Capillary electrophoretic techniques toward the metabolome analysis*

Shigeru Terabe¹,‡, Michal J. Markuszewski¹, Naoko Inoue¹, Koji Otsuka¹, and Takaaki Nishioka²

¹Faculty of Science, Himeji Institute of Technology, Kamigori, Hyogo, 678-1297 Japan; ²Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto, 606-8502 Japan

Abstract: Metabolome analysis is a systematic chemical analysis of metabolites, which may be used to investigate the metabolic activity in the cell. Capillary electrophoresis (CE) is one of the most promising techniques for the metabolome analysis, because it gives high-resolution separations in a reasonable time and requires a minimum amount of samples. General characteristics of CE are discussed from the viewpoint of metabolome analysis. Micellar electrokinetic chromatography (MEKC), a separation mode of CE, enables the separation of neutral analytes by using micelles as pseudostationary phases. MEKC is also powerful for the separation of ionic analytes to improve selectivity. To solve relatively poor concentration sensitivity with UV absorbance detection, on-line sample preconcentration techniques were developed resulting in up to few thousand-fold increases in sensitivity. Laser-induced fluorescence detection is another solution to increase concentration sensitivity, but most analytes are not natively fluorescent. Therefore, several derivatization reactions were performed to selectively detect a class of analytes with high sensitivity. Some preliminary results are shown with formic acid extracts of Bacillus subtilis.

INTRODUCTION

Genome sequencing projects have had strong impacts not only in academia but also in bioindustry and even general society. Whole sequences of genomes of several microorganisms have been published, and many interesting new facts are to be discovered. As for post-genome-sequencing projects, several large projects are running or planned. Proteome analysis or structural biology is the most active today, and metabolome analysis is soon to be developed. Metabolism is chemical reactions catalyzed by enzymes, and metabolites are transformed successively through complex pathway networks. Metabolism is never static but dynamic as the metabolic activity is regulated by gene expression of enzymes and also by activation or feedback inhibitions through metabolites. Genes coded in the genome are RNA genes and protein genes. The former consist of several hundreds of gene products, whereas the latter may total several thousands of genes, which include enzymes and a large number of unknown proteins. The number of enzyme genes increases with the size of genome and is about 1000 for Escherichia coli and Saccharomyces cerevisiae. Transferases and ligases are the major enzymes expected from genomes of microorganisms. The number of fundamental metabolic pathways and that of metabolites are nearly equal. They range from a few hundred to over a thousand metabolites or metabolic pathways for microorganisms. In order to maintain cellular homeostasis, it is necessary to keep living functions to


‡Corresponding author
regulate metabolism by enhancing or retarding the synthesis of a specific metabolite. The regulation can be done by controlling the amount of enzymes through enzyme gene expression or transcriptional regulation, or by activation or feedback inhibition of enzyme activity through metabolites. It has become possible to measure the enzyme gene expression by a DNA microarray chip.

Metabolome analysis is the systematic chemical analysis of metabolites present in a cell. Measurement of expression of the enzyme genes with a DNA microarray chip does not always show the activity of metabolism in the cell, because the activation or feedback retardation of metabolism is also regulated by the metabolites. Therefore, it is required to know the concentration of each metabolite in the cell. Metabolome analysis data can be used for (1) the simulation of the biological activity with genes coded in genome, (2) gene technology to produce valuable metabolites such as antibiotics or alkaloids, and (3) the study of functions of new genes or transcriptional regulation by using recombinant genes. In these studies, it is very useful to know the concentration profiles or changes of metabolite concentrations depending on conditions. The identification of new metabolites is also possible.

We have listed about 300 major metabolites in the cell [1], and they are classified according to the chemical structures except for water, hydrogen ion, and proteins in Table 1. These are all small molecules and many are ionic and not volatile and therefore, gas chromatography is not suitable for the comprehensive analysis of these metabolites without derivatization. We chose capillary electrophoresis (CE) to develop the comprehensive analytical techniques of these metabolites, because CE is a high-efficiency analytical separation technique with minimum sample requirements. The major problems to be solved in the metabolome analysis by CE will be detection sensitivity and identification of metabolites. In this paper, we will describe the general characteristic of capillary electrophoretic techniques and how we can apply the techniques to solve the problems. Some preliminary experimental results will be presented with cell extracts of Bacillus subtilis.

Table 1 Classification of major metabolic intermediates in the cell according to the chemical structure.

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>No. of compd.</th>
<th>Typical example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids, amines, and derivatives</td>
<td>54</td>
<td>L-glutamate, L-aspartate</td>
</tr>
<tr>
<td>Carboxylic acids</td>
<td>35</td>
<td>pyruvate, 2-oxoglutarate</td>
</tr>
<tr>
<td>Alcohols</td>
<td>3</td>
<td>glycerol</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>10</td>
<td>acetaldehyde, formaldehyde</td>
</tr>
<tr>
<td>Phosphate esters (excludes nucleotides)</td>
<td>33</td>
<td>d-glucose 1-phosphate</td>
</tr>
<tr>
<td>Nucleic acids and related compd.</td>
<td>37</td>
<td>ATP, ADP</td>
</tr>
<tr>
<td>Carbohydrates and related compd.</td>
<td>16</td>
<td>d-glucose, d-Fructose</td>
</tr>
<tr>
<td>Lipids, steroids, and fatty acids</td>
<td>19</td>
<td>estrone, cholesterol</td>
</tr>
<tr>
<td>Vitamins and coenzymes</td>
<td>45</td>
<td>NAD⁺, NADH</td>
</tr>
<tr>
<td>Inorganic ions</td>
<td>10</td>
<td>phosphate, nitrite</td>
</tr>
</tbody>
</table>

CAPILLARY ELECTROPHORESIS

General characteristics

CE is a relatively new analytical separation technique and is becoming popular among analytical chemists. Its advantages are listed as:

- The instrumental version of conventional electrophoresis and automated operation is easy.
- High-performance separation produces high plate numbers more than 100 000 up to 1 000 000, and analysis time is usually within 10 min or comparable to that of high-performance liquid chromatography (HPLC).
- Minimum sample amount requirement is usually less than pg or nL.
• There is a wide range of applications from small molecules to biopolymers and even particles such as polymer latexes of silica gels.
• Rinsing of the capillary is easy because in most techniques an open tubular fused silica capillary is used.
• Running cost is low because almost no organic solvent is used but only buffer solutions of small volumes (few mL) are required.

There are some disadvantages:
• Concentration sensitivity in detection is low, although the mass sensitivity is very high.
• Reproducibility in migration time and quantitation is slightly poor compared to HPLC, owing to slightly unstable electroosmotic velocity.
• Applications are impractical to preparative runs.

There are several separation modes in CE, and the most popular modes are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). These two modes must be the most suitable for the metabolome analysis because the metabolomes are usually small molecules.

Instruments

Several commercial CE instruments are available with a UV absorbance detector and automated sample introduction system. A fused silica capillary of 50–75-µm inside diameter and 50–100-cm length is used without or with inside surface modification. The capillary is usually thermostated at a constant temperature. Once sample solutions and running solutions are placed in relevant vials on the tray, sequential analyses are possible according to the program even under different condition for each sample. The detection sensitivity with an absorbance detector is not very high, and the concentration sensitivity is in the order of µM for most UV absorbing analytes. To improve the sensitivity, several techniques have been developed as discussed below. For non-UV-absorbing compounds, the indirect absorbance detection is available where a UV absorbing electrolyte is used as a visualizing agent and analyte peaks are detected as negative peaks. Extended pathlength absorbance detectors are commercially available, z-type [2] or bubble cells [3]: the former can generate about 10-fold and the latter few-fold increases in sensitivity without significant loss of resolution. To improve the detection sensitivity much more, laser-induced fluorescence (LIF) detection is very powerful, although available excitation wavelengths are limited and most analytes are nonfluorescent. For high-throughput analysis, a multiplex capillary electrophoresis instrument with an absorbance detection system that can run simultaneously 96 different samples under different separation conditions is available [4].

Capillary zone electrophoresis

CZE is the most versatile separation mode in CE, and only ionic or charged analytes can be separated according to the difference in charge and size of the analyte. Separation selectivity is mainly manipulated by the pH, and it is important to optimize the pH of the separation solution. The use of additives such as an organic solvent or cyclodextrin is also an effective technique to improve resolution. One of the advantages of CZE is easy optimization of resolution because changing separation conditions including running solutions are easily performed by the instrument. Prediction of the migration order is also easy if dissociation constants are known. Many enantiomeric pairs are successfully separated by using a cyclodextrin derivative as a chiral additive. Since the resolving power of CZE is high, even a minute difference in electrophoretic mobility causes the separation; e.g., oxygen isotopic benzoic acids (BAs), \( O^{16}\, O^{16}\text{-BA} \), and \( O^{18}\, O^{18}\text{-BA} \) are easily resolved in 11 min under optimized conditions due to ca. 2% difference in dissociation constants [5]. Even \( O^{16}\, O^{16}\text{-BA} \), \( O^{16}\, O^{18}\text{-BA} \), and \( O^{18}\, O^{18}\text{-BA} \) are resolved by manipulating the electroosmotic velocity.
Micellar electrokinetic chromatography

MEKC has been developed by the authors [6], and it is particularly useful for the separation of small molecules including neutral analytes. MEKC was originally developed for the separation of neutral analytes by introducing the chromatographic separation principle to electrophoresis. An ionic micellar solution is employed as a separation solution, and under the capillary electrophoretic condition the ionic micelle migrates at a different velocity from the bulk solution because the micelle is subjected to the electrophoretic migration. The micelle corresponds to the stationary phase in chromatography and therefore is called the pseudostationary phase. A fraction of the analyte is incorporated by the micelle in rapid equilibrium, having an effective electrophoretic mobility depending on the ratio of the incorporated analyte to the free analyte. The analyte free from the micelle migrates only by the electroosmotic flow, while the analyte totally incorporated by the micelle migrates at the velocity of the micelle or the sum of the electroosmotic velocity and the electrophoretic velocity of the micelle. Under neutral or alkaline conditions, the electroosmotic velocity is faster than the electrophoretic velocity of the micelle, and hence the micelle also migrates in the same direction as the electroosmotic flow. When an anionic micelle such as sodium dodecyl sulfate (SDS) is employed, all the neutral analytes migrate toward the cathode due to the strong electroosmotic flow: the less-incorporated analytes migrate faster than the more incorporated analytes by the SDS micelle. The fraction of the analyte incorporated by the micelle increases with the increase in hydrophobicity of the analytes.

Since MEKC is a chromatographic technique, the separation selectivity is manipulated by the chromatographic considerations. The choice of the surfactant, the pH and composition of the running solution, and the use of additive are important factors to manipulate selectivity. The chemical structure of the surfactant, in particular that of the polar group, affects significantly selectivity. For example, amino acid-derived chiral surfactants or bile salts can separate enantiomers. Highly hydrophobic analytes tend to be totally incorporated by the micelle and migrate at the velocity of the micelle, being unresolved. To resolve highly hydrophobic compounds by MEKC, several modifiers are developed: (1) cyclodextrin, (2) organic solvent such as methanol or acetonitrile, (3) high concentration of urea or glucose, most of which are to reduce the fraction of analytes incorporated by the micelle. Among these modifiers, cyclodextrin is particularly a useful additive for the separation of highly hydrophobic compounds as well as for the separation of enantiomers.

On-line sample preconcentration techniques

The relatively low concentration sensitivity in CE with the photometric detector is mainly due to the two reasons: the short optical pathlength nearly equal to the capillary diameter (50–75 µm) and the minute amount of sample injected (in the range of pg or nL). As mentioned above, several instrumental devices are available to enhance detection sensitivity in CE. Another approach to the enhancement of concentration sensitivity is on-line sample preconcentration. A large volume of the dilute sample solution is introduced into the capillary, and the sample zone is focused into a narrow zone followed by CE separation. Field-enhanced sample stacking and sweeping are the major preconcentration techniques. The former utilizes a high electric field observed in the sample zone by preparing the sample solution in a low electric conductivity matrix. Since the electrophoretic velocity is proportional to the field strength, analyte ions migrate at much faster velocity in the sample solution zone than in separation solution zone and stack at the boundary between the sample and separation solution zones. Deterioration of concentration efficiency in the sample stacking is caused by a mismatch of the electroosmotic flow. The electroosmotic velocity is also proportional to the field strength and must be different between the two zones due to the difference in electric field strength. However, owing to the continuity of the solution, the bulk electroosmotic velocity must be constant throughout the capillary. Therefore, mixing must occur at the boundary of the two zones. This discrepancy is minimized when the electroosmotic flow is suppressed.

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Sweeping is a preconcentration technique in MEKC recently developed by our group based on a new concept [7]. In sweeping, a homogeneous electric field is preferable different from the sample stacking, that is, the sample solution is prepared as a solution having the same conductivity as that of the separation solution or background solution (BGS). Sweeping utilizes the phenomenon that hydrophobic analytes tend to be incorporated into the micelle. As shown in Fig. 1, under a suppressed electroosmotic flow, when an ionic micelle like SDS enters continuously the long plug of the sample zone by electrophoresis from the inlet vial upon the application of the voltage, the analyte in the sample zone is picked and accumulated by the micelle at the front end of the micellar zone until the micelle reaches the end of the sample zone or the boundary between the sample zone and BGS zone. The analyte zone is focused into a very narrow zone if the interaction is strong between the analyte and the micelle, and separated by MEKC after the end of sweeping. Although sweeping is originally developed for MEKC with ionic micelles as pseudostationary phases, it is equally an efficient preconcentration technique in other electrokinetic chromatography using different pseudostationary phases such as microemulsions, charged cyclodextrins, and others. Ionic analytes are efficiently concentrated by MEKC using a micelle having opposite charge even if the separation of ionic analytes is successful without using micelles. Sweeping can increase the sensitivity up to 5000-fold in terms of concentration under optimized conditions as shown in Fig. 2. An advantage of sweeping is that sample matrix can contain relatively high concentrations of electrolytes because low conductivity is not required for the sample matrix. Although concentration efficiency is high when the electroosmotic flow is suppressed, sweeping is also powerful even under a strong electroosmotic flow. An increase of the concentration detection sensitivity of three orders of magnitude enables the UV photometric detector to detect nM ranges of concentration, which is comparable to or better than the sensitivity in HPLC with the

Fig. 1 Schematic of sweeping under the suppressed electroosmotic flow. The top two figures show the time when the application of the voltage is ready. The cathodic end of the capillary is filled with a long sample solution zone by pressure, and the electrophoretic voltage is applied after the cathodic end of the capillary is placed in the inlet vial containing the separation solution or background solution (BGS). The process of the sample concentration by sweeping is depicted in the bottom two figures. The concentrated sample zone is to be separated in the micellar zone following the focused sample zone by MEKC, because the micelle migrates faster than neutral analytes. The micellar vacancy zone migrates ahead of the analyte zones.

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absorbance detector. One disadvantage of sweeping is that it is not efficient for the preconcentration of hydrophilic analytes or weakly interacting analytes with the micelle.

**Application of on-line sample preconcentration techniques to aromatic carboxylic acids**

Many carboxylic acids are key metabolites in the cell, and to test the applicability of the sample preconcentration techniques, we chose six aromatic carboxylic acids expected to be found in the cell metabolites as test analytes because they can be easily detected with a photometric detector. They are cinnamic acid, 3-(4-hydroxyphenyl)pyruvic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylactic acid, protocatechuic acid, and 3,4-dihydroxyphenylacetic acid. These can be separated either by CZE or by MEKC. In Fig. 3, an alkaline solution (pH 9.47) was employed to keep the electroosmotic flow strong. The carboxyl groups are supposed to be totally ionized at the pH but phenolic hydroxyl groups are partially ionized to different extents causing the resolution of structurally related analytes. Figure 3 shows sensitivity enhancement by the field-enhanced sample stacking technique in comparison to the conventional CZE run. In Fig. 3B, the sample concentration was 10-fold diluted from the sample solution used in the conventional run (Fig. 3A) but 20 times larger volume was injected. The sensitivity enhancement was almost 20-fold, as can be estimated from the result in Fig. 3. The limits of detection with the sample stacking were from 0.06 to 0.24 ppm, or 0.4 to 2.0 \( \mu \)M at S/N ratio of 3. MEKC of these carboxylic acids was performed under acidic condition at pH 2.0 to increase the distribution of the carboxylic acids to the SDS micelle by suppressing the ionization. Sweeping and sample stacking techniques in MEKC were applied and almost the same sensitivity enhancements were observed in either technique as those obtained by CZE. These values are far less than the enhancement obtained for hydrophobic compounds by sweeping or sample stacking under suppressed electroosmotic flow. These preliminary results clearly show that detection sensitivity for many metabolites to be found in the cell extracts will not be strongly enhanced unless the analytes are very hydrophobic. To detect low concentration analytes in the cell extract, we have to employ high sensitive detectors such as the LIF detector or derivatize the analytes to more hydrophobic compounds.
PREPARATION OF THE CELL EXTRACT

*Bacillus subtilis* (strain 168 tryptophan requirement) was cultured in 100 mL of S6-glucose or S6-malate medium at 37 °C by shaking. The S6 medium (100 mL) contains 5 mM KH₂PO₄, 10 mM (NH₄)₂SO₄, 100 mM 3-(N-morpholino)propane sulfonate, 0.05 mg tryptophan, 20.3 mg MgCl₂·6H₂O, 10.29 mg CaCl₂·2H₂O, 0.99 mg MnCl₂·4H₂O, 0.014 mg ZnCl₂, 0.136 mg FeCl₃·6H₂O, and 25 mM glucose or 37.6 mM malate as the carbon source. The number of bacteria increased with the duration of culture, and when the cell concentration reached 4×10⁸ living cells mL⁻¹, 10 mL of culture medium was withdrawn and filtered by a glass membrane filter (Whatman GF/B 1 µm, 2.4 cm). Cells on the filter were rinsed with 3 mL of the rinse solution (100 mM K₂PO₄ pH 6.5) and immersed in 0.5 mL 1 M formic acid at 4 °C for 1 h. The total volume of the cells in 10 mL medium is 8.3 µL, and hence the volume of a single cell is 2.08 fL. The cell extract was kept at –20 °C and used for analysis.

ANALYSIS OF THE CELL EXTRACT

Survey analysis

To survey the compounds included in the cell extract, CZE analysis of the raw extract was performed using 200 mM borate buffer at pH 9.29 and absorbance at 210 nm. Only few peaks were observed with a conventional injection, and a large volume injection was needed. The results are shown in Fig. 4 for 1 M formic acid extract from *B. subtilis* cultured in both S6-glucose (Fig. 4A) and S6-malate media (Fig. 4B). About 30 peaks were observed in both electropherograms, and several minor peaks seemed identical between the two electropherograms, although significant differences in major peaks were apparent. As expected from the metabolite listed in Table 1, most of the metabolite will not absorb strongly UV, and it seems reasonable to observe the electropherograms shown in Fig. 4. We have not tried to identify these components yet. Most peaks observed in Fig. 4 were acidic or negatively charged.

compounds because they migrated slower than the electroosmotic flow. Figure 4 also suggests that the concentrations of UV absorbing neutral or basic compounds were lower than the detection limit.

**Laser-induced fluorescence detection of amino acids**

Amino acids are important metabolites in the cell, but most amino acids do not have strong chromophores for photometric detection. Therefore, derivatization is required to UV absorbing or fluorescent forms. To obtain high sensitive detection, we employed LIF detection with argon ion laser (488 nm) as an excitation source. There are several derivatizing reagents available for LIF detection with excitation at 488 nm. Fluorescein isothiocyanate (FITC), 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) and 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQA) were chosen in this study. To derivatize amino acids with these reagents, the formic acid extract was neutralized to slightly alkaline (pH 8.5) with potassium carbonate, and the labeling reagent was added together with other catalysts if necessary. FITC has the maximum excitation wavelength of 489 nm, which is close to 488 nm and generated low detection limits down to nM range. The disadvantages of FITC derivatization are a relatively long reaction time required and production of several by-products that emit fluorescence. Since the cell extract contains many components, the by-product peaks interfere with detection of some metabolites having the same migration times as the by-products. Therefore, FITC does not seem an appropriate derivatizing reagent for the purpose of the comprehensive analysis of the metabolites.

NBD-F has the maximum excitation wavelength of 475 nm lower than the argon ion laser emission. However, NBD-F reacted in a short time and only few by-product peaks were observed as shown in Fig. 5. The separation conditions are not optimized yet, but MEKC conditions were chosen because MEKC is superior to CZE in the separation of structure-related compounds. In derivatized amino acids, the labeling group is larger than the amino acid residues, and hence the difference in electrophoretic mobilities among the labeled amino acids must be small. However, in MEKC the separation selectivi-

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**Fig. 4** Electropherograms of formic acid extracts from the cultured *Bacillus subtilis* cells in S6-glucose (A) and S6-malate (B) media. Conditions: capillary, 50 µm i.d. × 64.5 cm (56 cm to the detector); running solution, 200 mM borate buffer (pH 9.29); injection, hydrostatic 20 s at 5 kPa, applied voltage, 20 kV; detection wavelength, 210 nm.
ty mainly depends on the difference in hydrophobicity, and the structural differences in amino acid residues contribute significantly to selectivity—even the residue groups are minor parts of the whole molecules. Essential amino acids were confirmed by spiking with standards; however, the identity of some peaks in Fig. 5 is not known yet. The concentrations found in the electropherogram are in the order of sub μM range, which is lower than expected, although the procedure is not validated yet.

The maximum excitation wavelength of CBQA is 468 nm and far less than 488 nm, but CBQA also showed good results for the detection of amino acids after derivatization. No by-product peak was found in the separation of a standard mixture of amino acids. Since we have not performed extensive analysis of the cell extracts yet, we do not have reliable quantitative data on amino acids in the cell. However, we estimate the concentrations of major amino acids in the cell extract are from sub μM to tens of mM, and, therefore, high sensitive detection methods are not always necessary. We have not applied the sample preconcentration techniques to the analysis of the derivatized analytes in the cell extract, but the combination of the derivatization and preconcentration will make the photometric detector powerful and useful because the derivatized analytes usually have higher hydrophobicity and tend to be more incorporated into the micelle.

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REFERENCES


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