Evaluation of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Rapid Identification of Bacteria in Processed Soybean Products

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been demonstrated as a rapid and reliable method for identifying bacteria in colonies grown on culture plates. Rapid identification of food spoilage bacteria is important for ensuring the quality and safety of food. To shorten the time of analysis, several researchers have proposed the direct MALDI-TOF MS technique for identification of bacteria in clinical samples such as urine and positive blood cultures. In this study, processed soybean products (total 26 test samples) were initially conducted a culture enrichment step and bacterial cells were separated from interfering components. Harvested bacterial cells were determined by MALDI-TOF MS and 16S rRNA gene sequencing method. Six processed soybean products (23%) were increased bacterial cells after culture enrichment step and they were successfully obtained the accurate identification results by MALDI-TOF MS-based method without colony formation.

Keywords: bacterial identification, MALDI-TOF MS, food spoilage

1. Introduction

Processed soybean products are an important part of diet not only in East Asia but also worldwide because of their beneficial health effects, particularly because they are an excellent source of high-quality proteins, isoflavones, and vitamins (Hettiarachchy & Kalapathy, 1997; Jacobsen et al., 1998). The potential health benefits of soy have been reported against cancer, heart diseases, and diabetes. However, processed soybean products are also associated with health hazards because of the incidence of food-borne illnesses caused by the presence of pathogenic bacteria (Fang et al., 1999; No et al., 2002). In addition, microbial spoilage of products is an important economic problem that discourages manufacturers.

The microbiota of soybean products are closely associated with the condition of raw materials, level of bacterial contamination, and preservation methods, which affect the growth and survival of bacteria. Thus, food-borne pathogenic and spoilage bacteria can be present as resident microbiota or can be introduced as contamination during processing. It should be noted that the bacterial species also play an important role in soybean food-borne illness and spoilage. Thus, quality and safety of processed soybean products is important (Katase & Tsumura, 2011; Tsumura & Tsuboi, 2012).

Based on the ribosomal proteins profiles of different bacteria, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been demonstrated as a rapid and reliable method for bacterial identification (Lay, 2001; Mazzeo et al., 2006). Databases of various pathogenic bacteria have been generated for use of this method in routine bacterial identification from plate cultures (Seng et al., 2009; Bizzini et al., 2010). However, this method generally requires at least 24-48 h to obtain colonies before MALDI-TOF MS. In addition, it is difficult to harvest sufficient cells because of cell damage or some cells may be viable but non-culturable (Rowan, 2004). Nevertheless, direct identification of bacteria may increase the utility of MALDI-TOF MS because it can significantly shorten the time required for bacterial identification (Ferreira et al., 2010).

The aim of the present study was to evaluate the potential usefulness on the direct identification of bacteria in processed soybean products using MALDI-TOF MS.

2. Materials and Methods

2.1 Samples

Twenty six of processed soybean products (soy milk; 14, tofu; 4, frozen tofu; 4, dried tofu; 4) were purchased from different retail outlets in the Osaka region of Japan and stored at 4°C until analysis. Except dried tofu, all samples were examined within 2 days of purchase. Soy milk (3.6% fat) for the contamination experiment was obtained from Fuji Oil Co., Ltd. (Osaka, Japan).

2.2 Bacterial Strains and Culture Media

Escherichia coli NBRC 3301, *Klebsiella pneumoniae* NBRC 14940 and *Citrobacter freundii* NBRC 12681 were were purchased from Biological Resource Center (NBRC), National Institute of Technology and Evaluation (Chiba, Japan) and used for model bacteria in the contamination experiment. Plate Count Agar (PCA, Nissui Seiyaku, Tokyo, Japan) was used for colony counts. Bacto™ Tryptic Soy Broth (TSB) was purchased from Nippon Becton Dickinson Company, Ltd. (Tokyo, Japan).

2.3 Contamination Experiment Using Soy Milk

To determine the identification of bacteria at low contamination levels using MALDI-TOF MS, soy milk was inoculated with *E. coli*, *K. pneumoniae*, or *C. freundii*, which were incubated in PCA at 35°C for 24 h before each experiment. Subsequently each bacterial strain was grown in TSB at 35°C for 24 h and harvested by centrifugation at 11,000 × g for 5 min (Himac CR22, Hitachi Koki, Tokyo, Japan). Sequential serial dilutions were made using fresh soy milk to achieve aliquots with bacterial counts ranging from 10^7 to 10^9 CFU/ml. About 100 μl of concentrated hydrochloric acid (36%, Kanto Kagaku,Tokyo, Japan) was added to 40 ml of each of these inoculated soy milk samples at pH 5 and incubated at 45°C for 5 min.Subsequently, most of soy proteins were coagulated by isoelectric precipitation method and the coagula were filtered using stomacher bags (Tempo bag, bioMérieux, Lyon, France). Aliquots were then filtered through 10-μm pore size syringe filters (PALL Life Sciences, Ann Arbor, Michigan) and centrifuged at 9,000 ×g for 5 min (Model 3500, Kubota Corporation, Tokyo, Japan). Bacterial cells were recovered and suspended in 400 μl of sterile water. A cell suspension (300 μl) was used for identification using MALDI-TOF MS, as described below. Total bacteria count was estimated using PCA at 35°C for 48 h.

2.4 Sample Preparation From Commercially Available Processed Soybean Products

We introduced a culture enrichment step before cell recovery because processed foods generally contain less viable bacterial cells. Twenty six samples of commercial soybean products were evaluated by culture enrichment prior to direct MALDI-TOF MS method. Increased bacterial cells in 6 of the 26 samples in total were determined by direct MALDI-TOF MS and 16S rRNA gene sequencing. Commercially available soy milk (8 ml) was mixed with 32 ml of sterile TSB and incubated at 35°C for 8 h. After this culture enrichment, bacterial cells were harvested according to the method described above and the cell suspensions were used for MALDI-TOF MS. In addition, the suspension was serially diluted and plated on PCA. The plate was incubated at 35°C for 48 h. All colonies grown in the plate were used for 16S rRNA sequence-based identification. All the experiments were performed in duplicate samples, and data shown are means values.

Ten grams of another processed soy product (i.e., tofu, frozen tofu and dried tofu) was mixed with 90 ml of sterile 0.9% NaCl solution and incubated at 35°C for 24 h. Next, 50 ml of this suspension was filtered through a stomacher bag (Tempo bag, bioMérieux) and centrifuged at 9,000 ×g for 5 min (Model 3500, Kubota Corporation). The harvested bacterial cells were suspended in 0.5 ml of sterile water and used for MALDI-TOF MS and 16S rRNA gene sequencing as the same manner.

2.5 MALDI-TOF MS

MALDI-TOF MS analysis was performed according to standard procedures (La Scola & Raout, 2009). A bacterial cell suspension (300 μl) was mixed with 900 μl absolute ethanol (99.5%, Kanto Kagaku, Tokyo, Japan) and centrifuged at 13,000 ×g for 2 min (Model 3615, Kubota Corporation). The supernatant was discarded and residual ethanol was removed after repeated centrifugations. Subsequently, 10 μl of formic acid (70%, Kanto Kagaku, Tokyo, Japan) was added to the pellet and mixed thoroughly by pipetting before the addition of 10 μl acetonitrile (98%, Kanto Kagaku, Tokyo, Japan). This mixture was centrifuged at 13,000 × g for 2 min. The supernatant (1 μl) was placed on a spot of the steel target and air dried at room temperature. The sample spots were overlaid with 1 μl of matrix solution [saturated solution of HCCA (α-cyano-4-hydroxycinamic acid) in an organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid)] and air dried at room temperature.

Measurements were performed using the Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with a 20-Hz nitrogen laser. Spectra were recorded in the linear, positive mode at a laser frequency of 20 Hz within a mass range from 2-20 kDa The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.5 kV, the lens voltage was 6 kV, and the extraction delay time was 250 ns. For each spectrum, 300 laser shots were collected and analyzed. The spectra were calibrated externally using the standard calibration mixture (Bruker Daltonics). For automated data analysis, raw spectra were processed using MALDI Biotyper 3.0 software (Bruker Daltonics) at default settings. To identify unknown bacteria, each peak that was generated was matched directly against reference libraries (3,995 strains) using the integrated pattern-matching algorithm incorporated in the Biotyper 3.0 software. Identifications obtained using MALDI-TOF MS were evaluated according to modified scores (ranging from 0 to 3) proposed by the manufacturer. A score of more than 1.7 indicated probable identification, and a score of less than 1.7 indicated no reliable identification.

2.6 16S rRNA Gene Sequencing Analysis

Genomic DNA was isolated from a pure colony. In brief, the bacterial cells were lysed with the PrepMan Ultra Reagent (Applied Biosystems, Foster City, California, USA) and then heated for 10 min at 98°C. Extracted DNA was amplified by 500 bp to the 5' end of the 16S rRNA gene using the MicroSeq 500 16S rDNA Bacterial Identification PCR kit (Applied Biosystems). PCR was performed according to standard procedures (Arosio et al., 2008). Amplicons were purified with Amicon ultra-centrifugal filters (Millipore, Bedford, Massachusetts, USA). The sequencing reactions were performed with MicroSeq 500 16S rDNA Bacterial Identification Sequencing kit (Applied Biosystems) and PCR products were purified using Performa DTR Gel Filtration Cartridges (Edge Bio, Gaithersburg, Maryland, USA).

The cycle sequencing products were analyzed on an ABI 3130 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The Basic Local Alignment Search Tool (BLAST) program in the DNA Data Bank of Japan (DDBJ) was used for sequence homology analysis.

3. Results and Discussion

To determine the identification of bacteria at low inoculum levels for accurate identification using MALDI-TOF MS, the recovery of bacteria from artificially contaminated soy food sample (e.g., soy milk) was investigated. As a model of bacteria,we used *Enterobacteriaceae* family and *Coliform* bacteria which are one of the most important indicators of sanitary quality of foods. Soy milk has many components, such as proteins, lipids and sugars, which interfere with MALDI-TOF MS analysis. In this study, most of the soy proteins were removed by isoelectric precipitation method that is commonly applied for production of soy protein isolate (Petenate & Glatz 1983; Tsumura et al., 2004) and the resulting supernatants were centrifuged to harvest bacterial cells. Good separation of bacteria from soybean food components was achieved with recovery rates between 50% and 90% (data not shown). Bacteria used for contamination experiment could be identified with adequate identification scores, providing a high reliability of these results (Table 1). In our contamination experiment, we obtained accurate identification results using soy milk samples that were contaminated with high bacterial count ($> 10^8$ CFU/ml). No significant differences in bacterial count range were observed between the test strains. These results coincided with previous studies that the bacterial count was an important factor for identification scores, and more than 10^7-10^8 CFU/ml bacterial cells were needed (Drancourt, 2010; Kroumova et al., 2011). At a lower bacterial count ($< 10^6$ CFU/ml), background peaks became more prominent and affected spectrum matching.

Table 1. MALDI-TOF MS scores for different inoculated bacterial counts in soy milk

Microorganisms	Inoculated bacterial count (CFU/ml)	MALDI-TOF MS score
E. coli NBRC 3301	3×10^9	2.0
	5×10^8	2.2
	5×10^7	NI
C. freundii NBRC 12681	2×10^9	2.4
	5×10^8	2.2
	7×10^7	NI
K. pneumoniae NBRC 14940	1×10^9	2.0
	1×10^8	1.8
	1×10^7	NI

NI, no reliable identification.

Although MALDI-TOF MS may become a popular identification method in food microbiology, there is little information on direct identification of bacteria in processed food products using MALDI-TOF MS (Angelakis et al., 2011; Böhme et al., 2011; Hochel et al., 2012). The separation of bacteria is conducted to harvest cells from complex food matrices that impede accurate detection (Benoit & Donahue 2003; Stevens & Jaykus, 2004). In the present study, a rapid and simple procedure to recover bacterial cells from contaminated soybean products was conducted and the bacterial cells obtained could be directly identified using MALDI-TOF MS.

To determine the feasibility of the proposed direct identification of bacteria using MALDI-TOF MS, processed soybean products such as soy milk and tofu were used. The most frequently occurring bacteria deduced in these samples using the 16S rRNA gene sequencing method were correctly identified by MALDI-TOF MS with adequate identification scores (Table 2).

Table 2. Comparison between MALDI-TOF MS and 16S rRNA gene sequencing identification techniques for bacteria in the processed soybean foods

| Product | CFU/ml | | MALDI-TOF MS [1] | 16S rRNA gene sequencing analysis [2] |
	Initial bacterial count	Bacterial count after culture enrichment		
Soy milk-1	3×10^3	9×10^8	*Leuconostoc lactis* (2.0)	*Leuconostoc lactis (57%)* *Lactococcus sp., Serratia sp.*
Soy milk-2	9×10^2	6×10^8	*Serratia liquefaciens* (2.1)	*Serratia liquefaciens (50%)* *Lactococcus sp., Enterobacter sp., Aeromonas sp.*
Tofu-1	$<1\times10^1$	1×10^8	*Bacillus cereus* (2.5)	*Bacillus cereus (100%)*
Tofu-2	2×10^3	7×10^7	*Leuconostoc lactis* (1.8)	*Leuconostoc lactis (100%)*
Frozen tofu	4×10^1	9×10^7	*Raoultella planticola* (2.3)	*Raoultella planticola (85%)* *Lactococcus sp., Enterobacter sp.*
Dried tofu	$<1\times10^1$	1×10^8	*Bacillus cereus* (2.4)	*Bacillus cereus (83%)* *Enterococcus sp.*

[1] MALDI-TOF MS scores indicate in parentheses.

[2] The ratio of identified bacteria indicates in parentheses. The names in the lower berth show the other bacteria identified.

Although pure cultures are generally employed for identification using MALDI-TOF MS, it has been reported that a mixed culture can be analyzed if the predominant bacterial count is sufficiently large (Maier & Kostrzewa, 2007; Christner et al., 2010). Moreover, improved software identification algorithms may increase the concordance scores with spectra from 2 or more different species (Wenzel et al., 2011).

Most frequently isolated bacteria were belonged to *Bacillus*, *Leuconostoc*, and *Enterobacteriaceae*, which are ubiquitous microbes commonly found in spoiled soybean food (Fang et al., 1999; No et al., 2002). The microbiota of soybean products are closely associated with the condition of raw materials, level of bacterial contamination, and preservation methods, which affect the growth and survival of bacteria.So It is assumed that these bacteria survived from raw materials or contaminated while processing or preservating. Processed soybean products examined in the present study were probably sterilized during manufacturing. *Enterobacteriaceae* do not usually survive under these conditions, although there may still be a risk of accidental cross-contamination during manufacturing, and the food may inevitably be contaminated during some stages of the process. Increased bacterial cell numbers were rare in most of the samples tested in the present study. This was probably the result of good manufacturing practices and strict hygiene procedures during manufacturing. However, it must be noted that only a limited number of samples were tested in this study.

4. Conclusion

MALDI-TOF MS used in the present study rapidly identified frequently occurring bacteria in contaminated food

samples. Thus, from a rapid quality assessment point of view, direct identification using MALDI-TOF MS could contribute to significant progress in the quality and safety of the food industry.

Acknowledgements

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