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Minireview

Profiling of primary metabolite by means of capillary electrophoresis-mass spectrometry and its application for plant science

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Abstract Metabolites in primary metabolic pathways, such as glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway, the Calvin cycle and amino acid biosynthetic pathways, are ionic compounds. It is difficult to analyze these metabolites simultaneously by established methods for metabolite profiling of biological samples such as gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry. Capillary electrophoresis-mass spectrometry (CE-MS) is a promising method for the comprehensive and quantitative analysis of ionic metabolites. This technique enables the simultaneous determination of over 1000 charged compounds. This review discusses the principles and practical procedures for conducting CE-MS, describes specific applications of CE-MS for primary metabolite profiling, and enumerates some of the technical problems associated with CE-MS at present.

Key words: Anionic metabolite, capillary electrophoresis, cationic metabolite, mass spectrometry, metabolite profiling.

In the current post-genomic era, metabolomics has attracted much attention of to the biologists (reviewed by Fukusaki and Kobayashi 2005; Schauer amd Fernie 2006). Metabolomics has been applied to diverse research areas such as examining the effects of genetic and environmental manipulations (Roessner et al. 2001), genotyping (Taylor et al. 2002), investigating unknown gene functions (Hirai et al. 2004), screening of novel biomarkers (Soga et al. 2006) and so on. Advances in analytical techniques for comprehensive and simultaneous analyses of primary metabolites, such as amino acids, amines, peptides, nucleosides, nucleotides, sugar phosphates, organic acids, coenzyme A (CoA) and so on, has promoted the field of metabolomics. These primary metabolites are intermediates of essential metabolic pathways including glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, the Calvin cycle and amino acid biosynthetic pathways. Metabolite profiles of these pathways are minimum requirement for consideration of intracellular metabolism.

Almost all of these primary metabolites are ionic and non-volatile compounds, properties that make comprehensive instrumental analysis difficult. Recently, gas chromatography-mass spectrometry (GC-MS) (Fiehn et al. 2000), liquid chromatography-mass spectrometry (LC-MS) (Tolstikov et al. 2003; Wilson et al. 2005), Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICRMS) (Aharoni et al. 2002; Brown et al. 2005), nuclear magnetic resonance spectrometry (NMR) (Ward et al. 2007) and other methods have been developed for application of the comprehensive analyses of metabolites. But these methods have many problematic properties. GC-MS can only analyze volatile compounds. The derivatization to increase a volatility of non-volatile metabolite is required but this process decreases recovery rate and lowers the accuracy of quantification. Reversed phased LC-MS does not achieve efficient separation of ionic compounds. Of course, MS analysis with direct injection, which is widely used in FT-ICRMS analysis, does not temporally separate metabolites. These methods are hard to distinguish a large number of metabolic isomers i.e. glucose 6-phosphate and fructose 6-phosphate. Insufficient separation of crude samples also leads to ion suppression and reduced accuracy in MS analysis. Ion chromatography (IC)-MS (van Dam et al. 2002; Sekiguchi et al. 2005), and ion pair (IP)-LC-MS (Luo et al. 2007) can separate ionic compounds; however, a membrane suppressor is required for the connection between the IC and MS in order to reduce the salt concentration of the eluent. In the case of IP-LC-MS,

Abbreviations: CE, capillary electrophoresis; CoA, coenzyme A; EOF, electroosmotic flow; ESI, electrospray ionization; MRM, multiple reactions monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TCA, tricarboxylic acid cycle; TOF-MS, time of flight mass spectrometry

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ion-pair salts lead ion suppression in MS analysis. NMR is insufficiently sensitive for primary metabolite profiling.

Capillary electrophoresis–mass spectrometry (CE-MS) is a promising technique for analyzing ionic compounds. In CE, the sample solution is first injected into a capillary. Next, high voltage (-30 kV) is applied to the capillary. Then, cationic and anionic compounds migrate to the cathode and anode, respectively, by electrostatic force. Electrophoretic mobility of the analyte is based on the ratio of charge to size of the solvate analyte ion. CE separates the analytes whose ratios of charge to size are different. Even structural isomers can be separated by CE, because the sizes of solvates of structural isomers, which possess same mass, are different. An electrospray ionization (ESI) source enables the connection between CE and MS. Mass spectrometers connected to CE measure the mass-to-charge ratio (m/z) of analyte ions, resulting high selectivity and sensitivity. Moreover, the system allows analysis without chemical derivatization that avoids reductions in recovery rate and quantification accuracy.

Despite these advantages, CE-MS has not been widely used. In this article, we introduce a primary metabolite profiling method using CE-MS, highlight the practical points for preparing samples and running CE-MS, and discuss potential technical problems.

Cationic metabolite analysis

Metabolites in biological samples possess various functional groups. The ionic functional groups in biological metabolites include amino, alcohol, phenol, carboxyl, thiol, phosphate and sulfate groups. All of these functional groups, except for sulfate, are protonated at pHs lower than 2. Therefore, the metabolites possessing amino and sulfate group(s) have positive and negative charge(s), respectively. All other metabolites have no charge at pHs lower than 2. CE-MS using a low pH electrolyte in a positive mode, which sets the anode at the inlet (CE side) and the cathode at the outlet (MS side), allows positive ions to migrate toward the MS and be separated. Based on the above-mentioned principle, metabolite profiling for amino acids, nucleosides, peptides and other amines is usually performed under such CE-MS analytical conditions (Soga and Heiger 2000; Soga et al. 2003; Sato et al. 2004; Harada et al. 2006a).

CE-ESI-MS with a low pH electrolyte is very stable for the following reasons. Silanol groups on the inner wall of fused-silica capillaries that are widely used for CE-ESI-MS, are also protonated completely at pHs lower than 2. Moreover, the tolerance of silica to low pH is very high. Therefore, the charge on the inner wall surface of the capillary is uniform. The presence of this uniform charge leads to a very slow and stable electroosmotic flow (EOF). The stable EOF ensures repeatability of migration time and peak area, parameters that correspond to levels of analyte.

In cases where a low pH is necessary for ESI-MS, volatile acids such as formic acid or acetic acid are commonly used as the electrolyte. Soga and Heiger (2000) showed that 1.0 M formic acid (pH 1.8) generated a high number of theoretical plates and resulted in complete resolution between isoleucine and leucine. Previously, our laboratory demonstrated that 1.5 M acetic acid added to 1.0 M formic acid resulted in even higher levels of resolution (Harada et al. 2006a).

A non-treated fused silica capillary is the most appropriate type for cationic metabolite profiling. There are no advantages to using physically or chemically modified capillaries. Moreover, fused silica capillaries are less expensive than other types of capillaries.

To obtain the best ionization efficiency and spray stability, the composition and flow rate of the sheath liquid should be optimized for CE-MS (reviewed by Ohnesorge et al. 2005). The polar organic solvents such as methanol and isopropanol are most frequently used as 1 : 1 mixtures diluted with water, resulting in a very robust sheath liquid. Furthermore, low amounts of volatile acids help to stabilize the spray as well as the CE current. Formic acid is preferred over acetic acid due to less ion pairing. The flow rate of the sheath liquid should be as low as possible to reduce dilution, but should not fall below a certain value, mainly to keep the spray stable. Flow rates of several μL $\min^{-1}$ for the sheath liquid are appropriate.

Cationic metabolite profiling by CE-ESI-MS succeeded in identifying specific metabolites whose levels were altered by knockout of genes with unknown functions or by chemical treatment. Soga et al. (2006) presented the profiling of mouse metabolites following acetaminophen-induced hepatotoxicity. CE-quadrupole-TOF-MS analysis revealed that ophthalmate levels in mouse liver increased as a result of acetaminophen treatment. In addition, these investigators found that serum ophthalmate is a sensitive indicator of hepatic glutathione depletion and may be a new biomarker for oxidative stress.

Watanabe et al. (2008) reported non-targeted metabolite profiling of T-DNA-inserted knockout mutants of *Arabidopsis* by CE-ESI-TOF-MS. Prior to this report, the physiological roles of the β-substituted alanine synthase (Bsas) gene family in plants had not been clear. The authors showed that the level of γ-glutamyl-β-cyano-alanine decreased in one of the Bsas mutants, *bsas3;1*. This result indicated that Bsas3;1 is primarily responsible for the synthesis of β-cyano-alanine and, subsequently, γ-glutamyl-β-cyano-alanine *in vivo*.

Anionic metabolite analysis

The intermediates of primary metabolic pathways including glycolysis, the TCA cycle, the pentose phosphate pathway, and the Calvin cycle are sugar phosphates, organic acids and CoA compounds. Nucleotides such as ATP, NAD and NADPH are important cofactors in these metabolic pathways. All of these metabolites possess carboxyl and/or phosphate groups. Cationic metabolite profiling by CE-MS is not able to separate isomers of sugar phosphates and organic acids. The method is not appropriate for detection of nucleotides and CoA compounds due to low ionization efficiency in the positive mode.

In order to transfer anionic metabolites to MS by electrostatic force, the polarity of CE should be set in a negative mode; the inlet of the capillary is at the cathode and the outlet is at the anode. In electrophoresis using a normal fused silica capillary, EOF occurs toward the cathode because the silanol groups on the capillary inner wall have negative charges. Moreover, the capillary outlet in a CE-MS system can not be immersed in the vial electrolyte. Therefore, in a negative mode, the EOF creates an air gap in the capillary at the outlet, resulting in a current drop.

The following two methods can prevent current drops during anionic metabolite profiling: (i) generating a reversed EOF, or (ii) setting the CE polarity to a positive mode and generating a fast EOF that allows anions to migrate toward the cathode. Soga et al. (2002a) developed the former method using a SMILE(+) capillary (Katayama et al. 1998), which is a cationic polymer (polybrene)-coated capillary. Because the capillary inner wall is positively charged, the capillary can constantly reverse EOF toward the anode, thus enabling the successive analysis of sugar phosphates and organic acids without a deleterious current drop in the negative mode. These investigators have also developed a pressure-assisted capillary electrophoresis technique using DB-1, a polydimethylsiloxane-coating on the inner capillary wall (Soga et al. 2002b). DB-1 suppresses EOF completely, thus application of air pressure to the electrolyte vial at the inlet provides electrolyte flow toward the MS instead of EOF. Comprehensive analysis of anionic metabolites from biological samples by using a combination of these systems was achieved. In addition, a polyethylene glycol-coated capillary instead of DB-1 has also been used for anionic metabolite profiling (Takahashi et al. 2006a).

Unfortunately, the CE-MS method using the SMILE (+) capillary can not accurately determine nucleotides and CoA compounds since the cationic polymer absorbs multivalent anions. The method employing capillaries with DB-1 coating does not obtain efficient separation of sugar phosphates and organic acids due to the following two disadvantages. First, the pressure-assisted flow causes peak diffusion. Secondly, a high pH electrolyte is required for separation of anionic metabolite isomers; however, electrolyte pH must be set near neutral due to low tolerance of DB-1 to high pH.

For the second method for avoiding a current drop, Sawada and Nogami presented a CE-MS method for the analysis of organic acids with a non-treated fused silica capillary and an alkaline running buffer in a CE set up with positive polarity (Sawada and Nogami 2004). High pH (>8.0) provides fast EOF in a fused silica capillary that facilitates stable electrophoresis without a current drop in the positive mode of CE. This method has been used for comprehensive anionic metabolite profiling (Harada et al. 2006b). We showed high pH condition that also provides high resolution anionic metabolite isomers. Moreover, we applied an electrolyte flow using an air pump after electrophoresis to prevent peak diffusion of organic acids. The method allowed 54 species of anionic metabolites to be measured in a single run.

In addition, we studied on a separation of CE by using sulfonated capillaries has also been investigated (Harada et al. 2008). Kodama et al. developed a sulfonated capillary that is chemically coated with sulfonated groups on the inner wall (Kodama et al. 2005). EOF of sulfonated capillaries is greater and more stable than that of untreated, fused silica capillaries within the range of pH 2–9. This coating type improves the repeatability of migration time of anionic metabolite profiling with CE-MS.

Anionic metabolite profiling by CE-ESI-MS has been especially useful in revealing metabolic responses in glycolysis and the pentose phosphate cycle. Kinoshita et al. (2007) showed the hypoxia-induced consumption of upstream glycolytic substrates, such as glucose 6-phosphate, and increased downstream products, such as phosphoenolpyruvate, in human erythrocytes. Ishii et al. (2007) reported on the response of *Escherichia coli* cells to genetic and environmental perturbations. Variations in metabolite levels of metabolic enzyme gene disruptants were smaller than the variations in mRNA and protein levels. This study suggested that *E. coli* seems to use complementary strategies that result in a metabolic network robustness against perturbations.

Takahashi et al. (2008) examined the metabolic shift from photoautotrophic to photomixotrophic conditions in *Synechocystis* sp. PCC 6803. Changes in levels of metabolites in glycolysis, the pentose phosphate pathway, and the Calvin cycle when glucose was added to the photoautotrophically grown culture were observed clearly. This study indicated the usefulness of CE-MS anionic metabolite profiling to the study of photosynthetic metabolism in plants.

Several studies on anionic metabolite profiling of plants, such as *Arabidopsis* (Takahashi et al. 2006b,

Watanabe et al. 2008), rice (Sato et al. 2004; Takahashi et al. 2006a) and *Catharanthus roseus* (Harada et al. 2008) have already been reported. In the future, we expect that the application of CE-MS anionic metabolite profiling to problems in plant biology will increase.

Sample preparation

In metabolite profiling, the sample preparation process is a very important issue. It is necessary to rapidly quench enzymatic activity and efficiently extract all targeted metabolites with a universal procedure. Sample preparation methods for plant tissues and cultured cells were well documented by Sato et al. (2004) and Harada et al. (2008). Freezing with liquid nitrogen is widely used to temporarily stop enzyme activity. Turnover of primary metabolites is very fast. Thus, reducing the time period from harvesting to freezing is a simple, but difficult problem. Researchers should pay special attention to this point. Because plant tissues and cells are very hard, homogenization should be performed. The use of a polar organic solvent, such as methanol, is acceptable for extracting highly polar metabolites. In order to break lipid membranes and inactivate enzymes, the extraction solvent should not include water. Hydrophobic compounds, such as lipids and chlorophylls, and proteins in the sample solution interfere with CE-MS analysis. Thus, removing these contaminants is also very critical. Liquid–liquid fractionation by the addition of water and chloroform to the methanol-extract solution allows the effective removal of contaminants. Ultrafiltration is an efficient method to remove proteins and can also remove liposomes that are generated by aggregation of phospholipids and chlorophylls. To obtain sufficient sensitivity for the monitoring of low-level metabolites, especially sugar phosphates and coenzymes, the prepared solution should be concentrated by evaporation or lyophilization. Solid phase extraction is often applied to sample preparation in GC-MS and LC-MS; however, this technique has disadvantages with respect to recovery rates of analytes and the introduction of complicated processes.

Problems

Although CE-MS is a promising method for metabolite profiling, this approach has not been popular compared to GC-MS and LC-MS due to the reduced stability and sensitivity of the system. As for the stability of CE-MS, the reduced repeatability of migration time is the most serious problem that affects the repeatability of quantifying values and also prevents system automation. Analyte migration time is affected by capillary temperature, the electrostatic state of the capillary inner wall, the extent of contaminants in the sample and so on. The operator should maintain a constant capillary temperature and attempt to improve the repeatability of migration time by physical and chemical modification of the capillary inner wall. Development of the sulfonated capillary is one example of how to control analyte migration time. Soga et al. also developed programs to align the analyte migration time and tools to display the difference. Such tools will accelerate the general use of CE-MS for analyzing plant metabolites.

Improvement of CE-MS is also an important issue. The absolute detection limit is about 0.1–100 fmol in our CE-MS system that is used for anionic metabolite profiling. This value is almost the same as the detection limit for LC-MS; however, the detection limit expressed as the analyte concentration is about 0.1–10 μM. Therefore, we are not able to conclude that CE-MS achieves a higher sensitivity than LC-MS. The injection volume in CE-MS analysis is about several nanoliters. The very small consumption of sample solution is one of the advantages of CE-MS analysis; however, this is disadvantageous with respect to sensitivity. Several microliters of sample solution must be prepared for injection to the CE-MS. Typically, operators are not able to concentrate sample solutions to a sufficiently small volume for injection. A technique in which only a small volume is prepared and injected into a CE-MS capillary would be very useful.

Additionally, sheathless CE-MS appears to be a promising technique for improving sensitivity (reviewed by Issaq 2004; Zamfir 2007). Generally, in CE-MS analysis, a sheath liquid that includes polar organic solvents and additive reagents is required for ionization of analyte in ESI and maintenance of electroneutrality at the capillary outlet. In the sheathless CE-MS method (Ishihama et al. 2002; Edwards et al. 2006a) an electrolyte is used that includes organic solvents to improve ionization of the analyte in ESI. The mechanical setting of the ESI source is also important. Although the sheathless CE-MS is not conventional at present, further development of this method is expected.

Perspectives

Applications of CE-MS for not only profiling of primary metabolites, but also for secondary metabolites (Bringmann et al. 2005; Edwards et al. 2006b; Wahby et al. 2006) and plant hormones (Ge et al. 2006) have been reported. These methods achieve sensitive and high resolution analyses with high throughput manner. Moreover, some recent investigations demonstrated the monitoring of stable isotope labeling in primary metabolites by CE-MS (Harada et al. 2006a; Toya et al. 2007), a technical advance that will accelerate accurate metabolic flux analyses. Combining these applications of CE-MS technology and primary metabolite profiling

should reveal new levels of details about plant metabolism.

Recently, the technology of LC-MS for profiling ionic metabolites has also been developed; however, the many advantages of CE-MS for profiling ionic metabolites are not provided by LC-MS. For the future, we expect that CE-MS will continue to contribute fundamental information about metabolic pathways for the biosciences, including the plant sciences.

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