Focusing Review

Capillary electrophoresis using chemical and physicochemical reactions

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Abstract

Chemical and physicochemical reactions can be carried out in a capillary tube on capillary electrophoresis. And utilizing in−capillary reactions on capillary electrophoresis allows quick determination of reaction rate constants and association constants between biological components with small samples. This paper summarizes "in−capillary derivatization" and "affinity capillary electrophoresis" as capillary electrophoretic analyses using chemical and physicochemical reactions.

Key words: Capillary electrophoresis, In−capillary derivatization, Affinity capillary electrophoresis, *o*−phthalaldehyde, carbohydrate

1. Introduction

Recently capillary electrophoresis is coming more popular to analyze not only general small compounds but also large molecules of biological samples. This separation tool requires just small amounts of samples and running buffers. Various modes of separation can be carried out only with filling a capillary tube with running buffers. We noticed this advantage of capillary electrophoresis what allows ultra−micro scale kinetic studies. In this paper chemical and physicochemical reactions carried out in a capillary tube on capillary electrophoresis were investigated multilaterally.

2. In−capillary derivatization

Derivatization in a capillary is an excellent technique to reduce a sample amount for conversion to a derivative amenable to the detection system installed in the apparatus. It can be carried out by three formats (at− inlet, zone−passing, and throughout−capillary) depending on the place where reaction takes. It should be stressed that all these formats allow rapid, automatic derivatization, by using only small amounts of samples (sample amount can be reduced to a thousandths of that in pre−capillary derivatization).

2. 1. At−inlet technique [1]

In−capillary derivatization at the capillary inlet named "at−in-

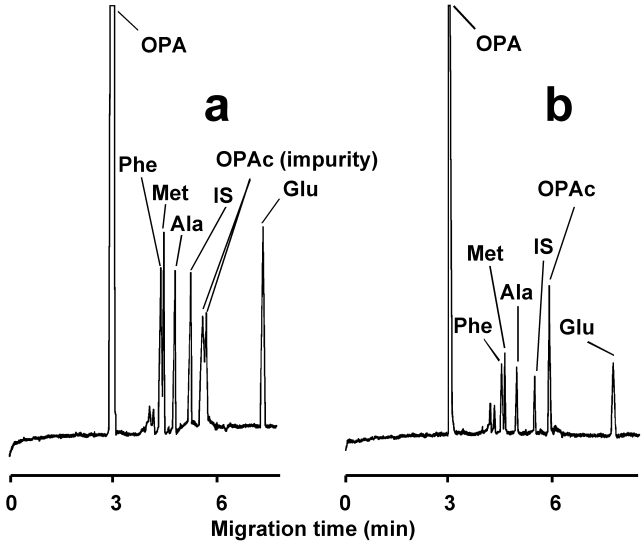

Figure 1. Electropherograms of selected amino acids derivatized with OPA by at−inlet (a) and pre−capillary (b) derivatization. Capillary, untreated fused silica (72 cm, 50 μm i. d.); running buffer, 50 mM borate buffer (pH 10.0); applied voltage, 30 kV; detection, UV absorption at 230 nm; reaction conditions, R (3 s)−S (1 s)−R (3 s)−B (5 s) were introduced in this order for (a), and 30 μL of R was added to 5 μL of S for (b), standing time for reaction was 20 min (S, sample solution, R, reagent solution, B, running buffer).

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let technique" is a similar format to pre−capillary derivatization and the simplest to analyze data among the three techniques of in−capillary derivatization. Solutions of a reagent and a sample are introduced successively into the capillary inlet, and stood for appropriate time followed by application of voltage for electrophoretic separation. Two introduction modes of each solution are available; tandem and sandwich modes. These two introduction modes with various introduction times were investigated on mixing and reaction efficiency. In this investigation, a mixture of selected amino acids was used as a sample solution and an *o*−phthalaldehyde (OPA) solution was used as a derivatization reagent. As a result the sandwich mode by reagent (3 sec)−sample (1 sec)−reagent (3 sec)−running buffer (5 sec) in this order gave the highest efficiency. This efficiency is similar to pre−capillary derivatization as shown in Figure 1. This technique of in−capillary derivatization has a special feature, that various solutions can be used as electrophoretic solutions. According to the advantage of this feature, fifteen of seventeen amino acids included in H−type amino acids could be well separated by using a phosphate buffer containing neutral detergent as shown in Figure 2.

2. 2. Zone−passing technique [2]

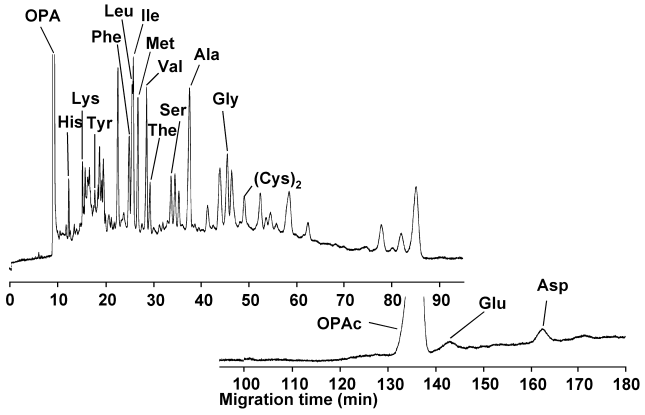


Figure 2. Separation of amino acids derivatized with OPA by the at−inlet technique of in−capillary derivatization. Running buffer, 50 mM phosphate buffer (pH 6.8) containing CHAPS at the concentration of 3.2 mM. The other conditions are as in Figure 1 a.

Amino acids will generally migrate more slowly than OPA reagent in an alkaline solution in the presence of electroosmotic flow because amino acids have negative charge. When a sample solution of amino acids is introduced followed by a reagent solution of OPA, the OPA zone will pass the sample zone. While these zones overlap, the derivatization reaction proceeds as shown in Figure 3. Reaction time can be easily controlled by changing introduction time of a reagent solution, hence, zone−passing technique of in−capillary derivatization is a convenient technique for kinetic studies

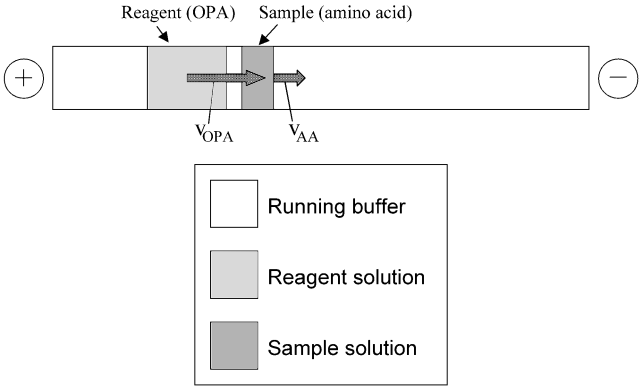


Figure 3. Schematic illustration of the zone−passing technique of in−capillary derivatization.

of fast reactions. Figure 4 shows the influence of the reagent introduction time on derivatization yields. The phenylalanine derivative gave a peak having half height compared with the peak of cinnamic acid added as an internal standard by introduction time of 10 sec. A similar and a higher peak height were given by 40 sec and 120 sec, respectively.

Reaction time on each electropherogram in Figure 4 can be es-

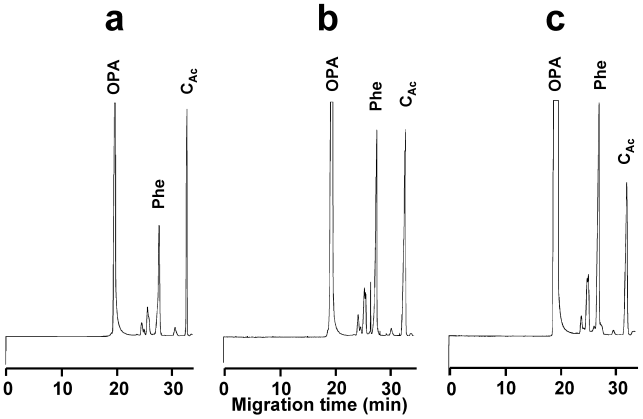


Figure 4. Electropherograms of phenylalanine derivatized with OPA by the zone−passing technique of in−capillary derivatization. Capillary, untreated fused silica (70 cm, 50 μm i. d.); running buffer, 50 mM borate buffer (pH 10.0); applied voltage, 5 kV; detection, UV absorption at 230 nm. Introduction of sample and reagent solutions; S (10 s)−B (10 s)−R (10 s) (a), S (10 s)−B (10 s)−R (40 s) (b) or S (10 s)−B (10 s)−R (120 s) (c) by the hydrostatic method (10 cm), where S, ES and R are sample solution, electrophoretic solution and reagent solution, respectively.

timated from half bandwidth of OPA and migration velocity distance between OPA and phenylalanie. Remaining phenylalanine concentration can be calculated from each peak response of phenylalanine−OPA derivative using a calibration curve. A natural loga-

rithm plot of remaining phenylalanine concentration to the reaction time gave a straight line with good linearity, consequently reaction rate constant can be obtained from the slope of this straight line.

2. 3. Throughout−capillary technique [3]

Throughout−capillary technique of in−capillary derivatization is the simplest technique. It can be performed by carried out electrophoretic analysis in a running buffer containing a reagent for the target samples. Figure 5 shows separation of selected amino acids derivatized by throughout−capillary derivatization. Each amino acid gave relatively broad peaks in comparison with at−inlet and pre−capillary derivatizations. The peak widening was likely to be caused by the detection time difference between derivatives reacted at the early stage and the late stage.

Figure 6 shows a magnification of a peak of phenylalanine

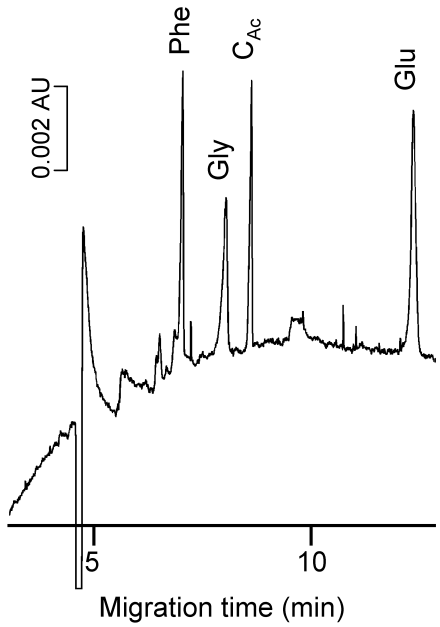


Figure 5. Analysis of a mixture of selected amino acids as OPA derivatives by the throughout−capillary technique of in−capillary derivatization. Capillary, untreated fused silica (70 cm, 50 μm i. d.); running buffer (reagent solution), 50 mM borate buffer (pH 10.0) containing OPA (50 mM); applied voltage, 20 kV; detection, UV absorption at 230 nm.

analyzed by throughout−capillary derivatization. It gave a fronting peak, because the derivative has stronger negative charge, and moves more slowly than the unreacted phenylalanine. From a data in Figure 6, amount of phenylalanine derivatized before the reaction time (t) can be calculated from the part peak area, marked ΔA. The reaction time (t) can be estimated from migration times of derivative and unreacted phenylalanine. A natural logarithm plot of remaining unreacted phenylalanine concentration to reaction time

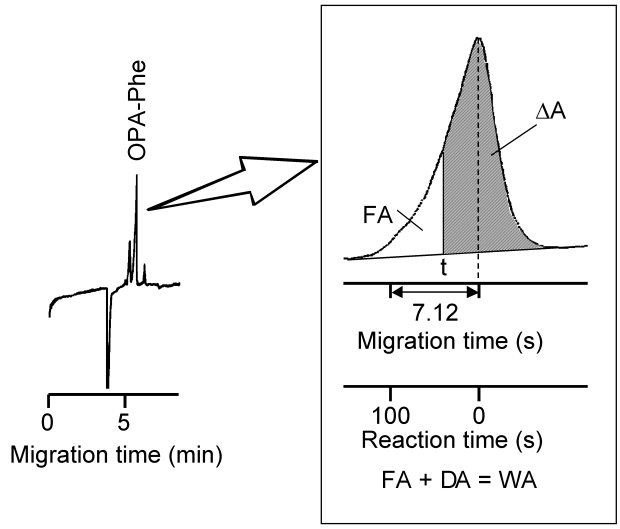


Figure 6. Analysis of phenylalanine as the OPA derivative by the throughout−capillary technique of in−capillary derivatization. The analytical conditions are as in Figure 5.

gave a straight line with good linearity, and from this straight line a reaction rate constant could be obtained with just one electrophoretic analysis.

3. Affinity capillary electrophoresis

Recently human gene mapping has been completed, hence studies of biological components having complex physical functions are becoming more important. Interactions of biological samples can be observed by capillary electrophoretic analyses called affinity capillary electrophoresis. Affinity capillary electrophoresis was investigated using interactions between proteins and carbohydrates as model systems. This technique has two systems. The first is “normal system” which uses proteins as samples and ionic carbohydrates as additives to running buffers. The second is “reverse system” which uses carbohydrate derivatives as samples and proteins as additives.

3. 1. Normal system [4,5]

In the normal system of affinity capillary electrophoresis, small molecules of carbohydrates are used as an additive to a running buffer. Neutral carbohydrates are unable to change the velocity of a protein having a large molecular weight, because they have no charge and also their small size. Accordingly, lactobionic acid having both a carboxylic group and a galactose residue was used as an additive to a running buffer. *Recinus communis* agglutinin (RCA_{60}) which is galactose specific lectin was used as a sample. This combination was used as a model interaction system for the basic study of the normal system. Figure 7 shows electropherograms of RCA_{60} in the absence and presence of lactobionic acid in neutral phosphate buffers. In the absence of lactobionic acid, this

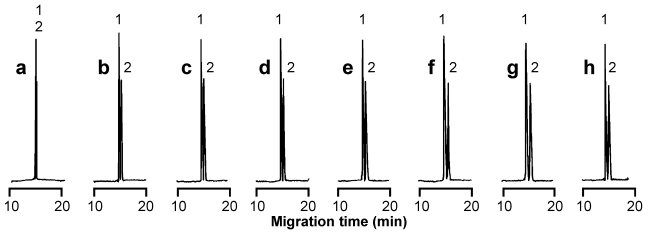
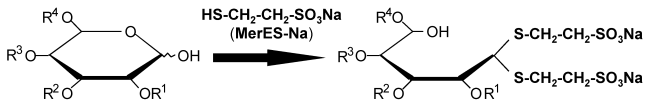


Figure 7. Concentration−dependent retardation of the RCA_{60} peak in running buffers containing lactobionic acid. Capillary, untreated fused silica (85 cm, 50 μm i. d.); running buffer, 50 mM phosphate buffer (pH 6.8) containing lactobionic acid at the concentrations of (a) 0, (b) 0.2, (c) 0.3, (d) 0.4, (e) 0.5, (f) 1.0, (g) 5.0 and (h) 10.0 mM; applied voltage, 20 kV; detection, UV absorption at 220 nm; sample solution, 50 mM phosphate buffer (pH 6.8) containing RCA_{60} and mesityl oxide to concentrations of 1 mg/ml and 1 mM, respectively. Peaks: 1= mesityl oxide (neutral marker); 2= RCA_{60}.

lectin gave a peak almost superimposed on that of a neutral marker. When the lactobionic acid was added to the running buffer, RCA_{60} was separated completely from the neutral marker by forming a complex with the negative charged ligand. A plot of migration time of RCA_{60} to concentration of the lactobionic acid gave a curve having a plateau at the concentration of more than 5 mM. Theoretical treatment to estimate an association constant is mentioned briefly below. The association constant (Ka) can be written as [P−C] / [P] [C] (P, protein; C, carbohydrate; P−C, complex). The migration time of the protein changes depending on the carbohydrate concentration in the running buffer. A plot of reciprocal migration time shift to reciprocal carbohydrate concentration gives a straight line. Ka can be estimated from the slope (A) and the Y−intercept (B) of this straight line and the migration time of the protein in the absence of the carbohydrate (t_1) by the equation $Ka = A^{-1}\, t_1^{-1}\, (Bt_1 + 1)$. The double reciprocal plot for RCA_{60} and lactobionic acid gave a straight line, and Ka could be estimated as a value of 3.3×10^3 (M^{-1}). The reproducibility of the determination of Ka was 3.6% as a relative standard deviation for five repetitions of a series of binding experiments on five successive days.

The association constant of interaction between a protein and an acidic carbohydrate can be determined as mentioned above. In this determination the carbohydrate as ligand must have an electric charge. A convenient method was investigated for conversion of neutral carbohydrates having no electric charge to a derivative having strong negative charge by dithioacetalation with 2−mercaptoethanesufonic acid sodium salt as shown in Figure 8. The yield of this derivatization was investigated for maltose series oligosaccharides. Maltose, maltotriose and maltopentaose gave the yields of 94.7%, 96.2% and 96.0%, respectively. These derivatives could be conveniently used as the negatively charged additives to running buffers for determination of their Ka values to a Lentil lectin (LCA) of glucose recognizing protein. The Ka values increased with their polymerization degree.



1. Dissolve a carbohydrate sample (40 μmol) in TFA (6 ml).
2. Add MerES-Na (250 mg),and hold the mixture for 5 min at room temperature.
3. Evaporate it to dryness in a desiccator containing NaOH.
4. Remove excess MerES-Na on a column of Sephadex G-10 (10 mm i.d., 100 cm) with water as eluate.

Figure 8. Procedure for introduction of 2−mercaptoethanesulfonate (MerES) tags to a carbohydrate.

3. 2. Reverse system [6,7]

It is difficult to separate carbohydrate derivatives having the same size by capillary zone electrophoresis. By adding a protein recognizing carbohydrate structures to a running buffer, oligosaccharide derivatives can be separated from each other. This separation technique, named “reverse system”, must be one of the separation modes to separate the compounds having micro heterogeneities. Separation of five kinds of disaccharides derivatized with 1−

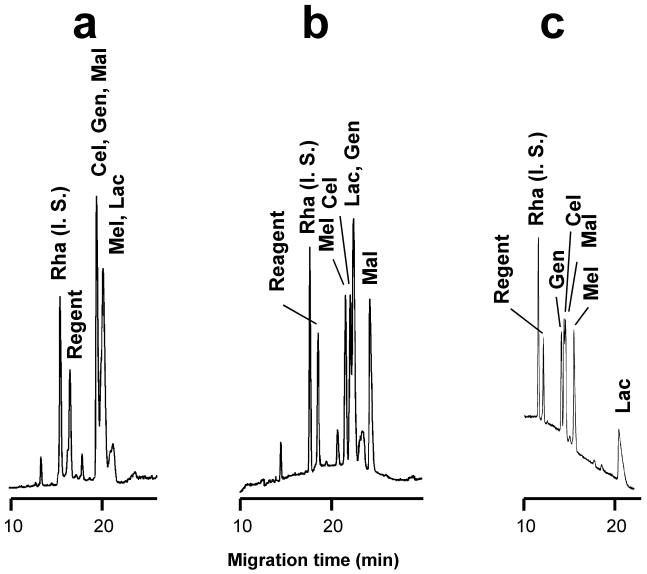


Figure 9. Separation of several disaccharides derivatized with PMP by capillary zone electrophoresis (a) and affinity capillary electrophoresis in the presence of LCA (b) or RCA_{60} (c). Capillary, linier polyacrylamide−coated fused silica (50 cm, 50 μm i. d.); running buffer, 50 mM phosphate buffer (pH 6.8) (a), 50 mM phosphate buffer (pH 6.8) containing LCA at a concentration of 10 mg/ml (b), 50 mM phosphate buffer (pH 6.8) containing RCA_{60} at a concentration of 5 mg/ml (c); applied voltage, −15 kV (a, b) or −18 kV (c); detection, UV absorption at 245 nm.

phenyl−3−methyl−5−pyrazolone (PMP) by capillary zone electrophoresis is shown in Figure 9 a. It gave group−separation of glucobioses and galactosyl glucoses. Addition of LCA having glucose and mannose specificity resulted in the splitting of these group−separated peaks into four peaks in total (Figure 9b). Addition of RCA_{60} having galactose specificity gave a quite different migration profile (Figure 9c). Since migration time delay of melibiose and lactose by addition of RCA_{60} were great, simultaneous determination of association constants of melibiose and lactose to RCA_{60} was examined by the same way as in the normal system. The plots of reciprocal migration time shifts of melibiose and lactose derivatized with PMP to reciprocal concentration of RCA_{60} gave straight lines, and determined Ka values of melibiose and lactose were 8.9 x 10^3 (M^{-1}) and 1.1 x 10^4 (M^{-1}), respectively. Based on this result a multiple solute system containing a larger number of ligands were examined. Figure 10 shows the electropherograms of 8−aminonaphthalene−1,3,6−trisulfonate (ANTS) derivatives of isomaltooligosaccharides having various degrees of polymerization in the absence (Figure 10a) and presence (Figure 10b) of LCA. Figure 10 indicated that the ANTS derivatives having any degree of polymerization were retarded in the presence of LCA, and association constants of each derivative could be determined simultaneously. It is notable that the small difference in Ka values was well differentiated and increasing tendency of Ka with degree of polymerization in these homologous series of oligosaccharides was clearly indicated.

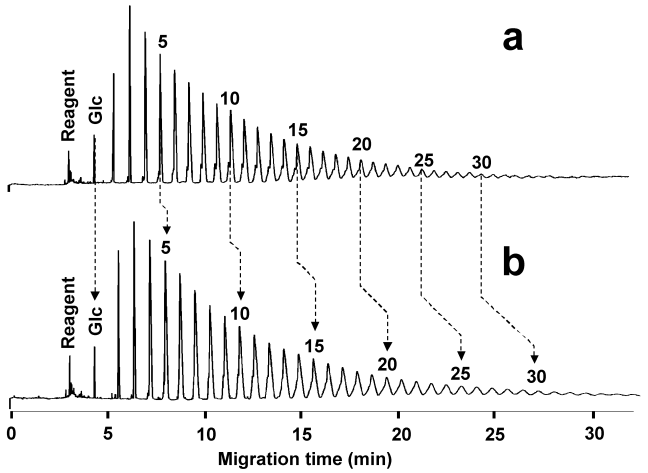


Figure 10. Electropherograms of isomaltooligosaccharides derivatized with ANTS in the absence and presence of LCA. Capillary, linier polyacrylamide−coated fused silica (50 cm, 50 μm i. d.); running buffer, 50 mM phosphate buffer (pH 6.8) (a) or the same buffer containing LCA at a concentration of 0.6 mg/ml (b); applied voltage, −15 kV; detection, UV absorption at 280 nm.

4. Conclusions

Various capillary electrophoretic analyses with chemical and physicochemical reactions were observed from our previous papers. Usefulness of in−capillary derivatization in the first half and affinity capillary electrophoresis in the latter half were mentioned in this review. The noticeable point is the convenience of carrying out various modes of analyses simply by filling appropriate running buffers and reagents into an open tubular fused silica capillary. This feature of capillary electrophoresis will allow more reliable ultra−micro scale analyses for complex functional studies.

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