Analysis of Health-related Microbes by Capillary Electrophoresis

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Analysis of health-related microbes called probiotics was performed by capillary electrophoresis. A rapid and easy characterization for two important probiotics, *Saccharomyces cerevisiae* and *Enterococcus feacalis*, was obtained in the running buffer containing poly(ethylene oxide). Quantitation of probiotic (*Saccharomyces cerevisiae*) shows a good linearity between the peak area versus the concentration of microbe. From the comparison of electropherograms of antidiarrhea, it was found that capillary electrophoresis could be employed for the quality control and quality assurance for the production of a medicine containing the probiotics.

Key Words : Capillary electrophoresis, Microbes, Probiotics, Health

Introduction

Some microbes are included in health products, medicines, and supplements as the active ingredients and generate the needed or useful compounds for human health.[1-3] Also, they can inhibit or retard the growth of fatal or pathological species. For example, the microbes such as *Lactobacillus acidophilus* and *Bifidobacterium infactis* in human gut produce organic acids, bacteriocin, H_2O_2 and reduce the growth or the attachment of toxic bacterium such as *Helicobacter pylori*.[4,5] The production of ammonia by urease from *Helicobacter pylori*, which can cause chronic gastritis, peptic ulcers and gastric cancer, is known to be neutralized by organic acids and H_2O_2 from *Lactobacillus acidophilus* and *Bifidobacterium infactis*.[6-8] *Lactobacillus acidophilus* has been also used for the diagnosis of women vaginitis.[9-11] *Saccharomyces boulardii*, *Enterococcus faecium*, *Lactobacillus* GG, *Bifidobacterium bifidum* have been employed to prevent diarrhea caused by the dosage of antibiotics.[12,13]

Microbes for good human health are termed as "probiotics" that are the living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition.[14,15] Probiotics have special effects on infections of mucosal surfaces such as the gut and vagina and become available as capsules and food supplements. The relative lack of side effects makes probiotics a good candidate for preventing antibiotic associated diarrhea.[16]

One of the problems associated with the production of medicines containing probiotics is that there are no effective assay methods for the active bacteria, which is important since the viability and the characteristics of bacteria determine the effectiveness.[17] Plate method has been used to identify bacteria by observing colonies after culture process. Although the formation of colony is clear, this method usually takes overnight and can misidentify a bacterium among mixed bacteria sample.[17,18] Polymerase chain reaction (PCR) has been applied to identify bacteria by inspecting unique genetic materials. PCR provides easy identification of bacteria, however, it requires a specific primer sequence and the whole procedure takes relatively long (> 3 hours).[19,20] Enzyme-linked immunosorvent assay (ELISA) test offers high sensitivity and high throughput for the identification of bacteria. However, it needs a specific antibody for a antigen and showed some difficulties to apply to micro-organisms.[17,21]

Capillary electrophoresis (CE) would be an excellent analytical technique for the analysis of microbes since it provides high separation efficiency, rapid analysis, high resolution, and easy automation capability.[22,23] Recently, capillary electrophoresis has been applied to the analysis of bacterial pathogens,[24,25] four types of human rhinovirus,[26] yeast cells,[27] and tobacco mosaic virus.[28] In this study, two important probiotics in the market (*Saccharomyces cerevisiae* and *Enterococcus feacalis*) were investigated by capillary electrophoresis for qualitative and quantitative analysis. Also, the content of probiotics as well as organic compounds in antidiarrhea from two different companies were compared.

Experimental Section

Chemicals and Instruments. Tris(hydroxymethyl)aminomethane (TRIZMA base), boric acid, ethylene-diaminetetraacetate (EDTA), poy(ethylene) oxide (PEO, Mw = 600,000), hydrochloric acid, and sodium hydroxide were purchased from Sigma-Aldrich Korea, Ltd (Yongin, Korea). A medicine containing *Saccharomyces cerevisiae* was obtained from S company. A pure *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich Korea, Ltd. as freeze-dried. Antidiarrhea containing *Enterococcus feacalis* was acquired from K and Y companies for comparison. Deionized water (Milli-Q reagent water system) was used throughout the experiment.

An UV detector (Lambda 1010, Bischoff, Nethelands) was employed for the detection of microbes. For the current monitoring of a capillary filled with buffer, DM-340 digital multimeter (MilTek, USA) was used. pH of the solution was measured by MP 220 pH meter (Mettler Toledo, USA). An ultrasonic cleaner (Model 1050, Chosun scientific, Seoul, Korea) was utilized for the dispersion of microbes and the degassing of a buffer. An ultracentrifuge (Micro 17TR, Hanil scientific industrial, Seoul, Korea) was used for the microbe preparation. A capillary with i.d. of 100 mm and o.d. of 375

mm was purchased from Polymicro Technologies (MJL Crysteck, Daejeon, Korea).

Pretreatment of Microbes. A tablet of the medicine containing *Saccharomyces cerevisiae* was first ground, then dissolved in 4.0 mL of 8 : 1 diluted 0.5X TBE (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH=8.4) buffer. It was vortexed vigorously for 2 min, then placed on the bench for 30 min to remove particulates. One milliliter of the probiotic solution was ultracentrifuged for 5 min at 3,400 rpm for the precipitation of microbes. The solution was decanted, then 1.0 mL of 8 : 1 diluted 0.5X TBE buffer was added. Finally, the microbes were dispersed in TBE buffer by the vortex for 2 min. The washing steps were repeated two times. Anti-diarrhea containing *Enterococcus feacalis* was treated by using the same procedure described above. *Saccharomyces cerevisiae* obtained from Sigma-Aldrich was dissolved in 8 : 1 diluted 0.5X TBE buffer at the concentration of 1.0 mg/mL. Then, it was ultracentrifuged for 5 min at 3,400 rpm and decanted. Finally, 1.0 mL of 8 : 1 diluted 0.5X TBE buffer was added and the sample was dispersed by vortexing for 2 min.

Capillary Electrophoresis. A 0.5X TBE buffer was diluted 8 : 1 by deionized water. Poly(ethylene)oxide (Mw = 600,000) was dissolved in this buffer at the concentration of 0.5%. The polymer solution was stirred for 12 h, then diluted to 0.0125%.

Electrophoresis was performed by a home-made capillary electrophoresis system. An UV detector with a high voltage power supply (Spellman) was equipped for running. The detection signal was transferred to the computer through LabView interface board. The system was controlled by in-house visual LabView program. A fused silica capillary with 100 μm i.d. was employed for the separation. Total length of the capillary was 50 cm with the effective length of 40 cm. The capillary was preconditioned by washing with 0.1 N hydrochloric acid for 3 min, deionized water for 2 min, 1.0 N sodium hydroxide for 2.5 min, then 0.0125% PEO running buffer for 2 min. Pre-electrophoresis run before microbe sample injection was carried out for 5 min for the stabilization of baseline. Each microbe sample was first dispersed by vortex and/or ultrasonication. Then, it was injected by siphoning (2.5 cm for 20 s) and run at 15 kV. Eluted sample was monitored by UV detector at 214 nm.

Results and Discussion

Saccharomyces cerevisiae has been used as a feed additive for the improvement of digestability of nutrients and production of livestock. Since *Saccharomyces cerevisiae* contains organic selenium, chromium G.T.F., vanadium, vitamins, enzymes, manna, and glucan, it has been also used as a dietary supplement and a major component for the medicine. A medicine used in this study is composed of organic compounds, enzymes, and *Saccharomyces cerevisiae*. Most of enzymes and organic compounds in the medicine were removed before the injection of *Saccharomyces cerevisiae* by ultracentrifugation (see experimental section). Figure 1 shows the electropherograms of *Saccharomyces cerevisiae* in the medicine (Fig. 1A) and the one as single component from Sigma-Aldrich (Fig. 1B). A major peak corresponding to *Saccharomyces cerevisiae* appeared around 23 min. A slight change of migration times in Fig. 1A and B was observed, however, it could be attributed to the sample composition. Armstrong *et al.* found the migration time shift of *E. coli* from urinary tract and it was explained that the higher concentration of dissolved material in the injected sample slowed the electrosmotic flow even though all other experimental conditions were the same.[29] In our experiment, there might be some enzymes and other impurities that were not completely removed by ultracentrifuge probably due to high molecular weight. Several minor peaks shown in Figure 1 could be originated from impurities such as high molecular weight enzymes (small peaks between 1 min and 10 min). Since the sample used in Figure 1B is presumably purer than that used in Figure 1A, small peaks and spikes appear to be relatively insignificant compared to the intensity of target peak. Though the compositions of the peaks were not thoroughly examined in this study, Figure 1B shows a good evidence. As shown in Figure 1A and B, the characterization of probiotic in medicine was simple and fast.

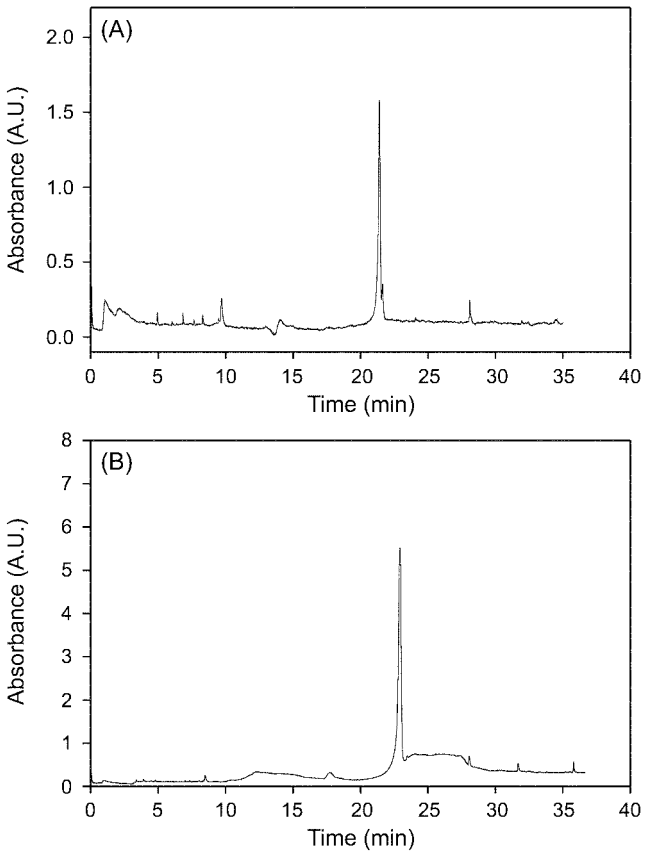

Figure 1. Electropherograms of *Saccharomyces cerevisiae* in the medicine (A) and *Saccharomyces cerevisiae* from Sigma-Aldrich (B). The probiotic was injected by siphoning (2.5 cm for 20 s) and run at 15 kV. Other conditions; a 100 μm i.d. fused silica capillary (50 cm with the effective length of 40 cm), 0.0125% PEO in 0.5X TBE buffer (pH 8.4), 214 nm UV detection.

For the separation of those microbes, it is important to

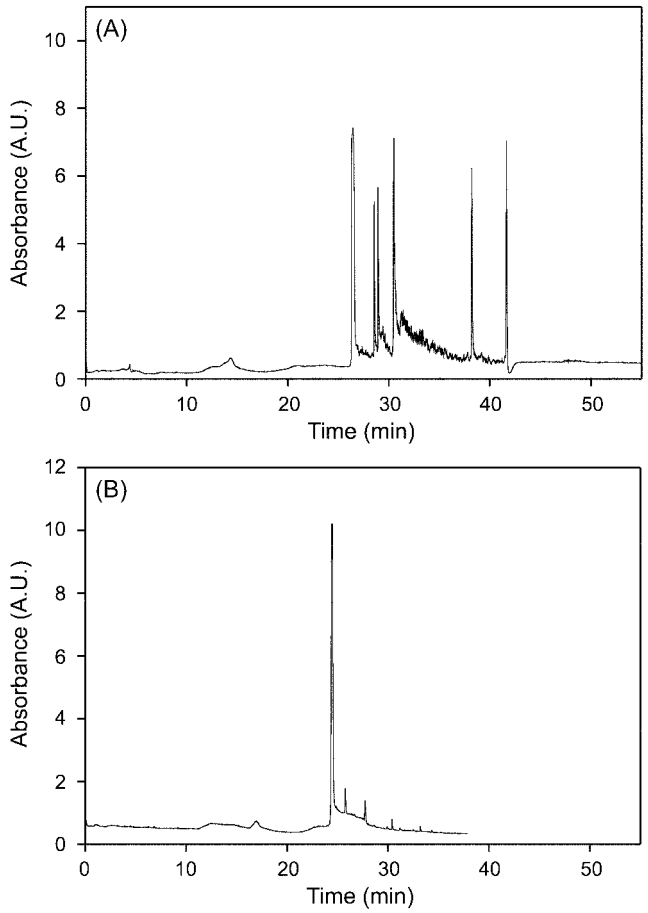

Figure 2. Electropherograms of *Saccharomyces cerevisiae* without cell dispersion (A) and with proper cell dispersion (B). Bacteria cells were dispersed by vigorous vortexing for 2 min and placing in ultrasound bath for 3 min before injection. Other conditions were the same as in Figure 1.

prepare the sample without the formation of chain or cluster that can cause many unwanted peaks, which can lead to false identification of microbes. We have shown that a proper dispersion of bacteria before the injection prevented the formation of chains or cluster.[24,25] A similar trend was observed for somewhat larger size microbe (~2 mm), *Saccharomyces cerevisiae*, as shown in Figure 2. Figure 2B explains that a vigorous dispersion procedure is necessary to obtain single peak corresponding to the probiotic.

Several techniques such as the plate method, polymerase chain reaction, and ELISA have been employed for the identification of microbes. Although real-time PCR and ELISA have shown good potential for quantitative analysis of analytes, whole processes are rather complicated and sometimes labor intensive. Besides, those methods take at least 3 hours to 2 days for the analysis of microbes. We have tested our technique for the possibility of quantitative measurement of the probiotic. Figure 3 shows the good linearity between the peak area versus the concentration of *Saccharomyces cerevisiae* (0.1 mg/mL~1.0 mg/mL). Although a linear dynamic range for the concentration was narrow in our study, we believe that our capillary electrophoresis

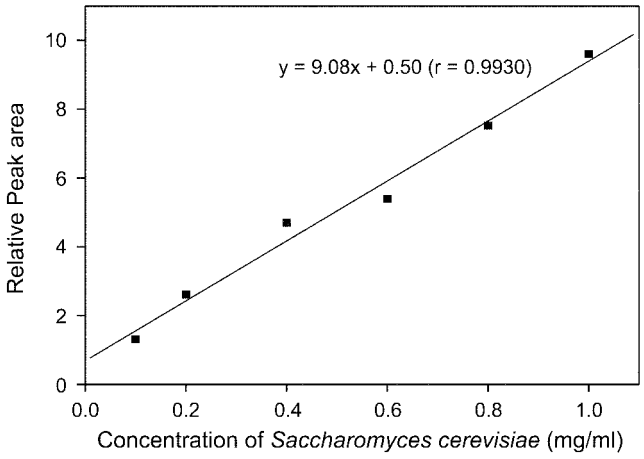

Figure 3. The relationship between the peak area and the concentration of *Saccharomyces cerevisiae*. Good linearity shows the high potential for the rapid and accurate quantitation of probiotics.

shows the high potential for the application of fast (<30 min) and quantitative analysis of the probiotics.

Enterococcus species belongs to the normal intestinal

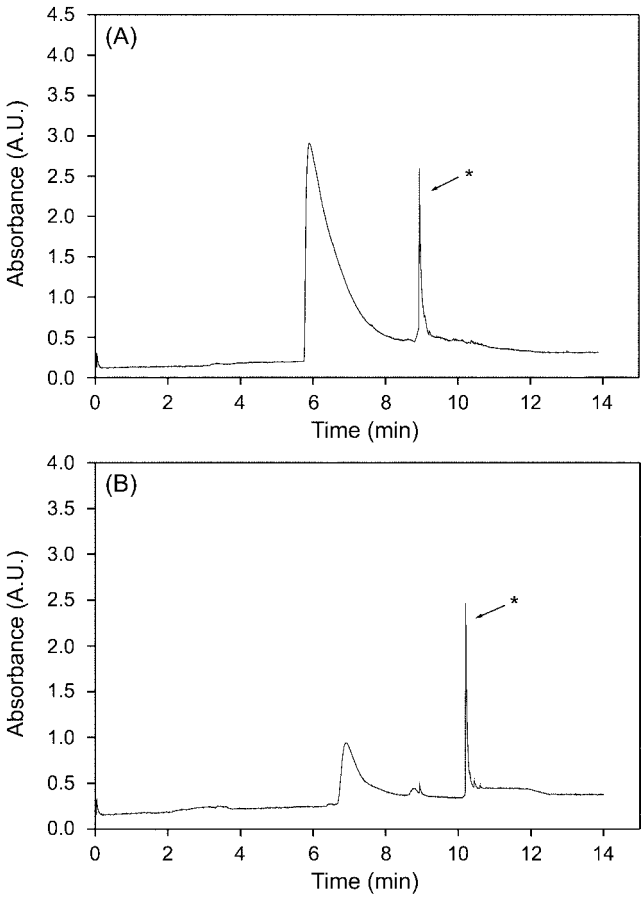

Figure 4. Electropherograms of *Enterococcus feacalis* (marked by *) in antidiarrhea from the company K (A) and the company Y (B). Relatively broad peaks shown before the peak of *Enterococcus feacalis* are organic compounds (loperamide hydrochloride, berberine chloride, and acrinol). Other conditions were the same as in Figure 1.

microflora and produces bacteriocins with anti-Listeria activity. It also has the high immunomodulation of the host and has been used to treat gastrointestinal and vaginal mucosal infections. Especially, it can reduce the incidence of acute diarrhea.[4,9,30] In our study, antidiarrhea containing *Enterococcus feacalis* was acquired from K and Y companies and investigated by capillary electrophoresis with the same experimental conditions. Figure 4 illustrates the separation of *Enterococcus feacalis* from two companies and shows the peak for the probiotic around 10 min. In this experiment, an equal volume of supernatant after centrifuge was remained to examine the organic content. The peak shown around 7 min corresponds to unresolved organic compounds (loperamide hydrochloride, berberine chloride, and acrinol). According to the information from two companies, the contents of those organic compounds and *Enterococcus feacalis* are the same. This means that the peak areas corresponding to organic compounds and *Enterococcus feacalis* should be the same in the electropherograms. Although the migration times were not exactly identical for both organic compounds and *Enterococcus feacalis* in Figure 4, it is clearly shown that the peak areas are different, meaning that the actual composition of both organic compounds and *Enterococcus feacalis* might be different. Here, it shows that our capillary electrophoresis technique has a potential for the quality control and quality assurance for the production of a medicine containing the probiotics.

Conclusions

Probiotics are important in the area of a feed additive, a dietary supplement, and a medicine since they have many special effects for the production of livestock and healthy food, the improvement of digestabilty of nutrients, and the fighting for the infections in the gut and vagina. Although there are several excellent techniques such as the plate method, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) for the identification of microbes, they have shown some difficulties for both qualitative and quantitative analysis of microbes. In this study, two important probiotics from the market, *Saccharomyces cerevisiae* and *Enterococcus feacalis*, were investigated by using capillary electrophoresis. A fast and easy characterization was performed for the probiotics and compared from the medicine and the pure form. The linear dynamic range between the peak area versus the concentration of bacterium was relatively narrow, however, our technique shows the great potential for the application of quantitative measurement for the probiotics. We also show that capillary electrophoresis technique has a potential for the quality control and quality assurance for the production of a medicine containing the probiotics. Study toward the wider dynamic range for the quantitative analysis of the probiotics and its application to Lab-on-a-chip is under progress.

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