by some pioneering groups and become popularized owing to the contribution by a number of groups. Apparatus for specialized areas of genomics and proteomics have been recently commercialized.

The place of separation in CE is inside a capillary, whereas that in ME is in a narrow channel on a microchip. Such devices can be fabricated on a thin plate of glass or quartz by a special technique of photolisography. The details are described in a few excellent reviews [45–48]. Our recent review [49] also summarizes device preparation, together with characteristic features of this technique and application. We also attempted application to carbohydrate analysis using a homemade apparatus, composed of a microscope and a computer-controlled power supply. Since high sensitivity detection is required in ME, at first we used argon laser-induced fluorescence for detection. Introduction of sample solution has solely relied on the electromigration method, because no other methods are available due to the difficulty in mechanically moving channel ends. Due to these limitations our first attempt was the analysis of amino sugars as 7-nitro-2,1,3-benzoxadiazole (NBD) derivatives. We could succeed in separating NBD derivatives of

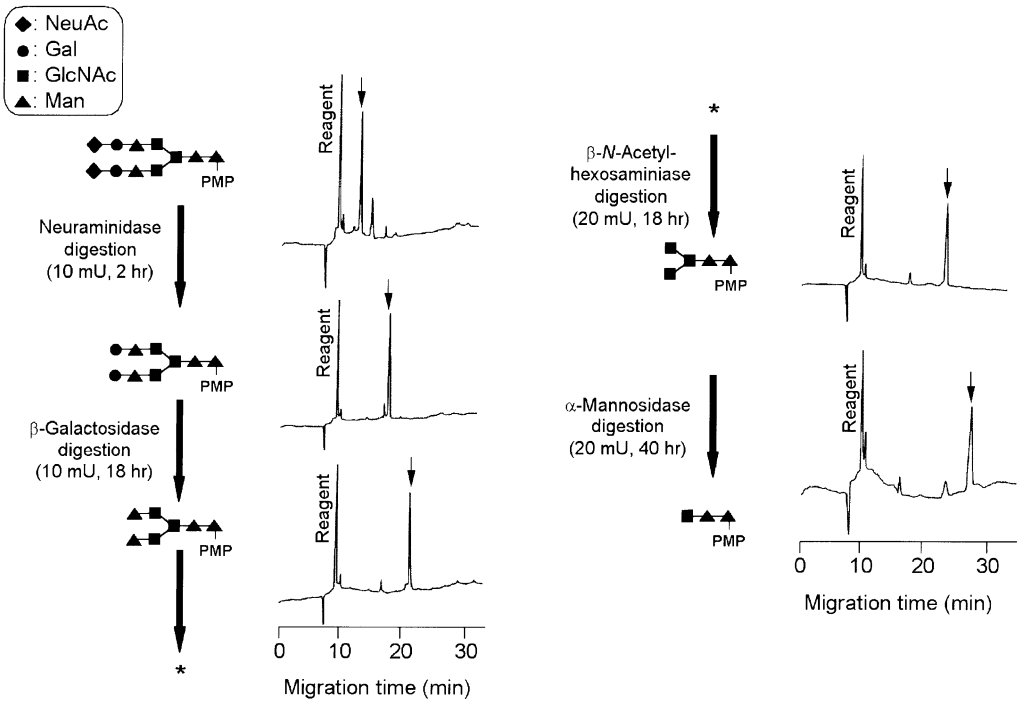

Fig. 17. Demonstration of the efficacy of the stepwise exoglycosidase digestion for sequencing oligosaccharides. A mixture of *N*-glycans released from human transferrin by digestion with PNGase F was subjected to consecutive digestions with neuraminidase, β-galactosidase, β-*N*-aetrylglucosaminidase, and α-mannosidase. The products were checked by CE at each step by the SDS micellar electrokinetic mode of CE. The arrowed peaks show the main products.

glucosamine, galactosamine, glucosaminitol and galactosaminitol using a quartz chip having cruciferous micro channels of a 20 μm depth and a 50 μm width). The reservoir to reservoir lengths of the longer (analytical channel) and shorter (sample introduction) channels were 12 mm and ca. 38 mm, respectively, and the distance between the cross point and the detection point was 25 mm.

The most difficult problem was the control of potentials, but we could conquer this problem by computer control using a Labview program (National Instruments Japan, Shiba-koen, Minato-ku, Tokyo). Under the optimized conditions the above amino sugars and their alditol derivatives could be completely separated from each other in only 45 s by the zone electrophoresis mode as phenylborate complexes (Fig. 18b).

Quite recently we also challenged ME of PMP derivatives of carbohydrates. Since PMP derivatives do not fluorescence by any, we had to use UV absorption. However, the sensitivity was not so high in UV absorption. Therefore, we tried to use a whole channel detection system of Shimadzu corporation (Nishinokyo-Kuwaharamachi, Nakakyo-ku, Kyoto), which is based on the integration of simultaneous detection throughout a channel. We analyzed PMP derivatives of isomaltooligosaccharides by zone electrophoresis as borate complexes in 100 mM phosphate buffer, pH 10.5 (unpublished result). We could obtain distance-based electropherogram and transformed it to time-based electropherogram. Fig. 19 shows an example obtained by 1024 times data acquisition.

The theoretical plate number for glucose was only 1100, but the height equivalent to a theoretical plate was ca. 10 μm, not greatly different from that in CE. Although the number of separable species was not so large as in CE, due to the shortness of separation length, the priority in

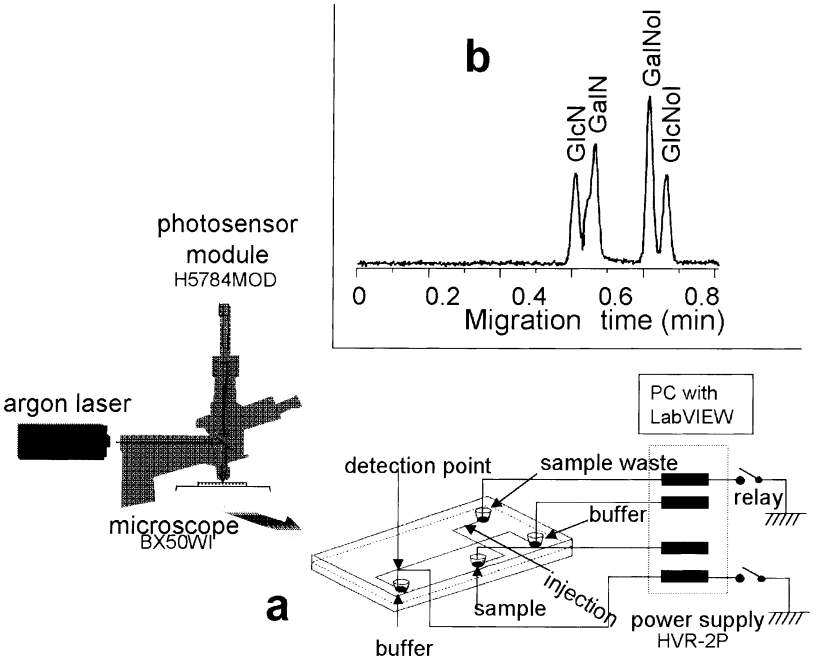

Fig. 18. (a) The layout of the microscope, microchip, and voltage control in a home-made apparatus for ME. (b) Analysis of hexosamines and hexosaminitols as NBD derivatives by ME with argon laser-induced fluorescence detection. Microchip, quartz (separation channel, 25 mm); running buffer, 100 mM phenylborate buffer (pH 7.5) containing acetonitrile (2%); applied voltage, 500 V. GlcN = glucosamine, GalN = galactosamine, GlcNol = glucosaminitol, GalNol = galactosaminitol.

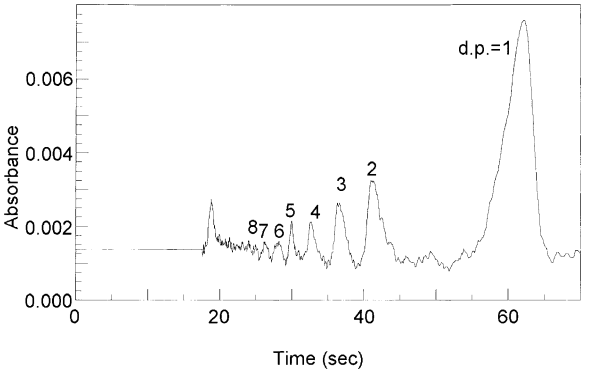

Fig. 19. Analysis of the PMP derivatives of isomaltooligosaccharides by ME with whole-channel UV detection. Microchannel, quartz (20 μm depth, 50 μm width, 25 mm); running buffer, 50 mM phosphate buffer (pH 7.0) containing acrylamide (1.0%|) and SDS (50 mM); applied potential, 500 V; detection, UV absorption at 245 nm; number of data acquisitions, 1024 times.

rapidity of analysis (within ca. 1 min) was remarkable. Application to other carbohydrate species by various separation modes is now going on.

14. Capillary electrochromatography

CEC is a hybrid of CE and LC in the sense that it is based on separation by LC with driving by EOF generated in the electric field. We challenged carbohydrate separation by this method for the first time and could succeed in fairly good separation of several monosaccharides commonly found in glycoproteins as PMP derivatives using a Hypersil ODS column (Hewlett Packard, obtained through Yokogawa Analytical Systems (Musashino-shi, Tokyo), as shown in Fig. 20 [50].

Since EOF has a plug flow nature unlike laminar flow in pressure-driven flow, better separation than in HPLC could be expected, but the realized separation was not as good as expected. Heavy tailing was observed due to the ionic interaction of the PMP derivatives having slightly negative charge with the remaining silanol group. It was ironic that the silanol group left untreated in order to generate EOF hampered resolution by heavy tailing due to adsorption. Reproducible

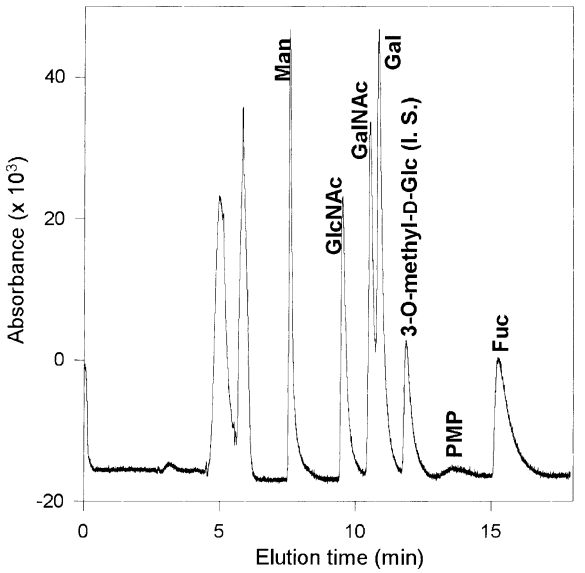

Fig. 20. Analysis of the PMP derivatives of the component monosaccharides in glycoproteins by CEC. Capillary, fused silica (100 μm, 25.5 cm) packed with Hypersil ODS (3 μm diameter); eluent, 50 mM HEPES/NaOH (pH 6.0)–acetonitrile (2.2:1, by volume); applied voltage, 25 kV; applied pressure, 7.5 kg/cm^2; detection, UV absorption at 245 nm.

separation was not easy to obtain, because preparation of high quality columns uniformly packed with small particles of ODS was difficult.

Under these circumstances our strategy has been to prepare columns by in-column modification of silica gel-packed columns, because packed silica gel columns of constant quality are relatively easy to obtain from commercial sources, and the packing technique for this material, involving creation of frits at both ends of the capillary, has been relatively well established. We could prepare an aminopropylated silica column according to this strategy by heating a packed silica column with 3-aminopropyltrimethoxysilane. Fig. 21 compares the separation of PMP derivatives of aldopentoses among a thus-prepared amino column (Fig. 21a) and columns prepared by normal packing of commercial amino silica particles into an uncoated (Fig. 21b) and 3-aminopropylytrimethoxysilane-treated (Fig. 21c) columns of the same size.

All columns were eluted commonly with a 2:1 (by volume) mixture of HEPES buffer (pH 6.0)–acetonitrile [51]. The peaks from the column prepared by in-column modification were much

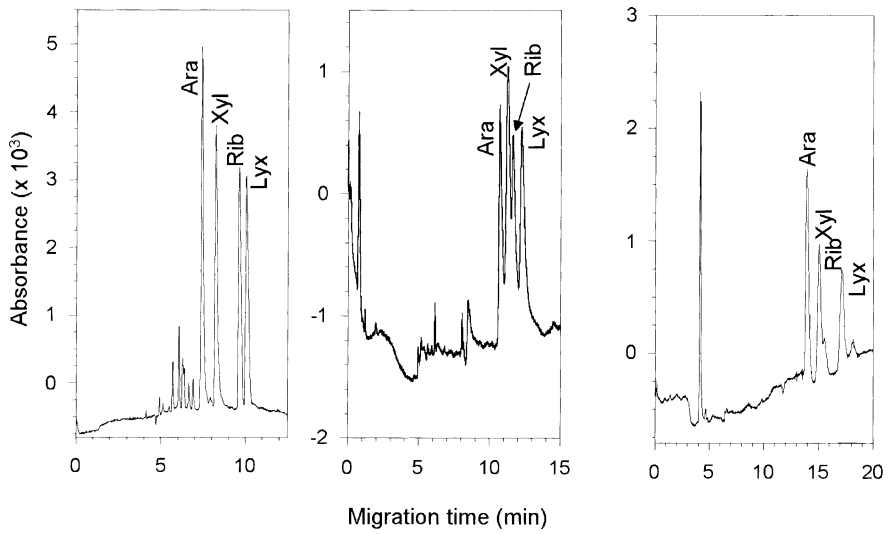

Fig. 21. Analysis of the PMP derivatives of aldopentoses by CEC using an amino column prepared by in-column modification (a). Panels b and c show references, obtained by using amino columns prepared by packing commercial amino silica gels into an uncoated and a 3-aminopropyltrimethoxysilane-treated columns of the same size, respectively. Analytical conditions: capillary, as above (100 μm i.d., 25 cm); eluent, 25 mM HEPES/NaOH buffer (pH 6.0)–acetonitrile (2:1, by volume); applied voltage, −20 kV; detection, UV absorption at 245 nm. Peaks: Ara = arabinose, Xyl = xylose, Rib = ribose, Lyx = lyxose.

sharper than those from the latter two columns, and the problem of peak tailing was overcome in this hydrophilic interaction mode.

The strategy of in-capillary modification has been extended to preparation of various columns of other types. New types of columns thus prepared generally allowed good separation. For more details please refer to the literature [50,51].

15. Conclusion

The PMP method could be widely used for derivatizing any kind of reducing carbohydrate, with quantitative yields of derivatives and without undesirable breakdown of samples. The derivatives could be more efficiently separated by CE than by HPLC. Diverse separation modes in CE and high reproducibility of relative mobility made reliable identification possible and the utilization of these advantages for multi-dimensional mapping without authentic specimens could be expected. The strong absorption in the UV region ensured routine analysis and the high sensitivity in ESI-MS was favorable for on-line CE-MS analysis of carbohydrates on the structure assignment level. Miniaturization to ME was also promising for routine analysis of carbohydrates. It will be widely used in the fields of clinical analysis, environmental analysis, quality control, etc. which require rapid and multi-sample analysis. Separation by CEC may be an alternative to HPLC and will give high efficiency separation, provided uniformly packed columns are provided by intelligent methods such as in-capillary modification.

A number of examples for application of the PMP methods have been shown, which will be useful for researchers working in the fields of glycobiology and many other related fields.

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