Anal. Chem. **2010,** *82,* 2113–2118

Determination of β-Lactamase Residues in Milk Using Matrix-Assisted Laser Desorption/Ionization Fourier Transform Mass Spectrometry

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A selective, fast, and effective enzyme assay based on matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) for quantifying β-lactamase, an illegal additive in milk products, has been reported. The strengths of the mass spectrometric assay are its response to all substrate and products, simple and direct detection of the conversion of substrate, and facile determination of enzyme activity. Also, MALDI MS is tolerant to many buffer salts and reagents without the requirement of complicated sample pretreatment procedures. In this study, the approach was used to detect the presence of β-lactamases (BLA) in milk samples. The amount of BLA that could be determined in a milk sample is 6×10^{-3} U·mL^{-1} by this approach. To test the strategy, it has been applied to the fortified milk (adding a BLA product known as an antimicrobial destroyer). It is then tested whether the pasteurization procedure of the milk process affects the activity of BLA in milk samples. This study offers a perspective into the utility of MALDI-FTMS as an alternative detection tool for BLA screening in milk.

In recent years, food safety has been of growing concern, especially after the emergence of a series of scandals (e.g., bovine spongiform encephalopathy, dioxins, avian influenza, and melamine). Therefore, in the field of food safety, it is important to identify any potential risks to consumers related to the consumption of food. Antibiotics are widely used in dairy cattle management for the treatment of disease and as dietary supplements. They may be administered orally as feed additives or directly by injection. Therapeutic use of antibiotics for treatment of mastitis in dairy cattle can result in contamination of milk with low amounts of the antibiotic if close attention is not paid to good therapeutic practice.[1,2] Antibiotics in milk could cause bacteria resistant to the same antibiotics used to treat people for infections, considered to be a potential health hazard.[3−5] The resistance to β-lactam antibiotics is of utmost importance because they are among the most frequently prescribed drugs. Avoidance of antibiotic residues in milk is an important focus of the dairy industry.

β-Lactamases (EC 3.5.2.6, BLA) are an important family of enzymes that catalyze the hydrolysis of β-lactam antibiotic produced by some bacteria[6] and are responsible for their resistance to β-lactam antibiotics. β-Lactamases catalyze the hydrolytic degradation of the amide bond of the four-membered β-lactam ring in β-lactam antibiotic (e.g., penicillins, cephalosporins, etc.), rendering them inactive. Since the 1980s, it has been extensively documented to use BLA for eliminating rudimental antibiotics in foodstuff.[7−9] However, it is reported that many pathogenic microbial strains have evolved an increased expression of β-lactamases, in this way decreasing the efficiency of antibiotic treatment.[10−13] In the most recent tainted milk scandal in China, a β-lactamase product known as an antimicrobial destroyer was introduced into contaminated dairy products for the destruction of antibiotic residue by an unethical manufacturer, in order to reuse the contaminated milk illegally. The introduction of β-lactamases into milk reduces the risk of antibiotics but exposes consumers to another public health threat, so rapid, unambiguous detection of trace amounts of β-lactamases in food products is of paramount importance.

β-Lactamases can be directly detected by an enzyme-linked immunosorbent assay (ELISA).[14,15] In addition to determine the

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10.1021/ac9019945
Published on Web 02/05/2010

enzyme itself directly, it is an alternative to measure enzymatic reactions catalyzed by β-lactamases to determinate the presence of enzyme. These strategies are based on identification of the formation of product or the consumption of substrate. Spectrophotometric assays of β-lactamases have been reported using a coupling reaction (such as an iodometric assay[16,17]) to detect the formation of product or have been based on direct UV monitoring.[18−22] In recent years, several strategies based on biocompatible hydrogels[23,24] and gold nanoparticles[25] have been developed for detection of β-lactamases. A modified cylinder plate method has been used for detecting β-lactamases in milk samples.[26] Martin et al have reported the use of electrogenerated chemiluminescence to detect β-lactam antibiotics and their hydrolysis catalyzed by β-lactamases.[27] More recently, a patent was reported to develop a β-lactamase detection kit for raw milk and milk products.[28] However, a simple, rapid, and accurate assay is still highly desirable because current assays have some major drawbacks, such as laborious manipulation and time-consuming processes.

The interest in leveraging mass spectrometry for studying enzyme activities derives from its high sensitivity and specificity. In recent years, mass spectrometry (MS)-based assays have attracted great attention for studying enzyme activities and inhibitor screening as well as for studying enzyme kinetics (for recent reviews see refs 29−31). MS offers the significant advantage that it does not require analytes to be labeled, either by direct attachment of fluorescent and radioactive labels or by binding of antibodies, and therefore offers greater flexibility in experiments. Thus, a mass spectrometry-based assay could be applied to any enzyme reaction system, providing that the substrates and products are distinguishable by molecular weight based on reasonable ionization efficiency. HPLC/ESI-MS techniques can detect the reaction products directly and quantitatively, rapidly expanding into the study of enzyme kinetics.[32−34] These methods are quite specific but have longer cycle times.

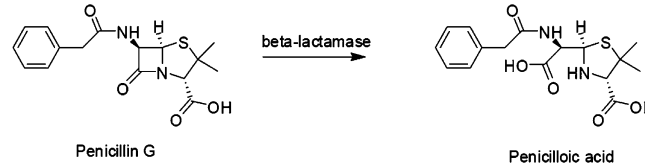

Figure 1. Hydrolytic degradation of the four-membered β-lactam ring in penicillins catalyzed by β-lactamases.

Due to its high speed, low sample consumption, and high sensitivity, quantitative MALDI-MS is now also applied as a tool for the quantification of low molecular weight compounds of biological interest. In a previous study, our laboratory developed a matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS)-based assay for kinetic measurements and inhibitor screening of acetylcholinesterase,[35] without addition of internal standards and preliminary separation. This method was accurate and reproducible and was successfully applied to the analysis of various real, raw samples. In addition, other groups have reported the successful implementation of MALDI-MS-based assays and the approach to screen enzyme reactions.[36−41]

The present paper describes a new screening method for the detection and presumptive identification of β-lactamases in commercial milk by MALDI-FTMS. The method is based on measurements of conversion of penicillin G (PEN G) to penicilloic acid (PA) (Figure 1). The use of these tracers that are hydrolyzed specifically by BLA permits the measurement and imaging of BLA activity in milk products. Due to the complexity of milk samples, the simplification of milk sample treatment is discussed at first. We demonstrated the applicability of the MALDI-FTMS-based assay in the determination of penicillin G and penicilloic acid. Furthermore, the general applicability of this approach for the determination of β-lactamases in milk is assessed using a fortified milk sample. Finally, the effect of the pasteurization process on the analysis are discussed. This method can be used to rapidly identify the presence of BLA and screen the β-lactam antibiotic in milk simultaneously with a high throughput potential.

EXPERIMENTAL SECTION

Chemicals and Materials. PEN G (sodium salt, 1500−1750 U·mg^{-1}) of USP grade was purchased from Amersco Inc. (Solon, OH). 2,5-Dihydroxybenzoic acid (DHB) and Tris were purchased from Sigma Chemical Co. (St. Louis, MO). β-Lactamase standard (3000 kU·mL^{-1}) was from Shanghai Institute for Food and Drug Control (Shanghai, China). The deionized water used in this study was from a Milli-Q water purification system (Millipore Corp.; Bedford, MA). Methanol was of HPLC-

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grade quality purchased from Merck (Darmstadt, Germany). Poly(ethylene glycol)-200 (PEG-200), PEG-400, and hydrochloric acid were bought from Shanghai Chemical Reagent Corporation. Stock solutions of all standards were made by dissolving 10 mmol of each compound in 1 mL of 10 mmol·L^{-1} Tris−HCl (pH 7.0). The stock solutions were stored at 4 °C in the dark. Working dilutions were prepared freshly on the day of use.

Incubation of Milk with β-Lactamases. All the milk samples were bought from a local super market. Commercial liquid milk products containing no β-lactamases were used as blank liquid milk samples. We did not find PA in milk samples when we directly analyzed milk samples by MALDI-FTMS. A volume of 400 μL of the milk sample (3.5% fat) was apportioned into 0.6 mL polypropylene centrifuge tubes. β-Lactamase solution was added to obtain concentrations of 0.06, 0.6, 6, 60, 600 mU·mL^{-1} (fortified sample). Moreover, a blank sample was performed (milk sample without spiking). Penicillin G (1 mmol·L^{-1}, 100 μL) was added to 10 μL of BLA-free (control) or BLA-containing milk samples. The tubes were closed and thoroughly vortexed for 30 s and incubated at 25 °C for 3 h, followed by centrifugation at 10 000 rpm for 30 min. The upper and bottom layers were discarded, and an aliquot of the middle part was collected and analyzed directly by MALDI-FTMS in triplicate.

Milk Pasteurization Conditions. A water bath was purchased from Shanghai Permanent Science and Technology Co. Ltd. (Shanghai, China). There are two methods of pasteurization, the low temperature long time (LTLT) process or high temperature short time (HTST) process. For the LTLT process, a quantity of milk in polypropylene centrifuge tubes is heated in at 62.8 °C for 30 min, followed by quick cooling to about 4 °C. For the HTST process, milk is heated at 71.7 °C for 15 s before being chilled back to 4 °C.

MALDI-FTMS Conditions. Experiments were conducted using an Ionspec 4.7 T HisRes MALDI-FTMS (Ionspec, Irvine, CA). The external Ionspec MALDI ion source used an air-cooled Nd:YAG laser (355 nm, New Wave, Fremont, CA) with a gradient filter for adjusting the UV-laser power. Ions, generated from a MALDI source, were transferred via a quadrupole ion guide to the capacitively coupled closed cylindrical cell. The intensity of MALDI-laser irradiation was varied between 20% and 30% as needed. The laser irradiation pulse time was set at 100 ms. The quadrupole guide had an applied voltage of 140 V (base to peak) at a frequency of 1260 kHz. The mass spectrometer was calibrated with PEG-200 and/or PEG-400 for each test. The acquisition mass range was m/z 100−500.

The matrix solution was deposited on the stainless steel target to produce a microcrystalline layer of matrix, followed by sample deposition onto the preformed matrix layer. After the sample spots had dried completely, each one was overlaid with 1.0 μL of matrix solution, which produced a thin film of homogeneous crystals.

RESULTS AND DISCUSSION

Selection of Sample Pretreatment. Milk is a complex matrix that contains a suspension of multiple components including fats, proteins, casein micelles, and lactose. The presence of big molecules such as protein micelles challenges the detection of trace amounts of β-lactamases in milk samples. Therefore, sample pretreatments are needed for detecting trace compounds in milk samples. Generally, sample treatment is the most tedious step in the analytical procedure. There is a need for simplification of this stage, trying to find simple methods which allow the simultaneous determination of a large amount of samples. Usual pretreatment strategies, such as solid-phase extraction (SPE) and liquid−liquid extraction (LLE) are laborious and time-consuming, which do not meet the requirements of high throughput screening. PEN G exhibits the good solubility in water. It was thought that the major effect on the detection of PEN G and PA is the water insoluble matter, such as fat globules in milk. We assume the simple centrifugation process will improve the mass spectrometry detection. The diluted milk was centrifuged at a high speed, resulting in the cream layer at the top, the aqueous supernatant, and a small pellet of leukocytes and other nonfat solids. As stated above, PEN G and PA have good solubility in water. Only the aqueous phase is further analyzed by MALDI-FTMS. Figure 2a,b displays the MALDI mass spectra of milk untreated and the middle aqueous phase. Compared to the milk sample unpretreated prior to detection (Figure 2a), the signal-to-noise (S/N) of the MS signal of the PEN G in the aqueous phase was improved (Figure 2b). The S/N of PEN G for centrifuged milk is 368, compared to a S/N of 26 for an unpretreated sample. It appears that the centrifugation procedure reduces the interference of other molecules in the mass spectrum. These results indicate that centrifugation is effective for the improvement of detection of PEN G.

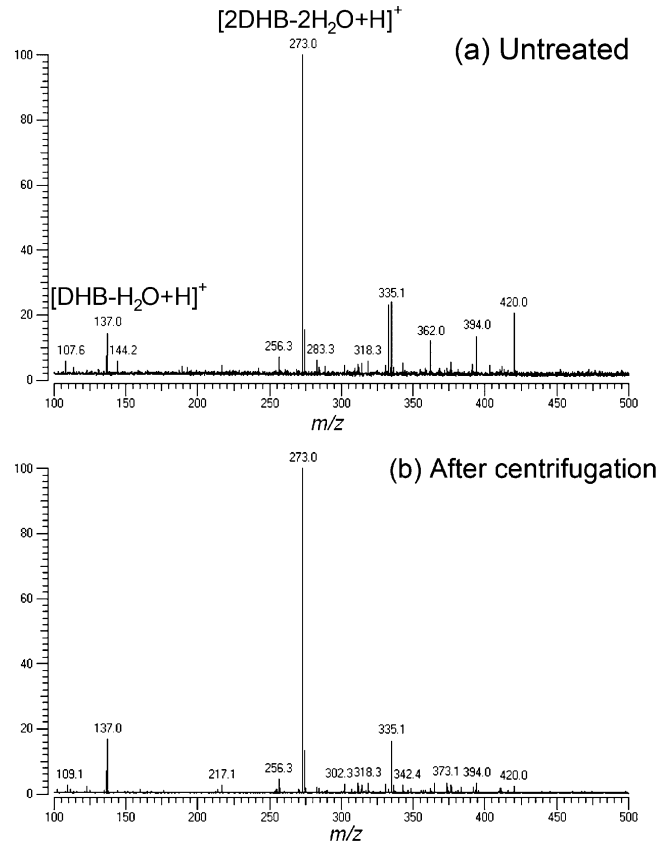

Figure 2. Mass spectra for the penicillin G-containing milk sample (a) unpretreated; (b) treated by centrifugation before MALDI-FTMS analysis.

Mass Spectrometry Analysis. The mass spectrometric behavior of all analytes was studied using MALDI-FTMS. Penicillin G is classified as a β-lactam antibiotic by its chemical structure.

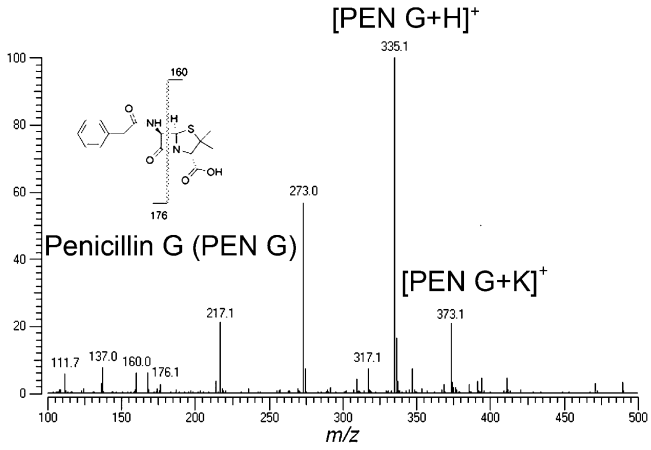

Figure 3. MALDI-FTMS spectrum obtained for penicillin G standard.

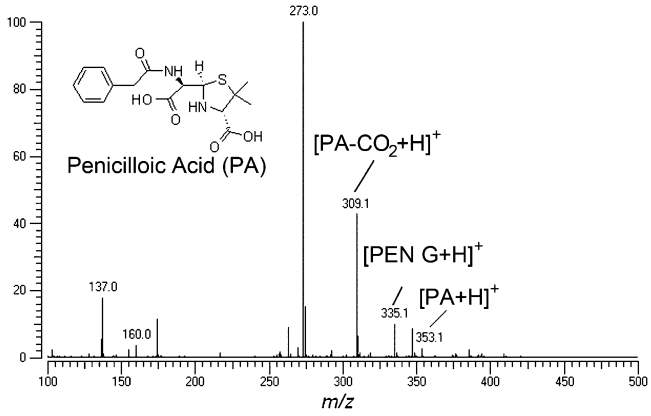

Figure 4. MALDI-FTMS spectrum for reaction solution of penicillin G catalyzed by BLA.

Its basic structure consists of a thiazolidine ring and a β-lactam ring. It has been reported that fragmental ions of PEN G were m/z 160 and 176 in the positive ion mode of ESI-MS/MS.[42−44] Figure 3 depicts a typical spectrum obtained for the PEN G standard. As shown, the relative signals of PEN G consist of protonated species ($[M + H]^+$, m/z 335), which exhibits a strong intensity, and the potassium cation adduct ($[M + K]^+$, m/z 373) of PEN G. The ions at m/z 273 and 137 are attributed to matrix used in MALDI-FTMS analysis (DHB). The fragment ions at m/z 160 and m/z 176, respectively, are also observed in the mass spectra under MALDI-FTMS, which is in accordance with the observation in ESI-MS/MS.[42−44] It is thought that the fragmentation observed for PEN G was induced by laser energy.

In the presence of β-lactamases, penicillin G is converted to penicilloic acid, corresponding to a change in molecular mass from 335 to 353. As shown in Figure 4, the signal of protonated penicilloic acid was clearly observed as a peak at m/z 353. Additionally, a signal at m/z 309 could also be observed. It is thought that this ion was generated by the elimination of carbon dioxide (44 Da) from the protonated penicilloic acid ion. This observation was similar to the fragmentation in the negative ion mode in literature reports.[45] Compared to other mass spectrometers, the use of FTMS as the detection system in this study allows the production of highly accurate mass spectra of target compounds. Accurate mass determination and elemental composition data calculated, thereof, can be used for structure elucidation. On the basis of the high resolution of FTMS profile data and high accuracy of mass measurements (Table 1), the signals from the enzymatic reaction were further confirmed. As the signal at m/z 309 exhibits higher peak intensity than protonated ion, it can be used as the reporter ion for the presence of PA when only a few peaks of PA are produced.

Table 1. Pencillin G and Penicilloic Acid Detected by MALDI-FTMS

ions	measured (m/z)	theoretical	elemental composition	error (ppm)	DBE
Penicillin G (PEN G)					
$[PEN\ G+H]^+$	335.1063	335.1060	$C_{16}H_{19}N_2O_4S^+$	0.9	8.5
$[PEN\ G+K]^+$	373.0624	373.0619	$C_{16}H_{18}N_2O_4SK^+$	1.4	9.0
fragment 1	160.0427	160.0427	$C_6H_{10}NO_2S^+$	0.1	2.5
fragment 2	176.0707	176.0706	$C_{10}H_{10}NO_2^+$	0.5	6.5
Penicilloic Acid (PA)					
$[PA+H]^+$	353.1177	353.1166	$C_{16}H_{21}N_2O_5S^+$	3.2	7.5
$[PA\text{-}CO_2+H]^+$	309.1271	309.1267	$C_{15}H_{21}N_2O_3S^+$	1.2	6.5

Determation of BLA in Milk. In order to develop the analysis of β-lactamases in milk, we plotted our strategy as follows: after the BLA are spiked into the milk sample, the antibiotics are added to the fortified milk samples. After a certain incubation time, the mixture was treated briefly and analyzed directly by MALDI-FTMS. The signals of PA in the spectra indicate the presence of β-lactamases in the milk sample. The enzyme reaction catalyzed by the BLA-containing milk sample was monitored by MALDI-FTMS analysis. According to our previous report,[35] it was possible to follow the reaction over time by monitoring the decrease of the intensity of the signal of substance and the increase of that of product. Relative peak−height ratios of substrate to product determined by MALDI-FTMS allow the semiquantitative analysis of enzymatic reaction for screening purposes. As shown in Figure 5, there was no significant difference in relative intensity (PEN G/PA ratio) between buffer and milk samples after being incubated for an appropriate time, while the ratio gradually decreased from the second hour to the sixth hour. It means the conversion of PEN G increases gradually as incubation time extends. In addition, the activity of BLA was not significantly changed in the milk matrix.

We further investigated the amount of β-lactamases in milk that can be detected by our method. Until the concentration of β-lactamases in the milk sample was diluted to 6×10^{-3} U·mL^{-1}, we could successfully detect the signals of PA, m/z 309 and m/z 353, which indicated the presence of BLA in the sample. To confirm this result, unfortified milk should be detected parallel to avoid the effect of BLA in milk itself. The concentration of β-lactamases that can be detected in milk by the proposed method is much lower than the result reported in literatures (4 U·mL^{-1}[46] and 15 U·mL^{-1}[47]), which indicates the

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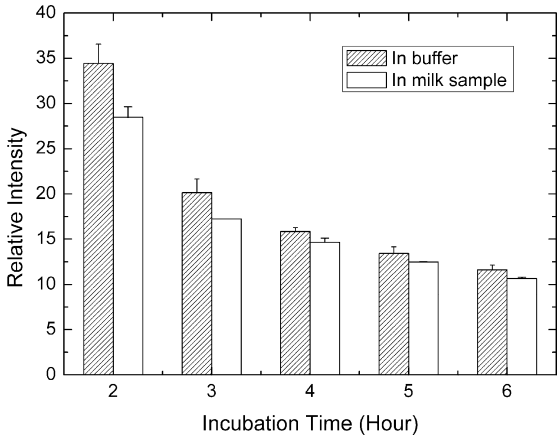

Figure 5. Relative intensity of $[PEN\ G + H]^+$ to $[PA\text{-}CO_2 + H]^+$ from mass spectra of reaction mixture (a) in solution; (b) in milk sample.

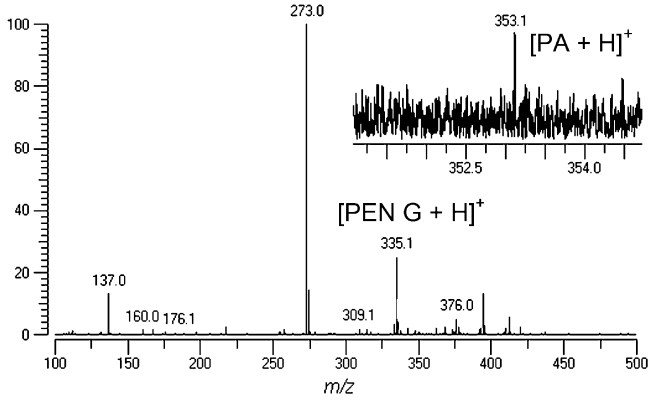

Figure 6. Mass spectrum of a β-lactamase positive sample.

good sensitivity of our method. As we monitor the hydrolytic reaction of PEN G to indicate the presence of BLA, we assume it is reasonable to prolong the incubation time of the enzymatic reaction in order to increase the sensitivity of method. Besides sensitivity, the high resolving powers of FTMS enable us to specifically measure ion intensities and circumvent or normalize for isobaric interferences, possibly caused by direct analysis of milk samples. During monitoring of commercial milk samples for the presence of BLA, a signal at m/z 335.3 was found in the spectrum, which could interfere with the determination of PEN G (m/z 335.1) if another mass spectrometry was used. In contrast, good separation of two signals can be gained easily by the use of FTMS. These advantages extend the MS-based screening to the complex matrix, without the need of a chromatographic separation step prior to MS analysis.

Furthermore, the described method was applied to characterize the commercial antimicrobial destroyer (the main component is BLA) in bovine milk samples. According to the package insert, 18.5 μL of antimicrobial destroyer was added to 1000 mL of milk. The production of PA can be detected under our conditions. It is clear that the MALDI-FTMS is sufficiently sensitive to assay BLA with good results. As an additional application, six real milk samples were tested by the proposed method. The data obtained indicates that one of them was found to be β-lactamase positive (Figure 6).

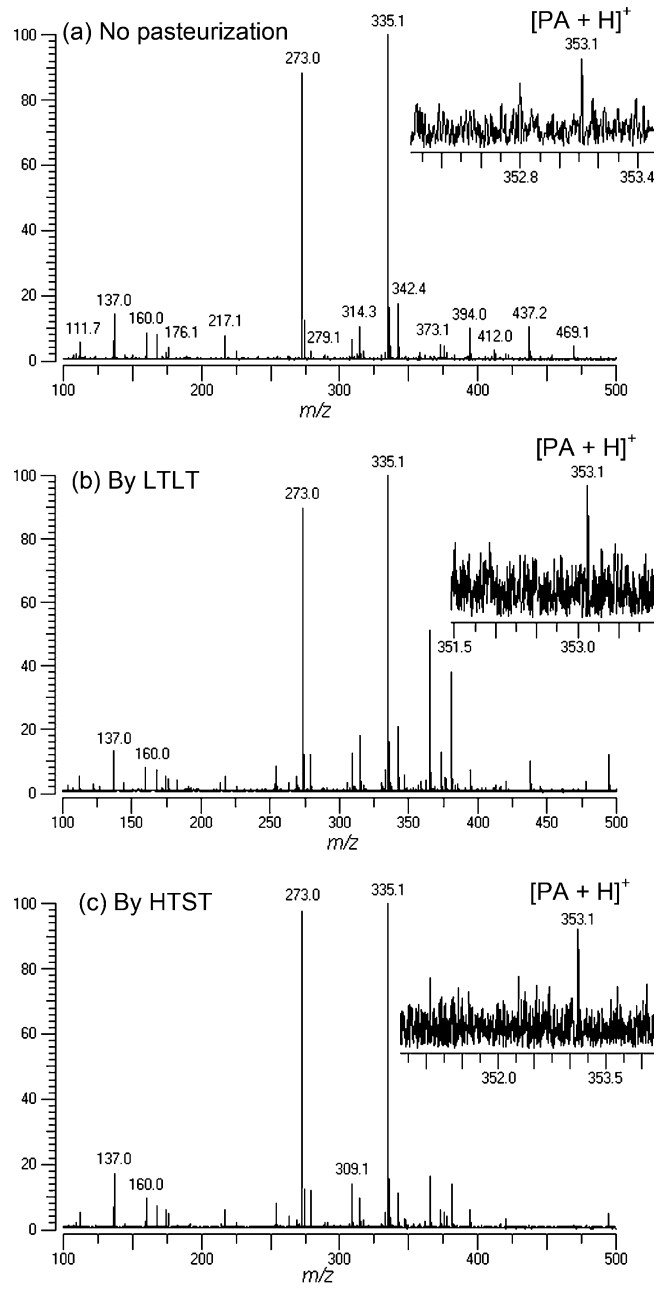

Figure 7. Mass spectra of the reaction mixture of PEN G catalyzed by BLA-containing milk (a) untreated; (b) treated by a low temperature long time pasteurization process; (c) treated by a high temperature short time pasteurization process.

Influence of Pasteurization Procedure. A further important point not adequately addressed in some methods for determining β-lactamases in milk is the issue of analyte stability during the pasteurization process. Raw milk is inherently dangerous, since it may contain many pathogens. Therefore, for the health benefits, pasteurization became mandatory for all milk for sale. Pasteurization is a process that destroys microorganisms by heating milk to a specific temperature for a set period of time. Generally, the β-lactamases are illegally added to raw milk just after the raw milk is collected from the dairy farm. It is necessary to investigate the effect of pasteurization on the β-lactamase activities because the

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goal of present study is to analyze the commercial milk sample directly. Pasteurization can be done as a low temperature long time (LTLT) or high temperature short time (HTST) process. In this study, both pasteurization conditions were investigated. In order to find whether any small differences of β-lactamase activity were made by pasteurization, fortified milk (concentration of BLA 6×10^{-3} U·mL^{-1}) was pasteurized according to the above procedures prior to the reaction with PEN G. As the concentration of BLA in milk investigated is a little low, the mini decrease of enzyme activity will contribute to the elimination of PA signals. Figure 7 displays the mass spectrum of the reaction mixture of PEN G catalyzed by different milk samples containing BLA. It appears that the heat treatment (62.8 °C for 30 min and 71.7 °C for 15 s) of the milk before the PEN G solution was added did not visibly change the the activity of BLA in the milk sample. The result indicates that the detection method can be used to analyze the BLA in commercial pasteurization milk samples.

CONCLUSION

We have established a simple, rapid, and simultaneous analytical method for BLA and penicillin G in milk samples using MALDI-FTMS. Centrifugation is applied to separate target analytes from a milk matrix. The method involves the following steps: addition of PEN G, incubation, purification with centrifugation, and mass spectrometric determination directly. In this study, to minimize as much as possible the handling of the milk sample prior to the MALDI-FTMS detection, while at the same time eliminating any major matrix effects, the sample pretreatment procedure was investigated. The extract mass and high resolution measurements using FTMS as the detection system make it possible to extend this methodology to complex samples, such as yogurt, cheese, etc. Furthermore, the method was successfully applied to characterize the BLA in milk. The presented data suggest that this method will help better monitor antibiotic residues and the BLA additive in foodstuff, which is useful for the assurance of food safety. The preliminary study has demonstrated that the MALDI-FTMS platform features universality, low cost, and ease of handling. The low cost of the materials and the relative short scanning time required make this method an alternative choice for high-throughput screening of a library of milk samples. We assume that this method might be useful for detection of enzyme in foodstuff and other matrixes.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (No. 20875097, 20902104), the ministry of Science and Technology of China (2009IM031300), and Shanghai basic research key project (09JC1417200).

Received for review September 4, 2009. Accepted January 25, 2010.

AC9019945