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Review

Applications of in-capillary reaction micellar electrokinetic chromatography in the food industry

Toshiro Watanabe[a,*], Shigeru Terabe[b]

[a]*Yaegaki Technology Development Laboratories, Yaegaki Bio-industry, Inc., 681 Hayashida, Himeji, Hyogo 679-4298, Japan*
[b]*Faculty of Science, Himeji Institute of Technology, Kamigori, Hyogo 678-1297, Japan*

Abstract

This review describes the quantitative analysis of in-capillary reactions by using capillary electrophoresis (CE) in the food industry. An electrophoretic analysis of products of an enzyme reaction of a substrate by in-capillary reaction was useful for the activity measurement of glucoamylase in *sake* rice *koji*. *p*-Nitrophenyl-β-D-maltoside was employed as a substrate and *p*-nitrophenyl-β-D-glucopyranoside was the product of the enzyme reaction. The glucoamylase activity of *sake* rice *koji* samples gave a good linear relationship with the peak area observed in the in-capillary enzyme reaction method. Also, in-capillary micellar electrokinetic chromatography (MEKC) was used for analyzing the *Monascus* pigment-mediated degradation of mutagenic 3-hydroxyamino-1-methyl-5H-pyrido[4,3-*b*]indole. During the electrophoresis, the mutagen and the pigment, due to their different migration velocities, mix for a certain period of time to interact, and then they are separated and quantitated. The in-capillary reaction MEKC method can be applied to the routine quality control of enzyme activities in the food industry and the evaluation of mutagenic compounds in food materials.

Keywords: Reviews; Food analysis; In-capillary reaction micellar electrokinetic chromatography; Micellar electrokinetic chromatography; *Monascus*; Rice; Glycoamylase; Amines, heterocyclic; Enzymes

Contents

1. Introduction

Recently, the microscale capabilities of capillary electrophoresis (CE) and the high specificity of enzyme reactions has been realized in biochemical analysis by combining CE and enzyme microreactors in a technique known as in-capillary electrophoresis reaction [1–25]. In in-capillary electrophoresis re-

*Corresponding author.

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action, different electrophoretic mobilities are used to merge distinct zones of analyte and analytical reagent under the effect of an electric field. The reaction is allowed to proceed within the region of mixed reagents either in the presence or absence of an applied potential, and the product migrates to the detector under the effect of an electric field. In this review two examples of applications of in-capillary reaction CE are introduced from the recent work by the authors.

2. Capillary electrophoresis

CE is a relatively new separation technique; it has an overall simplicity of instrumentation and can offer simpler method developments, minimal sample volume requirements, and lack of organic solvent waste. Numerous reviews and books have been published on various aspects of CE. A fundamental constituent of CE separation is electroosmotic flow (EOF). EOF is the bulk flow of liquid in the capillary and is a consequence of the surface charge on the inside capillary wall. Capillary zone electrophoresis (CZE) is the most popular separation mode for CE, where analytes are separated on the basis of differences in electrophoretic mobilities, which depend on the charge and molecular sizes. Simultaneous separation of both anionic and cationic analytes is possible by CZE due to strong EOF. Neutral solutes do not migrate by electrophoresis and all are coeluted with the EOF. Many of the applications of CZE have been in the bioscience area.

3. Micellar electrokinetic chromatography

Micellar electrokinetic chromatography (MEKC) is a method based on micellar solubilization employing the instrumental technique of CZE. In MEKC, a charged surfactant is added to the electrolyte solution, forming micelles, which work as a pseudo-stationary phase of chromatography. The anionic micelle, such as sodium dodecylsulfate (SDS), migrates toward the anode by electrophoresis. However, the strong EOF transports the bulk buffer solution and even anionic micelles toward the cathode under neutral or alkaline conditions. Neutral or charged compounds are incorporated into the micelles and as a result they move by electrophoresis in the capillary. Mixtures of neutral species as well as mixtures of charged and neutral compounds can be separated by MEKC. Many of the applications of MEKC have been applied to food constituents [26–41].

4. In-capillary electrophoresis reaction

Zhao and Gomez reported [7,21] on the quantitative analysis of in-capillary enzyme-catalyzed microreactions using CE. A plug of the substrate solution and that of the enzyme solution were injected separately into a capillary filled with a running buffer and the voltage was applied. Extent of reaction and product ratios were subsequently determined by CE. This concept is demonstrated using several model systems: the conversion of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide, reduced form (NADH) by glucose-6-phosphate dehydrogenase [7]. The point of the technique is based on the fact that electrophoretic mobilities of reagents and products are different under an electrophoretic condition. The technique was extended also to the double-catalyzed system [21].

Taga and co-workers reported [22–24] on the microreaction by in-capillary derivatization method. They proposed three techniques for in-capillary derivatization and evaluated them using amino acids and *o*-phthalaldehyde (OPA) as a model system [24]. In the at-inlet technique [22], the amino acids (sample) solution and the OPA (reagent) solution are introduced successively at the anodic inlet of the capillary and the two zones are allowed to mix and to react for a certain length of time (e.g., 20 min). The derivatized amino acids are then separated by applying voltage. The at-inlet technique gives better S/N ratios and moderate plate numbers [24]. In the zone-passing technique [23], a running solution zone is introduced between the sample and reagent zones mentioned above and the voltage is applied immediately after the introduction of the reagent zone. The derivatization reaction occurs while the reagent zone passes the sample zone and the derivatized amino acids are separated by electrophoresis. Therefore, the

reagent zone must migrate faster than the sample zone: OPA migrates by electroosmosis only, but the negatively charged amino acids migrate slower than the EOF. The technique gives low S/N ratios and low plate numbers among the three techniques [24]. Throughout the capillary technique [24], the whole capillary is filled with the reagent solution that is also the running solution, and the sample solution is introduced as a short zone at the anodic end. The amino acids react with the reagent during the migration by electrophoresis, but the migration velocities are different between the underivatized amino acids and OPA-derivatized amino acids, causing asymmetrical peaks for slow reacting amino acids. The technique is the simplest and gives moderate S/N ratios and better plate numbers among the three techniques [24].

There are many reports of in-capillary reaction methods for achieving enzyme assays by CE. The conditions of in-capillary reaction method are summarized in Table 1.

5. Enzyme activity assays by in-capillary micellar electrokinetic chromatography

An in-capillary reaction electrophoretic MEKC analysis of an enzyme reaction product was useful for the activity measurement of glucoamylase in *sake* rice *koji* [42,43]. The *sake* rice *koji* is the raw material used for *sake* brewing. *Sake* is the most popular alcoholic beverage in Japan. The mechanism for brewing *sake* is in the marriage of the *koji* mold (*Aspergillus oryzae*), which produces both α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3). These enzymes convert starch to glucose (Glc), and *sake* yeast (*Saccharomyces cerevisiae*), which is similar to the yeast used in brewing of beer, produces ethanol from Glc. Therefore, these enzymes are very important for the brewing process of *sake*. These enzyme activities in *sake* rice *koji* are usually measured by the official analytical method of the National Tax Administration Agency of Japan, although the measurement of glucoamylase activity needs a long time (2 days) for completion of the assay.

The principle of the in-capillary reaction MEKC method is shown schematically in Fig. 1. The PNP-Mal was converted to Glc and *p*-nitrophenyl-β-D-glucopyranoside (PNP-Glc) by glucoamylase, but the PNP-Mal was not converted to Glc and PNP-Glc by α-amylase in *sake* rice *koji* extract. The produced PNP-Glc and unreacted PNP-Mal were separated and detected by MEKC using sodium cholate (SC) micelles as the pseudo-stationary phase. This in-capillary MEKC method is similar to the at-inlet derivatization method of Taga and Honda [22], but the voltage is applied immediately after injection of the two zones. Therefore, this technique may be classified as the zone-passing mode proposed by Taga et al. [23]. It should be noted that both the unreacted substrate and the reaction products are detected, while the total amount of the substrate is kept constant. Therefore, an internal standard is not

Table 1
Representative applications of in-capillary reaction methods

Method	Enzyme	Product	Detection	Ref.
Electrophoretically mediated microassay (electrophoretic mixing)	Alkaline phosphatase, β-galactosidase	*p*-Nitrophenol	405 nm	[1]
Electrophoretically mediated microassay (electrophoretic mixing)	Glucose-6-phosphate dehydrogenase	NADPH	340 nm	[2]
Combination of on-column reaction and CE	Lactate dehydrogenase	NADH	Laser-induced fluorescence	[3]
On-column digestion	Pepsin	Digested peptides of β-lactoglobulin	Laser-induced fluorescence	[4]
Surface-immobilizing RNA-modifying enzymes	Ribonuclease T_1 and U_2	RNA fragments	258 nm	[8]
On-line microreactor	Trypsin	Digested peptides of oxidized insulin B-chain	CE-MS	[17]

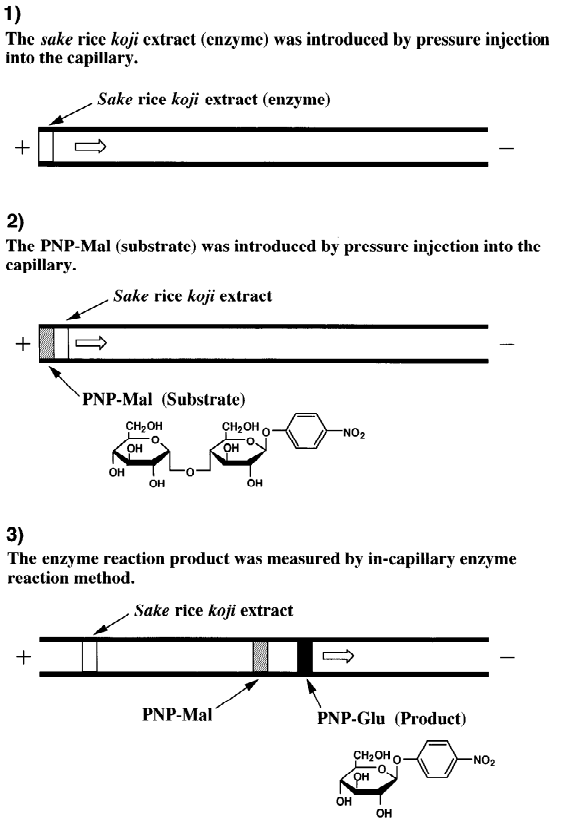

Fig. 1. Schematic representation of the measurement of glucoamylase activity by the in-capillary enzyme reaction method.

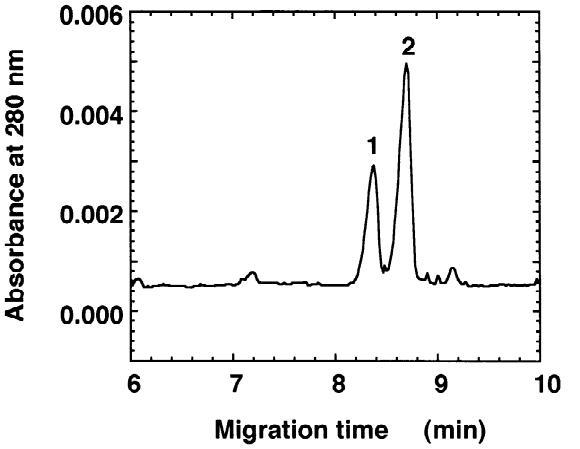

Fig. 2. Electropherogram of the hydrolysis mixture of PNP-Mal using *sake* rice *koji* extract by the in-capillary enzyme reaction method [43]. MEKC conditions: capillary, 36 cm×50 μm I.D.; running solution, 25 m*M* sodium cholate solution in 25 m*M* phosphate buffer at pH 7.0; injection, 70 mbar (pressure) for 1.0 s; applied voltage, 15 kV; temperature, 20°C; detection, 280 nm; peak identification, (1) PNP-Glc, (2) PNP-Mal; and glucoamylase activity, 218 units/g *koji*.

required. Fig. 2 shows in-capillary reaction MEKC of unreacted PNP-Mal and hydrolyzed PNP-Glc by the *sake* rice *koji* enzyme. This reaction was not repressed by the addition of SC. Dependence of PNP-Glc peak area on glucoamylase activities was measured with 20 fresh *sake* rice *koji* and five dried *sake* rice *koji* samples, with activities from 100 to 300 units/g *koji* by the in-capillary enzyme reaction MEKC method, giving a straight line (r=0.999). The glucoamylase activity of dried *sake* rice *koji* samples gave the same linear correlation as obtained for the extract from the native samples by this method. The glucoamylase activity in *sake* rice *koji* was measured more easily by this method than by the official method.

6. Evaluation of mutagenic compounds by in-capillary micellar electrokinetic chromatography

The authors studied [44] antimutagenic pigment-mediated decomposition of mutagenic 3-hydroxyamino-1-methyl-5H-pyrido[4,3-*b*]indole [Trp-P-

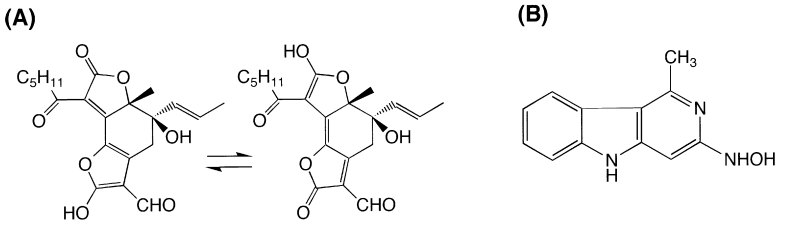

Fig. 3. Structures of xanthomonasin A (A) and Trp-P-2(NHOH) (B). The fungi *Monascus* produces the antimutagenic yellow pigment, xanthomonasin A.

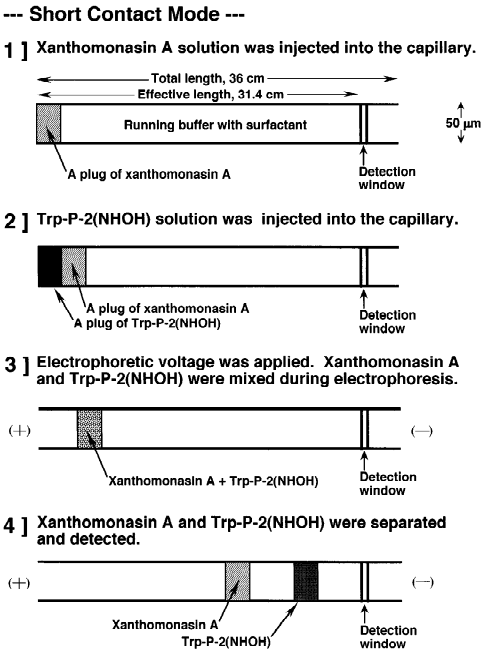

Fig. 4. Scheme for analysis using the short contact mode.

2(NHOH)] and 2-hydroxyamino-3-methylimidazo-[4,5-*f*]quinoline [IQ(NHOH)] by the in-capillary reaction MEKC. Izawa et al. reported [45] the inhibitory effects of *Monascus* yellow pigment (xanthomonasin A) against the direct-acting mutagenicity of Trp-P-2(NHOH), and of several heterocyclic amines, as assayed by the *Salmonella* mutagenicity test [46]. Structures of antimutagen (xanthomonasin A) and mutagen [Trp-P-2(NHOH)] are shown schematically in Fig. 3. The analysis of *Monascus* pigment-mediated degradation of mutagenic compounds, Trp-P-2(NHOH) and IQ(NHOH), using in-capillary MEKC were performed using two modes, and the principle of the technique is shown schematically in Figs. 4 and 5. The former mode, a short contact mode (Fig. 4), is similar to the zone-passing mode described by Taga et al. [23], although no buffer zone is introduced between the pigment zone and the mutagen zone. The latter mode, long

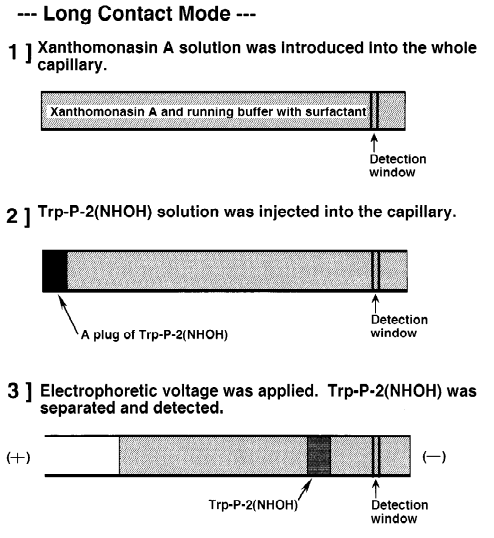

Fig. 5. Scheme for analysis using the long contact mode.

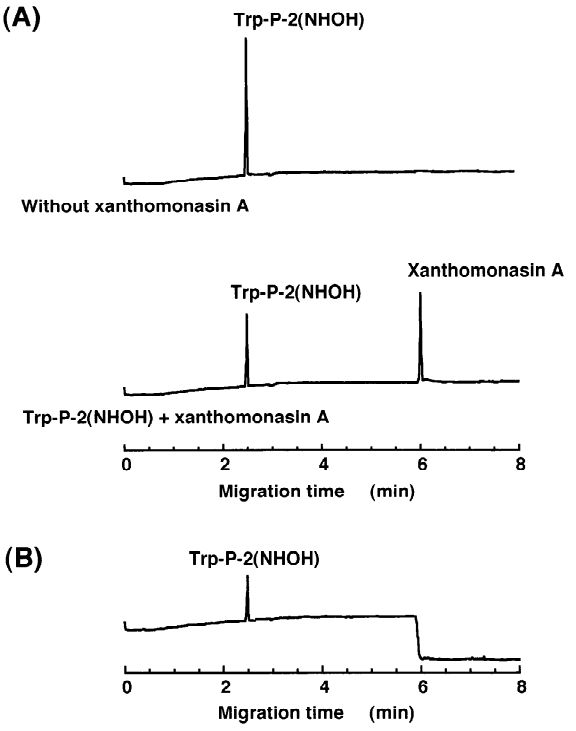

Fig. 6. Separation of xanthomonasin A and remaining Trp-P-2(NHOH) in the short contact mode (A) and in the long contact mode (B) [44]. (A) Short contact mode MEKC conditions: capillary, 36 cm×50 μm I.D.; running solution, 10 m*M* sodium cholate solution in 25 m*M* phosphate buffer at pH 7.0; applied voltage, 15 kV; temperature, 20°C; detection, 264 nm; and concentration, xanthomonasin A=0.1 m*M*, Trp-P-2(NHOH)=0.1 m*M*. (B) Long contact mode MEKC conditions: running solution, 10 m*M* sodium cholate solution in 25 m*M* phosphate buffer containing 0.1 m*M* xanthomonasin A at pH 7.0. The other conditions are the same as in the short contact mode.

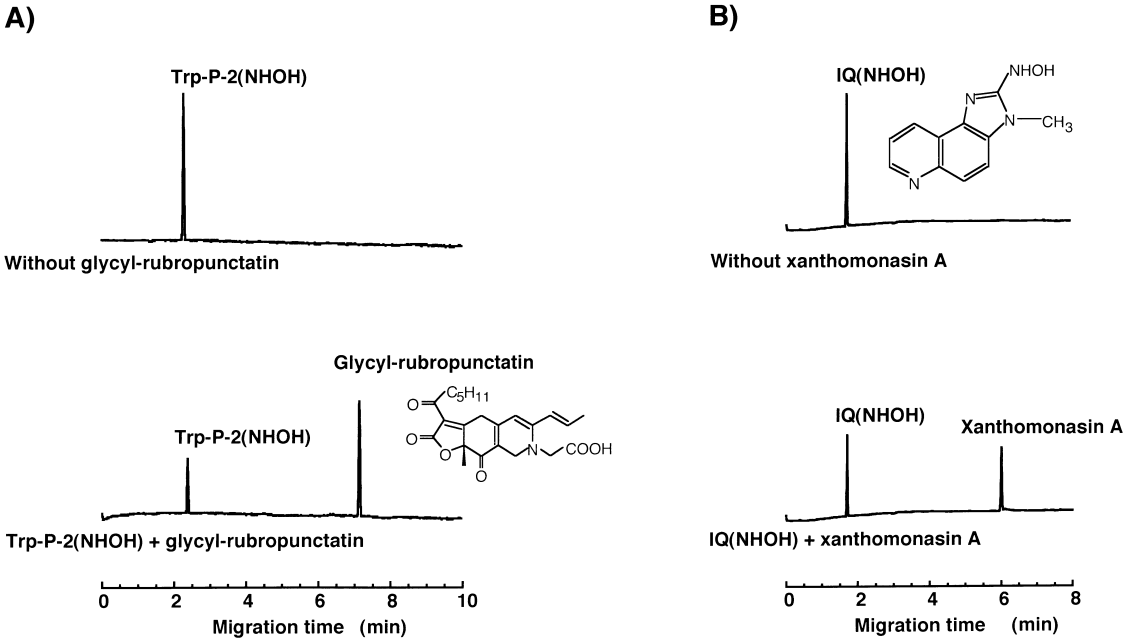

Fig. 7. Separation of glycyl-rubropunctatin and remaining Trp-P-2(NHOH) (A) and the separation of xanthomonasin A and remaining IQ(NHOH) (B) in the short contact mode [44]. Conditions as in Fig. 6.

contact mode (Fig. 5), corresponds to the throughout-capillary mode published by Taga et al. [24] and also by Oguri et al. [25].

The xanthomonasin A and Trp-P-2(NHOH) were successfully separated in less than 8 min both in the short contact mode and in the long contact mode, as shown in Fig. 6. It should be noted that the low voltage at 9 kV required an analysis time of about 15 min, and that the high voltage at 18 kV results in a significant tailing of the peak in spite of the fact that it takes only 6 min to complete the analysis. The other antimutagenic compound (glycyl-rubropunctatin) and mutagenic compound [IQ(NHOH)] were separated and evaluated both in the short contact mode and in the long contact mode, as shown in Fig. 7. Under the conditions employed (a short contact mode) the peak area of IQ(NHOH) was 78% compared to that of the control. No significant differences in the separation patterns and migration times were observed among these repeated runs. The decrease in peak area of Trp-P-2(NHOH) or IQ(NHOH) was proportional to the concentration or migration time of antimutagens. The ratio of the decreased peak area of the mutagen did not depend on the concentration of the mutagen between 0.1 and 0.5 m*M*. The decrease of the peak area was ascribed to the decomposition of the mutagen by the catalytic interaction of the antimutagen because the change in concentration of the antimutagen was not observed. However, no degradation product of the mutagen was identified probably owing to the production of polymeric materials.

7. Conclusions

In conclusion, the in-capillary reaction techniques were found to be useful for analysis in the food industry. The simple and rapid in-capillary reaction method is excepted to be widely applicable to the analysis of enzyme activities, antimutagenesis and other biological evaluation. CE is characterized by imaginative research into new separation techniques and mechanisms. CE will become one of the standard techniques in food industry analysis in the near future.

8. Nomenclature

CE	capillary electrophoresis
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CZE	capillary zone electrophoresis
EOF	electroosmotic flow
IQ(NHOH)	2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline
MEKC	micellar electrokinetic chromatography
NADH	nicotinamide adenine dinucleotide, reduced form
NAD	nicotinamide adenine dinucleotide
OPA	*o*-phthalaldehyde
PNP-Mal	*p*-nitrophenyl-β-D-maltoside
PNP-Glc	*p*-nitrophenyl-β-D-glucopyranoside
SC	sodium cholate
SDS	sodium dodecylsulfate
Trp-P-2(NHOH)	3-hydroxyamino-1-methyl-5H-pyrido[4,3-*b*]indole

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